Antimalarial Drug Discovery:
Exploring the MEP Pathway

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For the degree of

Doctor in Philosophy

By

Kathryn E Price

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Declaration

This thesis is the result of my own work. The material contained in the thesis has not been presented, or is currently being presented, either wholly or in part for any other degree or qualification.

Kathryn E Price

This research was carried out in the Department of Chemistry at The University of Liverpool and the Paediatric Department at Washington University School of Medicine in St. Louis.
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Abstract

The development of effective antimalarial chemotherapeutics remains a major challenge within drug discovery. Despite a significant scientific progress over the last two decades and an upscaling of antimalarial research programs, malaria is still a major threat to global health with almost half the world’s population at risk of contracting the disease. It is the adept ability of the *Plasmodium* parasite to develop drug resistance which so significantly hinders the fight against this disease. Indeed, the present acceleration of *Plasmodium* resistance development is seriously threatening the longevity of the existing antimalarial drug pipeline and the prospects of malaria eradication.

As the pursuit of new antimalarial drugs and novel drug targets has intensified, significant interest has arisen in the chemical manipulation of isoprenoid biosynthesis. Isoprenoids are a diverse class of natural products which play vital roles in many cellular functions and are essential for *Plasmodium* parasite survival. In contrast to mammals, *Plasmodium* parasites use the non-mevalonate (MEP) pathway to generate isoprenoid precursors, delivering a key biological selectivity that makes the MEP pathway highly attractive as an antimalarial target. Fosmidomycin, targeting the MEP IspC enzyme, has validated the pathway as a viable target for antimalarial drug discovery, driving our objective to deliver a lead candidate molecule as a selective inhibitor of *P. falciparum* IspD (*Pf*IspD). In particular, we ask the question: Can arrest of the MEP pathway through *Pf*IspD inhibition cause parasite death via disruption of isoprenoid precursor generation?

A targeted HTS has identified the 1,2-benzo[d]isothiazol-3(2H)-one (BITZ) chemotype as a promising inhibitor of the *Pf*IspD enzyme. Extensive SAR investigations have generated a diverse range of BITZ compounds, including three of the most potent BITZ *Pf*IspD inhibitors to date; demonstrating IC_{50} values as potent as 73 nM against the enzyme and efficacious phenotypic activity of 206 nM against *P. falciparum* parasite growth. Molecular modelling studies have illustrated a mechanism of action which relies on the formation of a covalent *Pf*IspD-BITZ adduct that renders the enzyme inactive; this model of inhibition is supported by robust evidence from biological and chemical investigations.

The covalent modification of proteins can be a risky strategy within the context of drug design. As a result, a distinct chemical chemotype, also known to inhibit the *Pf*IspD enzyme *via* an alternative non-covalent mechanism is also being explored. The 1R,3S-MMV-008138 diastereoisomer, a tetrahydro-β-carboline derivative, shows potent inhibition against *Pf*IspD and *Plasmodium* parasite growth with a high degree of specificity towards the *Pf*IspD target. Considering these two chemical chemotypes, developmental SAR and hit-to-lead optimisation aims to progress towards a lead candidate compound that is suitable for clinical development as a novel antimalarial agent, targeting the *Pf*IspD enzyme.
Publications

*Plasmodium* IspD (2-C-Methyl-d-erythritol-4-Phosphate Cytidyltransferase), an Essential and Druggable Antimalarial Target

Molecular Mechanism of Action of Antimalarial Benzoisothiazolones: Species-Selective Inhibitors of the *Plasmodium* spp. MEP pathway enzyme, IspD

Molecular interactions between *Plasmodium falciparum* IspD and the Malaria Box inhibitor, 1R,3S-MMV008138
Manuscript in preparation
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<thead>
<tr>
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<tbody>
<tr>
<td>1°</td>
<td>Primary</td>
</tr>
<tr>
<td>2°</td>
<td>Secondary</td>
</tr>
<tr>
<td>3°</td>
<td>Tertiary</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>Å</td>
<td>Angström</td>
</tr>
<tr>
<td>ABPs</td>
<td>Activity-based probes</td>
</tr>
<tr>
<td>ACTs</td>
<td>Artemisinin-based combination therapies</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism and excretion</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
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<tr>
<td>A. thaliana</td>
<td><em>Arabidopsis thaliana</em></td>
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<tr>
<td>AtIspD</td>
<td><em>A. thaliana</em> IspD</td>
</tr>
<tr>
<td>AstraZeneca</td>
<td>AZ</td>
</tr>
<tr>
<td>BITZ</td>
<td>1,2-benzo[d]isothiazol-3(2H)-one</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-Butyloxycarbonyl</td>
</tr>
<tr>
<td>br d</td>
<td>Broad doublet</td>
</tr>
<tr>
<td>br s</td>
<td>Broad singlet</td>
</tr>
<tr>
<td>Bu</td>
<td>Butyl</td>
</tr>
<tr>
<td>Bn</td>
<td>Benzyl</td>
</tr>
<tr>
<td>CCDC</td>
<td>Cambridge Crystallographic Data Centre</td>
</tr>
<tr>
<td>CDCI₃</td>
<td>Deuterated chloroform</td>
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<tr>
<td>CDP-ME</td>
<td>4-diphosphocytidyl-2C-methyl-ᴅ-erythritol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>CDP-MEP</td>
<td>4-diphosphocytidyl-2C-methyl-β-erythritol 2-phosphate</td>
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<td>CI</td>
<td>Chemical ionisation</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>CTP</td>
<td>Cytidine triphosphate</td>
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<td>cytB</td>
<td>Cytochrome b</td>
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<td>Cys</td>
<td>Cysteine</td>
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<td>d</td>
<td>Doublet</td>
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<td>DCM</td>
<td>Dichloromethane</td>
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<td>dd</td>
<td>Doublet of doublets</td>
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<td>DDT</td>
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<tr>
<td>ddd</td>
<td>Doublet of doublet of doublets</td>
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<td>DHFR</td>
<td>Dihydrofolate reductase</td>
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<td>Dihydroorotate dehydrogenase</td>
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<td>DHPS</td>
<td>Dihydropteroate synthase</td>
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<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
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<td>Dm</td>
<td>Doublet of multiplets</td>
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<td>DMAPP</td>
<td>4-Dimethylallyl pyrophosphate</td>
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<td>DME</td>
<td>Dimethoxyethane</td>
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<td>DMF</td>
<td>N,N-dimethylformamide</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DOXP</td>
<td>1-deoxy-α-xylulose-5-phosphate</td>
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<tr>
<td>Dscore</td>
<td>Druggability score</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
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<td>dt</td>
<td>Doublet of triplets</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>Dxs</td>
<td>1-deoxy-o-xylulose-5-phosphate synthase</td>
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<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<td>EcIspD</td>
<td><em>E. coli</em> IspD</td>
</tr>
<tr>
<td>em</td>
<td>Emission</td>
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<td>eq</td>
<td>Equivalents</td>
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<td>ES</td>
<td>Electrospray</td>
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<td>EtOAc</td>
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<td>Et$_2$O</td>
<td>Diethyl ether</td>
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<tr>
<td>ex</td>
<td>Excitation</td>
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<tr>
<td>FGI</td>
<td>Functional group inter-conversion</td>
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<tr>
<td>FPIX</td>
<td>Ferriprotoporphyrin IX</td>
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<tr>
<td>FRET</td>
<td>Föster resonance energy transfer</td>
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<tr>
<td>FSM</td>
<td>Fosmidomycin</td>
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<tr>
<td>GA</td>
<td>Genetic Algorithm</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<td>GSK</td>
<td>GlaxoSmithKline</td>
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<td>HATU</td>
<td>1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate</td>
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<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylases</td>
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<td>hERG</td>
<td>Human ether-a-go-go related gene</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HMBPP</td>
<td>2-methyl-2-(E)-butenyl diphosphate</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screening</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>IPP</td>
<td>Isopentyl pyrophosphate</td>
</tr>
<tr>
<td>iPPTase</td>
<td>Inorganic pyrophosphatase</td>
</tr>
<tr>
<td>iPr</td>
<td>Isopropyl</td>
</tr>
<tr>
<td>IPT</td>
<td>Intermittent preventive treatment</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor residual spraying</td>
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<td>IspC</td>
<td>1-deoxy-d-xylulose-5-phosphate reductoisomerase</td>
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<td>IspD</td>
<td>4-diphosphocytidyl-2C-methyl-d-erythritol cytidylyltransferase</td>
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<td>IspE</td>
<td>4-diphosphocytidyl-2C-methyl-d-erythritol kinase</td>
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<td>IspF</td>
<td>2C-methyl-d-erythritol-2,4-cyclodiphosphate synthase</td>
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<tr>
<td>IspE</td>
<td>4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase</td>
</tr>
<tr>
<td>ITNs</td>
<td>Insecticide-treated nets</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>Kₘ</td>
<td>Substrate concentration at half-maximal enzyme velocity</td>
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<tr>
<td>LC-MS</td>
<td>Liquid Chromatography–Mass Spectrometry</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>LLINs</td>
<td>Long-lasting insecticidal nets</td>
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</table>
m       Multiplet
MDA    Mass drug administration
Me     Methyl
MEcPP  4-diphosphocytidyl-2C-methyl-ᴅ-erythritol 2,4-cyclodiphosphate
MeOD   Deuterated methanol
MeOH   Methanol
MEP    Non-mevalonate pathway
MEP    2C-methyl-ᴅ-erythritol 4-phosphate
mg     Milligram
MgSO₄  Magnesium sulphate
mL     Millilitre
mM     Millimolar
MS     Mass spectrometry
MVA    Mevalonic acid
MW     Molecular weight
m/z    Mass to charge ratio
NBD    7-nitrobenza-2-oxa-1,3-diazole
NBD-Cl 4-chloro-7-nitrobenza-2-oxa-1,3-diazole
nM     Nanomolar
nm     Nanometres
NMR    Nuclear magnetic resonance
NOE    Nuclear Overhauser Effect
NOESY  NOE spectroscopy
NS     Number of scans
NSAIDs Non-steroidal anti-inflammatory drugs
o/n  Overnight

*P. berghei*  *Plasmodium berghei*

PBD  Phosphate-buffered saline

*P. falciparum*  *Plasmodium falciparum*

*PfCRT*  *P. falciparum* chloroquine resistance transported gene

*PfIspC*  *P. falciparum IspC*

*PfIspD*  *P. falciparum IspD*

Ph  Phenyl

P_i  Inorganic phosphate

PK  Pharmacokinetic

*P. knowlesi*  *Plasmodium knowlesi*

*P. malariae*  *Plasmodium malariae*

PNP  Purine nucleoside phosphorylase

*P. ovale*  *Plasmodium ovale*

Pol  Point of inflection

PP_i  Inorganic pyrophosphate

PPIs  Protein-protein interactions

ppm  Parts per million

*Propofol*  2,6-diisopropylphenol

Pt  Parasitemia

*P. vivax*  *Plasmodium vivax*

q  Quartet

R^2  R-squared

RBCs  Red blood cells

RMSD  Root-mean-square deviation
rt  Room temperature
RT  Retention time
s   Singlet
SAR Structure activity relationship
SD  Standard deviation
SDM Site direct mutagenesis
SDS Standard default settings
SEM Standard error of the mean
SNPs Single nucleotide polymorphisms
Sum FICs Sum fractional inhibitory concentrations
t  Triplet
\( t_{1/2} \)  Half-life
TB  Tuberculosis
TBVs Transmission blocking vaccines
TCAMS Tres Cantos Antimalarial Set
td  Triplet of doublets
TFA Trifluoroacetic acid
THF Tetrahydrofuran
tlc Thin-layer chromatography
TRS Traditional Random Screening
tt  Triplet of triplets
TTP Thiamine pyrophosphate
\( \mu \text{M} \)  Micromolar
UTI's Urinary tract infections
UV Ultraviolet
<table>
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<tr>
<th>Symbol</th>
<th>Definition</th>
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<tr>
<td>$V_{\text{max}}$</td>
<td>Maximum enzyme velocity</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>wt</td>
<td>Weight</td>
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Introduction
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1.1 Malaria: A Neglected Tropical Disease

The development of effective antimalarial chemotherapeutics remains a major challenge within drug discovery. Despite a 37% decrease in global malaria incidence between 2000 and 2015, 97 countries registered ongoing malaria transmission in 2014, with 214 million new cases of malaria worldwide in 2015.\textsuperscript{[1]} This highlights that the development of new antimalarial drugs and the battle to overcome increasing parasite resistance is a crucial and ongoing challenge.

Malaria is a life-threatening, vector-borne disease which causes over 200 million cases per year and is responsible for nearly half a million deaths therefore putting almost half the world’s population, about 3.2 billion people at risk of contracting the disease. In 2015, there were 214 million reported cases of malaria worldwide which lead to an estimated 438,000 deaths.\textsuperscript{[1]} Of these deaths, 90% occurred in Sub-Saharan African which carries a disproportionately high share of the total global malaria burden (Figure 1.1).\textsuperscript{[1]} Malaria is therefore endemic across the majority of tropical and subtropical regions since warm, humid climates promote ideal breeding conditions for Anopheles mosquitoes, the transport vectors of malaria, which breed in shallow, freshwater pools. Since 2000, it has been observed that declines in malaria incidence in the 15 sub-Saharan African countries where malaria transmission is ongoing, has significantly fallen behind that of other countries in South-East Asian and Mediterranean regions where the disease is less prevalent.\textsuperscript{[1]}

![World Distribution of Malaria in 2014](image)

*Figure 1.1 Geographical distribution of malaria in 2014; figure reproduced from [2].*
Malaria is a hematoprotozoan parasitic infection\cite{3} caused by five parasite species belonging to the *Plasmodium* genus and is transmitted exclusively through the bite of female *Anopheles* mosquitoes. *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium ovale* (*P. ovale*) and *Plasmodium malariae* (*P. malariae*) are the four human malaria species responsible for the spread of malaria from person to person which is facilitated by the *Anopheles* mosquito vector when taking a blood meal. Recently, human cases of malaria caused by *Plasmodium knowlesi* (*P. knowlesi*), the malaria-causing parasite species among monkeys, have also been recorded; however, this is currently thought to only be spread through zoonotic transmission.\cite{1} Of the protozoan parasite species infective to humans, *P. falciparum* and *P. vivax* present the greatest challenge to public health, contributing to the majority of malarial infections.\cite{4} *P. falciparum* however, has long been recognised as the deadliest of the parasite species due to its high mortality rates and significant prevalence within sub-Saharan Africa.\cite{1,5} Conversely, *P. vivax* has much lower incidence levels and very low transmission rates in African countries but has a much broader geographical distribution due to its ability to survive in cooler climates, compared to *P. falciparum*, and develop in the *Anopheles* mosquito at lower temperatures.\cite{5} *P. vivax* is therefore a substantial cause of morbidity in countries outside of African regions.

As well as the direct cost to health, malaria poses a huge economic burden, particularly in countries where the disease is endemic. Malaria is both a cause and a consequence of poverty,\cite{6} slowing economic growth and development in already underdeveloped regions which drives the vicious cycle of poverty primarily affecting the poor living in malaria-prone, rural areas where there are often few barriers against mosquitoes and malaria transmission.\cite{7} In African countries, malaria accounts for 40% of total public health expenditure placing a huge burden on health systems. Since 2001, malaria infection in these countries is estimated to be responsible for an annual cost of almost US$300 million in case management (diagnosis and treatment of infection) alone.\cite{3} However, estimates predict that annual investments in malaria control and elimination will need to increase by US$6.4 billion per year before 2020 if the goal of a 40% reduction in malaria incidence and mortality are to be met, as outlined by the *World Health Organisation* (WHO) within the proposed global technical strategy for malaria 2016–2030.\cite{4}

The intensity of malaria transmission is dependent on a number of factors including: the *Plasmodium* parasite, the mosquito vector, the human host and the specific environment within which these components are combined. Malaria transmission is often more intense in areas where the mosquito is long-lived due to increased parasite development within the mosquito. Insecticides are often used as methods of malarial vector-control however, increasing selective pressures have led to insecticide
resistance, particularly to the chlorinated hydrocarbon insecticide DDT (Figure 1.2), allowing the development of long-lived mosquitoes. It is therefore a combination of the long life span and strong human-biting habit of the African Anopheles mosquito vector which means 90% of the world’s malaria deaths occur in Africa.

![Figure 1.2 Chemical structure of the insecticide 1,1′-(2,2,2-trichloroethane-1,1-diyl)bis(4-chlorobenzene) (DDT).](image)

Malaria transmission is also dependent on climatic conditions, such as rainfall, temperature and humidity, which affect mosquito population sizes and make transmission seasonal with peaks during and just after the rainy season. The extent of malaria transmission and the risk of epidemics depends on levels of human immunity as well as climatic environment. Partial immunity can be developed over years of exposure to malaria but this doesn’t provide full protection against malaria infection or disease development. It is largely for this reason that children under the age of five are particularly at risk to infection since they have little opportunity to develop immunity and also loose maternal immune protection around 18 months of age. Of the estimated 584,000 worldwide malaria deaths in 2013, 78% of these were in children under five years of age. In addition, 20% of all child deaths in sub-Saharan Africa are due to malaria which remained the leading cause of death amongst this patient group in African regions between 2000 and 2015. Consequently this highlights the huge paediatric-specific mortality burden of malaria. Pregnancy is also a severe risk factor for contracting malaria and developing a severe form of the disease where pregnant women are three times more likely to contract malaria than non-pregnant woman and have a mortality rate close to 50% from severe malarial infection. Other groups at high risk of contracting malaria include patients with HIV (human immunodeficiency virus) or AIDS (acquired immune deficiency syndrome), non-immune travellers and mobile populations.

Malaria is an acute febrile disease from which symptoms are most likely to occur 10-15 days after an infective mosquito bite since asexual reproduction of Plasmodium parasites within human red blood cells (RBCs) (Section 1.2) is responsible for the clinical manifestations of the disease and its associated symptoms. Initial malaria symptoms are comparable to those of flu and can therefore be hard to recognise since they include fever, headaches, muscle aches, chills and vomiting and may be
mild. Such symptoms, with no signs of clinical severity or organ dysfunction, generally suggest a case of uncomplicated malaria which can be caused by all strains of *Plasmodium*. However, *P. falciparum* infection, the most prevalent and more severe of the *Plasmodium* infections, can rapidly progress to a severe form of the disease if not treated quickly. Severe malaria (or complicated malaria) occurs when untreated *P. falciparum* infection results in organ failure or blood abnormalities as a consequence of parasitic waste expulsion from erythrocytes, cytokine release and RBC sequestration during the asexual RBC stage of infection. Clinical manifestations of severe malaria include anaemia, hypoglycaemia (more severe amongst children), ketoacidosis, acute respiratory distress syndrome (ARDS) and kidney failure which, without rapid treatment can lead to death. Headaches, confusion and irritability may also proceed cerebral malarial, a further manifestation of severe malaria caused only by *P. falciparum* infection, which can proceed quickly to more severe cerebral symptoms such as coma, particularly in the cases of non-immune children.

### 1.2 Life Cycle of the *Plasmodium* Parasite

The life cycle of the *Plasmodium* parasite is highly complex, requiring specialised mechanisms to invade multiple cell types within the defence systems of both the human host and *Anopheles* mosquito vector. The life cycle of the malaria parasite ([Figure 1.3](#)) involves a sexual stage which takes place in the female *Anopheles* mosquito and an asexual stage which occurs in the human host. For this reason, it can be considered that humans are not the “definitive host” of *Plasmodia* since the *Anopheles* mosquito provides the site of sexual reproduction for *Plasmodia* parasites. From a parasite perspective, humans provide the means of accessing *Anopheles* mosquitoes where sexual recombination can occur but from the human perspective: the *Anopheles* mosquito is the vector by which *Plasmodial* malaria transmission occurs.

The bite of an infected female *Anopheles* mosquito transmits sporozoites, the infectious asexual forms of the parasite, from mosquito salivary glands to the human host during blood extraction when the mosquito takes a blood meal. Sporozoites swiftly enter human hepatocytes after inoculation into the blood where they begin pre-erythrocytic development in which multiplication occurs over 10-14 days in the human liver stage of the disease ([Figure 1.3](#)).
Sporozoites, known during the liver stage as schizonts, are able to remain concealed from the host’s immune system during occupation of hepatocytes. Schizonts, containing thousands of hepatic merozoites, are then liberated from the liver following rupture of parasitized liver cells. This releases the merozoites which initiate erythrocytic infection through invasion of host RBCs in the human blood stage of the life cycle (Figure 1.3). Intracellular development of these merozoites within the erythrocyte, from immature trophozoites (ring stage) into motile parasitic entities (mature trophozoites) allows further evasion of the human host immune system. To achieve rapid development, the parasite digests large amounts of erythrocyte haemoglobin (Hb) using aspartic proteases which degrade between 25-85% of total Hb from host RBCs. Degraded Hb is transported to the parasite food vacuole where the obtained amino acids are used for parasite growth and maturation.

A mature trophozoite can undergo mitosis, differentiating to release merozoites and gametocytes upon rupture of the red blood cell. New merozoites can then bind to and enter uninfected red blood cells causing magnification of the parasitic infection. The smaller fraction of gametocytes released on
Chapter 1

Cell rupture are able to complete the sexual stage of the parasite life cycle once re-ingested by the Anopheles mosquito when another blood meal is taken (Figure 1.3)\[18\] Erythrocyte rupture releases cell debris, parasite waste and infectious parasitic material into the human bloodstream which causes many of the observed clinical symptoms of malaria (Section 1.1).\[15\] In severe cases, death can occur within 48 hours of symptom presentation, corresponding to one asexual cycle of the P. falciparum blood-stage infection. This highlights that preventing maturation of early generation parasites into mature, more pathogenic parasite forms is essential in fighting malaria infection.\[3\]

P. vivax has a slightly different life cycle to P. falciparum within the human host as some sporozoites in P. vivax infections do not develop immediately schizonts within the liver stage. Unlike P. falciparum infections, where schizonts exit hepatocytes after one cycle of the human liver stage, schizonts of the P. vivax species are able to remain as silent single nuclear hypnozoites within hepatocytes over variable and extended time periods. Reactivation of these dormant parasitic forms, a consequence of unknown factors, produces further hepatic development weeks, months or years after the primary P. vivax infection, producing an exoerythrocytic multiplication cycle recognised as the ‘liver-form’ of the disease.\[18,20\] Treatment of P. vivax infections can therefore be difficult due to the potential for disease recurrence following initial infection. Consequently, P. vivax also poses a significant public health threat across many parts of the world, with estimations suggesting that this Plasmodium species was responsible for 13.8 million cases of malaria worldwide in 2015, which amounts to approximately half of the total malaria cases outside of sub-Saharan Africa.\[1\] Unlike P. vivax, P. falciparum parasites have no exoerythrocytic or ‘liver-persistent’ stage therefore highlighting the requirement for different treatment regimens and chemotherapeutic intervention methods to treat these two parasite species.\[16\]

1.3 Malaria Control and Elimination Strategies

1.3.1 Vector Control

Vector control is an essential component of successful malaria control and reducing malaria transmission rates. However economic, administrative and logistical issues often hinder the potential reductions in global malaria burden that could be achieved using these preventative methods.\[21\] Of the different strategies available, indoor residual spraying (IRS), insecticide-treated nets (ITNs) and preferentially, long-lasting insecticidal nets (LLINs)\[1\] are the most effective forms of vector control that can be used in a wide range of circumstances and are therefore fundamental in protecting individuals at risk of contracting malaria.\[21,22\] Early use of the organochloride insecticide, DDT (Figure 1.2) highlighted the proficiency of insecticides in dramatically reducing mosquito vector numbers and therefore reducing transmission rates. However, selective pressures led to insecticide
resistance and have consequently enabled the development of long-lived mosquitoes.\[9\] In recent decades, pyrethroid insecticides have been the choice chemical for malaria vector control and are currently the only insecticide recommended by the WHO for use on ITNs or LLINs. However, upscaling malaria vector control and increased use of these insecticides within agriculture has seen the development and spread of resistance against pyrethroids.\[22\] New insecticides are therefore urgently needed if this method of malaria vector control is to remain effective.

Considering other forms of vector control, upscaling LLIN distribution through mass distribution campaigns have resulted in approximately 49% of households in sub-Saharan African owning at least one ITN by the end of 2013, compared to only 3% in 2004.\[22\] The public health impact of this form of vector control is substantial: ITNs have been found to reduce the incidence of malaria cases in field trials by more than 50% and reduce malaria mortality rates in sub-Saharan Africa by an estimated 55% in children under five years of age.\[1\]

1.3.2 Antimalarial Chemotherapies

Whilst vector control methods can provide effective means of reducing disease transmission and the numbers of individuals contracting malaria, it is drug chemotherapy that has provided the most successful methods for the treatment and prevention of malaria infection.\[23\] Over the last century, a broad range of antimalarial treatments have been developed and administered as part of several malaria eradication campaigns which have achieved varying amounts of success.\[24\] Whilst a number of antimalarial chemotherapies have been significant in the progress and successes of malaria treatment and eradication efforts, chloroquine and artemisinin can perhaps be considered the most fundamental of these. A brief account of their identification, administration and subsequent impacts are presented below.

1.3.2.1 Chloroquine: Identification, Application and Resistance Development

The natural product quinine (Figure 1.4) was first isolated from the bark of the cinchona tree (Cinchona calisaya), native to South America, in France in 1820. The use of cinchona bark, a rich source of medicinal alkaloids, was used to treat malaria throughout the 17th century by Jesuit missionaries in Peru. However, it was only after successful extraction of the compound that the superiority of the pure alkaloid as an antimalarial agent was fully realised.\[4,25\] It was a fortuitous discovery by the German chemist, William Henry Perkins, in 1856 whilst attempting to synthesis quinine that identified the first aniline dye, mauveine. This discovery launched the development of the synthetic dye industries which swiftly lead to the birth of the pharmaceutical industry, in turn facilitating the production of the first synthetic antimalarial agents including chloroquine, mepacrine
(also known as quinacrine) and primaquine (Figure 1.4). These synthetic agents, belonging to the 4-aminoquinoline and aryl aminoalcohol chemical classifications, exert their antimalarial effects by interfering with the process of haem polymerisation.\textsuperscript{[24]} The efficacy demonstrated by these synthetic quinine derivatives meant they were able to replace the use of quinine itself, becoming the foundation of antimalarial chemotherapy over the next two decades.\textsuperscript{[4,16]}

![Chemical structures of quinine, chloroquine, mepacrine, and primaquine.](image)

Figure 1.4 Chemical structures of the natural product quinine and early synthetic antimalarials: chloroquine, mepacrine and primaquine.\textsuperscript{[16]}

The 4-aminoquinoline, chloroquine, is probably one of the most successful and important chemical entities ever developed against an infectious disease.\textsuperscript{[26]} Chloroquine was discovered in 1934 and became available as an antimalarial treatment in 1946 remaining highly effective as a front-line malaria chemotherapy throughout the mid-1900s. A combination its high clinical efficacy, low toxicity within the human host and affordable synthetic route made chloroquine a significant chemotherapeutic which had a huge impact in malaria therapy, saving thousands of lives and substantially reducing morbidity and mortality from the disease.\textsuperscript{[27]} It is thought that the efficacy of chloroquine stems from its ability to disrupt haematin detoxification in \textit{Plasmodium} parasites as they mature within host erythrocytes, causing fatal toxic effects towards parasite enzymes.\textsuperscript{[26]} It is generally accepted that chloroquine binds to ferriprotoporphyrin IX (FPIX), a Hb degradation product that is toxic to the parasite in high concentrations and released as the parasite digests host Hb within its food vacuole.\textsuperscript{[28]} Under normal conditions, FPIX is detoxified by the parasite through crystallisation into inert, non-toxic crystals of haemozoin to maintain unhindered parasite growth. Treatment with chloroquine leads to parasite death due to the parasites consequent inability to
complete this natural haem detoxification process following chloroquine-FPIX binding and the resulting accumulation of toxic FPIX.\textsuperscript{[26,28]}

Ultimately, the initial high success rates of chloroquine in reducing global morbidity and mortality from malaria, and its consequent heavy use across the decades, has led to the development of chloroquine resistance in \textit{P. falciparum} and \textit{P. vivax} parasite species.\textsuperscript{[26]} Therefore, despite the early success of chloroquine and its significant contribution to the 1955 WHO malaria eradication programme, its potent antimalarial efficacy is now significantly reduced following the emergence of resistant parasite strains in 1957, first documented at the Cambodia-Thailand border. Continued resistance development from then onwards means that today, more than 80\% of wild-type \textit{Plasmodium} isolates are resistance to chloroquine.\textsuperscript{[16]}

A single genetic mutation can be all that is required to impart resistance to antimalarial chemotherapies, or indeed any anti-infective agent, if mutation occurs within the molecular target of a specific chemical agent.\textsuperscript{[3,29]} Such is the case with the antimalarial agents: pyrimethamine and atovaquone (Figure 1.5). Pyrimethamine, introduced in the early 1950s for the therapy and prophylactic treatment of malaria, is a competitive dihydrofolate reductase inhibitor which impedes the synthesis of parasite DNA and proteins.\textsuperscript{[3]} Atovaquone belongs to the chemical family of naphthoquinones and operates its antimalarial effects through inhibition of cytochrome \textit{b} (\textit{cytB}) which is essential for maintaining a functional electron transport chain.\textsuperscript{[24]} \textit{Plasmodium} resistance to pyrimethamine can be induced by a single point mutation in the gene encoding dihydrofolate reductase (\textit{dhfr}) which has been found to emerge relatively frequently in the human malaria \textit{P. falciparum} parasite.\textsuperscript{[3,26,30]} Similarly, a single point mutation in the gene encoding cytochrome \textit{b(cytB)} is able to confer resistance to atovaquone.\textsuperscript{[16]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{antimalarials.png}
\caption{Chemical structures of antimalarials atovaquone and pyrimethamine.\textsuperscript{[24]}}
\end{figure}

In contrast, chloroquine resistance appears to involve higher genetic complexity than pyrimethathamine resistance since resistance to chloroquine took a number of years develop within initially limited foci and requires multiple mutations in the \textit{Plasmodium} chloroquine resistance
transporter gene (*PfCRT*) to arise.\[^{26}\] The Lys276Thr mutation in the parasite genome encoding the gene product *PfCRT* is principally responsible for chloroquine resistance. *PfCRT* is a transmembrane transport protein present in the digestive food vacuole of *Plasmodium* parasites. However, a genetic background where the parasite carries additional mutations within the same gene is also required to infer resistance development against chloroquine.\[^{31,32}\] The mutated form of the *PfCRT* transporter protein is able to reduce chloroquine accumulation in the parasite food vacuole where haem polymerisation occurs therefore reducing inhibition of parasite detoxification since chloroquine is unable to accumulate.\[^{24}\] In Malawi, following a halt in chloroquine use over the last 15 years, a recovery in chloroquine sensitivity has been observed. This suggests that the identified Lys276Thr mutation also confers a fitness disadvantage to *Plasmodium* parasites when this *PfCRT* gene mutation is present with the absence of drug pressure.\[^{33,34}\] However, as Malawi is surrounded by countries where high levels of chloroquine resistance remain, re-introduction of chloroquine treatment is not an option.\[^{32}\] This highlights the continued risk of resistance resurgence if these early antimalarial chemotherapeutics remain as front-line antimalarial agents within a number of limited treatment options due to failures to replace expended drugs with new malaria therapies that act against novel molecular targets.

### 1.3.2.2 Artemisinin: Identification, Application and Resistance Development

Comparable to the botanical origins of quinine, sweet wormwood *Artemisia annua* has been used in Chinese medicine, dating back over two millennia for the treatment of febrile illness.\[^{4}\] It wasn’t until the late 1960s when the Chinese government supported a research programme to identify antimalarial agents from known Chinese herbal medicines that the synthetic development of artemisinin began.\[^{4,35}\] Following the extraction of artemisinin (Figure 1.6) from the leaves of *Artemisia annua*, it was in the 1970s that the structure of this 15-carbon (sesquiterpene) peroxide, with its powerful, if unstable, antimalarial properties was identified and characterised. The development of semi-synthetic analogues soon followed demonstrating both increased potency and improved bioavailability than artemisinin, helping to remove initial scepticisms regarding the unusual structure of this new chemical entity.\[^{4,32}\]

Chemical exploration of semi-synthetic artemisinins was ongoing throughout the mid-1970s leading the development of artemether, a more stable methyl-ether derivative; artesunate, demonstrating improved water-solubility; and dihydroartemisinin (DHA), the active component of both artesunate and artemether, highlighting their identities as pro-drugs (Figure 1.6).\[^{4,36}\] Ultimately, these chemical derivatives replaced artemisinin as the parent drug due to superior antimalarial activity profiles and improved compound stability. Despite initial use as monotherapies, it was gradually realised that
these treatments should be administered in combination with additional drug entities, analogous to anti-tuberculosis and anti-retroviral chemotherapies. This aimed to minimise the development of drug resistance in areas where malaria is endemic and chemotherapies must therefore be used in elevated frequencies that create high levels of drug pressure. Such artemisinin-based combination therapies (ACTs) yield enhanced parasite killing through careful chemical combination: the artemisinin component of ACTs rapidly kills the majority of parasitic organisms quickly reducing initial parasitemia; the combined partner drug, commonly characterised by a longer half-life, is then able to slowly eliminate remaining parasites. There is therefore a dual benefit to ACTs, not only in optimising the therapeutic effect, but also in protecting both treatment components of the combined therapy, minimising the risk of resistance development against either chemical component. Today, ACTs are the mainstay of chemotherapeutic intervention for the treatment of *P. falciparum* malaria across the globe.

![Chemical structures of artemisinins and related compounds](image)

**Figure 1.6** Chemical structures of artemisinins and related compounds. Artemisinin is the natural compound produced by the plant *Artemisia annua*; artesunate, artemether and DHA are semi-synthetic derivatives of the natural product.

ACTs were first introduced in the mid-1990s for the treatment of uncomplicated malaria infection. Since 2005, in nearly all countries where the disease is endemic, ACTs have been the recommended frontline therapies against malaria after a change in treatment policy endorsed by the WHO. This follows a decade of increased malaria treatment failure rates with the use of chloroquine (Figure 1.4) and sulfadoxine-pyrimethamine combinations (Figure 1.5 and Figure 1.8) against *P. falciparum* infections. Sulfadoxine prevents folate biosynthesis through the inhibition of dihydropteroate
synthase (DHPS). Sulfadoxine is typically used in combination with pyrimethamine where this combination has been one of the widest used antimalarial chemotherapies, proven to deliver high efficacy as a prophylaxis and curative treatment. Today, clinical resistance to antifolate treatment is widespread but, remarkably, the administration of sulfadoxine-pyrimethamine is still seen to improve the outcomes of treatments in pregnant women when used as an intermittent preventative therapy.\[24]\n
Not only do artemisinin derivatives have an excellent safety profile, demonstrate high potency and display a rapid onset of action, but they are also show broad-stage specificity of action compared with other antimalarial agents, demonstrating activity across several stages of the \textit{Plasmodium} spp. life cycle.\[^{32,36,38}\] In addition, artemisinins are capable of killing immature and developing gametocytes therefore signifying activity against the sexual stages of the \textit{Plasmodium} spp. life cycle which gives these chemotherapies added value in their ability to reduce malaria transmission and infectivity.\[^{32}\] Current recommendations for treating malaria are consequently a three-day course of ACTs with varying drug partner combinations. Indeed, over the last half-decade, five different combinations of fixed-dose ACTs have been approved for use and administration. These include: artemether-lumefantrine, artesunate-amodiaquine, DHA-piperaquine, pyronaridine- artesunate and artesunate-mefloquine.\[^{39}\] Further discussion concerning the rationale, design and potential advantages of drug combination therapy is contained within \textbf{Chapter 5} (see \textbf{Section 5.3}).\n
There has been much debate regarding the mechanism of action by which artemisinins exert their robust parasite killing effects. Interestingly, like quinolones, artemisinins appear to target the parasite Hb degradation pathway by which parasites obtain amino acids from human host erythrocytes to facilitate growth and maturation during blood-stage infection.\[^{4}\] More specifically, it is the endoperoxide bridge, an unusual feature of the 1,2,4-trioxane structure, that distinguishes artemisinin and its derivatives and is responsible for the high potency of these antimalarial agents.\[^{36}\] The endoperoxide bridge is a known source of oxygen free radicals and it has been observed that artemisinin derivatives lacking this structural feature are devoid of antimalarial activity. Unbound ferrous haem, Fe\(^{2+}\), released during haemoglobin degradation is thought to be the catalyst responsible for activating artemisinins \textit{via} reduction of the endoperoxide bond. As a result of reduction by ferrous haem, the activated artemisinin structure undergoes rearrangement forming a carbon-centred radical species, which is capable of eliciting parasite-killing effects.\[^{4,36}\] Artemisinins therefore behave quite differently from other oxidant drugs, such as pirfenidone (an antifibrotic drug)\[^{40}\] and flavonoid antioxidants\[^{41}\], which often produce large quantities of oxygen free radicals.\[^{36}\] Whilst the importance of this initial endoperoxide bond reduction is widely accepted, the
subsequent downstream processes and specific mechanisms of parasite-killing have long been debated.\[^{[4]}\]

It is most likely that artemisinin derivatives exert their antimalarial effect at more than a single biological target and it is therefore reasonable that a generalised, two-step mechanism of action can be assumed.\[^{[42]}\] In the initial step, haem-mediated activation of artemisinin-based compounds produces carbon-centred free radicals and electrophilic alkylating species which are able to target specific proteins in the parasite. However, it is worthy to note that these electrophilic artemisinin species, unlike other alkylating agents, do not react with DNA.\[^{[37]}\] Instead, these reactive components operate in a number of ways: causing membrane damage in the vicinity of vital parasite enzymes, inducing lipid peroxidation, inhibiting parasites enzymes and linked biological processes and disrupting haem detoxification.\[^{[37,42]}\] Further to this, it has been outlined by Krishna et al. that the P. falciparum orthologue PfATPase6, the sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase (SERCA), represents a key alkylation target of reactive artemisinin species.\[^{[36,42,43]}\]

Collectively, these mechanisms compromise parasite viability which ultimately leads to parasite death.\[^{[44]}\]

Despite the high success rates of ACTs, which remain the recommended treatment for uncomplicated P. falciparum malaria infection and have substantially reduced global morbidity and mortality from malaria,\[^{[1]}\] continued progression of malaria elimination is again significantly threatened by resistance emergence against these therapies.\[^{[38]}\] Artemisinin resistance became established in western Cambodia more than a decade ago and is now prevalent across an ever expanding area of southeast Asia.\[^{[45]}\] These increasing levels of parasite resistance are characterised by slow parasite clearance rates following artemisinin-based treatment\[^{[46]}\] which is the consequence of a reduction in the susceptibility of ring-stage parasites to drug treatment.\[^{[47]}\] This is ultimately reflected in the increased ACTs failure rates, seen in both Cambodia\[^{[48]}\] and Thailand.\[^{[49]}\] Artemisinin resistance is developing in the same loci, the Thai-Cambodia boarder, that reared resistance development to the early mainstays of antimalarial treatments including: chloroquine, sulfadoxine-pyrimethamine and mefloquine (Figures 1.4 and 1.5).\[^{[50]}\] A combination of prevalent artemisinin monotherapies and poor-quality ACTs, sub-therapeutic dosing and the unregulated use of such agents in poorly developed economic regions of the world, in addition to logistical administration difficulties, are all factors contributing to the development and spread of artemisinin resistance.\[^{[38]}\]

Numerous studies have been conducted to determine molecular markers of resistance and the key determinants of parasite resistance to artemisinin therapy which is now prevalent across Southeast Asia.\[^{[3,15,16,32,37–39,45–50]}\] Most recently, studies have shown that P. falciparum resistance to artemisinin
is closely associated with mutations altering the propeller region of a kelch protein, encoded by \emph{kelch13}. A single nucleotide polymorphism (SNP) within \emph{kelch13}, referred to as K13, which causes up-regulation of unfolded protein response pathways, has been found as a determinate of artemisinin resistance and is consistently identified with a delayed and slow-clearance phenotype: a key indication of artemisinin resistance.\footnote{38,45} In further research conducted by Dogovski et al. K13 has been linked with enhancing a stress response with the cell which leads to a delayed onset of cell death in resistant parasites that exhibit low levels of ubiquitinated proteins as a consequence of the K13 resistance mutation.\footnote{51} However, it is worth considering that whilst artemisinin resistance is now prevalent across many areas of Southeast Asia and has the undeniable capacity to spread further, eroding treatment successes to date, \emph{Plasmodium} resistance to artemisinin-based therapy does not yet amount to clinical failure.\footnote{52}

In addition to increasing artemisinin treatment failure due to rapidly developing \emph{Plasmodium} resistance, artemisinin and its derivatives also suffer from short \emph{in vivo} half-lives, the requirement for combination treatment regimens and difficulties with synthesis and plant-derived extraction. As a result, much research has focused on the development of novel and completely synthetic endoperoxides, based around the 1,2,4-trioxane pharmacophore, with the aim of retaining potent antimalarial activity against \emph{P. falciparum} and \emph{P. vivax}, whilst exhibiting a good safety profile and improved synthetic tractability.\footnote{24,53}

Development of these synthetic peroxides (or ozonides) perhaps represents one of the best examples of a directed chemistry-based approach that took the chemical structure of a known antimalarial drug, developing SAR around the specified chemotype, to yield new chemical agents with superior antimalarial activity and pharmacology which function as new clinical compounds.\footnote{54} Subsequent ozonide derivatives retained the endoperoxide bridge, known to give artemisinin its potent blood-stage activity, but also included a bulky amantadine ring, to increase compound stability in plasma. The first-generation synthetic peroxide OZ277 (Figure 1.7), was the first ozonide to be evaluated in the clinic but did not demonstrate full efficacy in patients after seven days of treatment.\footnote{54}

![Figure 1.7 Chemical structured of synthetic endoperoxides: OZ277 and OZ439.\textsuperscript{53}](image)
OZ439 (Figure 1.7) is a second generation ozonide that demonstrates potent antimalarial activity with both a superior pharmacokinetic and efficacy profile than its predecessor OZ277. OZ439 is fast-acting against all *P. falciparum* blood stages *in vitro* and exhibits prolonged blood concentrations due to designed stabilisation of the pharmacophoric endoperoxide bond.\[53\] Since OZ439 has been shown to completely cure *Plasmodium berghei* (*P. berghei*) infected mice with a single 20 mg per kg dose, it is feasible that compounds in the OZ439 chemical series have the potential to provide single-dose antimalarial therapy in humans.\[24,53\] However, because ozonides are close structural variants of artemisinins where activity results from the endoperoxide bridge; these synthetic derivatives are also only active against blood-stages of malaria, causing concerns that reduced efficacy may be seen against artemisinin resistant parasites.\[54\]

### 1.3.2.3 New Antimalarial Drugs with Novel Mechanisms of Action Are Needed

A range of chemotherapeutics, spanning a number of pharmacophores, have been identified and optimised over the years: the 4-aminoquinolines, such as amodiaquine and chloroquine; the structurally related 8-aminoquinolines: primaquine and tafenoquine; quinine derivatives including mefloquine and lumefantrine; antifolate drug combinations such as sulfadoxine-pyrimethamine; naphthoquinone compounds such as atovaquone and antibiotics including ciprofloxacin and rifampicin, are to name a few (for representative examples, see Figure 1.8).\[16,24\] These agents work by diverse mechanisms of action and have been administered in varying treatment regimens, as part of a number of malaria eradications campaigns, all with varying success rates.\[24\] However, many of these chemotherapies have now been withdrawn or their administration greatly reduced due to the rise of *Plasmodium* resistance against each drug. Considering all of the examples of malaria treatment loss witnessed, resistance development to specific drugs has occurred at varying rates and across different loci where ultimately resistance has continued to spread and is currently tracked by increasing rates of morbidity and mortality from malaria infection.

This well-known cycle of drug identification, global administration and clinical success, swiftly followed by *Plasmodium* resistance development, increased recrudescence rates and treatment failure, continues to plight and compromise successful malarial treatment and the possibility of disease eradication. The vast history of antimalarial chemotherapies has been well reviewed and documented by many researchers and public health organisations which are summarised in the following publications.\[1,3,4,6,15,16,24,28,29,35,37,39,42,50,54–58\] Current antimalarial therapies are based on a handful of established antimalarial drugs, spanning only a few chemical chemotypes, with limited novel drugs and drug combinations in advanced or early stages of clinical studies. Representative
examples of the most common antimalarials (Figure 1.8) highlights that the number of chemical chemotypes within current chemotherapy options is somewhat limited, consequently limiting diversity of the associated mechanisms of action by which antimalarial drugs are able to elicit their effects.\textsuperscript{[16,24]}

![Figure 1.8](image_url) Representative compounds of antimalarial chemotypes and derivatives that are approved for clinical use; 1: Name; 2: Chemical family; 3: Mode of action; 4: Other drugs in the chemical family. Figure is adapted from \textsuperscript{[24]}.  

---

1. **Atovaquone**  
   2. Naphthoquinone  
   3. Mitochondrial cytochrome bc1 inhibitor  
   4. None

1. **Sulfadoxine**  
   2. Aminosulphonamide  
   3. Folate pathway inhibitor (DHPS)  
   4. Dapsone

1. **Pyrimethamine**  
   2. Diaminopyrimidine  
   3. Folate pathway inhibitor (DHFR)  
   4. Proguanil

1. **Chloroquine**  
   2. 4-aminoquinoline  
   3. Interferes with haem polymerisation  
   4. Amodiaquine

1. **Primaquine**  
   2. 8-aminoquinoline  
   3. Unknown  
   4. Tafenoquine

1. **Mefloquine**  
   2. Amino alcohol  
   3. Interferes with haem polymerisation  
   4. Lumefantrine

1. **Amodiaquine**

1. **Tafenoquine**

1. **Lumefantrine**

1. **Ciprofloxacin**  
   2. Antibiotic  
   3. Inhibition of DNA and RNA replication  
   4. Rifampicin

1. **Artemisinin**  
   2. Endoperoxide  
   3. Multiple mechanisms  
   4. Artesunate, artelmer, DHA (Figure 1.6)
1.4 Approaches to Antimalarial Drug Development

As highlighted by the current state of antimalarial chemotherapy and the known and challenging history of antimalarial drug discovery, it is clear that *Plasmodium* parasites are capable of developing drug resistance at unpredictable and varying rates against a wide range of structurally divergent chemotherapeutic motifs, which work by diverse mechanisms of action. There are global concerns that the efficacy of all current, and potentially pipeline, antimalarial agents will gradually erode. Consequently, there is urgent need to develop novel and inexpensive therapies that are effective in the treatment and prevention of malaria, enabling the replacement of current antimalarial agents against which resistance is growing.\(^{[59]}\) For this to be successful, replacement agents will need to display potent activity against new molecular targets and demonstrate chemical construct around novel chemotypes against which *Plasmodium* parasites have not yet developed resistance.

It can therefore be argued that researchers now face a single option: to adopt a united and two-pronged approach to continue and fortify antimalarial drug discovery efforts. In a collective effort to combat the growing levels of resistance against antimalarial agents, two research strategies should be conducted to drive progress towards improved antimalarial therapies and heighten clinical successes. Firstly, optimisation of ACTs and other currently available antimalarials (Figure 1.8) must be continued to prolong and maximise their efficacious lifespan. This approach requires the rational design of new antimalarial drug combinations using existing and available drugs, optimising ACTs regimens and minimising the use of monotherapies. The second arm of the attack needs to pursue research identifying new drugs that act against novel targets and can therefore gradually replace the current antimalarial therapies that are available.\(^{[6,54,60]}\)

Specific research priorities have been outlined which highlight: the development of front-line therapies for non-complicated *P. falciparum* and *P. vivax* malarial infections, drugs to treat severe malaria, drugs for intermittent preventive treatment (IPT) of highly at risk groups, chemoprophylaxis drugs for travel applications and anti-relapse drugs for the treatment of dormant liver-stage *P. vivax* infections, as those which should be most activity sought after.\(^{[56]}\)

A number of medicinal chemistry approaches can be utilised in the identification and development of novel antimalarial agents and new drug combinations. Optimisation of chemical scaffolds of known antimalarial drugs and chemotypes is one such approach and, as previously stated, should be used alongside complementary research to identify novel chemotherapeutic agents. However, continued remodelling and optimisation of known chemical motifs carries associated risks. Currently, no model antimalarial compound or pharmacophore possesses activity across all stages of the *Plasmodium* parasite life cycle so repurposing and compound ‘tweaking’ is perhaps unlikely to
generate a new compound series that could combat issues already faced. However, development of the synthetic ozonides, based on the 1,2,4-trioxane pharmacophore of artemisinin (Figure 1.6 and 1.8), provides an apt example of a compound class where a directed chemistry-based approach, modifying a known antimalarial structure, has proven highly successful (Section 1.3.2.1).[54]

Another rational chemistry-based approach involves the design of hybrid molecules where two or more known chemical scaffolds are combined and incorporated within a single therapeutic agent. Such an approach aims to combine chemical functionalities and pharmacophores to create a more ideal molecule with optimised features, such as stability, solubility and potency, which are contributed from the combined entities.[55] This strategy enables two distinct molecules, each with antimalarial activity, to be covalently linked producing a single hybrid molecule that displays dual activity.[54] Although such an approach may be an interesting exploration of chemical biology and synthesis, the antimalarial effect of hybrid molecules may also be achieved using an appropriately designed combination therapy. There is also no guarantee that a resulting hybrid molecule won’t display any of the chemical or pharmacological liabilities also possessed by component motifs.[54]

The identification of natural products which harness potent medicinal properties and their subsequent development into powerful pharmaceuticals is another approach to new medicines and drug design that has been used through history. As previously discussed (Section 1.3.2), two of the most effective antimalarial drugs, artemisinin and quinine, were derived from natural herbal remedies originally used to treat fever in China and South America.[4] Another example is atovaquone (Figure 1.5 and 1.8), a synthetic naphthoquinone, derived from the natural product lapachol, present in the bark of the pink Lapacho tree and therefore highlights another example of nature providing a chemical agent that is efficacious in the treatment of human disease.[54] However, many of these herbal remedies have been used for centuries, predating both the chemical and pharmaceutical industries, often without extraction into their pure form and before their full chemotherapeutic potential was realised. It therefore seems unlikely that a similar development scenario will now emerge and progress from a known natural product to a new, currently unimagined antimalarial agent.

More likely, in this modern era of research, is that new therapeutic agents and methods of intervention will be sought, using the ever improving technologies that are at our disposal. A key example of this is the current and on-going research into malaria vaccine development. To date, vaccination remains the most effective method of preventing infectious diseases and represents a huge contribution to immunology. Therefore, much optimism for moving towards the goal of malaria eradication has recently focused on the development of a malaria vaccine.[61] However,
vaccine development against the \textit{Plasmodium} parasite is a huge challenge made no smaller by the fact that, even after multiple infections, adults in malaria-endemic regions only acquire partial resistance to malaria-causing parasites which is then gradually lost on lessening exposure.\footnote{1} In total, three types of vaccine candidate, targeting different stages of the \textit{Plasmodium} parasite life cycle have been investigated including: transmission-blocking vaccines (TBVs), pre-erythrocytic vaccines and blood-stage vaccines. The liver stage of \textit{P. falciparum} is an attractive therapeutic target for the development of both oral antimalarial drugs and vaccines since it provides the opportunity to interrupt the parasite life cycle at an early stage, targeting the sporozoites which initiate hepatocyte infection.

Consequently, pre-erythrocytic vaccine development has progressed rapidly over the last decade with many vaccine candidates now targeting this stage of the parasite life-cycle. However, for a liver-stage vaccine to be efficient, it must be 100\% effective in humans who possess no natural immunity.\footnote{2} Malaria vaccines are species-specific; almost all of the 24 vaccines in the clinical pipeline target \textit{P. falciparum}, with a single exception targeting \textit{P. vivax}. The most advanced vaccine, Mosquirix, is the only vaccine in late-stage Phase III clinical trials and provides efficacy against malaria with 55\% protection in children, but only 33\% in infants.\footnote{3} It is therefore clear that further improvement is required in malaria vaccine development; particularly as the mass scale administration of any malaria vaccine will face the added challenges of affordability, feasible dosage and implementation strategies, along with issue of global administration and distribution. However, these research efforts contribute to the vital discovery approach which pursues development of novel therapeutics using innovative and under-explored chemical intervention methods with the ultimate aim of replacing the sub-optimal antimalarial agents currently available.

Target-based drug discovery is a popular medicinal chemistry approach that has been used extensively and with great success throughout a huge number drug development programmes; this approach has identified many chemical agents with applications in a broad range of indications. Over the last 10 years and in terms of antimalarial therapies alone, target-bases screens have identified several therapeutic agents capable of effectively inhibiting malaria growth.\footnote{4} Some of the most promising compounds identified by these methods include small molecule inhibitors of \textit{P. falciparum} histone deacetylases (HDAC)\footnote{5,6}; potent and selective biphenylamide and urea-based inhibitors of \textit{P. falciparum} dihydroorotate dehydrogenase (DHODH), the rate-limiting enzyme within pyrimidine biosynthesis,\footnote{7,8} and antifolate antimalarial compounds targeting \textit{P. falciparum} dihydrofolate reductase (DHFR).\footnote{9}
Molecular targets are typically cellular proteins with essential functions where their inhibition disrupts cellular processes and often leads to cell death. In the development of antimalarial chemotherapeutics, such molecular targets are selectively inhibited on the basis that they are essential for *Plasmodium* parasite viability. This is usually indicated when a molecular target is highly conserved across a species and is therefore likely to serve a specific cellular or metabolic function. Genetic knockout experiments can be used to genetically validate a molecular target and confirm that it is essential for an organism’s viability. Chemical validation, confirming a molecular target is susceptible to modulation by a chemical, can also support the selection of a target for chemical inhibition studies. Some examples of chemically validated antimalarial targets include *P. falciparum* dihydrofolate reductase (DHFR), inhibited by antifolates such as pyrimethamine and proguanil (Figure 1.5 and 1.8) and *P. falciparum* cytochrome bc1, inhibited by atovaquone (Figure 1.5 and 1.8) which belongs to the chemical class of naphthoquinones. Table 1.1 summarises the presumed molecular targets (or mechanism of action) of antimalarial compounds that were in preclinical and clinical development by the end of 2013.

<table>
<thead>
<tr>
<th>Presumed molecular target or mechanism of action</th>
<th>Chemical or Product name</th>
<th>Chemical Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome bc1</td>
<td>ELQ-300 (Preclinical)</td>
<td>Quinolone-3-diarylether</td>
</tr>
<tr>
<td>PNP (purine nucleoside phosphorylase)</td>
<td>BCX4945 (Preclinical)</td>
<td>Immucillin G</td>
</tr>
<tr>
<td>Haemoglobin digestion</td>
<td>OZ277 (Preclinical)</td>
<td>1,2,4-trioxolane</td>
</tr>
<tr>
<td></td>
<td>OZ439 (Phase IIa)</td>
<td>1,2,4-trioxolane</td>
</tr>
<tr>
<td></td>
<td>RKA182 (Preclinical)</td>
<td>1,2,4,5-tetraoxane</td>
</tr>
<tr>
<td>DHFR (dihydrofolate reductase)</td>
<td>P218 (Preclinical)</td>
<td>Diaminopyridine</td>
</tr>
<tr>
<td>DHOD (dihydroorotate dehydrogenase)</td>
<td>DSM265 (Preclinical and Phase I)</td>
<td>Triazolopyrimidine</td>
</tr>
<tr>
<td><em>Pf</em>Carl (cyclic amine resistance locus protein)</td>
<td>KAF156 (Phase IIa)</td>
<td>Imidazolopiperazine</td>
</tr>
<tr>
<td><em>Pf</em>ATP4 (P-type ATPase 4)</td>
<td>KAE609 (Phase IIa)</td>
<td>Spiroindolone</td>
</tr>
<tr>
<td>Haemozoin formation</td>
<td>Ferroquine (Phase IIa)</td>
<td>4-aminoquinoline</td>
</tr>
</tbody>
</table>

Table 1.1 Summary of antimalarial compounds in preclinical and clinic development in 2013; table reproduced from [54].

As previously acknowledged, the high complexity of the *Plasmodium* parasite life cycle (Section 1.2) and its numerous interactions with both the human host and mosquito vector means there is an abundance of potential drug targets which have the capacity to be targeted and exploited in the treatment of malaria infection and transmission. However, as highlighted in Table 1.1 and Figure 1.8, many of these potential targets remain un-explored, where only a handful of molecular targets...
were utilised within preclinical and clinical trials that were ongoing in 2013. In addition, the therapeutic agents under investigation, as inhibitors of this limited range of targets, span only a small number of chemical classes. Several other drugs, including some 8-aminoquinolines and natural product extracts, were also in preclinical and Phase I clinical trials in 2013 however, the molecular targets of these agents remain unknown. Whilst greatly concerning, perhaps it is unsurprising that so few molecular targets have been identified when current antimalarial agents and those under development span limited chemical space. It is therefore essential that validated, novel parasite targets are identified against which, new and novel chemical drug classes can be found showing potent, and ideally broad-spectrum antimalarial activity.

The current global portfolio of antimalarial medicines (Figure 1.9), as provided by MMV, exemplifies the current state of play within antimalarial drug development and clinical evaluation. It is immediately clear that the majority of chemical agents and drug combinations in the later stages of development and approval are those that have been known to the research community for a number of years. Of the six available treatments which have reached post-development approval, all are familiar antimalarial drugs that have long been recognised as standard malaria treatments and which continuing research efforts are urgently attempting to replace. Whilst there are several ongoing research projects in the stage of lead optimisation, only a limited number of new therapies are in translational research; where a particularly limited number of therapeutic agents are currently in human clinical trials. This further highlights that few novel antimalarial drug targets have been identified, or are currently being utilised within drug discovery programs; representing a significant issue in antimalarial drug discovery that must be overcome in the fight to gain control over malaria.
In broader terms, the number of known molecular targets which are targeted, not only by antimalarials, but more generally by anti-infective agents is relatively small and remains in the 2-digit range. Consequently, the majority of potential therapeutic gene products that exist throughout infective organisms are not addressed by antibiotic agents currently available. This suggests that either a large number of molecular targets are still available for discovery in anti-infective therapy, or there is only a small sub-set of gene products that can actually be targeted with gene therapy. If the latter is the case, it is likely that the majority of viable gene products have already been discovered and are now utilised, explaining many of the anti-infective success stories over the last decade.

Considering the anti-infective subgroup of Enterobacteriaceae, a large family of Gram-negative bacteria, including harmless symbionts and familiar pathogens such as *Escherichia coli* (*E. coli*); genome virulence studies have indicated that the set of potential gene products against which targeted therapies might be feasible is somewhat limited. However, an encouraging and optimistic outcome of these studies is that enzymes of the non-mevalonate (MEP) pathway, present within this Gram-negative bacteria family as well as in organisms outside this class and phylum,
appear prominent as gene products which have the potential to be successfully targeted in the development of new anti-infective agents.\[68\]

1.5 A Novel Antimalarial Target: Isoprenoid Biosynthesis and The MEP Pathway

The identification, development and administration of new drugs will ensure there is a sustained pressure on the *Plasmodium* parasite, enabling us to get closer to achieving malaria eradication.\[60\] With such aims at the forefront of research, the pursuit of novel antimalarial compounds and new antimalarial drug targets has again intensified. As a result, the biosynthesis of isoprenoid precursors has emerged as a metabolic, chemical process that is sensitive to chemical manipulation and represents a promising candidate for the use of drug intervention against the malaria parasite.\[70-74\] The MEP pathway, responsible for the biosynthesis of essential isoprenoid precursors in *Plasmodium* parasites, therefore provides a rich source of potential targets which may prove crucial in the fight against malaria.\[75\]

1.5.1 Isoprenoids

Isoprenoids represent the oldest and largest class of natural products and biomolecules, with over 35,000 distinct members identified in nature which span a highly diverse range of structures and functions.\[9,72,76,77\] Isoprenoids are essential to all organism classes, including Apicomplexan protozoans and therefore *Plasmodium* parasites, since they play vital roles in many cellular and biochemical functions. Some key examples include: the synthesis of ubiquinone (an isoprene-containing quinone), in the mitochondria of *Plasmodium* parasites to facilitate energy generation in the electron transport chain; protein prenylation, an essential post-translational protein modification to enable subcellular protein regulation; the production of sterols to ensure cellular membrane stability; and the generation of photosynthetic pigments, hormones and plant defence compounds.\[76\] Despite the divergent biochemical functions facilitated by isoprenoids, the biosynthesis of this highly diverse class of biological molecules requires only two universal five-carbon isoprene building blocks: isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP), (Figure 1.10). Subsequent metabolic reactions and assembly of these basic units, generates the vast compound class of naturally occurring isoprenoids which show enormous diversity and complexity.\[71\]
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Figure 1.10 Chemical structure of isopentenyl pyrophosphate and dimethylallyl pyrophosphate.\textsuperscript{71,72}

The isoprenoid precursors, IPP and DMAPP, are novel compounds biochemically synthesised by all organisms which comprise the fundamental units for many downstream biochemical processes which may be specific to individual organisms. Isoprenoids are therefore essential for the survival and biological function of many cellular processes across species. In addition, isoprenoids also form the largest group of diverse secondary metabolites, such as plant defensive terpenoids, which have significant commercial importance in the pharmaceutical, food and fragrance industries.\textsuperscript{72}

It was initially thought that the synthesis of isoprenoid precursors was ubiquitous across all organisms and living systems however, it is now recognised that two distinct and independent routes to IPP and DMAPP biosynthesis exist.\textsuperscript{76} Mammals and fungi synthesis IPP and DMAPP through the well-studied mevalonic acid (MVA) pathway which proceeds through a Co-enzyme A (CoA) dependent route, via a key mevalonate intermediate.\textsuperscript{72,74,78} The MVA pathway has long been established as an important drug target since the development of statins\textsuperscript{68} for the treatment and prevention of cardiovascular disease; statins inhibit cholesterol biosynthesis, a highly abundant isoprenoid, with key functions in hormone production and regulating membrane fluidity.\textsuperscript{75} Statins as cholesterol-lowering drugs, target HGM-CoA reductase which is the rate-limiting enzyme of the MVA pathway.\textsuperscript{71}

In contrast eubacteria, archaeabacteria, chloroplasts, plastid-containing eukaryotes, including \textit{Plasmodium} parasites and other protozoa, synthesise IPP and DMAPP ubiquitously via the non-mevalonate pathway (also referred to as the 1-deoxy-\textit{d}-xylose-5-phosphate (DOXP) or the 2C-Methyl-\textit{d}-erythritol 4-Phosphate (MEP) pathway), which is independent of mevalonic acid.\textsuperscript{75} Some notable exceptions include Gram-positive bacteria, such as staphylococci and streptococci, which have retained the MVA pathway for the biosynthesis of isoprenoid precursors.\textsuperscript{72} In contrast to mammals and organisms utilising the MVA pathway, \textit{Plasmodium} parasites are incapable of synthesising sterols, relying instead on scavenging these compounds, such as cholesterol, from host systems.\textsuperscript{75} Because sterol synthesis is not a \textit{de novo} biochemical process carried out by \textit{Plasmodium} parasites, sterol production does not contribute to the biological importance of isoprenoid biosynthesis in parasites, where it is essential for eukaryotic membrane stability.
However, many other isoprenoids have functions which are essential for parasite survival and cannot be obtained through other means; this makes their generation dependent on the synthesis of IPP and DMAPP via the MEP pathway.\textsuperscript{[75]} Examples include: protein prenylation, ubiquinone biosynthesis, the production of vitamin E and carotenoids; and the generation of dolichol for N-linked glycosylation, a post-translational protein modification required during intraerythrocytic parasite development.\textsuperscript{[75]} These diverse isoprenoid-dependent processes are essential for parasite development meaning that, inhibition of \textit{Plasmodium} isoprenoid precursor biosynthesis would disrupt these essential processes, severely compromising parasite growth and survival.\textsuperscript{[75]} This therefore highlights the biological importance of the MEP pathway and its promise as a novel antimalarial target.

1.5.2 The MEP Pathway

Despite slow identification of this independent biosynthetic route to IPP and DMAPP, the MEP pathway has now been extensively studied using combined efforts from enzymology, crystallography and biophysics meaning that the biochemistry of the pathway is now well documented following extensive genomic evaluation.\textsuperscript{[79–81]}

The MEP pathway produces IPP and DMAPP through a series of seven enzymatic steps (Scheme 1.1)\textsuperscript{[71,72,82]} and is localised to the apicoplast of \textit{Plasmodium} parasites which is often found proximal to the parasite food vacuole. The enzymes of the MEP pathway are present across all stages of intraerythrocytic \textit{P. falciparum} infection where they are nuclear encoded but transported to the apicoplast.\textsuperscript{[83]} The apicoplast is a plastid organelle which lacks photosynthetic function and is a unique biological feature of Apicomplexan species (members of the phylum, Apicomplexa), which includes \textit{Plasmodium} parasites.\textsuperscript{[75]} Unlike plastids present within plant species, the apicoplast is a secondary plastid derived from photosynthetic eukaryotes, which became incorporated into an ancestral apicomplexan that engulfed red alga. Despite its photosynthetic origins, the evolutionary divergence of the apicoplast makes it distinct from the plastids of green plants and Archaeplastida algae, making modulation of this organelle an exciting opportunity for targeted antimalarial drug development.\textsuperscript{[84]}

The apicoplast has an unusual non-mammalian metabolism and is known to support three specific metabolic functions including: Type II fatty acid biosynthesis, \textit{de novo} haem biosynthesis and isoprenoid biosynthesis. In contrast to fatty acid and haem biosynthesis, which are essential for liver and mosquito stages of parasite development, it has been shown that biosynthesis of isoprenoid precursors is the essential metabolic function of the apicoplast in the asexual intraerythrocytic stage of parasite development, which is also responsible for the clinical manifestations of malaria.\textsuperscript{[85,86]}

\textit{Chapter 1}
Scheme 1.1 The MEP Pathway.\textsuperscript{[71,72]}

The seven-step MEP pathway is initiated with the condensation of pyruvate and glyceraldehyde-3-phosphate, catalysed by the enzyme 1-deoxy-\(\beta\)-xululose-5-phosphate synthase (Dxs) with thiamine pyrophosphate (TPP) acting as co-factor, to afford 1-deoxy-\(\beta\)-xululose-5-phosphate (DOXP) in the rate-limiting step of the pathway.\textsuperscript{[75]} The second enzyme, 1-deoxy-\(\beta\)-xululose-5-phosphate reductoisomerase (IspC) then catalyses an intramolecular isomerisation and reduction of DOXP, producing 2C-methyl-\(\beta\)-erythritol 4-phosphate (MEP). MEP is the first dedicated metabolite of the MEP pathway, from which the biosynthetic route derives its name.\textsuperscript{[71]} The third step of the pathway involves a coupling reaction whereby 4-diphosphocytidyl-2C-methyl-\(\beta\)-erythritol cytidylyltransferase (IspD), a cytidyl transferase enzyme, catalyses the transfer of a diphosphocytidyl group from cytidine triphosphate (CTP) to MEP to generate 4-diphosphocytidyl-2C-methyl-\(\beta\)-erythritol (CDP-ME).\textsuperscript{[72]}

Phosphorylation of CDP-ME, mediated by an ATP-dependent kinase, 4-diphosphocytidyl-2C-methyl-\(\beta\)-erythritol kinase (IspE), produces 4-diphosphocytidyl-2C-methyl-\(\beta\)-erythritol 2-phosphate (CDP-MEP). IspE is the fourth enzyme and only ATP-dependent enzyme of the MEP pathway.\textsuperscript{[75]} Cyclisation of CDP-MEP is catalysed by the enzyme 2C-methyl-\(\beta\)-erythritol-2,4-cyclodiphosphate synthase (IspF) in the fifth biosynthetic step, generates 4-diphosphocytidyl-2C-methyl-\(\beta\)-erythritol 2,4-cyclodiphosphate (MEcPP). MEcPP is subsequently reduced in a two-electron reductive ring opening step by the enzyme 1-hydroxy-2-methyl-2-\((E)\)-butenyl-4-diphosphate (IspG) to form 2-methyl-2-\((E)\)-butenyl diphosphate (HMBPP). HMBPP is then further reduced by dehydroxylation to generate IPP and DMAPP, catalysed by the enzyme 4-hydroxy-3-methyl-2-\((E)\)-butenyl-4-diphosphate reductase (IspH), the most distal enzyme of the MEP pathway.\textsuperscript{[72,75]}
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These seven enzymes that construct the MEP pathway are particularly valuable in terms of drug development since each enzyme only actively functions when presented with its unique and defined substrate, then generating its specific product. Consequently, the functions and biochemical roles of these enzymes cannot be compensated for by any other enzyme since they are absolutely unique and essential to the pathway. Further to this, the MEP pathway is highly linear making each enzyme in the pathway a fundamental requirement for de novo isoprenoid biosynthesis. It can therefore be argued that inhibition of early pathway enzymes may produce greater or increasingly irreversible levels of inhibition than could be achieved by targeting a more distal enzyme. In addition, targeting multiple enzymes throughout the pathway may also achieve stronger levels of inhibition, compared with targeting a single enzyme that may be difficult to reverse or rescue.

The absence of the MEP pathway in humans and mammalian systems is a consequence of independent evolution from the MVA pathway. The two biochemical pathways have distinct origins and are phylogenetically separated, where each pathway uses different substrates to generate isoprenoid precursors. The absence of MEP pathway enzymes in the human host is highly favourable when considering toxicological aspects of chemotherapy since anti-infective drugs targeting enzymes of the MEP pathway shouldn’t produce target-related toxicity within the host system. The MEP pathway is therefore chemically and enzymatically distinct in its own right. This key biological distinction provides essential selectivity and enables specific inhibition of Plasmodium parasite enzymes with minimal toxicity risks to human cell lines.

So in summary, why is the MEP pathway a good biochemical target? In short, the MEP pathway displays a number of characteristics which make it highly favourable for antimalarial drug development. The MEP pathway is essential for Plasmodium parasite growth and none of the pathways enzymes are present within mammalian cells therefore minimising the risk of toxicity against human cells due to this species selectivity. The pathway has also been validated as amenable to chemical manipulation and inhibition by the small molecules (Section 1.5), highlighting that enzymes within the MEP pathway are indeed druggable and should be further explored within forward antimalarial drug design. Furthermore, the MEP pathway is present in many pathogens that are responsible for serious human pathologies including malaria, as well as tuberculosis and a number of sexually transmitted diseases. This makes the MEP pathway an obvious biochemical target due to its potential to support the development of a number of broad spectrum anti-microbial agents which target its component enzymes. Development of such therapies may ultimately hold the key to overcome the levels of drug resistance that we now see against many antimalarial and antimicrobial agents.
1.5.3 Known Inhibitors of The MEP Pathway

Small molecule inhibitors are known and have been described against each enzyme of the MEP pathway through numerous programs aiming to harness the commercial, agricultural and clinical potential of MEP pathway inhibition.\cite{74,87–89} The first enzyme of the pathway, Dxs, is known to be inhibited by the pyruvate derivatives and the chemical class of diphenylisoxazoles (Figure 1.11); acylated aminobenzothiazoles (5, Figure 1.16) and azolopyrimidine inhibitors (6a and 6b, Figure 1.16) are known to act against the IspD enzyme; and cytidine derived analogues (Figure 1.11) have been found to inhibit IspE.\cite{72,89}

Research by Crane et al.\cite{87} utilised molecular modelling techniques to develop the first designed inhibitors of the IspF enzyme in a structure-based lead generation study. In the absence of any known inhibitors of the IspF enzyme at the time of the study, known crystals structures of *E.coli* IspF (EcIspF) (PDB ID: 1GX1\cite{90} and 1JY8\cite{91}) were used to facilitate identification of CDP-substrate mimics with alkyl-linked aromatic side chains; fluorescent probes were used to determine the affinity of these designed compounds at EcIspF.\cite{87} Such results are very encouraging for the development of new inhibitors against MEP pathway enzymes using structure-based design which shows promise for the generation of new compound classes within antimalarial and antimicrobial therapies. This small selection of chemical inhibitors (Figure 1.11), demonstrating high structural diversity, highlight the breadth of chemical space that MEP pathway inhibitors have the potential to cover and therefore underlines the vast potential for antimalarial drug development targeting enzymes of this biochemical pathway.

The chemical structures shown (Figure 1.11) depict the state of play in 2012 and current knowledge within the field surrounding chemical agents known to act as inhibitors of enzymes within the MEP pathway.\cite{72}
Figure 1.11 Known inhibitors of the MEP pathway.\textsuperscript{[72]}

Many of the highlighted chemical agents and structural motifs (Figure 1.11) are weak inhibitors of MEP pathway enzymes with IC\textsubscript{50} values generally in the millimolar and upper-micromolar range, and with most compounds showing poor drug-like characteristics. However, these molecular templates are both tractable and structurally diverse again highlighting that a wide range of chemical agents may be developed as inhibitors of the MEP pathway and its component enzymes. This therefore
reiterates that the MEP pathway offers a wide range of molecular targets for modern drug discovery efforts against which diverse chemical agents may be developed and employed.\textsuperscript{[72]}

1.5.4 The IspC Enzyme and Fosmidomycin

The biological importance of the MEP pathway to \textit{Plasmodium} parasites has been demonstrated by the identification and exploration of the clinical candidate fosmidomycin, a potent antimalarial agent that inhibits isoprenoid precursor biosynthesis. Fosmidomycin (FSM) is probably the most known and best characterised inhibitor of the MEP pathway to date; and as a result, the molecular target of FSM, IspC, is likely the most extensively cited enzyme within the MEP pathway.

![Chemical structure of FSM and FR-900098](image)

**Figure 1.12** Chemical structures and activities of FSM and FR-900098 against \textit{P. falciparum} strain Dd2.\textsuperscript{[92,93]}

FSM is a phosphonic acid antibiotic (\textbf{Figure 1.12}) which was first isolated as a natural antibiotic from \textit{Streptomyces lavendulae} in the 1980’s; it was initially involved in clinical development for the treatment of urinary tract infections (UTI’s) as an anti-bacterial agent.\textsuperscript{[94]} More recently, FSM been identified as a substrate mimic and a competitive \textit{in vitro} inhibitor of IspC, the first dedicated enzyme of the MEP pathway.\textsuperscript{[82,95]}

The structure of IspC proteins in a wide number of microorganisms have been determined using X-ray crystallography, more than thirty crystal structures of the enzyme now known, including structures for \textit{E. coli}, \textit{M. tuberculosis} and \textit{P. falciparum} IspC. These studies have revealed that IspC proteins are C\textsubscript{2}-symmetric homodimers with a bivalent metal ion, magnesium (Mg\textsuperscript{2+}), cobalt (Co\textsuperscript{2+}) or manganese (Mn\textsuperscript{2+}), bound in the enzyme active site and which are essential for activity.\textsuperscript{[68,72]} IspC knockout experiments, disrupting the gene locus of IspC to assess the specific role of the enzyme, have validated it as essential for parasite survival.\textsuperscript{[96]} In addition, FSM-treated \textit{Plasmodium} parasites can be rescued by subsequent treatment with isoprenoid precursor supplemented growth medium, therefore providing evidence that FSM prevents \textit{Plasmodium} growth specifically through the inhibition of isoprenoid precursors biosynthesis.\textsuperscript{[95]}

The binding mode of FSM has been analysed within a number of crystallographic studies, all of which have found FSM to bind to IspC in a similar fashion to the natural enzyme substrate, DOXP.\textsuperscript{[72,82]} It
has been shown that the phosphonate group of FSM interacts with IspC in an analogous manner to DOXP. The flexible loop region of IspC, which shields the active site to bulk solvent molecules, interacts with the FSM carbon backbone whilst the hydroxamate entity of FSM interacts with several protein residues at the IspC active site as well as binding to the active site Mg$^{2+}$ ion in a bidentate fashion.$^{[72]}$ The structure activity relationship (SAR) between FSM and E. coli IspC can therefore be assessed with respect to these three FSM moieties, as a measure of in vitro P. falciparum growth inhibition.$^{[92]}$

FSM has shown strong clinical promise as an antimalarial agent, inhibiting recombinant P. falciparum IspC (PfIspC) and killing the malaria parasite. FSM has been used successfully in small clinical studies for the treatment of malaria where early studies demonstrated the high efficacy of FSM and its close derivative FR-900098 (Figure 1.12). Both compounds are able to achieve inhibition of P. falciparum growth in vitro at nanomolar concentrations, with FR-900098 showing almost twice the activity of FSM in both animal models and against cultured parasites.$^{[93]}$ However, FSM is not an ideal drug as it is a highly polar and water soluble molecule, therefore displaying poor pharmacokinetic (PK) properties. This suboptimal PK profile is characterised by: a short in vivo half-life, poor oral bioavailability,$^{[97]}$ cellular exclusion from erythrocytes and other pathogens, high recrudescence rates and indirect action at other MEP pathway enzymes. Indeed, FSM has shown inhibition of the downstream MEP enzyme IspD; however direct inhibition of IspD homologues has not been observed within in vitro studies.$^{[92,95]}$

Refinement of the antimalarial performance of FSM alone, or in combination with another chemical agent, is therefore needed to produce a more effective antimalarial therapy.$^{[92]}$ FSM entered Phase II clinical trials in combination with clindamycin, an antibiotic and semi-synthetic derivative of lincomycin,$^{[98]}$ for malaria therapy in 2004.$^{[99]}$ Unfortunately, high recrudescence rates were problematic with this drug combination, alongside poor efficacy, which was observed in the treatment of children under three years of age. Subsequent FSM combination studies have used the 4-aminoquinoline, piperaquine, as a partner drug; these studies are currently ongoing and at the stage of Phase II clinical trials treating patients over the age of five.$^{[39]}$ Several derivatives of FSM, as well as prodrugs which mask the polar phosphonate group of the molecule, have been developed in an attempt to improve PK properties, optimise half-life and improve drug uptake levels. A number of aryl and alkyl FSM analogous have shown improved activity against IspC with IC$_{50}$ values in the single digit nanomolar range.$^{[68]}$ However, further optimisations are still required and only a few IspC inhibitors have been identified that are structurally distinct from FSM and FR-900098; lipophilic phosphonate inhibitors containing pyridine or quinolone groups represent some of the most
promising examples 1-3 (Figure 1.13) which present IC\textsubscript{50} values in the low micromolar range against \textit{E. coli} IspC.\textsuperscript{[97]}

![Chemical structures and activities of lipophilic phosphonate inhibitors 1-3 of \textit{E. coli} IspC.\textsuperscript{[97]}}

Figure 1.13 Chemical structures and activities of lipophilic phosphonate inhibitors 1-3 of \textit{E. coli} IspC.\textsuperscript{[97]}

FSM itself demonstrates an impressive safety profile; it has a mouse LD\textsubscript{50} of > 11, 000 mgkg\textsuperscript{-1} showing that it is non-toxic to mammalian cells and, to date, no adverse effects have been identified in Phase I or Phase II clinical trials.\textsuperscript{[82,94,100]} However, the pharmacokinetics of FSM remain suboptimal\textsuperscript{[100]} meaning further chemical improvements to the antibiotic, its structural derivatives and combination formulations are needed. Nonetheless, FSM demonstrates the safety of targeting the MEP pathway and highlights that the component enzymes of the pathway are not only essential for parasite development, but also sensitive and amenable to small molecule chemical inhibition and manipulation.

1.5.5 The IspD Enzyme and Known Inhibitors

Despite the early success of FSM, no other MEP pathway inhibitors have progressed to clinical trials. Clearly, there are many unexplored opportunities to employ small molecule inhibitors against other MEP pathway enzymes as the pathway continues to be of considerable interest as a biological target for antibiotic, herbicidal and antimalarial therapies and applications. This has led to our objective to develop a lead candidate molecule suitable for clinical development as an antimalarial agent, targeting the third enzyme of the \textit{Plasmodium} MEP pathway, \textit{P. falciparum} IspD (\textit{PfIspD}), which catalyses second dedicated step of the pathway.

In contrast to IspC, IspD has been studied much less extensively. The first known inhibitor of IspD, L-erythritoyl 4-phosphate (Figure 1.14), showed weak millimolar activity against the bacterial enzyme homologue, for the pathogen \textit{Brucella abortus}.\textsuperscript{[72,101]}
Figure 1.14 Chemical structure and activity of L-erythritoyl 4-phosphate, the first known bacterial IspD inhibitor.\[72\]

To date, at least two crystal structures of IspD enzyme homologues have been solved and reported, with the majority of known structures encompassing \textit{M. tuberculosis} IspD (PDB ID: 3OKR) and several \textit{E. coli} IspD (EcIspD) enzyme strains. These structures show that the IspD enzyme is a C\textsubscript{2}-symmetric homodimer of which, each monomer is comprised of a single domain that adopts an α/β fold; from this fold a long β-arm extends and forms the majority of the dimer interface (Figure 1.15). The active site of the enzyme is located at this interface and is therefore solvent-exposed. Homologues of the IspD enzyme have largely been found to display substantial flexibility.\[72,102\]

Examination of substrate and product co-crystal bound structures, including \textit{M. tuberculosis} IspD co-crystallised with CTP (PDB ID: 2XWN\[103\]) and EcIspD co-crystallised with CDP-ME and CTP (PDB ID: 1IN\[104\] and 1IS2\[104\] respectively), have revealed that CTP and CDP-ME bind to a glycine-rich loop of IspD and more specifically, the protein residues Pro13 and Arg20. Subsequent mechanistic studies have shown that an ordered and sequential mechanism takes place between the IspD enzyme and its substrates in the transferase coupling reaction that it mediates. Mechanistic evaluations have shown that the Mg\textsuperscript{2+} divalent cation in the IspD active site first coordinates to EcIspD, followed by CTP protein binding and nucleophilic attack of the MEP phosphate on the α-phosphate of CTP.\[83\] The Mg\textsuperscript{2+} ion, known to be essential for activity, coordinates to the α, β and γ phosphate groups of CTP as well as the α-phosphate of CDP-ME (Figure 1.15).\[72,102\]
Figure 1.15 Tertiary structure of the *E. coli* IspD monomer with CDP-ME bound (PDB:1INI[104]); α-helices and β-sheets are shown in cyan and purple cartoon format respectively. In the close-up image of the CTP binding site; the cytosine binding region and residues are rendered pink; the ribose binding region and corresponding residues are rendered tan; and the phosphate and MEP binding residues are rendered yellow and orange respectively. CDP-ME is coloured by element; carbon is green. The Mg$^{2+}$ ion is shown as a grey sphere. [72,105]

The low lipophilic character and solvent-exposed nature of the IspD binding pocket represents a challenge for competitive-substrate inhibitor design. Indeed, over the last than five years, no potent IspD inhibitors had been reported and few substrate-competitive inhibitors targeting the IspD active site had been developed. Even *L*-erythritol 4-phosphate (Figure 1.14), a compound of high structural similarity to MEP, shows only very weak inhibition at the IspD enzyme. [102] This therefore indicates that there are many currently unexplored opportunities to develop novel chemical motifs that are capable of potently inhibiting the IspD enzyme.

Over the last few years, we and others, have reported small molecule inhibitors of IspD (4 and 5, Figure 1.16) confirming that IspD homologues are amenable to chemical inhibition. [88,106,107] These IspD inhibitors generally have high-micromolar activity, although some herbaceous *Arabidopsis thaliana* (*A. thaliana*) species-selective chemical motifs have also been identified (6a and 6b, Figure 1.16) which display nanomolar activity against the enzyme. [74]
Plants utilise both the MEP and MVA pathways as part of their biochemistry, each is localised in the plant chloroplast and cytosol respectively. MEP pathway function and regulation has been well explored in plant systems following extensive herbicidal research, further iterating the importance of the MEP pathway as a biological target. Such work has been exemplified by Kunfermann et al. who identified the pseudilins (4, Figure 1.16) as chemical inhibitors of plant IspD homologues.[88] Kunfermann et al. conducted a high-throughput screening (HTS) study which identified these highly halogenated marine alkaloids (4, Figure 1.16), displaying herbicidal activity in plant assays and anti-plasmodial activity in cell-based assays against the MEP pathway IspD enzyme. This attempt to discover new chemical entities acting as herbicidal agents against IspD, required the screening a medium library of 100,000 compounds using a photometrically monitored assay, which identified the pseudilins showing activity against *A. thaliana* IspD (AtIspD) in the nanomolar range. Kunfermann et al. used crystallographic studies to elucidate an allosteric mechanism of action whereby these pseudilin-type inhibitors were found to bind at an allosteric pocket of AtIspD, rather than at the IspD active site itself. Cell based assays also highlighted inhibition of *P. vivax* IspD and activity against *P. falciparum* with this class of compounds.[88]

A study conducted by Masini et al.[102] explored the druggability of enzymes within the MEP pathway. Druggability, as defined by Hopkins and Groom, refers to the capacity of a biological target
to bind a drug-like molecule, with acceptable absorption, permeability and binding affinity, in the nanomolar range.\textsuperscript{102,108} Evaluation of AtIspD (PDB ID: 2YC3\textsuperscript{107}) in this druggability study identified the allosteric IspD binding site which was attributed a druggability score (Dscore) of 0.79. The study assigned a Dscore of 0 to 1 to any identified enzyme pocket or enzyme sub-pocket following analysis of its geometrical and physicochemical properties relative to its potential binding capabilities.\textsuperscript{102} This is therefore highly encouraging; not only has the AtIspD allosteric binding site been identified as having good druggability, but distinct work conducted by separate researchers, has confirmed this with the identification of active inhibitors (4, \textbf{Figure 1.16}) against this specified binding region.

The druggability study conducted by Masini \textit{et al.} considered all enzymes of the MEP pathway; evaluating all druggable pockets, including active site and allosteric binding regions. Since the natural intermediates of the MEP pathway are all phosphorylated and therefore highly polar, the corresponding active sites of enzymes within the pathway are also of high polarity. Consequently, it must be acknowledged that the structure-based design of drug-like inhibitors against these enzymes may be challenging. However, Masini \textit{et al.} noted that the active site of the IspD enzyme is the least lipophilic of all enzymes analysed in the MEP pathway.\textsuperscript{102} Further to this, evaluation of PfIspC concluded that it was a druggable enzyme, assigning the PfIspC active site a Dscore of 0.80. We can therefore take confidence from the druggability analysis of PfIspD since it was given a slightly elevated Dscore of 0.86, compared to that of PfIspC. It therefore seems reasonable that mirroring the successes of FSM, as a potent PfIspC inhibitor, identifying novel and efficacious pharmacophores that are capable of targeting and inhibiting PfIspD are entirely possible. This gives confidence to the identification and selection of PfIspD as a specified MEP pathway enzyme target for novel antimalarial development.

A HTS study conducted by Witschel \textit{et al.}\textsuperscript{107} identified the 7-hydroxy-[1,2,4]triazolo[1,5-\textalpha]pyrimidine (6a, \textbf{Figure 1.16}) which demonstrates sub-micromolar activity against AtIspD and was also found to bind to the allosteric binding pocket of the enzyme. Despite a number of structural alterations around the heterobicyclic core, only replacement of the N atom with a C-linked cyanide group at the X position, generating 6b (\textbf{Figure 1.16}), was tolerated and gave slightly improved activity compared to the original hit 6a within this azoloprymidine series.\textsuperscript{107} In more recent research, conducted and published during the course of our studies, a chemical rescue screen identified 7 (1R,3S-MMV008138) from the Medicines for Malaria Venture (MMV) “malaria box” as a potent inhibitor of PfIspD. 7 displays nanomolar inhibition of recombinant PfIspD enzyme activity and sub-micromolar inhibition of parasite growth (\textbf{Figure 1.16});\textsuperscript{82,109} this chemotype therefore
Chapter 1

represents a highly promising structural motif which has great potential for SAR development into a potent and novel inhibitor series which is active against the PfIspD enzyme (Chapter 5).

Consequently, it is clear that there is much scope for the development of further inhibitors targeting the IspD enzyme. The limited, but conclusive research to date highlights that the IspD enzyme is amenable to chemical manipulation by a range of chemical structures and motifs at two potential binding pockets on the enzyme: both the IspD active site itself and the IspD allosteric binding site. The identification of PfIspD as a novel cellular target in an under explored, but chemically validated biochemical pathway,[96] is therefore highly exciting and represents an encouraging development in ongoing research efforts to identify novel antimalarial chemotherapeutics which act against new molecular targets.
1.6 Conclusions

The development of effective antimalarial chemotherapeutics remains a major challenge within drug discovery. Despite significant scientific progress over the last two decades, which saw a 30% decrease in the global incidence of malaria between 2000 and 2013, through up-scaling malarial interventions, malaria is still a major global health problem with almost half the world’s population at risk of contracting the disease. It is the adept ability of the *Plasmodium* parasite to develop drug resistance that so significantly hinders the fight against this entirely preventable disease. The continuation and acceleration of *Plasmodium* resistance development is seriously threatening the advances made in malaria treatment and prevention, as we are now facing the very real risk of widespread and irreversible *Plasmodium* resistance emerging against all current antimalarial therapies. With 95 countries registering malaria transmission in 2015, the development of new antimalarial drugs and battle to overcome parasite resistance has never been more crucial.

Very few novel drugs currently exist in the antimalarial drug pipeline and only a very limited number of antimalarial drug targets have been explored within drug development programs to date (Table 1.1 and Figure 1.8). The identification and development of new drugs is needed to ensure there is a sustained pressure on the *Plasmodium* parasite to enable the continuation of effective malaria treatment and progression towards malaria eradication. Circumventing the increasing problem of resistance against antimalarial agents requires the disease to be fought from several angles; it is not inconceivable that a continuous flow of innovative, novel and effective drugs will be needed to achieve complete eradication of the disease.

The intensified pursuit of new antimalarial drugs and novel molecular targets has caused the spotlight to fall on the chemical manipulation of isoprenoid biosynthesis. Isoprenoids represent the oldest and largest class of natural products which play vital roles in many cellular functions. In contrast to mammals; malaria-causing *Plasmodium* parasites use the non-mevalonate (MEP) biosynthetic pathway to generate isoprenoid precursors which is essential for parasite survival. Humans and other mammals use the mevalonate (MVA) pathway for this purpose. This observed biological selectivity makes the MEP pathway highly attractive as an antimalarial chemotherapeutic target, enabling enzymes within the pathway to be selectively inhibited with minimal risk of toxicity to the human host. FSM is currently the most potent and best-characterised MEP pathway inhibitor, targeting the first dedicated enzyme of the pathway, IspC. FSM has therefore validated the pathway as a viable target for antimalarial drug discovery efforts. As exemplified by the range of chemical motifs found to inhibit various enzymes of the MEP pathway, it
has been highlighted that the pathway is druggable and amenable to chemical manipulation and inhibition by small molecules.

Taken all together, this has led to our objective to deliver a lead candidate molecule suitable for clinical development, targeting the third enzyme of the MEP pathway, PfIspD. This enzyme represents an ideal biological target which is currently underexplored and shows real promise for advancing novel antimalarial drug development. Ultimately, we seek to answer the question: Can arresting the MEP pathway through PfIspD inhibition cause Plasmodium parasite death via disruption of isoprenoid precursor generation?
1.7 References


Chapter 1


Chapter 1


Chapter 2

Chemotype Identification; Development of Synthetic Methodologies and Initial SAR Investigations
2.1 HTS Studies – A Route to New Antimalarial Compounds

High-throughput screening (HTS) is a common strategy within modern drug discovery for identifying hit compounds against new or known molecular targets. With the pharmaceutical industry investing extensively on infrastructure to test ever-expanding compound collections, it now relies heavily on HTS for lead compound identification. The objective of screening large compound libraries is to maximise chances of discovering quality hit compounds for synthetic optimization of potential drug candidates. This uses the simple logic that the examination of a larger compound number can exponentially increase the chances of identifying a compound that acts efficaciously at a molecular target of interest. However, it should also be noted that the screening of large compound libraries, even on the 10 million compound scale, still only provides a small fraction and sample of the vast number of potential compounds that make up drug-like chemical space. It should therefore be realised that optimising the likelihood of identifying a compound which acts efficaciously at a molecular target, required the use of, not only a large, but also a structurally and chemically diverse library. Nonetheless, it is undeniable that many lead compounds have been discovered from HTS campaigns within modern drug discovery programmes.\[1\]

Since the 1990’s when the genomics era began, the main focus of drug discovery has been based around drug targets, typically proteins which have key roles in a specific disease pathogenesis, where modulation of target activity provides a rational basis for the discovery of new chemotherapeutics. However, before the introduction of target-based design approaches, drug discovery was propelled mainly by phenotypic assays where a limited knowledge of the molecular mechanism of disease pathology was often the case.\[2\] Phenotypic screening in discovery programmes measure the effect, or phenotype, that a compound induces in cells, tissues or whole organisms, identifying molecules that produce a desired biological effect or molecular change.\[3\] In comparison, target-based screens measure the effects of a compound on a specific, purified target protein using \textit{in vitro} assays.\[3\]

Today, these two drug discovery approaches are used throughout a vast number of diverse research programmes, both to varying extents and with varying levels of success. An advantage of a target-based approach is that knowledge of chemical and molecular mechanisms can be applied in order to investigate biological disease states and hypotheses concerning molecular mechanisms of action. However, the identified molecular solution to a suggested treatment strategy may ultimately not turn out to be relevant to the particular pathogenesis or provide an appropriate therapeutic index.\[2\]

In contrast, phenotypic drug discovery approaches do not require an understanding of the molecular mechanisms of action and often represent a less biased way of translating cellular assays into
therapeutic impact for a given disease state. Indeed, an arguable advantage to this discovery approach resides in the fact that a biological system can be interrogated without a predisposed bias towards a proposed mechanism of action.\textsuperscript{[4]} This lends to the identification of compounds that may interact with one or more molecular targets which are not anticipated by a single, mechanism-driven hypothesis. However, the challenge of phenotypic drug discovery lies in the complexity of differentiating compound profiles, gaining complete biological understanding of an observed phenotype and determining a defined mechanism of action.\textsuperscript{[4]} Incorporating new screening technologies into phenotypic approaches and optimising molecular properties of identified candidate drugs, without knowledge of a molecular mechanism of action, therefore remains highly challenging and possibly explains why phenotypic assays have lower throughput than target-based assays.\textsuperscript{[2]}

As \textit{P. falciparum} resistant strains continue to emerge against the limited number of available antimalarial drugs, it is clear that new and entirely novel antimalarial chemotherapies must be developed if we are to halt the resurgence in drug-resistant \textit{Plasmodium} parasites and maintain an effective antimalarial drug pipeline. The magnitude of this research problem has led to increased collaboration between industry and academia, placing emphasis on identifying new chemotypes through increased and extensive screening methods.\textsuperscript{[5]}

In 2010, both St Jude’s Children’s hospital\textsuperscript{[6]} and Novartis\textsuperscript{[7]} published the structures of thousands of compounds that were capable of inhibiting parasite growth, therefore significantly increasing the number of compounds instantly available for antimalarial drug discovery programmes. In the same year, GSK also published the Tres Cantos Antimalarial Set (TCAMS), comprising 13,533 compounds which resulted from the screening of around two million compounds from within the GSK’s corporate collection.\textsuperscript{[8]} The open and public availability of this large and potent set of drug-like, anti-plasmodial compound structures enables many important research questions to be addressed throughout an increasing number of antimalarial screening programmes and methodologies.\textsuperscript{[9]}

Indeed, a significant amount of work such as this is freely available within the ChEMBL-Neglected Tropical Disease archive\textsuperscript{[10]} which is an open access repository for primary screening and medicinal chemistry data focusing on neglected and endemic tropical diseases. A number of recent open access publications, involving compound screening and medicinal chemistry optimisation for the purpose of antimalarial drug discovery, can be found here\textsuperscript{[11–13]}. 
2.1.1 Phenotypic-based Screening: TCAMs

In 2011, Medicines for Malaria Venture (MMV) in partnership with GSK conducted a HTS programme using the TCAMS library to identify a small subset of compound structures that were made available to the research community as potential starting points for lead optimisation. This bold attempt to initiate novel antimalarial drug discovery, represents an example of a successful phenotypic screen since five separate chemical series were identified displaying potent antimalarial activity against *P. falciparum*.[8] The aim of the GSK/MMV study was to rapidly identify molecules that have a strong chance of successful antimalarial drug development and a low risk of attrition so as to fast track high quality, but currently unevaluated chemotypes, into lead optimisation. A schematic of this study is depicted below (Figure 1.2).[8]

![Figure 1.2](image)

**Figure 2.1** Representation of work flow for the identification of chemical starting points from the TCAMS data set for lead optimisation; figure reproduced from.[8]

The TCAMS library, of over 13,000 potential starting points, was first clustered using a series of algorithms which produced 2948 sets of data and could be further analysed based on compound similarity. Compounds were prioritised and consequently scored according to their *P. falciparum* (3D7 strain) antimalarial activity and lipophilicity, which was used as a preliminary measure of drug-likeness.[8] Once all compounds were evaluated, those with a score of less than 15 (out of a possible 30) were discarded. The remaining 3, 414 compounds were filtered to remove less “drug-like” molecules and compounds with chemical structures related to known antimalarials; this aimed to minimise the risk of developing a compound likely to rapidly develop resistance. Ultimately this led to the identification of 522 compounds which were then considered through a non-automated selection process.[8]

Five distinct chemical series were selected featuring indolines, aryl carboxamides, alkylpyrazoles, thienopyrazoles, and 4-aminopiperidines; all of which complied with the requirements of high potency, druggability, and chemical tractability. An exemplary compound from the aryl carboxamides series was TCMDC-125454 (Figure 2.2), a highly potent molecule with a *P.
falciparum IC₅₀ value of 60 nM, low cytotoxicity and favourable hERG and microsomal stability profiles.

![Chemical structure of TCMDC-125454.](image)

**Figure 2.2** Chemical structure of TCMDC-125454.

Other related compounds within the carboxamide analogue set were also attractive from a medicinal chemistry standpoint therefore emphasising the success of the fast-track TCAMS screening programme which identified a number of high quality starting points for antimalarial lead optimisation. However, as is the case with phenotypic screening, the molecular target (or targets) of identified compounds and chemotypes is not determined during this process.

There is therefore a requirement for subsequent target deconvolution to decipher the molecular target of compounds found to elicit desired antimalarial effects. This highlights the required compromise of a phenotypic screen: whilst such an approach has the potential to identify molecules that modify a disease phenotype, by acting on a target which may previously be unknown; the subsequent requirement to determine the relevant target (or targets) of the molecule can be very slow or even impossible. This often makes the process of drug optimisation following a phenotypic screen highly challenging, due to a lack of knowledge regarding the molecular mechanism of action. An example of a follow-up medicinal chemistry optimisation programme, using outputs from the TCAMS series, is well outlined here.

### 2.1.2 Target-based Screening

An alternative method of hit identification, which avoids the drawback of required target deconvolution following phenotypic assessment, is target-based screening. This, traditionally more common approach to drug discovery is generally hypothesis driven, where three hypotheses must be satisfied to enable successful identification of new drug molecules. Firstly, the selected target must be of significant importance in the specified human pathology; secondly, the molecular mechanism of action of the drug candidate at the selected target must elicit the required biological response; and finally, the activity observed in a preclinical screen that is used to select the drug candidate, must translate into clinical efficacy.
Target-based screens are often considered to be operationally simpler than phenotypic screens since they use pure proteins within biochemical assays. However, this approach requires selection of the target protein prior to screening, which means that the screen conducted can only detect candidate molecules capable of modulating the selected target.\[15\]

In contrast to Novartis and GSK, some pharmaceutical companies such as Roche’s Genentech have remained focused towards target-based screens due to the argument that a compound with a known molecular target will always be favoured within a drug discovery project due to the added chemical and molecular knowledge that is available and can be utilised and exploited.\[3\] Between 1999 and 2008, the use of target-based drug discovery led to the identification of 17 of the 50 first-in-class new molecular entities that were approved by the FDA during this period.\[2\] The identification of Eltrombopag, an anti-thrombin agent, was achieved through the screening of small molecule libraries to detect compounds with the ability to activate a reporter molecule of thrombopoietin-dependent cell lines. In research conducted by Kuter et al.\[16\] hit compounds from this initial screen were optimised to improve pharmaceutical properties and advance biological efficacy, which led to the development of Eltrombopag.\[2,16\] This research highlights that cellular assays and biological investigation can be used effectively to discriminate different compound profiles identified in a target-based HTS screen, which can then drive structure-activity relationship (SAR) development and generate efficacious chemotherapeutic treatments.

In a distinct drug discovery programme, aimed at discovering non-peptide endothelin receptor agonists, a class of aryl-sulphonamidopyrimidine compounds were identified from a diverse compound library which led to the identification of Bosentan, a dual endothelin receptor antagonist for the treatment of pulmonary hypertension.\[2\] A screen of several thousand compounds, carried out by Hoffmann-La Roche, identified this class of compounds which, after chemical and structural modifications, promoting potency, oral activity and binding affinity, resulted in the most potent analogue of the class, Bosentan.\[17\]

The development of six of the new molecular entities, discovered through target-based methodologies between 1999 and 2008, also involved the rationalisation of their molecular mechanism of action at their specified target which was first selected for study and initial screening. Examples include: imatinib (Glivec), a BCR-ABL kinase inhibitor and targeted anti-cancer drug;\[18\] the HIV-Integrase inhibitor, raltegravir for the treatment of HIV-AIDS infection;\[19\] and maraviroc, a selective allosteric inhibitor of the chemokine receptor CCR5, also used in the treatment of HIV\[20\] (Figure 2.3).
Figure 2.3 Chemical structures of maraviroc, raltegravir and imatinib (Glivec): providing examples of clinically approved chemotherapies that were lead optimised from hit structures identified in HTS studies as part of target-based drug discovery programmes.\(^{18-20}\)

These identified inhibitors therefore demonstrate some of the successes of target-based screening, and also highlight the potential that this drug discovery approach facilitates in the determining the molecular mechanism of action at a molecular target, which may not be apparent on initiating the discovery programme.\(^{2}\)

To try and replicate some of these drug discovery successes within our own body of research, a target-based screening approach was employed to identify new lead-like inhibitors of the novel \(P. falciparum \) IspD (\(Pf\)IspD) enzyme. The use of this drug design approach facilitates the screening of a diverse area of chemical space with the aim of identifying novel chemical structures active against the selected \(Pf\)IspD enzyme target which was identified prior to compound screening. Furthermore, the selection of a target-based screening approach also facilitates and recognises future requirements to consider inhibitor mechanism of action studies.

### 2.2 Chemotype Identification

For this body of research, a novel hit-finding strategy was consequently initiated to explore chemical space using chemoinformatics and virtual screening techniques with the aim of identifying a rational set of diverse chemical compounds, selected from a larger compound library. Cellular assays were subsequently used to profile the inhibitory activity of the identified focused compound library against the \(Pf\)IspD enzyme in a HTS study. This screen identified a number of compounds and chemical distinct chemotypes with which we could initiate hit-optimisation and SAR development.
2.2.1 Chemoinformatics: Chemotype and Compound Selection

Chemoinformatics techniques of virtual screening, diversity selection, activity prediction, compound clustering and filtering can be employed as a rational alternative to more Traditional Random Screening (TRS) approaches. TRS generally produces high hit rates but also tends to identify compounds that may be less optimal for SAR studies.\[21\]

In an attempt to combat the issues of low chemotype diversity and poor drug-likeness within known PfISP\(_D\) inhibitors, a novel hit finding strategy was developed to explore chemical space using a number of chemoinformatic methodologies. Similarity searching was used to explore and ultimately expand chemical space around compounds known to be active against PfISP\(_D\), alongside the employment of scaffold hopping methods which together aimed to identify new, undiscovered chemotypes. These approaches enabled the selection of a focused ligand library for sequential application in a HTS regimen; a representative scheme depicting the described research strategy is shown below (Figure 2.4).

\[22\]
The initial selection of a focused ligand library was based on the two parallel approaches of ligand-based and structure-based virtual screening. This combined chemoinformatics strategy ultimately led to the selection of 5000 compounds from the divergent “BioFocus Combined Library” of around 500,000 compounds which was used as the molecular compound library in this virtual screening study. The “Biofocus Combined Library” is a concatenated library of the BioFocus Diverse and BioFocus SoftFocus libraries which together provide close to 540,000 compounds.[22]

Focused chemoinformatic compound selection uses a number of methods but is fundamentally based on the similarity principle which specifies that “structurally similar compounds are more likely to exhibit similar properties”[23]. Chemoinformatic similarity searching was therefore used to try and achieve scaffold hopping across chemical chemotypes.

Scaffold hopping is a strategy for discovering structurally novel compounds, starting from compounds known to be active, and ending with a novel chemotype through modifications to the central core of the original molecule.[24,25] Scaffold hopping therefore enables the identification of equipotent compounds with novel chemical backbones that display improved molecular properties.[26] This technique was consequently employed to move away from known chemotypes and chemical structures within current literature, and identify novel PfIspD inhibitors that would expand and develop the limited number of known PfIspD inhibitors at this time, which generally displayed relatively poor mM activity.[27–31]

Query libraries were constructed around several compound categories (Table 2.1) to facilitate similarity searching against which, all compounds in the diverse “Biofocus Combined Library” were assessed. These categories included natural substrates of the IspD enzyme, since substrate mimetics may possess inhibitory enzyme activity; natural substrates of the IspC enzyme using the rationale that additional IspC inhibition may prevent resistance development against an inhibitor capable of dual inhibition; as well as known IspD and IspC inhibitors, active at concentrations of ≤ 1 μM.

*E. coli* IspD (*Ec*IspD) is a metalloprotein with a Mg^{2+} ion present in the CTP binding site which is essential for IspD activity.[27,32] Based on the assumption that the *Pf*IspD homologue is also likely to contain an active site Mg^{2+} ion, similarity searches were performed against metal binding groups and fragment structures, such as sulphonamides and hydroxamic acids, since metal binding groups may prevent *Pf*IspD enzyme turnover. Known biophosphate isosteres, such as CF_{2}-linked phosphonic acid, tetrazole and O-linked malonyl, were also used as query molecules since phosphate groups are present in the natural substrates, MEP and CTP, of the IspD enzyme (Chapter 1, Scheme 1.1)[32,33],[22]
Table 2.1 summarises the compound categories of the query molecules used for similarity searching within the 500,000 compounds of the “Biofocus Combined Library”.

<table>
<thead>
<tr>
<th>Compound Query Category</th>
<th>Example structure(s)</th>
<th>Number of compounds known in literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>IspD and IspC natural substrates</td>
<td><img src="image" alt="IspD and IspC Example" /></td>
<td>3</td>
</tr>
<tr>
<td>Known IspD inhibitors</td>
<td><img src="image" alt="Known IspD Example" /></td>
<td>5</td>
</tr>
<tr>
<td>Known IspC inhibitors</td>
<td><img src="image" alt="Known IspC Example" /></td>
<td>41</td>
</tr>
<tr>
<td>Metal binding groups</td>
<td><img src="image" alt="Metal Binding Example" /></td>
<td>146</td>
</tr>
<tr>
<td>Biophosphate isosteres</td>
<td><img src="image" alt="Biophosphate Example" /></td>
<td>7</td>
</tr>
</tbody>
</table>

Table 2.1 Example query structures and associated categories used for similarity searching.

Fingerprint similarity and scaffold-hopping methods were some of the chemoinformatics techniques used to select compounds from the “Biofocus Combined Library” that were assessed as being similar to the defined query groups. Compounds with a Tanimoto coefficient > 0.7 compared to any query compound (as defined by ECFP4, FCFP4[36] or MDL[37] public key fingerprints) or compounds with an average ECFP4 Tanimoto coefficient between 0.2 to 0.4, used to achieve scaffold hopping,[38] were selected from the “Biofocus Combined Library”.

Fingerprint similarity methods use molecular fingerprints as a way of encoding the structure of a molecule. The most common type of fingerprint is a series of binary digits (or bits) that represent the presence or absence of particular substructure within a molecule. The comparison of fingerprints between two given structures allows the similarity of the two molecules to be determined and therefore identifies matches to query structures. The similarity between two molecules is most
frequently quantified using the Tanimoto coefficient which gives a measure of the number of fragments in common between two molecules using binary vectors. The Tanimoto coefficient can range from a value of zero to one: a value of one indicates that selected molecules have identical fingerprint representations, but may not necessarily be structurally identical, whereas a value of zero indicates no similarity between compared molecules.\textsuperscript{[39,40]}

Scaffold-hopping describes the identification compounds which show similar activity to a specified query compounds but which represent different chemotypes of chemical constructs.\textsuperscript{[24,26]} This chemoinformatics methodology is therefore valuable since it facilitates the identification of alternative compound series which may lack certain chemical motifs which cause suboptimal pharmacokinetics or difficult chemistry within existing development programmes.\textsuperscript{[24,39,41]} Together, the use of these chemoinformatic selection methods identified 23,000 unique compounds from the diverse “Biofocus Combined Library” in this chemoinformatic virtual screen.\textsuperscript{[22]}

To filter these compounds further, this subset of selected molecules were docked against known crystal structures of the IspC and IspD enzymes.\textsuperscript{[42]} Specifically for the IspD protein docking, known protein structures were used to facilitate compound evaluation at the \textit{E. coli} IspD CDP-ME binding site (PDB: 1IN1\textsuperscript{[43]}) and also the \textit{A. thaliana} IspD allosteric binding site (PDB: 2YC3\textsuperscript{[30]}). Binding (or fitness) scores, a measure of a compounds binding affinity at a given protein binding site (\textbf{Chapter 4, Section 4.1}),\textsuperscript{[44]} using the GOLDScore scoring function\textsuperscript{[45–47]} were assigned to each compound after docking the structures into the IspD and IspC protein complexes. This provided a measure of predicted binding strength of each compound at the given enzymes, allowing compounds to be ranked based on their expected inhibitor activity, as evaluated by their measured binding score.

The 23,000 compounds were then filtered according to calculated compound solubility (see \textbf{Section 2.9.1.3}) since acceptable solubility is required for reliable assay readout and inhibitor development. Therefore, compounds with LogS (solubility log) values of > -3.5 were selected to comprise our focused ligand library.\textsuperscript{[48]} The docking validation studies were therefore used to confirm that no outstanding compounds, as evaluated by the described docking studies, were not taken forwards and considered experimentally as a result of compound filtering by the defined LogS solubility threshold. Ultimately, a focused ligand library of 5000 compounds from the original “Biofocus Combined Library” were selected on the basis of optimal predicted binding affinities and molecular and physicochemical properties from the combined results of these chemoinformatics methodologies.\textsuperscript{[22,42]} This focused library of 5000 compounds was then entered into our designed HTS study.
2.3 \textit{Pf}IspD HTS

In order to profile the inhibitory activity of the selected 5000 compounds against \textit{Pf}IspD, a HTS was conducted by Biofocus DPI screening the selected compound library. To facilitate this HTS study, a \textit{Pf}IspD enzymatic assay was developed and transferred from the laboratories of our collaborator Audrey Odom at the Washington University School of Medicine, St Louis, USA (Section 2.2.3).[49] The 5000 compounds identified through the previously described chemoinformatics methods, were tested directly against purified \textit{Pf}IspD to determine their inhibitory activity against the enzyme. Biofocus DPI optimised the \textit{Pf}IspD enzyme assay for the purpose of HTS, enabling each of the 5000 selected compounds to be screened and evaluated in terms of \textit{Pf}IspD inhibition; this resulted in the successful screening of 316 plates, comprising the 5000 compounds.

2.3.1 \textit{Pf}IspD Enzymatic Assay

The Odom lab developed a \textit{Pf}IspD enzyme assay that operates by a linked enzymatic reaction detecting the release of inorganic pyrophosphate by the IspD enzyme (Scheme 2.1).[49] \textit{Pf}IspD is reacted in the presence of its two substrates, methylerythritol phosphate (MEP) and cytidine triphosphate (CTP) to produce the product, CDP-ME and inorganic pyrophosphate (PPI) as a natural by-product of the reaction.

![Scheme 2.1](image)

\textit{Scheme 2.1} IspD-catalysed biosynthesis of CDP-ME: the third enzymatic reaction of the MEP pathway.[29,50]

In order to examine this enzyme-catalysed process, the release of PPI can be monitored, first through reaction with inorganic pyrophosphatase (iPPTase), producing inorganic phosphate (Pi) which can then react with the synthetic substrate, 2-amino-6-mercapto-7-methylpurine riboside (MESG). MESG is converted enzymatically by purine nucleoside phosphorylase (PNP), in the presence of Pi, resulting in the release of a ribose-1-phosphate and 2-amino-6-mercapto-7-methyl purine that has a maximal absorption of 360 nm[51] (Scheme 2.2). The measured absorption at 360 nm allows quantification of the Pi consumed in the second enzyme-linked reaction. Since Pi consumption is proportional to the formation of 2-amino-6-mercapto-7-methyl purine; this in turn corresponds to
the quantity of PPI released by PfIspD, therefore providing a quantifiable measure of PfIspD enzyme activity.\textsuperscript{49}

\[
\text{MEP + CTP} \rightarrow_{\text{IspD}} \text{CDP-MEP + PPI} \rightarrow_{\text{IPPTase}} 2\text{Pi} + \text{MESG} \rightarrow_{\text{PNP}} \text{Abs 360 nm}
\]

\textbf{Scheme 2.2} A) The PfIspD enzymatic assay; B) Structural schematic of the enzymatic conversion of MESG to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by PNP.\textsuperscript{49,51}

The PfIspD enzyme assay is a rapid and quantitative assay which is performed in 96-well plates for standard small-scale laboratory throughput.\textsuperscript{49} It has been verified that the assay is linear with respect to time and enzyme concentration and that assay activity is dependent on the presence PfIspD and both the CTP and MEP enzyme substrates (\textbf{Figure 2.5}).\textsuperscript{49}
The PfIspD enzyme assay (Scheme 2.2) can be modified for the HTS of large compound libraries, as verified by the Biofocus HTS, and is suitable for both steady-state and stopped-flow kinetic analysis of enzymes.\(^{[49]}\)

### 2.3.2 HTS Outputs and Chemotype Selection

The selected 5000 compounds were profiled for PfIspD activity using the PfIspD enzymatic assay in a HTS regimen conducted by Biofocus. Active compounds were defined as those displaying IC\textsubscript{50} values of \(\leq 20\ \mu\text{M}\) at the PfIspD enzyme: this led to the initial identification of 76 compounds with PfIspD inhibitory activity. An expression of the activity of a drug or compound, in terms of the concentration required to produce a defined effect at a specified molecular target, can be given as an IC\textsubscript{50} measurement when the compound being administered is an antagonist.\(^{[52]}\) The term IC\textsubscript{50} therefore represents the molar concentration (or dose) of administered compound (antagonist) that is required to cause half-maximal inhibition of the total enzymatic or biological activity.\(^{[53,54]}\)

Activity readouts were considered for two methods of IC\textsubscript{50} measurement: both point of inflection (Pol) and \(y=50\) interpretations.\(^{[55]}\) Pol IC\textsubscript{50} readings report the IC\textsubscript{50} value as the data value on the x-axis which corresponds to the point of inflection on the dose-response curve (Figure 2.6, A). Alternatively, the \(y=50\) method of measurement can be used to report IC\textsubscript{50} values: here the reported IC\textsubscript{50} value is the value on the x-axis that corresponds to a 50% response on the y-axis (Figure 2.6, B). Using either method of measurement, the resulting IC\textsubscript{50} value was required to meet a defined threshold of \(\leq 20\ \mu\text{M}.\)\(^{[22,42]}\)
Figure 2.6 Graphical representation of the difference in IC50 values using different methods of measurement. A: Pol method of IC50 measurement; B: y=50 method of IC50 measurement.

Following LC-MS (Liquid Chromatography-Mass Spectrometry) analysis, 54 compounds from the original 76, with acceptable levels of purity (≥ 75% pure as measured by LC-MS\cite{22,56}), were selected as HTS hit compounds and classified as active against PfISP\textsubscript{D}. These 54 active compounds (Appendix 1), comprising more than 10 structural chemotypes, displayed a broad range of activities which varied from the specified 20 μM threshold to sub-micromolar activity levels (Figure 2.7)\cite{22,56}.

Figure 2.7 Bar graph depicting the range of PfISP\textsubscript{D} IC50 activities, using Pol IC50 values (given in μM), of the 54 hit compounds from the HTS\cite{56}.

Significantly, the 1,2-benzo[d]isothiazol-3(2H)-one (BITZ) chemotype was repeatedly identified (three times) within a number of hit structures, including the most active hit from the screen: DT2009-0168020 which displayed an inhibitory PfISP\textsubscript{D} IC50 value of 484 ± 149 nM (Figure 2.8). In addition, the hit DT2009-0097223, based around a thiazole phenyl-linked sulphonamide chemotype also displayed encouraging inhibitory PfISP\textsubscript{D} activity with an IC50 value of 775 nM (Figure 2.8). Both hit compounds have a molecular weight (MW) and associated ligand efficiency (LE) appropriate for further exploration.
LE is a method of comparing molecules in terms of their average binding energy per atom. Kuntz et al.\textsuperscript{[57]} described LE as the free binding energy of a molecule divided by its size, as defined by the number of heavy (non-hydrogen) atoms in the molecule.\textsuperscript{[58]} Therefore, molecules which achieve a given potency with fewer heavy atoms are more efficient.\textsuperscript{[1]} Lipinski’s “Rule of five” indicates that for compounds to have good absorption and permeability, they should meet his specified molecular properties as a good oral drug profile is unlikely when one of these guidelines is not met.\textsuperscript{[59]} Therefore, in a successful drug discovery setting, and according to Lipinski, compounds should: have no more than five hydrogen bond donors, no more than 10 hydrogen bond acceptors, a MW of less than 500 and a calculated Log P (ClogP) of less than five.\textsuperscript{[59]} However, Lipinski also points out that a drug candidate may still display a good oral drug profile when only one of these parameters is not met by a specified molecule.

Following preliminary investigations around the DT2009-0097223 hit, subsequent compound analogues were found to be inactive at PfIsPD and did not demonstrate a tractable SAR [based on the synthesis of 14 compounds derived from DT2009-0097223, all of which displayed PfIsPD IC\textsubscript{50} values > 50 µM against PfIsPD]. As a result, further inhibitor development based around the thia diazole phenyl-linked sulphonamide chemotype was terminated.

![Chemical structure, PfIsPD enzyme activity and ligand efficiency of the two most active HTS hit compounds DT2009-0168020 and DT2009-0097223.](image)

\textbf{Figure 2.8} Chemical structure, PfIsPD enzyme activity and ligand efficiency of the two most active HTS hit compounds DT2009-0168020 and DT2009-0097223.

The BITZ chemotype was therefore selected as the hit chemotype to take forward for PfIsPD inhibitor design and SAR development. The inhibitory activity of DT2009-0168020 at PfIsPD was highly encouraging and therefore represented a good starting point for analogue development. A preliminary SAR around the DT2009-0168020 hit was also evident from outputs of the HTS, thus increasing our confidence in this chemical motif for the development of a tractable series. At the time of hit identification, a series of literature searches found no references relating the BITZ
chemotype to IspD enzyme inhibition or antimalarial drug development of any kind. With these facts in mind, we proceeded in developing the BITZ chemotype with confidence that this chemical motif represented a distinct starting point for hit-to-lead optimisation.

2.4 Synthetic Strategies to Generate the 1,2-benzo[d]isothiazol-3(2H)-one Chemical Motif

A number of synthetic pathways have been identified which facilitate generation of the 1,2-benzo[d]isothiazol-3(2H)-one chemical motif and subsequent analogues of the chemotype. Each synthetic route has been applied with varying success and synthetic efficiency with the reproducibility of each method depending on analogue complexity. The first synthetic requirement was to generate close structural analogues and simplified compound structures of the DT2009-0168020 HTS hit, facilitating further PfIspD enzymatic testing of these structures to enable validation of the selected chemical motif.

2.4.1 Synthetic Strategy: Route 1

To enable full and extensive SAR development around the BITZ motif there was a requirement for the rapid generation of BITZ analogues in high numbers. Availability of starting materials, ease of chemical synthesis and low cost of component chemicals were therefore key considerations in determining appropriate syntheses. Considering DT2009-0168020 (Figure 2.8) from the perspective of retrosynthetic analysis, it was quickly apparent that an analogue series, structurally related to this hit compound, was synthetically plausible (Scheme 2.3). A decision was made to remove the ortho-morpholine functionality present within the DT2009-0168020 hit to generate a more simplified starting point from which to determine a plausible synthetic route towards the BITZ chemotype and initiate SAR explorations around the motif. This decision was also supported by observations made in parallel investigative SAR studies on-going at this time (Section 2.5.1). Analysis of the chemical cleavage points within DT2009-0168020 (having removed the ortho-morpholine group) brought us to the commercially available starting material of 2-(methylthio) benzoic acid which, through forward synthesis could quickly be progressed to the required BITZ entities using a thionyl chloride mediated ring closure as the key and final step of BITZ motif generation (Scheme 2.4).
Scheme 2.3 Simplification and retrosynthetic evaluation of the HTS hit, DT2009-0168020.

A linear, three-step synthesis of BITZ compounds analogues was devised (Scheme 2.4) involving an acid-chloride mediated amide coupling of 2-(methylthio) benzoic acid with appropriately substituted aniline derivatives to generate (methyl-thio)benzamide compounds 1a-b.[60,61] This was followed by oxidation of intermediates 1a-b to the corresponding sulfoxides, methylsulfinyl benzamides 2a-b,[60] which were then cyclized using thionyl chloride[62] to give the required inhibitor structures 3 and 4. Depending on the structural design of the side chain of specified BITZ structures, additional synthetic steps can be incorporated into this synthesis (Scheme 2.4) in order to generate a wide range of structurally diverse BITZ compounds.

Scheme 2.4 Synthetic Route 1: Thionyl chloride mediated generation of BITZ compounds 3 and 4.

To test the reliability and scope of this synthesis, analogues 3 and 4 (Figure 2.9) were generated by the synthetic route outlined in Scheme 2.4. 4 is a simplified analogue of the DT2009-0168020 hit in which the morpholine functionality ortho to the BITZ headgroup has been removed. 3 is a simple BITZ core derivative with an un-substituted N-phenyl ring as a basic side chain. 3 is therefore a highly
simplified BITZ derivative of the original DT2009-0168020 HTS hit and therefore aids rational SAR development by providing a distinct point of reference against which the activity and structural development of all subsequent, more complex, BITZ inhibitors can be evaluated.

![Chemical structures of simplified BITZ analogues 3 and 4.](image)

**Figure 2.9** Chemical structures of simplified BITZ analogues 3 and 4.

To generate 4, the morpholine sulfonyl side chain was first synthesised in a straightforward two-step reaction (**Scheme 2.5**) using established methodology. Accordingly, reaction between morpholine and 3-nitrobenzene sulfonyl chloride gave 5 followed by Pd-catalysed hydrogenation, converting the nitro functionality into a 1° amine. This gave the *meta*-substituted aniline derivative 6 for use in the initial amide couple step of **Route 1** (**Scheme 2.4**). For the synthesis of 3, un-substituted aniline was used directly in the first synthetic step.

![Scheme 2.5] Synthesis of 6.

All steps of the combined syntheses (**Schemes 2.4 and 2.5**) proceeded in moderate to high yields, over short reaction times with straightforward isolation and purification of all target compounds and intermediates. The final step of thionyl chloride mediated ring closure, generating the closed BITZ motif and final analogues 3 and 4, performed consistently well with moderate yields that were not compromised by the increased structural complexity of 4 compared to 3. Indeed, the mechanism by which this ring closure proceeds may be considered slightly unusual but can be described in the following manner (**Scheme 2.6**).
Initial attack of the sulfoxide of 2a on thionyl chloride generates a positively charged intermediate. The N-S bond is formed in a rapid 5-membered ring cyclisation which is accompanied by the entropically favourable loss of gaseous SO$_2$. This is followed by sulfur demethylation via an S$_n$2-mediated reaction with chloride to generate the BITZ motif and analogue, restoring a +2 oxidation state at the sulfur atom.$^{[62]}$

This synthetic route was used to generate a number of BITZ inhibitors as, despite a linear pathway, this synthesis is easily modified to incorporate varying chemical constructs to generate a range of BITZ side chain derivatives.

### 2.4.2 Synthetic Strategy: Route 2

An alternative synthesis, constructing the BITZ motif via a copper-mediated heteroatom coupling reaction, using copper(I) iodide and sulfur powder, has also been explored. This copper-mediated sulfur-nitrogen heteroatom coupling, outlined by B. S. Bhakuni et al.$^{[65]}$ has been proposed as ‘an environmentally benign method’ of accessing a diverse library of aryl benzoisothiazolones from readily available substrates including 2-halo-arylamides, as hyper-valent iodine reagents, sulfur powder and the ligand 1,10-phenanthroline. The catalytic cycle has been proposed to proceed via a LCu-NR amide complex, where the S atom inserts into the LCu-NR bond which then reacts intramolecularly via reductive elimination to give the benzoisothiazolone ring and regenerated copper(I) iodide complex (Scheme 2.7).$^{[65]}$
Scheme 2.7 Proposed mechanism for the copper-mediated synthesis of S-N heterocycles\textsuperscript{[65]}.

This chemistry was applied to generate 3 and 4 via a significantly shorter synthetic route than Route 1 (Scheme 2.4), using readily accessible and low cost starting materials (Scheme 2.8). The commercially available starting material, Benodanil (2-iodobenzenilide), was used for the straightforward, single-step generation of 3 which proceeded rapidly with a short reaction time of three hours in a clean reaction which gave 3 in a 70% yield. For the synthesis of 4, and to determine the tolerance of Route 2 (Scheme 2.8) to increased molecular complexity (additional functional groups), a short two-step synthesis was employed. To generate the 2-halo-arylamide reagent 7, required to yield the side chain of 4, a simple amide coupling step, preceding the copper-mediated ring closure was used. The synthesis of intermediate 7 required coupling the acid chloride, 2-iodobenzyl chloride, with 6 (Scheme 2.5).
Scheme 2.8 Synthetic Route 2: Copper(I) iodide-mediated sulfur-nitrogen heteroatom coupling for the generation of BITZ compounds 3 and 4.\[65\]

Despite the ease of synthesis and high yield achieved in the synthesis of 3, this was not reproduced with 4 where only a 9.9% yield was observed in the copper-mediated heteroatom coupling step. This indicated that this synthetic route is less tolerant to the increased reagent complexity needed for more structurally complex BITZ analogues, despite the consistent yields reported by B. S. Bhakuni et al. with analogues explored in their study.\[65\]

2.4.3 Synthetic Strategy: Route 3

Identification of a third synthetic pathway, using a stable and isolatable acid chloride 8, enabled the rapid synthesis of many BITZ analogues, supporting the incorporation of a varied and diverse range of side chains. The generation of the 2-chlorothiobenzoyl chloride intermediate 8 occurs in a rapid two-step, one-pot synthesis using the disulphide, 2,2-dithiobenzoic acid.\[66\] Condensation of 8 with an appropriately substituted aniline derivative then enables the required BITZ scaffold to be easily and swiftly obtained (Scheme 2.9).

Scheme 2.9 Synthetic Route 3: Acid chloride-mediated amide coupling and simultaneous heteroatom ring closure for the generation of BITZ derived compounds.\[66\]

For the generation of 8, thionyl chloride initially converts the two carboxylic acid components of 2,2-dithiobenzoic acid to the corresponding acid chlorides. This is then followed by cleavage of the
disulphide bond, mediated by sulfuryl chloride in the second step, producing two molecules of 8 (Scheme 2.9).\textsuperscript{[66]}

\textbf{Scheme 2.10} Proposed mechanism for the disulfide bond cleavage of 2,2-dithiobenzoic acid, mediated by sulfuryl chloride in the generation of 8.

This rapid and efficient synthesis of 8 is low in cost, uses readily available starting materials and is capable of producing large quantities of the acid chloride in quantitative yield, without the need for purification before use in subsequent reactions. Since 8 appears to be stable under a N\textsubscript{2} atmosphere over a period of several weeks, this methodology offers a point of divergence within the synthesis of BITZ derived compounds. The outlined synthetic pathway facilitates divergent syntheses since the N-linked side chain adjacent to the BITZ core can be synthesised discretely, without requirement for component structure of the BITZ warhead to be present. As a result, this broadens synthetic possibilities, enabling the development of more chemically complex and diverse side chains as any structural component carrying a 1° amine functionality can be incorporated as a BITZ core side chain in a single synthetic step with 8: a simple amide coupling and simultaneous ring closure (Scheme 2.9). Consequently, the length of synthesis for the generation of target BITZ compounds via this synthetic route is dependent only on the side chain complexity of each designed inhibitor.

Synthetic Routes 1 and 3 have been used frequently throughout this research, generating a diverse range of small molecule inhibitors based around the BITZ chemotype and core motif.

\textbf{2.5 Assessment of Compound Activity Against the PfIspD Enzyme and \textit{P. falciparum} Growth in Culture}

The PfIspD enzyme assay\textsuperscript{[49,51,67]} was utilised throughout this body of research in order to assess and determine the inhibitory activity of designed BITZ compounds against the PfIspD enzyme. Application of the enzyme assay provided biological data measuring the inhibitory PfIspD activity of BITZ derived compounds, therefore enabling SAR to be developed around the selected chemotype and motif.
2.5.1 Assessing \textit{P. falciparum} Growth Inhibition in Culture

Following assessment of compounds in the \textit{PfIspD} enzymatic assay and subject to the inhibitory activity measured, \textit{P. falciparum} whole cell assays were performed to evaluate the activity of designed compounds against \textit{P. falciparum} growth in culture. Prior to measurement of the inhibitory activity of a compound against the parasite, an acceptable measure of activity against the \textit{PfIspD} enzyme, now specified as a threshold IC$_{50}$ value of $\leq 5 \, \mu$M, should first be observed.

Ideally, we aim to observe potent \textit{PfIspD} enzyme inhibition in the initial enzyme assay which subsequently translates into comparable inhibition of \textit{P. falciparum} whole cell growth as this would highlight an effective antimalarial agent that targets the \textit{PfIspD} enzyme. To adhere to target candidate profiles outlined by MMV, we have defined $\leq 1 \, \mu$M as a suitable threshold for phenotypic activity.$^{[68]}$ Compounds which display phenotypic activity at $\leq 1 \, \mu$M are therefore defined capable of inhibiting \textit{P. falciparum} whole cell growth in the early stages of hit to lead optimisation. Indeed, if BITZ compounds display a strong correlation between inhibitory activity against the \textit{PfIspD} enzyme and \textit{P. falciparum} growth, this suggests that these compounds are able to prevent \textit{P. falciparum} parasite growth as a result of inhibiting the \textit{PfIspD} enzyme target.

As stated, all compounds that demonstrate acceptable inhibition of the \textit{PfIspD} enzyme (IC$_{50} \leq 5 \, \mu$M) were subsequently be evaluated against the intraerythrocytic stage of \textit{P. falciparum} in culture. A standard in vivo protocol will be used to quantify \textit{P. falciparum} parasite growth, facilitated by application of the DNA fluorophore PicoGreen$^{\circledast}$.\textsuperscript{[69]} This is a method that is regularly used in the microbiology lab of our collaborators at Washington University and is consequently well validated.$^{[49]}$ Consequently, it was a simple decision to continue utilising this assay to determine the inhibitory activity of novel BITZ compounds against \textit{P. falciparum} parasite growth within this body of work.

This established in vivo methodology quantifying \textit{P. falciparum} parasite growth represents an efficient and accurate method to assess antimalarial agents, based on the intercalation of the PicoGreen$^{\circledast}$ fluorophore into \textit{Plasmodium} DNA. This method detects the amount of \textit{Plasmodium} DNA in a short-term culture, using a 96-well plate assay format, following incubation with potential antimalarial agents, therefore facilitating a quantitative measurement of anti-plasmodial activity across a large number of samples.$^{[69]}$

PicoGreen$^{\circledast}$ is an ultra-sensitive nucleic acid dye that intercalates with double-stranded DNA (dsDNA) therefore enabling its detection in solution. Using a spectrofluorometer and the appropriate $\lambda_{ex}/\lambda_{em}$ wavelengths, as little as 25 pg/ml of dsDNA can be detected when intercalated with PicoGreen$^{\circledast}$.\textsuperscript{[69]} Parasites are first incubated with a potential antimalarial agent for 72 hours,$^{[49,67]}$ followed by the
addition of PicoGreen® and a further incubation period of between five and 30 minutes before measurement of the resulting fluorescence.

The extent of parasite replication is directly proportional to the amount of fluorescence; a linear relationship between the two is observed in assays that maintain a parasitemia (Pt) of between 0.1% and 15%.\[69\] The amount of resulting fluorescence, corresponding directly to plasmodial DNA content, can be easily read using a fluorescent Microplate reader. It has been demonstrated that cultures of both synchronised and non-synchronised parasites can be used with no observed differences in fluorescence output.\[69\] In addition, it has been shown that it is possible for assay samples to be stored at -20°C before measuring cellular fluorescence without a significant change in the observed signal strength.\[69\] This therefore highlights a further practical advantage of this methodology.

The described microfluorimetric assay for detecting anti-plasmodial compounds in an in vivo Plasmodium assay holds a number of advantages over previous in vivo methodologies which monitored the incorporation of \(^3\)H-hypoxanthine by Plasmodium parasites.\[69,70\] Measurement of intercalation between the PicoGreen® fluorophore and Plasmodium DNA is highly appropriate for antimalarial drug discovery programs within developing countries, which is in contrast to previous anti-plasmodial radioactive isotope assays which were often not suitable for initiating drug discovery programs in developing regions due to financial restrictions and limited access to resources.\[69–71\]

In terms of data analysis, the inhibitory concentration (IC\(_{50}\)) determined by application of this assay is defined as the drug concentration that produces 50% of the net fluorescence observed with non-treated control cultures. In addition to regular and successful use of this methodology by researchers at the microbiology laboratory at Washington University, the assay has also been validated through the assessment of known antimalarial drugs by others.\[69,72\] It has been shown that the dose-response for Chloroquine, as determined by the microfluorimetric method (IC\(_{50}\) = 31 ± 0.7 nM, mean ± SD) was comparable and in line with previously reported dose-response values determined by \(^3\)H-hypoxanthine incorporation (IC\(_{50}\) = 29 ± 9 nM).\[69,72\]

It is worth noting that the final dilution of anti-plasmodial agents in these assays contains less than 0.1 DMSO of the total assay solution since this amount has been found to have no measurable effect on parasite survival within the described system.\[69\] This is therefore a parameter that is to be maintained throughout all applications of this assay.
2.6 Chemotype Validation: The BITZ Motif is Required to Achieve PfISP\text{D} Inhibition

Having identified a number of synthetic routes by which to generate the BITZ motif, as well as the enzymatic and whole cell readouts to assess the potency of generated analogues, further validation of the chemotype and its chemical construct was required. To confirm the requirement for the selected BITZ chemotype and that this chemical headgroup is essential for achieving PfISP\text{D} enzyme inhibition, some small molecule manipulations were made around the BITZ core (Table 2.2). These included heteroatom replacements within the BITZ headgroup, substituting the sulfur heteroatom for alternative functionalities, and also determining the activity of a ring open analogue of the BITZ motif 1a at PfISP\text{D}.

Replacement of heteroatoms in the thiazoline ring of the BITZ headgroup with alternative atoms enables the importance of each specific atom to be assessed. The assessment of each atom, as a measure of biological activity, provides a clear illustration of the importance of each heteroatom in maintaining PfISP\text{D} activity due to its specified position within the headgroup. Measuring the inhibitory PfISP\text{D} activity of an open ring BITZ derivative provides an assessment of the requirement for the closed BITZ motif in achieving PfISP\text{D} inhibition. These chemotype validation studies also have the potential to inform mechanism of action studies (Chapter 4).

<table>
<thead>
<tr>
<th>Compound Structure</th>
<th>PfISP\text{D} Inhibition IC\text{50} (μM)</th>
<th>Whole Cell Growth (3D7) Inhibition IC\text{50}(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Structure 3" /></td>
<td>0.599</td>
<td>5.49</td>
</tr>
<tr>
<td>2-phenylisoindoline-1,3-dione</td>
<td>&gt;100</td>
<td>-</td>
</tr>
<tr>
<td><img src="image" alt="Structure 1b" /></td>
<td>&gt;350</td>
<td>&gt;80</td>
</tr>
</tbody>
</table>

Table 2.2 Chemical structures and corresponding inhibitory activity against PfISP\text{D} and *P. falciparum* 3D7 growth in culture (IC\text{50} given in μM) of compounds representing small molecular manipulations around the BITZ core.

Testing the simplest derivative of the BITZ core 3 proved to be highly encouraging since it was found to inhibit PfISP\text{D} with comparable level of activity to that of the DT2009-0168020 hit. This not only
provides an optimum reference point to our hit structure, against which evaluation of all future inhibitors can be made, but it also validates the selection of the BITZ chemotype, highlighting the importance of this chemical structure for PfIspD inhibition.

Replacement of the sulfur heteroatom in the BITZ core with a carbonyl functionality, exemplified by 2-phenylisoindoline-1,3-dione, was shown to completely abolish inhibitory PfIspD enzyme activity of the resulting structure (Table 2.2). This therefore highlights the requirement for the specific chemical construct of the BITZ headgroup and in particular, the presence of the sulfur atom adjacent and directly bound to the nitrogen atom, within the thiazolone ring system. Further to this, 1b, the ring-open structure of 4, confirms the absolute need for a closed and intact BITZ ring to infer inhibitory activity against the PfIspD enzyme. Since the presence of the unbroken N-S heteroatom bond within the BITZ motif is clearly required for inhibitory PfIspD activity, it seems reasonable that the sulfur atom and N-S bond contribute to the mechanism of inhibitory activity of this chemotype (Chapter 4).

The assessment of alternative heteroatom replacements within the BITZ headgroup, replacing sulfur with other atoms and small functional groups, as well as similar evaluation regarding the nitrogen atom, has also been considered (Table 2.3). This question of specific heteroatom replacement around the BITZ headgroup has been addressed by a number of individuals in our research group (*indicated) where the results, thus far, are summarised in Table 2.3.
The generation of designed structures (Table 2.3) has not been possible across all specified compounds within research to date, as a result of synthetic difficulties and a lack of commercial availability of the described compounds. However, further testing of a second ring-open compound (2-Amino-N-phenylbenzamide) and a rearrangement of the BITZ headgroup, exchanging the nitrogen and sulfur atom positions and removing the linked N-phenyl, to give benzo[c]isothiazol-3(1H)-one, resulted in a complete loss of inhibitory activity against PfISP-D. This again highlights that the BITZ core headgroup of 3 (Figure 2.9) is explicitly required to achieve inhibition of the PfISP-D enzyme. Therefore, these structures provide interesting points of comparison to enable distinct SAR studies around the core BITZ headgroup using a range of heteroatom replacements and considering alternative atoms and functional groups. This work represents an area of ongoing research.

### Table 2.3 Chemical structures of resulting compounds considering the replacement of specified heteroatoms with the thiazolone ring of the BITZ headgroup; PfISP-D inhibitory activity of compounds generated given in μM where synthesis was successful.


<table>
<thead>
<tr>
<th>Compound Structure</th>
<th>PfISP-D Inhibition IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="2-phenylisodindolin-1-one.png" alt="structure_1" /></td>
<td>Not synthesised</td>
</tr>
<tr>
<td><img src="2-phenylbenzo%5Bd%5Disoxazol-3(2H)-one.png" alt="structure_2" /></td>
<td>Not synthesised</td>
</tr>
<tr>
<td><img src="2-amino-N-phenylbenzamide.png" alt="structure_3" /></td>
<td>&gt; 200</td>
</tr>
<tr>
<td><img src="benzo%5Bc%5Disothiazol-3(1H)-one.png" alt="structure_4" /></td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>

*Figure 2.9*
2.7 Preliminary SAR development

In order to rapidly determine an early picture of SAR around our selected hit and BITZ chemotype, a number of compounds, structurally similar to our HTS hit DT2009-0168020 were purchased and tested to determine their activity against the PfISPd enzyme. The aim of this was to initiate preliminary considerations concerning structural modifications that may optimise BITZ derived inhibitors. Compounds 9-11 (Table 2.4) were specifically selected due to their subtle chemical and structural differences to the DT2009-0168020 hit. Assessment of these structures were able to highlight quickly some specific and essential structural requirements that must be preserved in subsequent BITZ inhibitor series to ensure activity against PfISPd. In addition, the biological testing results of 9-11 (Table 2.4) also revealed some areas of the hit motif that might be more amenable to structural variation and chemical modifications.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Chemical Structure</th>
<th>PfISPd Inhibition IC₅₀ (µM)</th>
<th>Whole Cell Growth (3D7) Inhibition IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td><img src="attachment" alt="Chemical Structure" /></td>
<td>1.00</td>
<td>5.193</td>
</tr>
<tr>
<td>10</td>
<td><img src="attachment" alt="Chemical Structure" /></td>
<td>0.401</td>
<td>2.774</td>
</tr>
<tr>
<td>11</td>
<td><img src="attachment" alt="Chemical Structure" /></td>
<td>5.789</td>
<td>16.04</td>
</tr>
</tbody>
</table>

Table 2.4 Chemical structures and corresponding inhibitory activity against PfISPd and *P. falciparum* 3D7 growth in culture (IC₅₀ given in µM) of compounds 9-11 that are structurally similar to the HTS hit DT2009-0168020. (Compounds purchased for testing).

As shown by the inhibitory effects of compounds 9 and 10, variation in the size of substituents around the C ring is tolerated to varying extents, highlighting that there is room for SAR development around this central ring. The loss of enzymatic activity demonstrated by para-substituted compound 11 indicates the requirement to maintain a meta-substitution pattern across the C ring, compound 4 (Figure 2.9) is the representative comparison. This observation may reflect
key spatial constraints at the PflspD binding site. The molecular ‘kink’ across the C ring (Figure 2.9) that appears to be required within inhibitor structures suggests that linear, rigid molecules might not be tolerated at the PflspD active site.

In addition to the small selection of purchased compounds 9-11, other work within the group[73] examined close structural derivatives of the DT2009-0168020 hit (Figure 2.8). Within this series of compounds, the meta-sulphonamide group present in the original DT2009-0168020 hit was maintained, varying the amine component of this functionality using a range of amine heterocycles. Small structural variations were also made at the ortho position of the C ring, replacing the original ortho-morpholine group with smaller hydroxy and methoxy functionalities. In addition, both directly attached and methylene-linked side chains were considered within this series. An overall summary of these structural explorations and the resulting compounds that were synthesised and tested for activity against the PflspD enzyme and parasite growth is given below (Figure 2.10).

![Figure 2.10](image)

Figure 2.10 Initial analogues synthesised and tested against the PflspD enzyme and P. falciparum parasite growth as close structural derivatives of the DT2009-0168020 hit.[73]

Following identification and selection of the BITZ chemotype and chemical motif, subsequent compounds based around the BITZ core will only be considered active inhibitors of the PflspD enzyme if they display a PflspD IC\textsubscript{50} value of < 5 μM. Whilst some compounds within the described series (Figure 2.10) demonstrated enzymatic activity against PflspD close to that of the original DT2009-0168020 hit, a number of compounds, close to a quarter of the 21 compounds generated, displayed very weak inhibitory activity against the PflspD enzyme with IC\textsubscript{50} values > 5 μM and are therefore considered to be inactive (Appendix 2).

Further analysis of biological data also revealed that almost half of the compounds generated, displaying activity against PflspD under the 5 μM threshold, only displayed weak inhibition with
PfiSpD IC₅₀ values between 1-5 μM. In addition to the distinct lack of inhibitory PfiSpD activity, sub-optimal whole cell activity was consistent across all 21 analogues with 3D7 whole cell inhibition spanning a broad and weak activity range of ~ 2.5 - 33 μM. Only a single derivative, compound 12 (Figure 2.11), displayed sub-micromolar activity against 3D7 whole cell parasite growth with an EC₅₀ value of 0.98 μM; however, this was not supported by enzymatic PfiSpD activity which was > 1 μM. Despite the general lack of activity identified against the PfiSpD enzyme and P. falciparum parasite growth with this analogue series (Figure 2.10), these results are somewhat encouraging as they demonstrate that SAR exits around the selected chemotype, and that further development of SAR may lead to potent and selective inhibitors of the PfiSpD enzyme and P. falciparum parasite growth.

Figure 2.11 Chemical structure of 12.[73]

Taken all together, these studies highlight that analogues comprised of this specific chemical construct (Figure 2.11 and Appendix 2) do not have potential as small molecule PfiSpD inhibitors or, more broadly as antimalarial agents. An initial outcome of these results was to remove any substitution at the ortho position of the C ring, adjacent to the BITZ headgroup. It was also decided to remove the sulphonamide group from subsequent inhibitor structures since this functionality has not be seen to significantly contribute to the activity of this BITZ inhibitor series (Appendix 2), compared to activity of simple BITZ derivative 3. These results therefore add to our knowledge and SAR understanding gained from the evaluation of 9-11 (Table 2.4), facilitating an in-depth SAR exploration initiated from a simplified BITZ compound, such as 3 (Figure 2.9), from which more complex analogues can be derived. In addition, 4 provides a measure of the inhibitory effect of the sulphonamide group, relative to 3, which can be reintroduced if necessary following subsequent SAR development.

The preliminary SAR map below (Figure 2.12) provides a summary of the SAR requirements identified in the structural explorations discussed in this chapter (Section 2.4 and 2.5). Figure 2.12 denotes the known SAR requirements of the BITZ chemotype and motif learnt from these initial studies, following chemotype selection and therefore represents our knowledge of this chemical motif at the point of progression into hit-to-lead optimisation studies (see Chapter 3).
Figure 2.12 Preliminary BITZ SAR Map.

Structural refinement, including precise embellishment and chemical functionalisation, is clearly required around this simplified BITZ framework; however, we were confident that it represents an optimal foundation from which to address subsequent SAR questions and initiate more detailed PfSpD inhibitor design and development.
2.8 Conclusions and Ongoing Work

Virtual screening and cheminformatics studies generated a focused ligand library of 5000 compounds, selected from the ‘Biofocus Combined Library’, to facilitate a target-based screen with the aim of identifying novel compounds that inhibit the PfISPd enzyme. The 5000 compounds were subjected to a HTS study, facilitated by upscaling the PfISPd enzymatic assay which was designed by collaborators in the Molecular and Cellular Biology Research Group at Washington University in St Louis. The HTS screen resulted in the identification of 54 novel compound structures which showed activity against the PfISPd enzyme; each compound met the specified IC_{50} value of ≤ 20 μM which was used to define compounds as active against PfISPd. This small series of 54 compounds spanned a diverse range of chemical space and was made up of compounds structured around 10 different chemical motifs. Of these chemotypes (or motifs), the 1,2-benzo[d]isothiazol-3(2H)-one (BITZ) chemotype was repeatedly identified within a number of hit structures and therefore selected as our hit chemotype to take forward for PfISPd inhibitor design and SAR development.

Subsequent SAR investigations, facilitated by organic synthesis, have been able to confirm that the specific chemical construct of the selected BITZ headgroup is essential for achieving inhibition of the PfISPd enzyme. This has been rationalised through a series of small molecule manipulations around the thiazolone ring (B ring) of the BITZ headgroup, highlighting that alteration of the specific BITZ construct abolishes inhibitory PfISPd activity in resulting compounds. The question of tolerated chemical and structural variation around the BITZ headgroup has been addressed by several members of our group; research efforts are currently ongoing and aim to further evaluate heteroatom replacement within the BITZ core motif and the extent to which it may be tolerated. Overall, it is hoped that these studies will contribute to deciphering the mechanism of action by which BITZ compounds exert their inhibitory effects at the PfISPd enzyme. Indeed, the importance of the sulfur heteroatom and N-S bond within the thiazolone ring has already been realised.

A number of synthetic strategies have been identified that enable the successful and efficient generation of the BITZ chemotype and derived inhibitors. In particular, two syntheses seem highly promising as they are shown to be efficient, with tolerance to varying chemical groups, and offer scope for further synthetic modifications, facilitating the generation of chemically diverse BITZ compounds. The two most appealing synthetic methodologies are represented by Route 1 and Route 3, (Section 2.3) which correspond to a thionyl chloride-mediated generation of the BITZ motif and the condensation of a stable and isolatable acid-chloride with various primary amines. Use of these identified syntheses, with appropriate modifications, will facilitate the generation of a large number of BITZ-derived compounds, comprising various analogue series.
Preliminary SAR investigations, conducted by myself and others, have provided an early picture of SAR around the BITZ core motif and selected HTS hit, DT2009-0168020. This has delivered a structural foundation on which further, more precise SAR development can be initiated. Following these preliminary studies, exploratory SAR development will probe more specific questions concerning the chemical construct of BITZ inhibitors. The particular influence of selected molecular functionalities will be questioned, assessing the effects that certain chemical groups may have on inhibitory compound activity, against both the PfIspD enzyme and P. falciparum parasite growth. As more in-depth SAR analysis is instigated, facilitating hit-to-lead optimisation, a revised activity threshold is now specified where an IC$_{50}$ value of ≤ 5 μM will define a compound as active against the PfIspD enzyme.

The identified synthetic methodologies, together with a strong preliminary SAR platform, will support and enable an efficient transition into the next stage of compound development which will begin with more detailed SAR investigations. Hit-to-lead optimisation will therefore enable knowledge of the selected BITZ chemotype to develop and expand, promoting chemical and structural properties of BITZ derived compounds, aiming to enhance inhibitory effects against the PfIspD enzyme and P. falciparum parasite growth.
2.9 Experimental
2.9.1 General Methodologies
2.9.1.1 General Chemical Methods

The following general chemical techniques were used for the synthesis and characterisation of all chemical compounds synthesised and described in the experimental sections throughout this thesis.

Reagents

All reagents were purchased from FluoroChem, Alfa Aesar or Sigma-Aldrich and were used without purification unless otherwise indicated. Reactions that were air and moisture sensitive were performed in dry solvent, under a nitrogen atmosphere in oven dried or flame dried glassware.

Solvents

Anhydrous solvents were obtained from commercial sources or dried and distilled prior to use. Distillation was carried out under the flow of dry nitrogen; THF was distilled from sodium; dichloromethane (DCM) was distilled from calcium hydride.

Thin Layer Chromatography (TLC)

Analytical thin layer chromatography was performed with 0.25 mm Merck silica gel 60 F-254 plates. Chromatograph plates were visualised by UV at 254 nm light or treated with potassium permanganate, p-anisaldehyde, ninhydrine or iodine, as appropriate and developed using a heat gun.

Flash Column Chromatography

Purification of products was achieved by flash column chromatography unless otherwise stated. Flash column chromatography was performed using the required amount of silica (particle size 40 – 63 μm, supplied by Aldrich) made into a slurry with the appropriate volume of the desired eluent system. The slurry was added to the column over a thin base layer of sand. Crude material was applied to the column, either dissolved in a minimum volume of eluent system or pre-absorbed onto silica. The column was then eluted with the eluent and fractions were collected and analysed by TLC.

Nuclear Magnetic Resonance Spectroscopy (NMR)

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker AMX-400 spectrometer ($^1$H 400 MHz; $^{13}$C 101 MHz) or on a Bruker Avance III HD spectrometer ($^1$H 500 MHz; $^{13}$C 101 MHz) in deuterated solvents as indicated with the experimental data. Chemical shifts are reported in parts per million ($\delta$, ppm)
downfield from an internal standard of tetramethylsilane (TMS); coupling constants ($J$) are reported in hertz (Hz).

**Mass Spectrometry and Elemental Analysis**

Mass spectrometry (MS) and High Resolution Mass Spectrometry (HRMS) were recorded using an in house service within the Chemistry Department at the University of Liverpool. MS and HRMS was conducted on a VG analytical 7070E machine, Frisons TRIO mass spectrometers or Agilent QTOF 7200 using electron ionisation (EI), chemical ionisation (CI) or electrospray (ES). Elemental analysis (%C, %H, %N and %S where specified) were determined by the University of Liverpool Microanalysis Laboratory.

**2.9.1.2 General Biological Methods**

The following biological methodologies were used for the enzymatic and whole cell growth assays of chemical compounds tested in these described assays. Biological testing has been conducted in the Molecular and Cellular Biology lab at Washington University School of Medicine, St Louis, coordinated by our collaborator Audrey Odom.

**Purification of IspD proteins**

Plasmids derived from pBG1861, containing wildtype or mutant alleles of *Plasmodium* spp. or *E. coli* IspD, were used to transform Artic Express (DE3) RIL *E. coli* cells (Stratagene). Cells were grown in LB broth with 100 μg/mL ampicillin at 37°C and 200 rpm. During mid-logarithmic growth, cultures were cooled to 8°C, and protein expression was induced with 1 mM IPTG for 16 hours.

After induction, cell pellets were lysed by sonication in lysis buffer (25 mM Tris pH 7.5, 250 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol (DTT), 20 mM imidazole, 10% glycerol, 0.1% Triton X-100, 200 μM PMSF, 1 mg/mL lysozyme, 0.3 U/mL benzonase nuclease (Novagen), and Roche Complete EDTA-free protease inhibitor). 6His-tagged IspD proteins were purified from soluble lysate over Ni-NTA resin (Goldbio). Beads were washed with 250 mM NaCl, 25 mM Tris pH 7.5, 1 mM MgCl₂, 20 mM imidazole, and protein was eluted with 250 mM NaCl, 25 mM Tris pH 7.5, 1 mM MgCl₂, 300 mM imidazole. Affinity purification of *Pv*IspD proceeded similarly to *Pf*IspD purifications, except that 0.1% Triton X-100 and 10% glycerol were not present in the lysis buffer. 10% glycerol was also absent from the lysis buffer used in purifying wildtype and A14C *E. coli* IspD, and lysis buffer used in purifying wildtype and C202A *Pf*IspD used in mechanistic studies (Chapter 4, Figures 4.21 to 4.25).
Affinity-purified proteins were further purified over a HiLoad 16/60 Superdex 200 gel filtration column (GE Health Sciences), using an AKTA Explorer 100 FPLC (GE Health Sciences). FPLC buffer contained 250 mM NaCl, 25 mM Tris pH 7.5, 1 mM MgCl₂. Fractions containing purified protein (>90% pure as evaluated by SDS-PAGE) were pooled and concentrated by centrifugation using Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore). Concentrated protein was supplemented with 10% glycerol and 1 mM DTT (although DTT was omitted from wildtype and C202A PfIspD preparations used in mechanism of action studies, (Chapter 4, Figures 4.21 to 4.25), flash-frozen in liquid nitrogen, and stored at -80 °C. Protein concentration was measured using a BCA protein assay kit (Thermo Scientific).

**Enzymatic IspD assay conditions**

Phosphate released by IspD was quantified using the EnzChek Phosphate Assay Kit (Invitrogen, Life Technologies), as previously described.[67] 2-Amino-6-mercaptop-7- methylpurine riboside (MESG) and purine nucleoside phosphorylase (PNP) were diluted and stored according to the manufacturer’s instructions. Final concentrations of reagents were as follows: 100 mM NaCl, 25 mM Tris pH 7.5, 7.5 mM MgCl₂, 1 U/ml PNP, 0.1 U/mL yeast inorganic pyrophosphatase (New England Biolabs). Unless otherwise specified, IC₅₀ assays contained 50 nM enzyme, 400 µM CTP (Sigma), 200 µM MEP (Echelon Biosciences), 2% DMSO (vehicle), and 200 µM MESG. Reactions were performed in 50 µL final volumes in 96-well clear flat bottom plates. In general, enzymes, buffers, and inhibitors were pre-warmed approximately 15 minutes at 37 °C, and reactions were then initiated by addition of the MEP substrate. Timed inhibitor IC₅₀ assays (5, 20, 35, and 50 minute incubations) and comparisons between behaviour at 100 µM and 400 µM CTP were initiated by the addition of the CTP substrate and contained 100 nM enzyme. Assays using E. coli IspD used 7.5 nM Eclsp-D-wt or 75 nM Eclsp-D-A14C. Absorbance at 360 nm was measured over time on a BMG POLARStar plate reader, preheated to 37°C. Nonlinear regression analysis was performed using GraphPad Prism software. Values reflect the mean and standard error from ≥ 3 independent experimental and were compared using the Student’s t-test (two-tailed). Slopes of changing absorbance values were converted to (µM MEP) (µM enzyme)^-1 s^-1 using a phosphate standard curve.

**P. falciparum culture**

Unless otherwise specified, *P. falciparum* parasites were cultured as previously described,[74,75] in a 2% suspension of human erythrocytes in RPMI-1640 medium (Sigma Aldrich) supplemented with 27 mM sodium bicarbonate, 11 mM glucose, 5 mM HEPES, 1 mM sodium pyruvate, 0.37 mM hypoxanthine, 0.01 mM thymidine, 10 µg mL⁻¹ gentamicin and 0.5% Albumax (Life Technologies). Cultures were maintained in 5% O₂/5% CO₂/90% N₂ at 37°C. Parasite growth was monitored by
microscopy of Giemsa-stained parasites. 3D7 strain parasites were obtained from the Malaria Research and Reference Reagent Resource Center, ATCC, Manassas, Virginia. D6 and 7G8 strains were obtained through BEI Resources Repository, NIAID, NIH: *Plasmodium falciparum*, Strain D6, MRA-285, contributed by D.E. Kyle; *Plasmodium falciparum*, Strain 7G8, MRA-152, contributed by David Walliker. The IPC 5202 strain was provided by the Malaria Research and Reference Reagent Resource Center (MR4) for distribution by BEI Resources NIAID, NIH: *Plasmodium falciparum*, Strain IPC 5202, also known as CAM3.IR539T; MRA-1240, contributed by Didier Ménard.

### 2.9.1.3 Predictions of $C_{\text{log}D}$ and $C_{\text{aqSol}}$

The distribution coefficient ($C_{\text{log}D}$) and aqueous solubility ($C_{\text{aqSol}}$) were predicted for all BITZ derived compounds that were synthesised and then tested in the enzymatic IspD assay. AstraZeneca have previously described the design of good models for the accurate prediction of the $\log D$ and solubility of organic compounds.\[76,77\] Collaboration between AZ and The University of Liverpool has provided access to these models which have been used to predict $C_{\text{log}D}$ (at pH 7.4) and $C_{\text{aqSol}}$ (given in µM) for all designed and evaluated BITZ compounds; where all $C_{\text{log}D}$ and $C_{\text{aqSol}}$ predictions were conducted by collaborators at AstraZeneca, Alderley Park.

### 2.9.2 Organic Synthesis

**Preparation of 4-((3-Nitrophenyl)sulfonyl)morpholine 5**

![Chemical Structure]

To a solution of 3-nitrobenzene sulfonyl chloride (2.0 g, 9.02 mmol) in DCM (40 ml) under a N$_2$ atmosphere, morpholine (1.0 ml, 10.8 mmol, 1.2 eq) and triethylamine (1.5 ml, 10.8 mmol, 1.2 eq) were added. The resulting solution was allowed to stir at room temperature for 2 hours. The crude material was diluted with brine (40 ml), the organic layer extracted into DCM (3 x 30 ml), washed with 2N HCl (30 ml), dried over MgSO$_4$ and concentrated under reduced pressure to give 5 as white crystalline solid in a 91.1% yield. $R_f = 0.31$ (1:1 EtOAc:hexane). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.60 (t, $J = 1.9$ Hz, 1H), 8.50 (ddd, $J = 8.2$, 2.2, 1.0 Hz, 1H), 8.09 (ddd, $J = 7.8$, 1.6, 1.1 Hz, 1H), 7.81 (t, $J = 8.1$ Hz, 1H), 3.78 (t, $J = 4.6$ Hz, 4H), 3.08 (t, $J = 4.8$ Hz, 4H); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 148.9, 138.2, 133.6, 131.0, 127.9, 123.3, 66.4 (2C), 46.4 (2); **Elemental analysis** (%) found: C, 43.92; H, 4.43; N, 10.18; S, 11.78%. C$_{10}$H$_{12}$N$_2$O$_5$S requires C, 44.11; H, 4.44; N, 10.29; S, 11.77%; ES+HRMS m/z 295.0373 [M+Na]$^+$ C$_{10}$H$_{12}$N$_2$O$_5$S requires 272.05.
Preparation of 3-(Morphpolinosulfonyl)aniline 6

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{S} \\
\text{O} \\
\text{O}
\end{array}
\]

10% Pd/C (0.05 g, 10% by mass) was added to a solution of 5 (0.5 g, 1.83 mmol) in 50:50 EtOAc-MeOH. The solution was put under a hydrogen atmosphere at room temperature overnight after which time the reaction was complete. The solution was filtered through Celite under vacuum and resulting material was washed with 50:50 EtOAc-hexane and concentrated under reduced pressure to give 6 as an orange-brown solid in a 98.4% yield. \( R_f = 0.14 \) (1:1 EtOAc:hexane). \(^{1}H\) NMR (400 MHz, CDCl\(_3\)) \( \delta \): 7.32 (t, \( J = 7.9 \) Hz, 1H), 7.12 (ddd, \( J = 7.7, 1.3, 0.8 \) Hz, 1H), 7.06 (t, \( J = 1.9 \) Hz, 1H), 6.92 (dd, \( J = 8.1, 1.6 \) Hz, 1H), 3.74 (t, \( J = 4.7 \) Hz, 4H), 3.01 (t, \( J = 4.8 \) Hz, 4H); \(^{13}C\) NMR (101 MHz, CDCl\(_3\)) \( \delta \): 146.9, 136.3, 130.5, 120.0, 118.3, 114.2, 66.5 (2C), 46.5 (2C); Elemental analysis (%) found: C, 49.69; H, 5.87; N, 11.48%. \( \text{C}_{10}\text{H}_{14}\text{N}_{2}\text{O}_{3}\text{S} \) requires C, 49.57; H, 5.82; N, 11.56%; CI+MS \( m/z \) 243.1 [M+H]\(^+\). \( \text{C}_{10}\text{H}_{14}\text{N}_{2}\text{O}_{3}\text{S} \) requires 242.07.

Preparation of 2-(Methylthio)-N-phenylbenzamides 1a and 1b

**General Procedure (A)**

To a stirring solution of 2-(methylthio)benzoic acid 1 (1.0 eq) in anhydrous toluene (10 ml/mmol) under a \( N_2 \) atmosphere, thionyl chloride (3.0 eq) was added followed by 3 drops of \( N,N \)-dimethylformamide: the resulting solution was refluxed for 2 hours. Solvent and thionyl chloride were removed under reduced pressure to give the acid chloride intermediate. The appropriate 1° amine (1.2 eq) was dissolved in anhydrous THF (10 ml/mmol), to which triethylamine (2.0 eq) was added. The crude acid chloride was re-dissolved in anhydrous THF (10 ml/mmol) and added drop-wise to the amine and base solution over 10 minutes at 0°C. After a further 10 minutes, the solution was warmed to room temperature and allowed to stir for 20 hours. The solvent was removed under reduced pressure; the resulting crude material diluted with water and extracted into EtOAc (3 x 20 ml). The combined organic extracts were washed with 1M HCl (15 ml), saturated aq. NaHCO\(_3\) (15 ml) and brine (20 ml), dried over NaSO\(_4\) and concentrated under reduced pressure to give the pure products.

**1a**

\[
\begin{array}{c}
\text{O} \\
\text{N} \\
\text{R}
\end{array}
\]

R = H

**1b**

\[
\begin{array}{c}
\text{O} \\
\text{S} \\
\text{O} \\
\text{O}
\end{array}
\]

R = morpholinosulfonyl
Preparation of 2-(Methylthio)-N-phenylbenzamide 1a

The general procedure (A) was followed using aniline (0.65 ml, 7.13 mmol) to give 1a as an off-white solid in a 93.8% yield. $R_f = 0.70$ (50:50 EtOAc:hexane). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.31 (s, 1H, NH), 7.73 (d, $J = 7.5$ Hz, 1H), 7.66 (d, $J = 7.8$ Hz, 2H), 7.44 (td, $J = 8.0$, 1.5 Hz, 1H), 7.41 – 7.35 (m, 3H), 7.29 (dd, $J = 7.5$, 1.3 Hz, 1H), 7.16 (t, $J = 7.4$ Hz, 1H), 2.50 (s, 3H).

Preparation of 2-(Methylthio)-N-(3-(morpholinosulfonyl)phenyl)benzamide 1b

The general procedure (A) was followed using 6 (0.22 g, 0.926 mmol) to give 1b as an off-white solid in a 85.5% yield. $R_f = 0.27$ (6:4 EtOAc:hexane). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.64 (s, 1H, NH), 8.04 (br s, 1H), 7.98 (dt, $J = 7.4$, 1.9 Hz, 1H), 7.78 (dd, $J = 7.7$, 1.4 Hz, 1H), 7.60 – 7.52 (m, 2H), 7.48 (td, $J = 7.3$, 1.5 Hz, 1H), 7.41 (dd, $J = 7.9$, 1.1 Hz, 1H), 7.31 (td, $J = 7.6$, 1.3 Hz, 1H), 3.76 (t, $J = 4.6$ Hz, 4H), 3.07 (t, $J = 4.8$ Hz, 4H), 2.52 (s, 3H); $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 166.5, 139.2, 137.1, 136.5, 134.7, 132.0, 130.4, 129.9, 128.7, 126.4, 124.7, 123.9, 119.4, 66.5 (2C), 46.4 (2C), 17.5; Elemental analysis (%) found: C, 54.73; H, 5.31; N, 7.01; S, 16.27%. $C_{18}H_{20}N_{2}O_{4}S_{2}$ requires C, 55.08; H, 5.14; N, 7.14; S, 16.34%; ES+HRMS m/z 415.0761 [M+Na]$^+$ $C_{18}H_{20}N_{2}O_{4}S_{2}$ requires 392.09.

Preparation of 2-(Methylsulfinyl)-N-phenylbenzamides 2a and 2b

General Procedure (B)

A solution of sodium periodate (1.3 eq) in water was added dropwise to the appropriate 2-(methylthio)-N-phenylbenzamide in MeOH at room temperature. The biphasic solution (10 ml/mmol; 1:1 water:MeOH) was heated to 50°C for 4-5 hours before the solvent was removed under reduced pressure. The crude precipitate was diluted with water, filtered through a sinter and washed with water before drying for a desiccator to give the pure products.

Preparation of 2-(Methylsulfinyl)-N-phenylbenzamide 2a

The general procedure (B) was followed using 1a (0.5 g, 0.215 mmol) to give 2a as an off-white solid in quantitative yield. $R_f = 0.08$ (50:50 EtOAc:hexane). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.95 (s, 1H, NH), 8.12 (dd, $J = 7.4$, 1.6 Hz, 1H), 7.78 (d, $J = 7.1$, 1.3 Hz, 1H), 7.69 (d, $J = 7.9$ Hz, 2H), 7.64 – 7.54 (m, 2H), 7.41 (t, $J = 7.6$ Hz, 2H), 7.20 (t, $J = 7.4$ Hz, 1H), 2.93 (s, 3H).
Preparation of 2-(Methylsulfinyl)-N-(3-(morpholinosulfonyl)phenyl)benzamide 2b

The general procedure (B) was followed using 1a (0.1 g, 0.255 mmol) to give 2b as a cream solid in a 86.6% yield. \( R_f = 0.10 \) (9:1EtOAc:hexane). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 9.65 (s, 1H, NH), 8.14 (br s, 1H), 8.11 (dd, \( J = 7.8, 1.0 \) Hz, 1H), 8.07 (dt, \( J = 7.6, 1.6 \) Hz, 1H), 7.95 (d, \( J = 7.5 \) Hz, 1H), 7.65 (td, \( J = 7.6, 0.9 \) Hz, 1H), 7.62 – 7.54 (m, 3H), 3.76 (t, \( J = 4.4 \) Hz, 4H), 3.07 (t, \( J = 4.7 \) Hz, 4H), 2.96 (s, 3H).

Preparation of 2-Phenylbenzo[d]isothiazol-3(2H)-ones 3 and 4

**General Procedure (C)**

Thionyl chloride (1.3 eq) was added dropwise to a stirring solution of the required 2-(methylsulfinyl)-N-phenylbenzamide (2a or 2b, 1.0 eq) in DCM (15-20 ml) under a N\(_2\) atmosphere. The solution was heated to reflux at 50°C for 1.5 hours. Solvent and excess thionyl chloride were removed under reduced pressure to give the crude products.

Preparation of 2-Phenylbenzo[d]isothiazol-3(2H)-one 3

The general procedure (C) was followed using 2a (0.25 g, 0.964 mmol) to give 3, as a white crystalline solid, following purification by trituration with Et\(_2\)O, in a 51.0% yield. \( R_f = 0.69 \) (6:4 EtOAc:hexane). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.12 (dt, \( J = 7.9, 0.8 \) Hz, 1H), 7.74 – 7.70 (m, 2H), 7.68 (td, \( J = 8.3, 1.2 \) Hz, 1H), 7.60 (dt, \( J = 8.1, 0.8 \) Hz, 1H), 7.51 – 7.43 (m, 3H), 7.33 (tt, \( J = 7.5, 1.3 \) Hz, 1H); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 164.5, 140.3, 137.6, 132.8, 129.8 (2C), 127.6, 127.5, 126.2, 125.3, 125.0 (2C), 120.5; **Elemental analysis** (%) found: C, 68.53; H, 4.07; N, 6.07; S, 14.21%. \( \text{C}_{13}\text{H}_9\text{NOS} \) requires C, 68.70; H, 3.99; N, 6.16; S, 14.11%; **ES+HRMS** m/z 250.0299 [M+Na]^+ \( \text{C}_{13}\text{H}_9\text{NOS} \) requires 227.04.

Preparation of 2-(3-(Morpholinosulfonyl)phenyl)benzo[d]isothiazol-3(2H)-one 4

The general procedure (C) was followed using 2b (0.09 g, 0.233 mmol) to give 4 purified by flash column chromatography, as an pale orange solid in a 63.2% yield. \( R_f = 0.65 \) (85:15 EtOAc:hexane). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.12 (t, \( J = 1.0 \) Hz, 1H), 8.05 (d, \( J = 7.9 \) Hz, 1H), 7.94 (dt, \( J = 6.7, 2.3 \) Hz, 1H), 7.68 – 7.59 (m, 3H), 7.56 (t, \( J = 7.2 \) Hz, 1H), 7.42 (td, \( J = 7.9, 0.8 \) Hz, 1H), 3.70 (t, \( J = 4.6 \) Hz, 4H), 3.03 (t, \( J = 4.7 \) Hz, 4H); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 164.7, 139.9, 138.8, 137.0, 133.4, 130.6, 128.5, 127.8, 126.7, 124.0, 124.8, 123.6, 120.7, 66.5 (2C), 46.4
(2C); **Elemental analysis** (%) found: C, 54.28; H, 4.49; N, 7.22; S, 17.05%. C_{17}H_{16}N_{2}O_{4}S_{2} requires C, 54.24; H, 4.28; N, 7.44; S, 17.03; **ES+MS** m/z 399.0447 [M+Na]^+ C_{17}H_{16}N_{2}O_{4}S_{2} requires 376.06.

### Preparation of 2-Iodo-N-(3-(morpholinosulfonyl)phenyl)benzamide 7

![Chemical structure of 2-Iodo-N-(3-(morpholinosulfonyl)phenyl)benzamide 7]

To a solution of 6 (0.49 g, 2.03 mmol, 1.2 eq) in THF (8 ml) under a N₂ atmosphere, triethylamine (0.45 ml, 3.38 mmol, 2.0 eq) was added. The solution was cooled to 0°C before the dropwise addition of 2-iodobenzoylchloride (0.45 g, 1.69 mmol, 1.0 eq) dissolved in THF (8 ml). The resulting solution was allowed to warm to room temperature and stir overnight. Solvent was removed under reduced pressure and the crude material re-suspended in 50:50 EtOAc:water and extracted into EtOAc. The combined organic extracts were washed with 2N HCl (30 ml), NaHCO₃ (25 ml) and brine (25 ml), dried over MgSO₄ and concentrated under reduced pressure to give 7 as a white-yellow crystalline solid in a 91.1% yield. Rᵣ = 0.24 (4:6 EtOAc:hexane). **¹H NMR** (400 MHz, CDCl₃) δ 8.04–7.98 (m, 2H), 7.92 (d, J = 7.7 Hz, 1H), 7.88 (br s, 1H), 7.58 (t, J = 7.9 Hz, 1H), 7.54 (dd, J = 6.1, 1.3 Hz, 2H), 7.46 (td, J = 7.5, 0.8 Hz, 1H), 7.18 (td, J = 7.7, 1.7 Hz, 1H), 3.74 (t, J = 4.6 Hz, 4H), 3.04 (t, J = 4.6 Hz, 4H); **¹³C NMR** (101 MHz, CDCl₃) δ 167.8, 141.7, 140.6, 138.9, 136.5, 132.3, 130.5, 129.1, 128.9, 124.7, 124.2, 119.5, 92.6, 66.5 (2C), 46.4 (2C); **ES+HRMS** m/z 494.9839 [M+Na]^+ C_{17}H_{17}N_{2}O_{4}S requires 472.00.

### Preparation of 2-Phenylbenzo[d]isothiazol-3(2H)-ones 3 and 4

#### General Procedure (D)

To a solution of 1,10-phenanthroline (0.25 eq) in DMF (10 ml) under a N₂ atmosphere, copper(I) iodine (0.25 eq) was added at room temperature and the resulting solution allowed to stir for 15 minutes. The appropriate 2-iodo-N-phenylbenzamide was then added, followed by sulfur powder (1.25 eq) and K₂CO₃ (1.25 eq). The N₂ atmosphere was restored and the solution stirred at room temperature for a further 15 minutes before heating to reflux at 110°C for 3 hours. The crude solution was allowed to cool to room temperature before pouring into brine (50 ml) and stirring for 1.5 hours. The organic layer was then extracted into EtOAc (3 x 25 ml), dried over Na₂SO₄ and
concentrated under reduced pressure. The crude products were purified by flash column chromatography.

Preparation of 2-Phenylbenzo[\(d\)]isothiazol-3(2\(H\))-one 3

The general procedure (D) was followed using 2-iodo-\(N\)-phenylbenzamide (0.2 g, 0.619 mmol) to give 3 as a white crystalline solid in a 70.0% yield. \(R_f = 0.49\) (4:6 EtOAc:hexane). \(^1\text{H} \text{NMR}\) (400 MHz, CDCl\(_3\)) \(\delta\) 8.11 (ddd, \(J = 7.9, 0.8, 0.3\) Hz, 1H), 7.74 - 7.68 (m, 2H), 7.68 – 7.64 (m, 1H), 7.59 (dt, \(J = 8.1, 0.8\) Hz, 1H), 7.52 – 7.42 (m, 3H), 7.33 (tt, \(J = 7.4, 1.1\) Hz, 1H); \(^{13}\text{C} \text{NMR}\) (101 MHz, CDCl\(_3\)) \(\delta\) 164.6, 140.3, 137.6, 132.8, 129.8 (2C), 127.6, 127.5, 126.2, 125.3, 125.0 (2C), 120.5; \text{Elemental analysis (\%)} found: C, 68.49; H, 4.06; N, 6.07; S, 14.26%. \(\text{C}_{13}\text{H}_9\text{NOS}\) requires C, 68.70; H, 3.99; N, 6.16; S, 14.11%.

\(\text{ES}^+\text{HRMS}\) \(m/z\) 250.0295 [M+Na]\(^+\) \(\text{C}_{13}\text{H}_9\text{NOS}\) requires 227.04.

Preparation of 2-(3-(Morpholinosulfonyl)phenyl)benzo[\(d\)]isothiazol-3(2\(H\))-one 4

The general procedure (D) was followed using 7 (0.4 g, 0.847 mmol) to give 4 as a pale-orange solid in a 9.97% yield. \(R_f = 0.32\) (6:4 EtOAc:hexane). \(^1\text{H} \text{NMR}\) (400 MHz, CDCl\(_3\)) \(\delta\) 8.20 (t, \(J = 1.6\) Hz, 1H), 8.12 (dd, \(J = 7.9, 0.7\) Hz, 1H), 8.03 – 7.99 (m, 1H), 7.72 (td, \(J = 8.2, 1.2\) Hz, 1H), 7.70 – 7.67 (m, 2H), 7.64 (t, \(J = 7.5\) Hz, 1H), 7.49 (td, \(J = 8.0, 0.9\) Hz, 1H), 3.78 (t, \(J = 4.7\) Hz, 4H), 3.10 (t, \(J = 4.8\) Hz, 4H); \(^{13}\text{C} \text{NMR}\) (101 MHz, CDCl\(_3\)) \(\delta\) 164.7, 139.9, 138.8, 137.0, 133.4, 130.6, 128.5, 127.8, 126.7, 124.0, 124.8, 123.6, 120.7, 66.5 (2C), 46.4 (2C); \text{Elemental analysis (\%)} found: C, 53.69; H, 4.44; N, 7.35; S, 15.79%. \(\text{C}_{17}\text{H}_{16}\text{N}_2\text{O}_4\text{S}_2\) requires C, 54.24; H, 4.28; N, 7.44; S, 17.03%; \(\text{ES}^+\text{HRMS}\) \(m/z\) 399.0459 [M+Na]\(^+\) \(\text{C}_{17}\text{H}_{16}\text{N}_2\text{O}_4\text{S}_2\) requires 376.06.

Preparation of 2-(Chlorothio)-benzoyl chloride 8

Thionyl chloride (22.8 mmol, 7.0 eq) and \(N, N\)-dimethylformamide (3 drops) were added to a solution of 2,2'-dithiobenzoic acid (3.26 mmol) in anhydrous dichloroethane (25 ml) under a \(N_2\) atmosphere at room temperature. The stirring solution was heated to reflux at 80°C for 1.5 hours. Sulfuryl chloride (4.24 mmol, 1.3 eq) was added to the cooled solution at 25°C before heating the solution to 50°C for 45 minutes. Solvent, excess thionyl chloride and excess sulfuryl chloride were removed under reduced pressure to generate 9, a yellow-green solid as pure product in quantitative yield. \(R_f = 0.79\) (50:50 EtOAc:hexane). \(^1\text{H} \text{NMR}\) (400 MHz, CDCl\(_3\)) \(\delta\) 8.34 (dd, \(J = 8.0, 1.2\) Hz, 1H), 7.93 (dd, \(J = 8.2, 0.4\) Hz, 1H), 7.77 (dt, \(J = 8.5, 1.3\) Hz, 1H), 7.42 (dt, \(J = 8.0, 0.8\) Hz, 1H); \(^{13}\text{C} \text{NMR}\) (101 MHz, CDCl\(_3\)) \(\delta\) 169.5, 146.1, 135.8, 134.7, 127.4, 125.8, 124.4.
2.10 References


Chapter 2


Chapter 3
Developing an Understanding of SAR Around the BITZ
Chemotype and Biological Evaluations of BITZ
Compound Activity
3.1 Medicinal Chemistry Strategy - TCAMS

This chapter will present the research that has been conducted, taking the DT2009-0168020 HTS hit (Chapter 2, Section 2.2.4, Figure 2.8) towards an optimised lead structure via SAR exploration and through the optimisation of physicochemical properties. As an introduction to this work, first presented is an example of a medicinal chemistry program carrying out a similar approach of hit to lead optimisation within drug design, from which we have taken inspiration.

As discussed in Chapter 2 (Section 2.1), a phenotypic screen of the 13,533 chemical structures published by GSK in 2010 comprising the Tres Cantos Antimalarial Set (TCAMS) compound library was performed. It identified 47 high quality starting points of then unevaluated chemotypes to fast track into lead optimisation studies with the purpose of initiating open and innovative antimalarial drug discovery.¹ Five compound series were ultimately selected, each complying with the chief requirements of antiplasmodial potency, druggability and synthetic tractability.

A series of indoline compounds was one of the five selected compound series, in which; the primary hit was TCMDC-139046 (Figure 3.1), a highly potent compound with good in vitro antimalarial activity (IC₅₀ value of 80 nM against P. falciparum 3D7A in a 48-hour assay).² Whilst the TCMDC-139046 hit is metabolically stable with little cytotoxicity, the molecular weight (MW) and lipophilicity of the compound were high and, as part of the indoline series, also known to have activity against the 5-HT₂c receptor.¹,³

Inhibition of the 5-HT₂c receptor is associated with the treatment of depression and anxiety and therefore may complicate clinical development of an antimalarial compound series; consequently, minimising activity at this receptor type was a priority for subsequent SAR development around the TCMDC-139046 hit.² Work by Calderon et al. addressed this challenge in a double SAR study that aimed to drive antimalarial potency at both P. falciparum 3D7 and P. falciparum Dd2, whilst avoiding interaction with 5-HT₂c receptors.² Optimisation of TCMDC-139046 also aimed to improve the physicochemical profile of the compound, already known to have a poor pharmacokinetic (PK) profile of high lipophilicity and low solubility in aqueous media.²

A diverse range of functional group substitutions and chemical modifications were made to the TCMDC-139046 hit, summarised in Figure 3.1, in a full SAR analysis conducted by Calderon et al. Functional group substitutions were explored at R¹ to determine if the lipophilic trifluoromethoxy group could be replaced with more desirable polar groups such as F, H and CF₃.
Figure 3.1 SAR development and optimisation of the TCAMS TCMDC-139046 hit to a lead antimalarial compound: figure reproduced from [1] and [2].

In addition, varying the pyridyl ring structure at $R^2$ was also examined. However, replacement of the pyridyl ring with various functionalities, including H, Br and Me, showed a loss in antiplasmodial activity; substitution with a pyrimidyl ring gave a slightly improved physicochemical profile, due to a small reduction in lipophilicity, but showed no great improvement in terms of potency. Overall, SAR analysis around the left hand side of the molecule, as drawn, revealed that the trifluoromethoxy group at $R^1$ represented the best substitution for maintaining antimalarial activity and that the 3-pyridyl ring at $R^2$ represents the optimal functionality for minimising activity at the 5-HT$_2c$ receptor.

Calderon et al. acknowledged that a wide variety of substituents would be tolerated in the indole ring, as highlighted by the diversity seen in the TCAMS. Subsequently, a wide range of substitutions were made at a number of positions in the indoline core (Figure 3.1), comparing antimalarial activity results to an unsubstituted indoline structure to determine clearly the effect of each structural modification. SAR development around the right hand side of the TCMDC-139046 structure was therefore extensive and ultimately led to the development and rationalisation of the optimised lead compound (Figure 3.1) where $R^5 = Cl$, $R^6 =$ piperidine and $R^7 = H$. The resulting structure, depicted above, therefore represented the most promising compound since it was able to demonstrate a divergent SAR between in vitro antimalarial activity and affinity for the 5-HT$_2c$ receptor. It also had an acceptable physicochemical profile in terms of oral absorption, lipophilicity and in vitro clearance; the next stage of research from this point aimed to improve in vivo efficacy.
The work conducted by Calderon et al.\textsuperscript{[2]} therefore represents a good example of a successful medicinal chemistry optimisation program with full SAR analysis, moving from a hit starting point to an optimised lead compound. Medicinal chemistry optimisation was performed identifying progressively optimised compounds, designed to display specific and divergent activity at different molecular targets, as required by the specifications of this research problem. Altogether, a clear progression was made moving from a HTS hit to an optimised lead compound; advancing into an area of improved chemical space and optimal biological performance.

Relating this to our own antimalarial research question, we took inspiration from the work of Calderon et al.\textsuperscript{[2]} and aimed to mirror this successful medicinal chemistry approach in the application of hit to lead optimisation around the identified of 1,2-benzo[d]isothiazol-3(2H)-one (BITZ) chemotype.

3.1.1 Aims

Having identified the BITZ chemotype and derivatives as novel, small-molecule inhibitors of the \textit{P. falciparum} IspD (\textit{Pf}IspD) enzyme, the aims of research were to undertake a medicinal chemistry optimisation program. The long term aims were to deliver a lead candidate molecule targeting \textit{P. falciparum} methylerythritol cytidyltransferase (\textit{Pf}IspD) that is suitable for clinical development as an antimalarial drug. With organic synthesis facilitating structural modifications to the BITZ side chain, the resulting inhibitors aim to develop SAR around the BITZ chemical chemotype and \textit{Pf}IspD enzyme active site which are informed and guided by \textit{Pf}IspD enzymatic activity assays and 3D7 whole cell activity assays of resulting BITZ compounds.

The primary objectives of research were to enhance the efficacy and pharmacokinetic properties of the HTS hit, \textbf{DT2009-0168020}. Therefore, optimisation of drug-like properties, removal of metabolic and pharmacokinetic vulnerabilities, probing chemical space, determining the nature of the \textit{Pf}IspD enzyme target and the role of BITZ inhibitor in achieving enzyme inhibition were key developmental priorities.

3.1.2 SAR Map: Summary of Synthetic Explorations and BITZ Compound Series

Several series of BITZ compounds, with defined structural variations and specific chemical modifications, have been designed and synthesised around a simple BITZ compound framework (\textbf{Figure 3.2}). SAR development and hit to lead optimisation was initiated from this simplified BITZ structure since it was close to a “leadlike” starting point therefore allowing growth in molecular weight whilst being able to optimise drug-like properties.\textsuperscript{[4]} Each BITZ compound series has a specific and well-defined design rationale with the aim of informing SAR and developing an understanding of optimal
functionality, promoting inhibitory activity against PfISP. These structural explorations and design rationales, along with their outcomes, are outlined and discussed throughout this chapter.

**Figure 3.2** Summary of BITZ compound series developed around the selected chemotype.

Based around the synthetic explorations discussed in Chapter 2 (Section 2.3), distinct synthetic strategies have been used to generate more than 30 BITZ compound analogues which make up the eight discrete compound series, shown in Figure 3.2. A series of structural modifications have been made to the C and D rings in the side chain of the hit scaffold with the aims of varying lipophilicity, improving solubility and introducing functionality to promote potency against PfISP. Each BITZ analogue generated has been tested in both the PfISP enzymatic assay (Chapter 2, Section 2.2.3) and the 3D7 whole cell parasite growth assay (Chapter 2, Section 2.3.5) to determine the extent of inhibitory compound activity against PfISP and Plasmodium parasite growth.

### 3.2 Biphenyl BITZ Analogue Series

#### 3.2.1 Design Rationale and Synthesis

The first of BITZ compound series was established on the basis of knowledge gained from preliminary SAR investigations around the HTS hit, DT2009-0168020 (Chapter 2, Section 2.5). The sulphonamide group of the DT2009-0168020 hit was therefore removed and the required meta-substitution pattern
across the C ring was maintained, but with all C ring ortho-functionalities removed. The purpose of
the biphenyl analogue series was to determine the optimal D ring for BITZ derived PfIspD inhibitors
and which functional group substituents were best tolerated. Consequently, in this series of BITZ
analogues, comprised of seven derivatives, the D ring is specified as a para-substituted phenyl ring
with the inclusion of various electron-donating and accepting groups to explore the effects of various
para-substitutions in the aromatic D ring. A single exception to this structural motif exists in the case
of compound 10 where the phenyl D ring was replaced with a thiophene ring (Figure 3.3).

Figure 3.3 Chemical structure and substitution pattern of BITZ compounds 4-10.

All compounds in this series were synthesised using a synthetic scheme which was derived from Route
1 (Chapter 2, Section 2.3.1, Scheme 2.4), facilitating generation of the BITZ motif and related
analogues from the commercially available starting material, 2-(methylthio) benzoic acid (Scheme
3.1).

The BITZ headgroup itself was generated in the final synthetic step of the synthesis, via the previously
discussed thionyl chloride-mediated ring closure of methylsulfanyl benzamide intermediates (Chapter
2, Section 2.3.1, Scheme 2.6). In order to incorporate the para-substituted D ring of desired inhibitor
structures (4-10), Suzuki-Miyaura cross-coupling reactions\(^5\) were incorporated into the synthetic
procedure, producing a representative four-step synthesis of compounds 4-10, as depicted below.

Scheme 3.1 General synthetic strategy for BITZ compounds 4-10.
Acid-chloride mediated amide coupling of 2-(methylthio) benzoic acid and 3-iodoaniline, generated the (methylthio)benzamide 1. Oxidation of 1 to the corresponding sulfoxide, 2, followed by Suzuki-Miyaura cross-coupling reactions, using a range of para-substituted phenyl boronic acids, was employed to generate intermediates 3a-h. The methylsulfinyl benzamides intermediates 3a-h were finally cyclized using thionyl chloride-mediated ring closure to give the required BITZ derivatives 4-10. All synthetic steps proceeded in moderate to good yields with the straightforward purification of all intermediates and target compounds by trituration or flash column chromatography.

### 3.2.2 PfIspD Enzymatic and Whole Cell Biological Assays

All of the compounds in this series demonstrated low micromolar to nanomolar inhibitory activity against PfIspD as well as low micromolar activity against Plasmodium parasite growth; highlighting the efficacy of these compounds and their developmental potential. These initial results show that para-substitution in the phenyl D-ring, using electron releasing or withdrawing groups (4-10) was well tolerated, with PfIspD IC₅₀ values as low as 73 ± 20 nM and a parasite EC₅₀ of 1.04 ± 0.164 µM for the para-chloro analogue 5.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>R¹/D ring</th>
<th>PfIspD IC₅₀ (µM)</th>
<th>Whole cell 3D7 EC₅₀ (µM)</th>
<th>MW (gmol⁻¹)</th>
<th>ClogD</th>
<th>CaqSol (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>OCH₃</td>
<td>0.206 ± 0.089</td>
<td>0.920 ± 0.066</td>
<td>333.08</td>
<td>4.72</td>
<td>1.40</td>
</tr>
<tr>
<td>5</td>
<td>Cl</td>
<td>0.073 ± 0.020</td>
<td>1.080 ± 0.164</td>
<td>337.03</td>
<td>4.98</td>
<td>0.56</td>
</tr>
<tr>
<td>6</td>
<td>CF₃</td>
<td>0.273 ± 0.015</td>
<td>0.646 ± 0.021</td>
<td>371.06</td>
<td>5.26</td>
<td>0.38</td>
</tr>
<tr>
<td>7</td>
<td>OCF₃</td>
<td>0.290 ± 0.195</td>
<td>0.411</td>
<td>387.05</td>
<td>5.55</td>
<td>0.26</td>
</tr>
<tr>
<td>8</td>
<td>CO₂CH₃</td>
<td>0.393 ± 0.096</td>
<td>1.00 ± 0.237</td>
<td>361.08</td>
<td>4.82</td>
<td>0.53</td>
</tr>
<tr>
<td>9</td>
<td>OH</td>
<td>0.660</td>
<td>2.450</td>
<td>319.07</td>
<td>4.33</td>
<td>3.61</td>
</tr>
<tr>
<td>10</td>
<td>Thiophene</td>
<td>0.265 ± 0.021</td>
<td>2.877</td>
<td>309.03</td>
<td>4.49</td>
<td>3.71</td>
</tr>
</tbody>
</table>

Table 3.1 Inhibitory activity of compounds 4-10 against PfIspD and P. falciparum growth in culture (strain 3D7); predicted ClogD and CaqSol values calculated using algorithms from AstraZeneca (as predicted for all BITZ compounds).[[8,9]]

Whilst para-chloro analogue 5 was seen to be the most potent inhibitor in series against PfIspD, the para-methoxy analogue 4 and para-trifluoromethyl analogue 6 were found to possess comparable enzymatic PfIspD activity of 206 ± 89 nM and 273 ± 15 nM respectively. This encouraging enzymatic activity was also translated into whole cell growth inhibition, with 3D7 EC₅₀ values of 920 ± 66 nM and 646 ± 21 nM displayed by 4 and 6 respectively. Importantly, it should be pointed out that the most potent inhibitor of the biphenyl series against PfIspD, para-chloro analogue 5, demonstrates inhibition of the enzyme at a potency six-fold higher than that of the original HTS hit, DT2009-0168020 (Figure
3.15), highlighting that a substantial amount of structural optimisation has been achieved. These results are therefore encouraging as they not only show that BITZ compounds are capable of potently inhibiting the PfIspD enzyme target but that this also translates into whole cell antimalarial activity.

In slight contrast to inhibitors 4-7, a reduction in activity against both the PfIspD enzyme and Plasmodium parasite growth was observed with the thiophene derivative 10. This result suggests that a substituted aromatic phenyl ring, as the D-ring of the inhibitor side chain, may be preferable to the slightly smaller, aromatic thiophene heterocycle. This may also reflect spatial requirements and inhibitor shape-fit at the PfIspD active site however, further SAR investigation is needed to support this inference.

A consistent issue across the biphenyl BITZ series is high compound lipophilicity, tracking with poor predicted aqueous solubility, which was particularly apparent with some of the most potent inhibitors in the series, 4-8 (Table 3.1). The ability of a drug to penetrate biological membranes and tissues is a key factor which mediates the extent of drug interactions with a biological system and therefore the ability of chemical agents to achieve their desired effect. Consequently, optimisation of aqueous solubility has remained a key priority throughout BITZ compound development with acceptable aqueous solubility defined as > 50 μM; which is consistent with accepted pharmacokinetic (PK) parameters as defined in literature. In order to gain an awareness of the probable lipophilicity and solubility of the designed BITZ compounds, we requested predicted distribution coefficient and solubility measurements from AstraZeneca (AZ) for all BITZ compounds synthesised within this research. In these papers AZ highlight the design of good predictive models for the logD and aqueous solubility of organic compounds. The strategic collaboration between The University of Liverpool and AZ for research into tropical diseases enabled our access to these in silico models, allowing us to obtain pharmacokinetic (PK) predictions for our BITZ derived compounds.

### 3.2.3 Lipophilicity

Compound polarity, electronic and steric parameters, as well as molecular weight and geometry are important physicochemical factors which are strongly correlated with biological activity. However, lipophilicity is possibly one of the most important physicochemical properties to be considered in the development of potential drugs due to its major role and influence in compound solubility, absorption and membrane penetration, plasma protein binding and distribution within biological systems.

Lipophilicity represents the affinity of a molecule for a lipophilic environment and is measured by its distribution behaviour within a biphasic system. Lipophilicity, most commonly measured as LogP, is a component of Lipinski’s Rule of Five for predicting the drug-likeness of molecules. LogP values
provide the partition coefficient of a neutral (or unionised) molecule between aqueous and water phases, usually octanol and water (Figure 3.4). A partition (or distribution) coefficient signifies the tendency of a molecule to differentially compartmentalise between two immiscible phases.[15]

\[
LogP_{oct/wat} = \log \left( \frac{[\text{compound}]_{\text{octanol}}}{[\text{compound}]_{\text{water}}} \right)
\]

**Figure 3.4** The octanol/water partition coefficient (logP) defining the intrinsic lipophilicity of a drug molecule.[16]

Since many known drug molecules are ionisable species, and the lipophilicity of a compound changes as a function of pH, LogD (distribution coefficient) is often used as a measure of lipophilicity in drug discovery and development programs. The distribution constant LogD is pH dependent and therefore provides an appropriate description of molecule lipophilicity at any specified pH. Since ionisable groups within drug molecules are likely to be charged at physiological pH (pH 7.4), LogD provides a more accurate representation of a compounds lipophilicity when present within a biological system.[16]

In order to adhere with literature guidelines and optimise the prospect of developing compounds that display optimal PK profiles, we aim to generate compounds that observe our target parameters for ClogP and ClogD. More specifically, designed compounds should ideally demonstrate ClogP values of < 5 in order to conform to Lipinski’s guidelines and enhance the likelihood of achieving optimised drug-likeness and compound bioavailability.[14,17,18] Considering parameters for ClogD (pH 7.4), values between 1.1 and 3.1 represent a suitable target range for compound design since our compounds are likely to span the MW category of 300 – 450 g mol\(^{-1}\); and it is now well documented in literature that compounds with distribution coefficients inside these parameters tend to have good solubility and permeability (i.e. favourable oral absorption and cell membrane permeability).[19]

### 3.3 Biphenyl Benzyl-linked BITZ Analogue Series

#### 3.3.1 Design Rationale and Synthesis

Compound lipophilicity has long been recognised as important for aqueous solubility of compounds, where a lack of sufficient solubility is a significant and often challenging obstacle in medicinal chemistry optimisation.[19–21] Therefore, to reduce the highly lipophilic nature of the biphenyl BITZ analogues 4-10, as well as improve aqueous solubility, a new compound series (13-17) was designed. This biphenyl benzyl-linked series, comprised of five compounds, incorporates a methylene link between the phenyl D ring and various polar heterocyclic groups, maintaining para-substitution in the terminal D ring (Figure 3.5).
Figure 3.5 Chemical structures and substitution pattern of BITZ compounds 13-17.

The main aim of this molecular addition was to promote aqueous solubility and reduce compound lipophilicity through introduction of a basic protonation site. In addition, the introduction of this basic protonation site from the attachment of heterocycles offers the possibility of administering the resulting compounds as salts, to facilitating solubility for drug administration.\(^\text{[22]}\) Furthermore, extension of the BITZ inhibitor side chain potentially enables additional protein-ligand contacts to be made, at the PfISPd active site, therefore potentiating increased strength of BITZ compound binding. The benzyl linker adds a degree of ligand flexibly to 13-17 which may enable an increased number of ligand binding poses and inhibitor orientations to be adopted within the PfISPd binding site. This may therefore support new protein-inhibitor contacts and promote the strength of inhibitor binding and therefore the magnitude of enzyme inhibition.

The synthesis of this inhibitor series was approached using two distinct synthetic routes. Initially, inhibitors 14 and 15 were generated using the previously described synthetic route (Section 3.2.1, Scheme 3.1) where yields were found to be consistent with those previously observed. However, larger and more complex boronic acid species were required to incorporate the benzyl-linked side chains of 16 and 17 within the Suzuki-Miyaura cross-coupling synthetic step. To maintain this synthetic approach using more complex boronic acid species, the chemical reagents required became notably higher in cost, increasing the total expense of analogue production. Since high developmental costs are not in line with the affordability ethos of antimalarial drug development programs and aims for economic viability,\(^\text{[23,24]}\) a revised synthesis was sought.

As a result, an alternative three-step route, using the previously discussed acid chloride-mediated synthesis (Chapter 2, Section 2.3.3), was used to generate the morpholine-linked analogue 13 (Scheme 3.3). The synthesis of 2-(chlorothio)-benzoyl chloride \(X\) occurs via a rapid and quantitative two-step, one-pot reaction using the disulphide; 2',2'-dithiobenzoic acid (Scheme 3.2).\(^\text{[25]}\) In addition, \(X\) is air stable at room temperature making it a versatile chemical intermediate to incorporate since it can be isolated and stored over short periods of time.
Scheme 3.2 Synthesis of X and the subsequent synthetic strategy for the generation of BITZ derived compounds by acid chloride-mediated amide coupling and ring closure between X and appropriate aniline derivatives.\cite{25}

Towards the synthesis of BITZ compound 13, 11a was generated via nucleophilic displacement using morpholine and the corresponding benzylbromine.\cite{26} This was then subject to a Suzuki-Miyaura cross-coupling reaction with 3-bromoaniline to generate the intermediate 11b. Cyclization of 11b with acid chloride X (Scheme 3.2),\cite{25} in a simultaneous amide coupling and ring closure mechanism, produced 13\cite{27,28} with all reactions proceeding in high yields.

Scheme 3.3 Synthetic strategy for compound 13.

In order to generate BITZ analogues 16 and 17, a separate synthesis was devised due to the requirement for Boc-protection of the piperazine heterocycle and the subsequent sulfonylation of compound 16 to give 17. As a result, generation of 16 and 17 required a linear, five-step synthesis which is shown in Scheme 3.4.
**Scheme 3.4** Synthetic strategy for compounds 16 and 17.

Nucleophilic displacement of a 4-(bromomethyl)benzene boronic acid by 1-Boc-piperazine gave 12a\[^{26}\] which, analogous to the conversion of 11a to 11b, underwent a Suzuki-Miyaura cross-coupling reaction with 3-bromoaniline to generate the intermediate 12b. 12b was then cyclized with X via the described simultaneous amide coupling and ring closure mechanism to produce 12c.\[^{27,28}\] Removal of the Boc-protecting group was achieved under acidic conditions, using TFA, in high yield to reveal the free piperazine heterocycle and generate 16. Compound 16 was subsequently converted to 17 in moderate yield by simple nucleophilic substitution with methanesulfonyl chloride,\[^{29}\] generating the methylsulfonyl-substituted piperazine heterocycle and the final target of this series (17). Despite the linear nature of this synthesis (**Scheme 3.4**), all synthetic steps proceeded in reasonable to good yield with the straightforward isolation and purification of all intermediate compounds.

In accordance with the aim of this BITZ compound series, incorporation of the piperidine heterocycle intended to promote the aqueous solubility of 16 and lower the predicted distribution coefficient of the compound. Specifically, the piperidine functionality adds an additional hydrogen bonding capacity to 16 since it is hydrogen donating and therefore provides a subtle contrast to the hydrogen bond acceptor capabilities of the morpholine and N-methyl piperazine functionalities of 13 and 15. This BITZ compound series has therefore utilised a range of saturated heterocyclic rings, providing both hydrogen bond donating and accepting capabilities, intended to facilitate hydrogen bonding...
interactions at the *PfIspD* active site and promote compound solubility. In addition, compound 17 facilitates further extension of the BITZ side chain, together with increased structural flexibility. This side chain extension could enable increased protein-ligand interactions at *PfIspD* through increased inhibitor bulk and therefore greater occupation of the *PfIspD* active site.

### 3.3.2 *PfIspD* Enzymatic and Whole Cell Biological Assays

All compounds in this series 13-17 demonstrated low micromolar activity against *PfIspD* which also translated into comparable low micromolar activity against parasite growth in the case of compounds 13-15. Compound 14 showed the most potent parasite killing effects with a 3D7 EC₅₀ value of 0.323 ± 0.092 μM (Table 3.2). Biological analysis and physicochemical property predictions highlight that incorporation of benzyl-linked heterocycles into the BITZ side chain improves the solubility and lowers the lipophilicity of resulting inhibitors. This is particularly notable with compounds 16 and 17 which also demonstrate inhibitory against the *PfIspD* enzyme and *Plasmodium* parasite growth.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>χ¹</th>
<th><em>PfIspD</em> IC₅₀ (μM)</th>
<th>Whole cell 3D7 EC₅₀ (μM)</th>
<th>MW (gmol⁻¹)</th>
<th>ClogD</th>
<th>CaqSol (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>O</td>
<td>0.400 ± 0.082</td>
<td>0.413 ± 0.082</td>
<td>402.14</td>
<td>4.13</td>
<td>2.51</td>
</tr>
<tr>
<td>14</td>
<td>CHF</td>
<td>0.862 ± 0.107</td>
<td>0.323 ± 0.092</td>
<td>418.15</td>
<td>4.64</td>
<td>1.86</td>
</tr>
<tr>
<td>15</td>
<td>NCH₃</td>
<td>0.446 ± 0.075</td>
<td>0.416 ± 0.006</td>
<td>415.17</td>
<td>3.80</td>
<td>56.23</td>
</tr>
<tr>
<td>16</td>
<td>NH</td>
<td>0.160 ± 0.007</td>
<td>3.25</td>
<td>401.16</td>
<td>2.56</td>
<td>52.86</td>
</tr>
<tr>
<td>17</td>
<td>NSO₂CH₃</td>
<td>0.523 ± 0.09</td>
<td>1.47</td>
<td>479.13</td>
<td>3.99</td>
<td>1.08</td>
</tr>
</tbody>
</table>

**Table 3.2** Inhibitory activity of compounds 13-17 against *PfIspD* and *P. falciparum* growth in culture (strain 3D7); calculated ClogD and CaqSol values given.

Particularly encouraging from this series is the significantly enhanced aqueous solubility of 15; likely a consequence of the *N*-methyl piperazine heterocycle which introduces two basic protonation sites into the molecule. Indeed, compound 15 showed a 100-fold increase in predicted aqueous solubility compared to our most active *PfIspD* inhibitor 5 (Table 3.1), a reduction in ClogD from 4.98 to 3.80 and ~ 3-fold enhanced activity against cultured *P. falciparum*. In addition, compound 16, incorporating the unsubstituted piperazine heterocycle, also displayed significantly enhanced aqueous solubility compared to other analogues, as well as potent *PfIspD* enzymatic activity with a corresponding IC₅₀ value of 0.160 μM. Disappointingly, the enzymatic activity of 16 was not translated into whole cell 3D7 growth inhibition, as achieved with compounds 13-15. This may be a consequence of the reduced lipophilicity of 16 since a reduction in lipophilicity can be mirrored by reduced compound permeability.
within a biological system.\textsuperscript{[10,31]} Taken together, these results demonstrate that the inhibitory activity of BITZ compounds can be maintained whilst enhancing the drug-like properties of analogues through structural modification and chemical manipulations.

Following much debate and research, it is now commonly recognised that the optimal logD parameters for any compound are dependent on molecular weight.\textsuperscript{[19]} However a general assumption can be made that the optimal logD for a potential drug molecule lies in the narrow range of ~ 1-3.\textsuperscript{[32]} Considering this compound series, the calculated distribution coefficient of each analogue currently lies outside of this range. Further inhibitor modifications were therefore required to try and reduce ClogD values further, whilst maintaining PfIspD enzymatic and whole cell activity, alongside compound solubility.

3.4 Mono-phenyl Benzyl-linked BITZ Analogue Series

3.4.1 Design Rationale and Synthesis

Guided by previous results, a series of mono-phenyl inhibitors 19-21 were designed and synthesised, maintaining meta-benzylic heterocycle substitution at the C ring (Figure 3.6). The aromatic phenyl D ring of previous analogues was removed to reduce lipophilicity in this resulting mono-phenyl series; while the heterocycle functionalities were conserved to retain compound solubility. The overall aim was to retain inhibitor activity and optimise aqueous solubility whilst lowering inhibitor lipophilicity. Therefore, this analogues series may provide a means of exploring the extent or lack of lipophilicity is tolerated in the SAR of BITZ analogues. Given that a phenyl ring has an associated Clog value of 2.14,\textsuperscript{[33]} removal of the phenyl D ring moves the mono-phenyl series into our target zone for ClogP and ClogD. In other words, the compound series (19-21) was used to assess if inhibitory whole cell growth activity correlates with increased lipophilicity, which would likely be as a result of enhanced membrane penetration.

![Figure 3.6 Chemical structures and substitution pattern of BITZ compounds 19-21.](image)

The mono-phenyl inhibitor series was generated using a straightforward three-step synthesis. The required inhibitor side chain was first constructed in the initial two steps of the synthesis which was
completed by acid chloride-mediated amide coupling and ring closure to generate the BITZ motif (Scheme 3.5).

\[ \text{3-nitrobenzyl bromine} \xrightarrow{\text{iPr}_2\text{EtN, THF}} \text{intermediates 18ai-ii} \xrightarrow{\text{hydrogenation}} \text{amines 18bi-iii} \]

Scheme 3.5 Synthetic strategy for mono-phenyl BITZ compounds 19-21; 18bi (3-morpholine-4-yl methyl-phenylamine) was purchased from Sigma Aldrich.

The first synthetic step involved nucleophilic displacement of 3-nitrobenzyl bromine by treatment with the appropriate heterocycle, in the presence of diisopropylethyl amine (iPr\(_2\)EtN), to yield the 3-nitrobenzyl heterocycle intermediates 18ai-ii. A subsequent hydrogenation reaction converted the nitro functionality of intermediates 18ai-ii into the corresponding 1° amines, giving 3-aminobenzyl heterocycles 18bi-iii. In the final synthetic step, the acid chloride X\(^{[25]}\) facilitated simultaneous amide coupling and ring closure\(^{[27,28]}\) introducing the BITZ headgroup into the final inhibitor structures 19-21.

3.4.2 PfISPDE Enzymatic and Whole Cell Biological Assays

The mono-phenyl BITZ derivatives demonstrated a reduction in lipophilicity, the immediate consequence of removing an aromatic ring, and improved solubility, which overall is likely due to reduced molecular size and retention of the saturated heterocyclic ring. Considering these favourable physicochemical parameters it seemed likely that this compound series would be able demonstrate good permeability in biological systems due to average molecular weights < 400 and predicted AZ\(\log D\) parameters of > 1.3.\(^{[19]}\)
Table 3.3 Inhibitory activity of compounds 19-21 against PfISP\textsubscript{D} and \textit{P. falciparum} growth in culture (strain 3D7); calculated C\textsubscript{log}D and C\textsubscript{aq}Sol values given.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>X\textsuperscript{2}</th>
<th>PfISP\textsubscript{D} IC\textsubscript{50} (μM)</th>
<th>Whole cell 3D7 EC\textsubscript{50} (μM)</th>
<th>MW (gmol\textsuperscript{-1})</th>
<th>C\textsubscript{log}D</th>
<th>C\textsubscript{aq}Sol (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>O</td>
<td>0.540</td>
<td>5.71</td>
<td>326.11</td>
<td>2.60</td>
<td>119.9</td>
</tr>
<tr>
<td>20</td>
<td>CHF</td>
<td>0.530 ± 0.027</td>
<td>2.18 ± 0.340</td>
<td>342.12</td>
<td>3.16</td>
<td>71.89</td>
</tr>
<tr>
<td>21</td>
<td>NCH\textsubscript{3}</td>
<td>0.524</td>
<td>1.88</td>
<td>339.14</td>
<td>2.18</td>
<td>1343</td>
</tr>
</tbody>
</table>

Enzymatic PfISP\textsubscript{D} activity of mono-phenyl compounds 19-21 was highly comparable across the series and also close to that of the equivalent biphenyl compounds 13-15. The mono-phenyl compound 20, the 4-fluoropiperidine analogue, demonstrated an inhibitory PfISP\textsubscript{D} IC\textsubscript{50} value of 0.530 μM which demonstrates a 1.6-fold increase in potency compared to the equivalent biphenyl compound 14 (Table 3.2 and Table 3.3). Despite increased and comparable potency against PfISP\textsubscript{D}; this was not translated into efficacious activity against whole cell parasite growth with the observation of only mild 3D7 growth inhibition. Indeed, all mono-phenyl inhibitors displayed whole cell EC\textsubscript{50} values > 1 μM (Table 3.3) which is in the range of six to 18 times less potent than the whole cell inhibitory activity of 14 (Table 3.2). This result therefore contradicts predictions of high cellular permeability that was thought to be achievable with this compound series since the predicted \textit{log}D values are notably lower than earlier biphenyl benzyl-linked BITZ inhibitors (Section 3.3).

3.5 Biphenyl Pyridinyl BITZ analogue series

3.5.1 Design Rationale and Synthesis

In order to try and restore the 3D7 growth inhibition lost with inhibitors in the mono-phenyl compound series, the biphenyl BITZ side chain was revisited. Having evaluated the effects of solubilising saturated heterocycles on inhibitor structures; this functionality was again removed in order to determine the effects of alternative structural motifs. Again considering the D ring, the phenyl ring was converted to a pyridine ring with functionality \textit{ortho} to the ring nitrogen; this therefore maintained aromaticity throughout the side chain but varied the electronics within the terminal D ring (Figure 3.7). Conversion of an aromatic phenyl ring to an aromatic pyridine ring is a classic isosteric substitution that aids compound solubility and helps to reduce hydroxylation of resulting rings through the reduction of metabolic bioactivation.\cite{34} Bioisosteres are structurally distinct compounds that provide alternative and often subtle variations in physiochemical properties, but which are recognised similarly by biological systems. Typically bioisosteres are less exact than structural compound mimetics but are generally more alike in biological and physical properties.\cite{34}
The incorporation of a pyridine ring into inhibitor structures served a number of purposes, the most important of which is lowering lipophilicity and enhancing compound solubility; a key requirement having removed the saturated heterocycle functionality. The pyridine nitrogen atom provides an additional protonation site and hydrogen bond acceptor group which has the potential to facilitate protein-ligand interactions at the PfIspD active site, promoting the strength of ligand binding.\cite{34,35}

Where additional functional group substitution in the pyridine ring was included (24-26), the incorporated functionalities were selected to enable a head to head comparison with compounds 4-6 since there three compounds were the most potent BITZ-derived inhibitors generated thus far.

All analogues in the pyridine series were synthesised in a rapid two-step synthesis which involved Suzuki-Miyaura cross-coupling reactions between 3-bromoaniline and various pyridinyl boronic acids\cite{36} to generate the required analogue side chain. This was followed by incorporation of the BITZ core by reaction of the pyridinyl aniline intermediates 22a-d with X\cite{25,27} generating target compounds 23-26 (Scheme 3.6). The specific conditions of the Suzuki cross-coupling reaction were altered slightly for the synthesis of 23-26 compared to previous conditions (Scheme 3.1 and Scheme 3.3). Dimethoxyethane (DME), rather than a system of H₂O/THF, was used as the solvent, enabling the reaction to be refluxed at a slightly elevated temperature of 90°C. The base was also exchanged (previously K₂CO₃, now Na₂CO₃) according to methods outlined in literature.\cite{36}

\[ \text{Pyridinyl boronic acid species} \]

\[ \begin{array}{c}
\text{3-bromoaniline} \\
\text{[Pd(PPh₃)₃]₂, Na₂CO₃} \\
\text{DME, reflux, 20 hours} \\
\text{70 - 97%} \\
\end{array} \]

\[ \begin{array}{c}
\text{H₂N} \\
\text{X, Et₃N, DCM} \\
\text{0°C - rt, 20 hours} \\
\text{21 - 63%} \\
\end{array} \]

\[ \text{Pyridinyl boronic acid species} \rightarrow \text{3-bromoaniline} \rightarrow \text{H₂N} \rightarrow \text{X, Et₃N, DCM} \rightarrow \text{Pyridinyl boronic acid species} \]

**Scheme 3.6** Synthetic strategy for biphenyl pyridinyl BITZ compounds 23-26.

Generation of the pyridinyl aniline intermediates (22a-d) via the described Suzuki cross-coupling reactions\cite{36} proceeded without issue and in high yield in the case of each pyridinyl boronic acid
derivative used. In contrast, a significant reduction in yield was observed in the final acid-chloride mediated cyclisation step\textsuperscript{[27]} compared to previous analogue syntheses. This outcome may be the result of the pyridine ring reducing the reactivity the 1° amine, although no unreacted starting material was detected in these reactions, or alternatively, a consequence of the age of the acid chloride used in these specific reactions. Whilst the acid chloride X can be isolated and is therefore highly stable in comparison to many acid chlorides, it shows a gradual loss of reactivity over time which may be a result of slow decomposition. This consequently suggests that X should be prepared at regular intervals when using over extended time periods and syntheses to ensure consistent reaction yields and reaction efficiency.

3.5.2 PfsPd Enzymatic and Whole Cell Biological Assays

The predicted distribution coefficients of this biphenyl pyridinyl compound series (Table 3.4) was comparable to the BITZ analogues containing heterocyclic functionalities 13-15 (Table 3.2). This therefore highlights that, in this case, inclusion of a pyridine ring is able to circumvent a significant increase in compound lipophilicity which would generally accompany the removal of six-membered ring saturated heterocycles. In other words, the pyridine system is able to sustain comparable physiochemical properties to those achieved with saturated heterocycles in a comparable systems and compounds.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Y</th>
<th>Z</th>
<th>R\textsuperscript{2}</th>
<th>PfsPd IC\textsubscript{50} (μM)</th>
<th>Whole cell 3D7 EC\textsubscript{50} (μM)</th>
<th>MW (gmol\textsuperscript{-1})</th>
<th>ClogD</th>
<th>CaqSol (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>C</td>
<td>N</td>
<td>-</td>
<td>5.94</td>
<td>3.65</td>
<td>304.07</td>
<td>3.57</td>
<td>14.14</td>
</tr>
<tr>
<td>24</td>
<td>N</td>
<td>C</td>
<td>OCH\textsubscript{3}</td>
<td>58.5</td>
<td>1.67</td>
<td>334.08</td>
<td>4.30</td>
<td>4.088</td>
</tr>
<tr>
<td>25</td>
<td>N</td>
<td>C</td>
<td>Cl</td>
<td>&gt; 100</td>
<td>2.34</td>
<td>338.03</td>
<td>4.24</td>
<td>3.431</td>
</tr>
<tr>
<td>26</td>
<td>N</td>
<td>C</td>
<td>CF\textsubscript{3}</td>
<td>&gt; 100</td>
<td>1.91</td>
<td>372.05</td>
<td>4.33</td>
<td>2.576</td>
</tr>
</tbody>
</table>

Table 3.4 Inhibitory activity of compounds 23-26 against PfsPd and P. falciparum growth in culture (strain 3D7); calculated ClogD and CaqSol values given.

Biological testing results for this compound series were somewhat unexpected since the pyridinyl analogues 23-26 showed a significant loss of activity at the PfsPd enzyme, but maintained inhibition of 3D7 whole cell parasite growth at levels close to that seen with the previous mono-phenyl analogue series 19-21 (Table 3.3). The fact that a complete loss of PfsPd enzyme activity was witnessed with some analogues in this series (24-26), alongside a reduction in compound lipophilicity, perhaps suggests that the SAR around the BITZ chemotype may be assisted by increased compound lipophilicity. In addition, these could also results suggest that BITZ compounds 23-26 may be involved
in off-target contributions that lead to an expression of antimalarial potency, since inhibitory activity against parasite growth is retained when \( Pf\text{IspD} \) activity is lost.

Increased lipophilicity in a molecule can sometimes increase the observed potency at the target; however, this is at the risk of increasing off-target interactions and altering observed phenotypic activity.\[^{12}\] Since loss of \( Pf\text{IspD} \) enzymatic activity was observed across 23-26 but a level of antimalarial potency sustained, a scenario of increased off-target interactions due to altered lipophilicity may here be the case. A direct comparison can therefore be made with the mono-phenyl compounds 19-21 which have lower predicted lipophilicities than pyridinyl analogues 23-26 and display similar potencies against both \( Pf\text{IspD} \) and \textit{Plasmodium} parasite growth (Table 3.3).

### 3.6 Biphenyl BITZ Compound Mimetics

#### 3.6.1 Design Rationale and Synthesis

In order to determine if levels of \( Pf\text{IspD} \) enzyme activity correlate with increased compound lipophilicity; a small inhibitor series was designed comprised of two BITZ analogues which display significantly lower \( C\log D \) values than any BITZ compounds considered to this point. In attempt to generate compounds with reduced molecular weight and lipophilicity, notably distinct side chains were incorporated to explore a contrasting BITZ inhibitor structure with complete replacement of the aromatic D ring, 28 and 30 (Figure 3.8).

![Figure 3.8 Chemical structures of BITZ compounds 28 and 30.](image)

Increasing compound potency, at any specified molecular target, is often achieved by increasing the molecular weight and lipophilicity of compounds; since increasing compound lipophilicity, and therefore molecular permeability, eases the access of a compound to a defined molecular target.\[^{20}\] However, potency often has an unhealthy correlation with molecular obesity (defined as high molecular weight and lipophilicity)\[^{20}\] and may also be consistent with increasing non-specific protein-ligand interactions, which is an undesirable consequence of amplifying these two parameters.\[^{37}\]
As a result, this small compound series aimed to reduce BITZ compound lipophilicity, consequently promoting analogue solubility, whilst preserving an appropriate molecular size of < 400 Da, which is highlighted in the GSK ‘4/400’ rule as a likely promoter of favourable ADMET compound properties. More specifically, the GSK ‘4/400’ rule suggests that compounds which have a ClogP of > 4, alongside a molecular mass of > 400 Da, are likely to demonstrate a less favourable ADMET profile.

The biphenyl side chain of previous analogues 13-17 is likely to show limited rotation (considering the side chain component itself) and therefore may adopt a relatively linear projection or trajectory in the PfISPd binding site. A further aim of this analogue series is therefore to increase the extent of flexibility in the BITZ side chain by introducing a structural motif which displays an increase in degrees of freedom. Such a motif would consequently be capable of increased rotation and flexibility, potentially promoting new and improved protein-inhibitor interactions at PfISPd due to reduced ligand rigidity.

In the case of 28 and 30, the phenyl D ring was replaced with distinct functionalities, both of which mimic the previous biphenyl side chain by occupying a comparable area of space but which have the potential to adopt varying and more diverse conformations and structural orientations. Considering compound 28, an ether-containing hydrocarbon chain links the phenyl C ring to a terminal morpholine heterocycle, notably increasing rotatable bond number within the compound. Indeed, the side chain of 28 contains four rotatable bonds compared to the side chain of 4 (Figure 3.3 and Table 3.1), which has only two. In compound 30, the phenyl D ring is replaced with the aromatic and polar pyrazole ring, a generally well tolerated and metabolically stable group, which is linked directly to piperidine. The respective morpholine and piperidine heterocycles were incorporated as terminal heterocyclic rings to aid compound solubility.

For the synthesis of compounds 28 and 30, two separate synthetic routes were developed, outlined in Scheme 3.7 and Scheme 3.8 respectively. As has been the case for many BITZ compounds, synthesis was initiated with the generation of the specified side chains of 28 and 30, before attachment of the BITZ motif in a final synthetic step using the acid chloride X (Scheme 3.2).

To generate 28, a three-step synthesis was required, initiated by a Sn2-substitution between commercially available 4-(2-chloroethyl)morpholine and 3-nitrophenol to give the 4-(2-(3-nitrophenoxy)ethyl)morpholine intermediate 27a (Scheme 3.7). 27a was subject to hydrogenation, converting the meta-nitro group of 27a into the corresponding 1° amine 27b; the final acid-chloride mediated amide coupling and cyclisation with X produced the target biphenyl BITZ mimetic 28 (Scheme 3.7).
Scheme 3.7 Synthetic strategy for BITZ biphenyl mimetic compound 28.

The synthesis of 28 was relatively straightforward with high yielding reactions for the first two synthetic steps. However, the final acid-chloride coupling and cyclisation was particularly low yielding, which is generally inconsistent with the synthesis of other analogues. In contrast, the synthesis of 30 proceeded with moderate to high yields across all steps. Compound 30 also required three synthetic steps, initiated by a Suzuki-Miyaura cross-coupling reaction between 3-bromoaniline and a pinacol ester boronic acid derivative (Scheme 3.8) to generate the Boc-protected side chain 29a. 29a was coupled to the BITZ core via reaction with the acid chloride X\textsuperscript{25,27} which proceeded in high yield to give the Boc-protected structure 29b. The final synthetic step involved the acid-mediated Boc-deprotection of 29b to generate BITZ compound 30 (Scheme 3.8).

Scheme 3.8 Synthetic strategy for BITZ biphenyl mimetic compound 30.
3.6.2 PfIspD Enzymatic and Whole Cell Activity Data

As was the aim with these biphenyl side-chain mimetics, the distribution coefficients of compounds 28 and 30 were significantly reduced compared to previous biphenyl BITZ derivatives 4-10, 13-15 and 17 (Table 3.1 and Table 3.2). The significant decrease in distribution coefficients was also represented by a large increase in the predicted aqueous solubility of compounds 28 and 30 (Table 3.5). In particular, incorporation of the pyrazole ring and polar piperidine moiety into 30 resulted in a compound that displays a significantly lower predicted distribution coefficient than one of the most potent BITZ inhibitors to date, the biphenyl analogue 6, with greater than a 3-fold reduction in predicted ClogD and more than a 400-fold increase in aqueous solubility.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>PfIspD IC₅₀ (μM)</th>
<th>Whole cell 3D7 EC₅₀ (μM)</th>
<th>MW (g/mol⁻¹)</th>
<th>ClogD</th>
<th>CaqSol (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>0.340 ± 0.018</td>
<td>5.15</td>
<td>356.12</td>
<td>2.52</td>
<td>119.1</td>
</tr>
<tr>
<td>30</td>
<td>0.232 ± 0.002</td>
<td>8.17</td>
<td>376.14</td>
<td>1.64</td>
<td>159.9</td>
</tr>
</tbody>
</table>

Table 3.5 Inhibitory activity of compounds 28 and 30 against PfIspD and P. falciparum growth in culture (strain 3D7); calculated ClogD and CaqSol values given.

The resulting biological activities of compounds 28 and 30, show that both analogues have nanomolar activity against the PfIspD enzyme (Table 3.5). These results were highly encouraging, particularly in the case of 30, since the level of measured enzymatic activity is equivalent to that displayed by the methoxy and trifluoromethyl biphenyl BITZ analogues 4 and 6 (Table 3.1), which are two of the most potent PfIspD BITZ inhibitors generated to date. This therefore demonstrates that compounds with reduced lipophilicity are also capable producing potent inhibition of the PfIspD target. This is highly encouraging for forward inhibitor design and the improvement of physiochemical compound properties for lead optimisation. Compounds 28 and 30 both conform to Lipinski’s Rule of Five, defining parameters for likely compound bioavailability,[18] and the GSK ‘4/400’ guideline, relating compounds with a ClogP of < 4 and a molecular weight of less than 400 with more favourable ADMET profile.[38]

However, neither 28 nor 30 were able to translate observed activity against PfIspD into whole cell parasite growth inhibition (Table 3.5). This is potentially an effect of reduced compound lipophilicity and a consequent decrease in cellular permeability within biological systems; despite a significant increase in aqueous solubility that may have been thought to aid compound absorption.
Chapter 3

3.7 Variations to the Inhibitor C Ring

3.7.1 Design Rationale and Synthesis

Having considered a number of structural modifications involving functional changes around the D ring of the BITZ side chain, a discrete compound series was developed to assess the effects of structural variations at the C ring of the BITZ inhibitor construct. Alterations were made to the C ring system itself, as well as revisions to the linker functionality between the BITZ core and the adjacent C ring (Figure 3.9) within a strategy that aimed to mirror a design approach outlined by Luo et al.[41]

![Chemical structures of BITZ compounds 33-35.](image)

Figure 3.9 Chemical structures of BITZ compounds 33-35.

Work by Luo et al. describes the synthesis and evaluation of multi-target-directed ligands, generated by fusing the cholinesterase inhibitor donepezil and the antioxidant Ebselen (Figure 3.10), for the treatment of Alzheimer’s disease.[41] Ebselen (or 2-phenyl-1,2-benisoselenazol-3(2H)-one) is an organoselenium compound, categorised chemically as an electrophile, and also a lipid-soluble molecule which can readily enter the cells of biological systems.[41,42] Ebselen is a definitive example of a glutathione peroxidase mimic, catalysing the reduction of reactive oxygen species through reactions with specific cysteine thiol groups in proteins, and is therefore a promising synthetic antioxidant.[42,43] Ebselen is a clinically safe compound with a well-known pharmacology profile. It is currently part of a number of clinical trials for the prevention and treatment of various disorders including cardiovascular disease, arthritis and cancer.[44]
Figure 3.10 Design strategy and chemotype fusion conducted by Luo et al. towards the generation of novel compounds for the treatment Alzheimer’s disease; figure reproduced from \cite{41}.

Luo et al. adopted a design principle that involved the fusion of the two pharmacophores, Donepezil and Ebselen, generating new hybrid molecules and a fused ligand construct, without major structural modifications to the selected pharmacophores (Figure 3.10). The resulting hybrid molecules possessed the strong acetylcholinesterase inhibitory activity of Donepezil as well as many of the pharmacological benefits of Ebselen: notably its antioxidant and anti-inflammatory properties which are closely related to the aetiology of Alzheimer’s disease and is therefore highly appropriate to this indication.\cite{41}

Combination of the core Ebselen motif with the Donepezil side chain produced a fused ligand structure with a saturated heterocyclic ring as a surrogate structure to the adjacent phenyl ring system of Ebselen, with an alkyl linker connecting the two entities (Figure 3.10). Luo et al. subsequently demonstrated that combination of these structural components produced optimal pharmacology, including appropriate parameters of lipophilicity and solubility, whilst retaining potent acetylcholinesterase inhibitor activity.\cite{41} As a result of this research, structural modifications were introduced to BITZ inhibitors to mirror the scaffold of the fused inhibitors designed by Luo et al.

The close structural similarity between Ebselen (Figure 3.10) and our simplified BITZ chemotype core inhibitor structure 31 (Figure 3.11), was an additional driving force behind the rationale of this approach. The phenyl C ring of BITZ inhibitors was replaced with a saturated, non-aromatic piperidine group with a methylene linker to a conserved aromatic D ring where no further substitution was present (Figure 3.9).
Further to replacement of the C ring system, variations to the length of the alkyl linker between the BITZ core and heterocyclic C ring were also made: n was varied between 0 and 2 generating the corresponding BITZ compounds 33-35 (Figure 3.9 and Table 3.6). The overall aim of this compound series was to mirror the successes outlined by Luo et al.\cite{41} through introduction of a non-aromatic, surrogate C ring to determine the direct effects on PfISP\(D\) enzyme activity. This inhibitor series also aimed to introduce increased ligand flexibility by extension of the alkyl linker and optimise the physiochemical properties of resulting analogues with reduced lipophilicity and enhanced solubility.

Due to the commercial availability of the required benzyl-piperidine 1° amine compounds 32a-c (Scheme 3.9), the synthesis of each target compound within this series required only a single synthetic step. The purchased amino benzyl-piperidine starting materials 32a-c were reacted with the stable acid-chloride X in the previously discussed simultaneous amine coupling and BITZ ring closure\cite{25,27} (Chapter 2, Section 2.3.3) to generate the designed BITZ analogues 33-35 (Scheme 3.9) in moderate to good yields.

![Scheme 3.9 Synthetic strategy for BITZ analogues 33-35.](image)

### 3.7.2 PfISP\(D\) Enzymatic and Whole Cell Activity Data

Whilst the distribution coefficients of analogues 33-35 are low, with predicted ClogD values of consistently less than three; extension of the alkyl linker between the BITZ headgroup and inhibitor side chain was accompanied by a notable decrease in the aqueous solubility of resulting compounds (Table 3.6). Most significantly, 34 is ~ 2.5 times less soluble than analogue 33, which is the consequence of introducing a methylene linker between the BITZ core and side chain, rather than
direct attachment as with 33. In comparison, introducing an ethylene linker group (35) between the BITZ core and C ring produces ~ a 3.25-fold loss of aqueous solubility, comparing 33 with 35, but which only corresponds to a further decrease in aqueous solubility of about a third when considering replacement of the methylene linker, 34, with the ethylene group, 35. Generally, the molecular weight of compounds in this series is slightly below that of previous biphenyl mimetic BITZ analogues.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>n</th>
<th>PfIspD IC₅₀ (μM)</th>
<th>Whole cell 3D7 EC₅₀ (μM)</th>
<th>MW (gmol⁻¹)</th>
<th>ClogD</th>
<th>CaqSol (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>0</td>
<td>8.22</td>
<td>13.28</td>
<td>324.13</td>
<td>2.94</td>
<td>225</td>
</tr>
<tr>
<td>34</td>
<td>1</td>
<td>40.1</td>
<td>7.45</td>
<td>338.15</td>
<td>2.84</td>
<td>88.59</td>
</tr>
<tr>
<td>35</td>
<td>2</td>
<td>&gt; 100</td>
<td>2.00</td>
<td>352.16</td>
<td>2.90</td>
<td>69.21</td>
</tr>
</tbody>
</table>

*Table 3.6 Inhibitory activity of compounds 33-35 against PfIspD and P. falciparum growth in culture (strain 3D7); calculated ClogD and CaqSol values given.*

Disappointingly, biological testing highlighted that none of the analogues 33-35 can be considered active inhibitors of either the PfIspD enzyme or whole cell 3D7 growth since all PfIspD IC₅₀ values were > 5 μM and whole cell 3D7 growth inhibition was measured in the mid to low micromolar region.

Whilst it is therefore clear that 33-35 are inactive at both the PfIspD enzyme and the level of the whole cell, directly opposing activity trends can clearly be seen when comparing enzymatic and whole cell biological activity (*Table 3.6*). Lengthening the alkyl chain between the BITZ core and piperidine C ring resulted in a rapid loss of PfIspD enzyme activity, as highlighted by 34 and 35 which are increasingly inactive against PfIspD. In contrast, a slight improvement in parasite growth inhibition is observed as the alkyl chain linker is extended through n = 0 to n = 2. This possibly reflects a slight increase in lipophilicity caused by extending the alkyl linker region which will aid the permeation of resulting compounds on exposure to a biological system, likely facilitating enhanced whole cell inhibition due to improved compound access to the cell and improved permeation of biological membranes.

These results therefore suggest that maintaining aromaticity in the C ring of BITZ inhibitors is required to conserve both inhibition of the PfIspD enzyme and Plasmodium parasite growth. Furthermore, resulting data highlights that direct Naryl attachment between the BITZ core and side chain represents an optimal structural arrangement.
3.8 Alkyl-amino Biphenyl BITZ Analogues

3.8.1 Design Rationale and Synthesis

As a result of the micromolar PfIspD inhibitory activity of 9 (Table 3.1), the biphenyl para-hydroxy BITZ compound, a series of alkyl amino derivatives of this analogue have been synthesised and evaluated. In addition to encouraging inhibitory enzyme activity, 9 displays slightly enhanced physiochemical properties compared to other analogues in the biphenyl series (Table 3.1). Inhibitor 9 has the lowest predicted ClogD value of all the biphenyl analogues and slightly improved aqueous solubility, almost 10-fold of that displayed by the potent CF₃ analogue 6. Therefore, the generally poor aqueous solubility of biphenyl BITZ analogues (15 and 16 are exceptions) supported the decision to synthesise a further BITZ compound series, comprising three analogues 37-39 (Figure 3.12) which incorporate an alkyl-amino substituent ortho- to the para-hydroxy group in the D ring. The rationale of this design motif was to improve the aqueous solubility of resulting structures and promote parasite growth inhibition through improved cellular accumulation due to increased BITZ compound concentration at the PfIspD enzyme target.

Figure 3.12 Chemical structures of BITZ compounds 37-39.

Further support of this design rationale was provided by work conducted by Cooke et al.\[45\] which demonstrated the successful use of similar alkyl-amino groups in generating water-soluble compounds that retained the inhibitory activity of their more lipophilic counterparts, in a drug discovery program based around the anaesthetic activity of propofol analogues.\[45\] Propofol (2,6-diisopropylphenol) is used extensively as an intravenous anaesthetic which acts on GABAₐ receptors as a positive allosteric modulator of γ-aminobutyric acid (GABA).\[46\] However, the administration of propofol is complicated by the fact that is must be formulated as an emulsion since it lacks water solubility.\[47\] Consequently, research by Cooke et al. aimed to produce a number of water-soluble propofol analogues, containing para-alkylamino substituents which were found to successfully maintain good intravenous anaesthetic activity in rodents.\[45\]
Figure 3.13 Chemical structure of water-soluble Propofol analogues designed and synthesised by Cooke et al. R₂ is representative of a variety of tertiary amines. Figure reproduced from [45].

Within this research, synthesis of 37-39 was achieved using the biphenyl methoxy BITZ inhibitor 4 as a starting point. De-methylation of 4 generated the corresponding biphenyl hydroxy inhibitor 9 which was able to undergo Mannich base reactions with various 2° amines to generate the required alkyl-amino biphenyl BITZ derivatives 37-39 (Scheme 3.10). In contrast to initial syntheses of 4 (Scheme 3.1), the alternative acid-chloride X mediated synthesis of BITZ derivatives[25,27] was employed to generate the required quantities of 4 for the described synthesis towards 37-39 (Scheme 3.10). The generation of 4 by this alternative route was highly successful: both the Suzuki-Miyaura cross-coupling reaction between 3-bromoaniline and 4-(methoxy)phenyl boro nic acid, generating the side chain 36, and the subsequent amide coupling and ring closure with acid chloride X, to generate compound 4, proceeded without complication and in high yield.

Scheme 3.10 Synthetic strategy for alkyl-amino BITZ analogues 37-39.

De-methylation of 4 to 9 using boron tribromide[48] proceeded rapidly but in low yield. Conversion of 9 to BITZ alkyl-amino derivatives 37-39 was achieved using a Mannich base synthesis. This involved reaction of 9 with aqueous formaldehyde solution (37 wt. % in H₂O) and the appropriate 2° amine in a 7:3 ethanol:water mixture under reflux conditions.[45] The Mannich reaction is a classic method of preparing β-amino ketones and aldehydes and is widely used as a chemical transformation which facilitates C-C bond formation and the construction of nitrogen-containing compounds.[49,50] Whilst the synthesis and isolation of 37-39 was achieved using the outlined synthetic strategy (Scheme 3.10),
the yields of the Mannich base reactions were consistently poor ranging from 6-40% over the synthesis of the three analogues 37-39. The isolation and purification of these compounds was also very challenging; it is therefore likely a combination of poor reaction yields, the generation of side products and high compound polarity made purification difficult. In addition, all three Mannich base syntheses had to be performed on small reaction scales due to the poor yield of the previous de-methylation step, further hindering the ease of synthesis and successful product isolation.

3.8.2 PfISPd Enzymatic and Whole Cell Activity Data

The design rationale of the alkyl-amino biphenyl BITZ motif was to promote the aqueous solubility of compounds within the series. Whilst compounds 37-39 demonstrated acceptable distribution coefficients, only the pyrrolidine analogue 39 was predicted to have good aqueous solubility, almost equivalent to that of the heterocycle-linked biphenyl BITZ analogues 15 and 16 (Table 3.2).

<table>
<thead>
<tr>
<th>Compound number</th>
<th>NR&lt;sub&gt;z&lt;/sub&gt;</th>
<th>PfISPd IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>Whole cell 3D7 EC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>MW (gmol&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>ClogD</th>
<th>CaqSol (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>Morpholine</td>
<td>0.501</td>
<td>0.736</td>
<td>418.14</td>
<td>3.96</td>
<td>7.245</td>
</tr>
<tr>
<td>38</td>
<td>Diethylamine</td>
<td>2.33</td>
<td>0.438</td>
<td>404.16</td>
<td>3.61</td>
<td>33.53</td>
</tr>
<tr>
<td>39</td>
<td>Pyrrolidine</td>
<td>0.507</td>
<td>0.133 ± 0.016</td>
<td>402.14</td>
<td>3.42</td>
<td>52.59</td>
</tr>
</tbody>
</table>

Table 3.7 Inhibitory activity of compounds 37-39 against PfISPd and *P. falciparum* growth in culture (strain 3D7); calculated ClogD and CaqSol values given.

Interestingly, a slight loss in PfISPd enzymatic activity was observed with this compound series, particularly in the case of the dialkylamino analogue 38, which showed more than a five-fold reduction in activity against PfISPd compared to 15 (Table 3.2). This is somewhat surprising given that the predicted physiochemical properties of analogues 37-39 are largely comparable to those predicted for the heterocycle-linked biphenyl series (Section 3.3, Table 3.2), which generally show improved activity at the enzyme.

In contrast to this observation, the measured whole cell 3D7 activity of the alkyl-amino compound series (Table 3.7) is more potent than that of the heterocycle-linked biphenyl series (Section 3.3, Table 3.2), with the most potent pyrrolidine analogue 39 demonstrating a 3D7 EC<sub>50</sub> value of 133 nM. Indeed, alkyl-amino analogues 37-39 demonstrate increased activity against the parasite, producing noticeably improved inhibitory activity against 3D7 parasite growth than the PfISPd enzyme. This observation may be an effect of improved compound solubility, although this is not uniform across the series, but is best supported in the case of the pyrrolidine analogue 39 which displays enhanced predicted aqueous solubility and the most potent whole cell growth inhibition within the series.
Alternatively, heightened inhibitory activity against the whole cell compared to that seen at the PfIspD enzyme may lead to the suggestion that these compounds are capable of exerting inhibitory effects at additional biological targets within *P. falciparum* (i.e. off target effects that are not specific to the PfIspD enzyme). Further exploration of this suggestion is needed since off-target activity can complicate and confuse observed phenotypic effects, making the interpretation of biological activity difficult, as well as increasing the risk of unwanted associated toxicities.\[^{51}\]

### 3.9 Aminoquinoline-BITZ Hybrid Compounds

#### 3.9.1 Design Rationale and Synthesis

The exploration of an alternative design rationale led to the design and synthesis of a series of BITZ-aminoquinoline hybrid analogues. This structurally distinct BITZ compound series incorporates the BITZ chemotype, using various hydrocarbon linkers (R^3\), into the familiar antimalarial chemotype of 7-chloro-4-aminoquinoline (Figure 3.14).

Molecular hybridisation is a useful strategy in drug design and provides an attractive and alternative approach to the design of antimalarial chemotherapies as requirements shift to focus on the development of novel and affordable drugs which can prevent resistance development.\[^{52}\] There are now many examples of this design approach within antimalarial drug discovery programs which have been well reviewed and discussed extensively throughout literature; some key applications of this design approach are summarised in the following publications.\[^{52-55}\]

Molecular hybridisation aims to combine different pharmacophores of biologically active and distinct materials to produce new hybrid compounds that show affinity at the molecular targets of the component pharmacophores and therefore increase phenotypic efficacy compared to the individual pharmacophores of the hybrid structure.\[^{56,57}\] This methodology identifies covalent linkage points between two or more pharmacophoric motifs, enabling the stable assembly of specific functionalities to generate a single unit and new drug entity which is designed to perform as a dual-acting compound. Resulting hybrid molecules are typically larger than the component drugs and, as a result, are often more lipophilic. It is therefore important that the incorporated linker region or functional group between component pharmacophores is as simple as possible.\[^{52}\]
Figure 3.14 Chemical structures of aminoquinoline-BITZ hybrid compounds 42-47.

Such an approach to compound design is particularly appropriate for the development of new antimalarial agents which aim to combat the onset of drug-resistant *Plasmodium* parasites by generating multi-targeted and pharmacologically enhanced antimalarial compounds.\[58\] In this context, the hybridisation strategy aims to incorporate key pharmacophoric features (and the associated biological behaviours) from existing antimalarial drugs, and design novel molecules of new chemical structure which display efficacy and an improved resistance profile. This approach has been successful within a number of drug design efforts, generating novel and effective chemical agents against protozoan parasites.\[54\]

Combination therapies which employ the simultaneous use of two or more antimalarial drugs, administering two or more chemical agents with differing modes of action, have frequently been used as a therapeutic approach to reduce the effects of *Plasmodium* parasite drug resistance. New generations of hybrid antimalarial compounds provide an alternative to combined administration therapies of two or more individual drugs, and have the associated advantage of lower drug-drug interaction risks since two pharmacophores are combined in the same molecular entity rather than co-administered. In addition, this approach can also promote improved treatment adherence.\[54,59,60\]

Applying the concept of hybrid agents to antimalarial drug design can be challenging for a number of reasons. Firstly, the complexity of the *Plasmodium* parasite life cycle means only a small number of validated targets are known across the life cycle. Added to this, an incomplete understanding of *Plasmodium* parasite biology may compromise and add difficulty to hybrid drug design in antimalarial therapy.\[52\] However, if these obstacles are overcome, the molecular outcomes and resulting therapeutic effects can be highly rewarding as outlined in reviews by Walsh\[55\] and Muregi\[54\] as well as in the work carried out by Pinheiro *et al.*\[60\] Pinheiro *et al.* demonstrated the success of the hybrid design approach in aiming to enhance to the anti *P. falciparum* activity of quinolone derivatives by designing of a series of hybrid quinolones containing the pharmacophoric groups of 7-chloroquinoline and arylsulfonamide. Pinheiro *et al.* connected these two structural entities with a linker group that
was distinct from the pharmacophore framework of either parent drug: chloroquine (CQ) and sulfadoxine.\[^{[60]}\]

To mirror this approach, a series of synthetic hybrid BITZ-aminoquinoline compounds (42-47, Figure 3.14) have been designed with the aim of generating dual-targeted antimalarial agents with enhanced activity against *P. falciparum* parasite growth and which show good PK and resistance profiles. Inclusion of the 7-chloroquinoline pharmacophore was desirable due to its presence in CQ, representing one of the most successful antimalarial compounds within the history of antimalarial drug design.\[^{[24,61,62]}\] The BITZ headgroup remained the selected chemotype of choice following our HTS screen and identification of the DT2009-0168020 hit (Figure 3.15).

**Figure 3.15** Rational approach to drug design of hybrid compounds 42-47.

Extensive research over the last few decades has highlighted that the 7-chloro-4-aminoquinoline nucleus (Figure 3.14) is essential for the antimalarial activity of 4-aminoquinoline compounds, enabling the inhibition of β-haematin formation and accumulation of this antimalarial chemotype at the target site of the parasite food vacuole.\[^{[63]}\] In contrast, the BITZ chemotype is proposed to target and inhibit the *Pf*IspD enzyme within the MEP pathway, thereby disrupting isoprenoid precursor biosynthesis and preventing parasite growth, which ultimately causes parasite death. The combination of these two pharmacophores therefore aims to produce a series of compounds which are capable of behaving by a dual-mechanism of action. To adhere to suggestions from literature, a range of simple linker groups have been incorporated to covalently join the two pharmacophoric
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systems. These include simple alkyl chains, aromatic rings, branched alkyl chains and saturated ring systems (Figure 3.14 and Table 3.8).

The synthetic route to generate 42-47 is shown in Scheme 3.11 and is comprised of three synthetic steps. The commercial availability of a number mono-Boc-protected diamines enabled the synthesis of 42-47 to be initiated from mono Boc-protected hydrocarbon linker groups (R³) that were incorporated into each analogue. The presence of a second free amine within these N-Boc-protected diamines (Scheme 3.11) facilitated amide coupling with acid-chloride X (Scheme 3.2) to generate the BITZ pharmacophore and Boc-protected intermediates 40a-f. TFA-mediated deprotection of intermediates 40a-f revealed a free 1° amine functionality in the resulting structures 41a-f, facilitating the final substitution step of the synthesis.

Scheme 3.11 Synthetic strategy for the aminoquinoline-BITZ hybrids 42-47.

To afford the target BITZ-aminoquinoline hybrid structures, an aromatic nucleophilic substitution was employed as the final synthetic step of the synthesis. A similar synthetic approach was previously outlined by Apelt et al. in their preparation of non-imidazole histamine H₃ receptor ligands.[64] To generate the histamine H₃ receptor antagonists, Apelt et al. combined the required amino molecular component with a range of chloroquinolines, including 4,7-dichloroquinoline, using phenol as the solvent and heating to 140°C for 12 hours. A comparable synthesis, using phenol and heating to 140°C in a sealed tube, was successfully employed for the generation of 42 and 43. However, a significant drop in reaction yield following the synthesis of 43 highlighted that alternative reaction conditions should be considered for the more sterically bulky R³ linkers. Indeed, notable alterations were needed regarding the solvent and reaction temperature to generate BITZ-hybrid analogues when R³ was saturated or bulky.
Apelt et al. suggested that the use of phenol as a solvent increases the reactivity of the quinolone component by enhancing stability of the leaving group. It was also found that when using 4,7-dichloroquinoline, aromatic substitution only took place at the 4-position due to the low electrophilic activity at the 7-position. Whilst phenol achieved aromatic nucleophilic substitution between 4,7-dichloroquinoline and 41b and 41a, with poor to moderate yields respectively, extensive refinement of synthetic conditions was needed in order to generate 44-47.

For the synthesis of 44, using 41c and 4,7-dichloroquinoline, anhydrous ethanol was used in replacement of phenol due to its lower boiling point, easing solvent removal and purification of the crude material. In the synthesis of 45 and 46, no reaction was observed between 4,7-dichloroquinoline and the required amines 41d and 41e after 12 hours. Therefore, two equivalents of DIPEA were added to each reaction and the resulting solutions heated to a temperature of 110°C in a sealed tube for two days. DIPEA was introduced with the aim of promoting the nucleophilic substitution between the non-aromatic, saturated amine intermediates 41d and 41e and 4,7-dichloroquinoline: however, this was not achieved.

Replacement of ethanol with isopropanol (IPA), with the addition of triethylamine (4.0 eq) and extension of the reaction time to four days at 140°C, was trialled in the synthesis of 45 and 46. IPA was proposed to improve reagent solubility and achieve better material dissolution under reflux; however, no reaction was observed with either analogue.

Since the use of high temperatures over extended reactions times and the use of various solvents was unsuccessful in the generation of 45-47, harsher reaction conditions were evidently required. To circumvent the issue of slow and sluggish nucleophilic substitution, microwave reactor-mediated syntheses were attempted using the appropriate BITZ-amine intermediate 41d-f and 4,7-dichloroquinoline in IPA, in the absence of base which proved to be a successful method for the generation of 45-47. Two short heating intervals at 190-200°C was used in the microwave-mediated generation of 45; whereas a single temperature elevation to 190°C, over 45 minutes to 1 hour, was optimal for the generation of 46 and 47.

3.9.2 PfIspD Enzymatic and Whole Cell Activity Data

It was anticipated that designed aminquinoline-BITZ hybrid compounds 42-47 (Figure 3.13) would retain activity against the PfIspD enzyme due to the presence of the BITZ pharmacophore which remained accessible as a chemical entity in the hybrid structures. However, it was also considered probable that increased activity against Plasmodium parasite growth, compared to PfIspD, could be seen with these compounds. Furthermore, it was plausible that 42-47 may display greater efficacy
against *Plasmodium* parasite growth than the *Pf*ispD target itself due to the hybrid nature of the compounds, designed for the purpose of dual-activity.

As expected with the approach of compound hybridisation, **42-47** are larger in molecular size than earlier BITZ analogues, which are based around the inhibitory BITZ motif alone (see Table 3.1 for representative examples **4-10**). The average MW of hybrid compounds **42-47** is 407.41 g mol⁻¹, which contrasts with the lower average MW of BITZ inhibitors **4-10** which is 345.34 g mol⁻¹. Comparing predicted PK parameters of these two inhibitor classes, aminoquinoline-BITZ hybrid compounds **42-47** and biphenyl BITZ derivatives **4-10** display similar distribution coefficients (ClogD) and solubility (CaqSol) values, as predicted by AZ⁸,⁹ (Table 3.1 and 3.8). It should also be noted that the aminoquinoline-BITZ hybrids consistently display poor predicted aqueous solubility (Table 3.8). CaqSol values of < 10 μM were predicted across **42-47** which does not meet our defined aqueous solubility threshold of > 50 μM (Section 3.2.2).

<table>
<thead>
<tr>
<th>Compound number</th>
<th>R²</th>
<th><em>Pf</em>ispD IC₅₀ (μM)</th>
<th>Whole cell 3D7 EC₅₀ (μM)</th>
<th>MW (g mol⁻¹)</th>
<th>ClogD</th>
<th>CaqSol (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>Ethyl</td>
<td>19.0</td>
<td>-</td>
<td>355.05</td>
<td>3.73</td>
<td>6.47</td>
</tr>
<tr>
<td>43</td>
<td>1,5-Phenyl</td>
<td>5.84</td>
<td>-</td>
<td>403.05</td>
<td>4.28</td>
<td>0.67</td>
</tr>
<tr>
<td>44</td>
<td>2-Methyl-1,5-phenyl</td>
<td>&gt; 10</td>
<td>-</td>
<td>471.07</td>
<td>4.50</td>
<td>0.71</td>
</tr>
<tr>
<td>45</td>
<td>1,4-Cyclohexyl</td>
<td>3.757 ± 0.881</td>
<td>0.091 ± 0.048</td>
<td>409.10</td>
<td>3.93</td>
<td>3.22</td>
</tr>
<tr>
<td>46</td>
<td>1,6-Cyclohexyl</td>
<td>&gt; 15</td>
<td>&gt; 100</td>
<td>409.10</td>
<td>4.25</td>
<td>3.17</td>
</tr>
<tr>
<td>47</td>
<td>2-Dimethyl-propane</td>
<td>3.673 ± 0.564</td>
<td>0.015 ± 0.006</td>
<td>397.10</td>
<td>4.40</td>
<td>3.41</td>
</tr>
</tbody>
</table>

Table 3.8 Inhibitory activity of compounds **42-47** against *Pf*spD and *P. falciparum* growth in culture (strain 3D7); compound MW and calculated ClogD and CaqSol values are given.

As anticipated, the aminoquinoline-BITZ hybrid series displayed enhanced activity against *P. falciparum* growth compared to activity against *Pf*spD; where compounds **45** and **46** explicitly demonstrate this effect. Compound **45** displays activity against *Plasmodium* parasite growth (EC₅₀ = 0.091 ± 0.048 μM) which is 41 times more potent than that measured against the *Pf*spD enzyme (EC₅₀ = 0.091 ± 0.048 μM). Even more striking are the effects of **47** which most strongly demonstrates this activity distinction. **47** has an IC₅₀ value of 3.673 ± 0.564 μM against *Pf*spD, compared to an EC₅₀ =
0.015 ± 0.006 μM against *Plasmodium* parasite growth, representing a 245-fold increase in potency against the parasite compared to the PfISP-D enzyme target.

Considering the strong activity distinction shown by 45 and 47, the measured activity of 46 could be considered an anomalous result as 46 shown to be inactive against both PfISP-D and *Plasmodium* parasite growth. Since 46 is a regioisomer of 45, it could reasonably be expected to demonstrate comparable biological activity. However, the lack of inhibitory activity demonstrated by 46 may be a consequence of significant steric clashes and the bulky construct of the compound, a consequence of the 1,6-disubstitution pattern within the cyclohexane R3 linker (Figure 3.15 and Table 3.8). The 1,6-disubstitution pattern holds the aminoquinoline and BITZ pharmacophores in very close proximity, generating a large amount of steric bulk and potential molecular clashes within the analogue; this could potentially hinder the performance of 46 in both the PfISP-D enzymatic and *Plasmodium* parasite whole cell growth assays.

Hybrid compounds 42-44 all performed poorly against PfISP-D with activity data outside the required threshold of < 5 μM as a definition of activity against the PfISP-D enzyme. Furthermore, the very poor solubility of these analogues, particularly 43 and 44, meant whole cell activity data couldn’t be obtained for 42-44, which was a disappointing outcome within this analogue series.

### 3.10 Further SAR Explorations

This thesis describes a distinct body of work that was conducted as part of a collaborative research project between the Medicinal Chemistry research group at The University of Liverpool, coordinated by Dr Neil Berry and Professor Paul O’Neill, and the Molecular and Cellular Microbiology group, coordinated by Audrey Odom, at the School of Medicine at Washington University in St Louis. Additional SAR studies have therefore been conducted around the BITZ chemotype by other researchers in the medicinal chemistry group at the University of Liverpool which have focused on structural modifications to the A and B rings of the BITZ core (Figure 3.2).

A series of BITZ compounds have been synthesised, incorporating functional group substitutions to the A ring of the BITZ core (Figure 3.16). The aim of these analogues was to assess if the metabolism profile of BITZ compounds could be altered by the introduction of fluorine or nitrogen into the aromatic A ring, whilst maintaining PfISP-D enzymatic and phenotypic activity. Fluorine is known to modulate the metabolism profile of compounds by reducing the extent Phase I metabolism, through reduced hydroxylation and therefore increased compound stability; whilst the presence of nitrogen within a ring can be used to block a site of metabolism on related analogues.[35,65]
Figure 3.16 Chemical structure of BITZ analogues with modifications to the A ring.

Work within the Liverpool University medicinal chemistry group has therefore generated a number of BITZ compounds, incorporating the described A-ring modifications alongside variations to the BITZ side chain, as depicted by the R group in the C ring (Figure 3.16). Whilst it is not possible to discuss the full scope of this work and range of syntheses here, it can be broadly summarised that A ring substitutions were not well tolerated, producing only moderate to poor levels of activity against the \textit{Pf}IspD enzyme and showing significant losses of whole cell activity against parasite growth.

In other work carried out within the group, the structure of the Ebselen motif was revisited. The close structural similarity between Ebselen (Figure 3.10, Section 3.7.1) and our simplified BITZ chemotype core 31 (Figure 3.11) promoted a series of compounds to be generated, based around the BITZ motif but replacing the sulfur heteroatom with selenium (Se) (Figure 3.17), therefore mirroring the core Ebselen construct. Not only are 31 (Figure 3.11) and 48 (Figure 3.17) highly similar in structure, but Ebselen (2-phenyl-1,2-benzisoselenazol-3(2\textsubscript{H})-one), an electrophilic antioxidant possessing glutathione peroxidase-like activity, has been shown to mediate this activity due through its ability to induce cysteine-targeted oxidation of cellular proteins.\textsuperscript{43} The demonstration of cysteine reactivity is therefore relevant to our own research, since our proposed mechanism of BITZ activity at \textit{Pf}IspD involves formation of a covalent adduct to a cysteine protein residue in the \textit{Pf}IspD active site (see Chapter 4). These comparable methods of protein binding and enzyme inhibition therefore strengthened our interest in the Ebselen chemotype and led to an SAR study generating a series of complimentary BITZ analogues, incorporating selenium as a replacement atom to sulfur in the B ring of the BITZ headgroup (Figure 3.17).

Figure 3.17 Chemical structure of Selenium (Se) BITZ analogues.

Direct comparison of the biological activities of 31 and 48 highlights that replacement of sulfur with selenium in the BITZ motif does not improve phenotypic or \textit{Pf}IspD enzymatic activity (Table 3.9). Whilst both enzymatic and phenotypic activity levels are comparable for 48, a significant, 15-fold loss
of \( PfIspD \) inhibitory activity was observed comparing 48 to the simple BITZ scaffold 31. From this direct comparison, and as an initial assumption, this result provides further evidence that the BITZ core is specifically required to achieve consistent inhibition of the IspD enzyme.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>( PfIspD ) IC(_{50}) (( \mu )M)</th>
<th>Whole cell 3D7 EC(_{50}) (( \mu )M)</th>
<th>MW (gmol(^{-1}))</th>
<th>ClogD</th>
<th>CaqSol (( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>0.5995</td>
<td>5.49</td>
<td>227.04</td>
<td>2.97</td>
<td>112.4</td>
</tr>
<tr>
<td>48</td>
<td>9.374</td>
<td>9.65</td>
<td>274.98</td>
<td>2.48</td>
<td>84.9</td>
</tr>
</tbody>
</table>

Table 3.9 Inhibitory activity of 31 and 48 against \( PfIspD \) and \( P. falciparum \) growth in culture (strain 3D7); MW and calculated ClogD and CaqSol values are given.

Further analogues of the selenium compound series were made with the aim of mimicking the construct of previous BITZ compounds to provide direct measures of activity. Generally, all of the resulting selenium analogues displayed very poor phenotypic activity with consistently weak activity against the \( PfIspD \) enzyme itself. Displaying an exception to this trend are compounds 49-51 (Figure 3.18) which were designed as parallel analogues to the biphenyl benzyl-linked BITZ compounds 13-15 (Figure 3.5 and Table 3.2).

\[
\begin{align*}
\text{Chemical structures of selenium compounds 49-51.}\n\end{align*}
\]

Compounds 49-51 displayed inhibitory \( PfIspD \) activity close to the 100 nM region; however, this was not translated into phenotypic activity, measured at > 2 \( \mu \)M for all three compounds. Considering the biological activities of other selenium analogues generated, it was concluded that SAR around these BITZ mimetics was not tractable since consistently poor phenotypic activity was demonstrated. Further development of this compound series therefore wasn’t continued.
3.11 Biological Evaluation of BITZ Compound Activity at the IspD Enzyme and in Malaria Parasites

3.11.1 BITZ Compound Activity at Alternative Plasmodium spp. Enzymes

Having synthesised and tested a wide range of BITZ derived compounds as targeted inhibitors of the PfIspD enzyme, the potential for broad-spectrum antimalarial activity of this compound class was also considered.

The known and observed capabilities of Plasmodium parasites to develop drug resistance against a wide variety of structurally diverse chemotherapeutic motifs has led to the current state of play with global concerns questioning the longevity and continued efficacy of the current antimalarial pipeline. As previously discussed (see Chapter 1), there is urgent need to develop inexpensive and novel chemotherapies that are effective in the treatment and prevention of malaria, enabling the replacement of current antimalarial agents.\(^66\) It is also essential that new and novel chemical drug classes are identified which show both potent and broad-spectrum antimalarial activity.\(^67\)

Of the protozoan parasite species infective to humans, *P. falciparum* and *P. vivax* present the greatest challenges to public health, contributing to the majority of malarial infections.\(^62\) *P. vivax* infections have lower incidence levels and very low transmission rates compared to *P. falciparum* infections within African countries.\(^68\) However, the geographical distribution of *P. vivax* infections is very broad due to the parasites ability to survive in cooler climates. Consequently, *P. vivax* poses a significant public health threat across many parts of the world with estimations suggesting that this *Plasmodium* species was responsible for 13.8 million cases of malaria in 2015, accounting for approximately half the total malaria cases outside sub-Saharan Africa in that year.\(^69\) Many current antimalarial drug development campaigns focus on the well-studied blood stage of the *Plasmodium* parasite life cycle; however, the *Anopheles* mosquito vector and liver stages of the parasite life cycle also represent good options for eradication campaigns, more specifically targeting *P. vivax* and *P. ovale* species parasites.\(^66\)

In light of this, some of our most potent PfIspD BITZ inhibitors were selected and tested against the purified and recombinant *P. vivax* IspD (PvIspD) enzyme. The selected BITZ compounds (4-6 and 13-15, Table 3.9) demonstrated low micromolar to nanomolar activity against both the *P. falciparum* and *P. vivax* IspD enzymes, successfully inhibiting the two enzyme species. This consequently highlights, not only the efficacy of these compounds, but also their potential to act as broad spectrum antimalarial agents.
### Table 3.9

<table>
<thead>
<tr>
<th>Compound number</th>
<th>PfIspD IC₅₀ (μM)</th>
<th>Whole cell 3D7 EC₅₀ (μM)</th>
<th>PvIspD IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.206 ± 0.089</td>
<td>0.920 ± 0.066</td>
<td>0.054 ± 0.003</td>
</tr>
<tr>
<td>5</td>
<td>0.073 ± 0.020</td>
<td>1.080 ± 0.164</td>
<td>0.057 ± 0.013</td>
</tr>
<tr>
<td>6</td>
<td>0.273 ± 0.015</td>
<td>0.646 ± 0.021</td>
<td>0.041 ± 0.018</td>
</tr>
<tr>
<td>13</td>
<td>0.400 ± 0.082</td>
<td>0.413 ± 0.082</td>
<td>0.050 ± 0.016</td>
</tr>
<tr>
<td>14</td>
<td>0.862 ± 0.107</td>
<td>0.323 ± 0.092</td>
<td>0.240 ± 0.100</td>
</tr>
<tr>
<td>15</td>
<td>0.446 ± 0.075</td>
<td>0.416 ± 0.006</td>
<td>0.062 ± 0.026</td>
</tr>
</tbody>
</table>

Table 3.9 Inhibitory activity of 4-6 and 13-15 (mean and SEM; n ≥ 3) against PfIspD and PvIspD enzyme homologues and corresponding inhibitory activity against *P. falciparum* parasite growth in culture (strain 3D7).

All the selected BITZ compounds displayed encouraging inhibitory activity against PvIspD, with a number of compounds (4 and 6-13) showing enhanced potency at the PvIspD enzyme compared to PfIspD. Interestingly all BITZ compounds examined, apart from 14, displayed potent activity against PvIspD with IC₅₀ values consistently < 100 nM (Table 3.9). Also of note, all BITZ compounds evaluated in Table 3.9, with the exception of 5, demonstrate sub-micromolar to nanomolar activity against both the PfIspD and PvIspD enzymes, as well as *P. falciparum* parasite growth. 5 is in fact the most potent BITZ inhibitor identified against the PfIspD enzyme with an IC₅₀ value of 73 ± 20 nM, but which demonstrates a slight loss of activity against parasite growth (Table 3.9). In particular, this study highlights that overall BITZ compounds 4 and 6 demonstrate the most potent and consistent inhibition against both the PfIspD and PvIspD enzymes as well as against whole cell 3D7 parasite growth. 4 and 6 can therefore be considered as two of the most potent IspD enzyme inhibitors identified to date, demonstrating strong potential as broad-spectrum antimalarial agents.

### 3.11.2 Correlation Between PfIspD Enzyme Inhibition and Inhibition of *Plasmodium* Parasite Growth

To further evaluate 4 and 6, and reiterate that the observed potency against PfIspD and PvIspD translates directly into antimalarial activity, the relationship between PfIspD enzymatic and whole cell parasite growth inhibition was directly evaluated (Figure 3.19).
Figure 3.19 Correlation of PfIspD enzymatic inhibition and anti-parasitic activity for BITZ inhibitors 4-6 and 13-15.

Dose-dependent inhibition of PfIspD enzyme activity and P. falciparum parasite growth was measured for 4-6 and 13-15 (Table 3.9) and half-maximal inhibitory concentrations determined prior to least-squares linear regression analysis (coefficient of determination, $r^2 = 0.91$; GraphPad Prism: mean activity values given with SEM; $n \geq 3$). Overall, BITZ compounds 4-6 and 13-15 demonstrated good correlation between PfIspD enzyme inhibition and inhibition of Plasmodium parasite growth ($r^2 = 0.91$, Figure 3.19) which indicates that arrest of PfIspD activity corresponds to an impediment of parasite growth. It can therefore be suggested that inhibiting the MEP pathway, through inhibition of the PfIspD enzyme, is able to cause parasite death.

3.11.3 BITZ Compounds Inhibit Isoprenoid Metabolism in Malaria Parasites

To test this indication and establish that observed antiparasitic activity is the result of MEP pathway inhibition: targeted metabolic profiling of MEP pathway intermediates was performed in P. falciparum parasites that were treated with our BITZ PfIspD inhibitors. Early-stage cultures of P. falciparum were treated with BITZ compound concentrations at ~ five times the IC$_{50}$ of inhibitor 4 for 10 hours. To enable a comparative study, separate P. falciparum cultures were also treated with fosmidomycin (FSM) for 10 hours. The MEP pathway intermediates DOXP and MEcPP (Figure 3.12) present within the treated cultures, were quantified by LC-MS/MS (Liquid Chromatography Tandem Mass Spectrometry) analysis and compared to the metabolic levels observed in untreated P. falciparum cultures, as described in previous work by Odom et al. [70]
Scheme 3.12 The MEP Pathway.\textsuperscript{[71,72]}

Similar to the MEP inhibitor, FSM (Chapter 1, Section 1.5.1), BITZ inhibitor 4 produced a significant decrease in MEcPP levels ($p = 0.0042$ \textbf{Figure 3.20}) which are comparable to those observed under treatment with FSM ($p = 0.0038$ \textbf{Figure 3.20}). However, treatment with 4 did not affect the levels of the upstream metabolite, DOXP ($p = 0.94$: \textbf{Figure 3.20}). These results again mirror those seen under comparable treatment of \textit{P. falciparum} cultures with the established IspC inhibitor, FSM,\textsuperscript{[73]} and therefore confirm that BITZ compounds are capable of inhibiting the \textit{Plasmodium} MEP pathway at levels that affect asexual parasite growth.

\textbf{Figure 3.20} Bar graph demonstrating that, comparable to FSM, BITZ compound 4 inhibits the MEP pathway in \textit{P. falciparum} shown with the targeted metabolic profiling of MEP pathway intermediates. Mean and standard error values from three separate experiments displayed. Asterisks (*) indicate significance threshold (alpha) = 0.05.
This study and observed data therefore highlights that the treatment of *P. falciparum* with growth-inhibitory concentrations of BITZ compounds is sufficient to disrupt *de novo* production of isoprenoid precursors through MEP pathway inhibition. Consequently, these findings strongly indicate that BITZ compounds inhibit parasite growth through cellular inhibition of the MEP pathway, by targeting the *Pf*IspD enzyme.

### 3.11.4 BITZ Compound Activity in the Presence and Absence of IPP Supplementation

In malaria parasite cultures, supplementation with the isoprenoid precursor, isopentyl pyrophosphate (IPP) ([Scheme 3.12](#)) has been demonstrated to rescue MEP pathway dysfunction. Indeed, IPP supplementation has been shown to allow the continuation of *Plasmodium* parasite growth during treatment of parasite cultures with the IspC inhibitor, FSM, and the IspD inhibitor, 1R,3S-MMV008138.[74–76] In order to determine whether BITZ compound inhibition is specific to activity against the MEP pathway, we tested for observed IPP rescue during treatment of *P. falciparum* cultures with BITZ compound 8.

Parasite growth inhibition assays were performed in both the presence and absence of supplementation with the MEP pathway product and isoprenoid precursor, IPP. Over the course of this study, *Plasmodium* parasite growth rescue was not observed following the supplementation of *P. falciparum* cultures with IPP that had initially been treated with 8 ($p = 0.75$, [Table 3.10](#)). The previously observed and acknowledged correlation between *Pf*IspD enzymatic inhibition and parasite growth inhibition ([Figure 3.19](#)), as well as metabolic analysis of MEP pathway intermediates ([Figure 3.20](#)), suggest that parasite growth inhibition by treatment with BITZ compounds involves inhibition of the IspD enzyme. However, the observed lack of rescue by IPP supplementation suggests that off-target activity may also contribute to parasite growth inhibition.[77]

<table>
<thead>
<tr>
<th><em>Plasmodium</em> parasite growth conditions</th>
<th>Whole Cell 3D7 Growth Inhibition EC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No supplementation</td>
<td>0.92 ± 0.066</td>
</tr>
<tr>
<td>200 µM IPP</td>
<td>0.98 ± 0.24</td>
</tr>
</tbody>
</table>

(Table 3.10) Activity of 8 against *Plasmodium* parasite growth in both the presence and absence of IPP supplementation; mean EC₅₀ data and SEM given for $n \geq 3$.[77]

Although side-by-side experiments conducted with the IspC inhibitor, FSM, demonstrated IPP rescue of FSM-treated parasite cultures, we did not observe rescue of *Plasmodium* parasites that were first treated with BITZ compound 8 ([Table 3.10](#)). Since IPP rescue is not observed, it must therefore be rationalised that the apparent inhibition of *P. falciparum* parasite growth by BITZ derived compounds does not only result from the established activity against the IspD enzyme. However, further studies
can be conducted that may shed light on additional targets which BITZ compounds may be capable of inhibiting, potentially contributing towards the observed inhibition of *Plasmodium* parasite growth (see Chapter 6).

### 3.11.5 BITZ Activity Against Resistant Parasites

Given the ongoing and widespread issues of multiple drug resistance to *P. falciparum* infections,\[^{60}\] it is critical that existing drug-resistant parasites are not cross-resistant to novel antimalarial therapies currently in development. Taking account of this, the antimalarial potency of BITZ compounds was evaluated against a series of lab-adapted field isolates of *P. falciparum*, which included parasite strains resistant to chloroquine, mefloquine and artemisinin: the archetypes of antimalarial chemotherapy.\[^{78}\]

Following growth inhibition studies, it was identified that 4, one of the most potent BITZ inhibitors against both the PfISPd enzyme and parasite growth, also effectively inhibits the growth of three lines of drug-resistant *P. falciparum* parasites (Table 3.11).

<table>
<thead>
<tr>
<th><em>P. falciparum</em> strain</th>
<th>Whole Cell Growth Inhibition EC(_{50}) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D7</td>
<td>0.92 ± 0.066</td>
</tr>
<tr>
<td>D6</td>
<td>0.80 ± 0.12</td>
</tr>
<tr>
<td>7G8</td>
<td>1.02 ± 0.25</td>
</tr>
<tr>
<td>IPC 5202</td>
<td>1.38 ± 0.19</td>
</tr>
</tbody>
</table>

Table 3.11 Inhibitory activity of 4 against drug-resistant *P. falciparum* parasites grown in culture; mean EC\(_{50}\) data and SEM given for ≥ 3.

The inhibitory activity of BITZ compound 4 was found to be in the low micromolar region against three drug-resistant *P. falciparum* parasite strains and consistent with observed inhibitory activity against 3D7, the pan-sensitive *P. falciparum* strain (Table 3.10). Of the drug-resistant *P. falciparum* parasite strains examined, including: the mefloquine-resistant D6 strain,\[^{79}\] the multidrug (chloroquine)-resistant 7G8 strain\[^{80}\] and the artemisinin-resistant IPC 5202 strain;\[^{81}\] 4 showed the greatest inhibition against drug-resistant *P. falciparum* strain D6, with a parasite EC\(_{50}\) of 800 ± 120 nM. This level of whole cell growth inhibition was actually slightly more pronounced than that observed against the 3D7 *P. falciparum* strain with a measured parasite EC\(_{50}\) value of 950 ± 72 nM. These results demonstrate that BITZ compounds are able to inhibit parasite growth in *P. falciparum* parasite strains with acquired resistance against current antimalarial drugs. This therefore highlights the promise of BITZ derived compounds as novel antimalarial therapies which have the potential to show broad spectrum antimalarial activity across both drug-resistant and non-drug-resistant *Plasmodium* parasites.
3.12 Conclusions and Future Work

This chapter has provided an account of the SAR explorations that have been conducted around the of 1,2-benzo[d]isothiazol-3(2H)-one (BITZ) chemotype for the development and optimisation of novel PfispD inhibitors, as inspired by previous medicinal chemistry programs demonstrating successful hit-to-lead optimisation. This chapter provides the details of SAR developments and corresponding syntheseses that I, myself, have designed and performed, as well as some of the key SAR studies and findings that have been carried out by others in the Liverpool medicinal chemistry group.

A diverse range of BITZ derived compounds have been synthesised, comprising a wide variety of chemical constructs which have been generated using a number of distinct synthetic routes. Ultimately, this work has identified, to the best of our knowledge, three of the most potent BITZ derived inhibitors against the IspD enzyme to date, all of which belong to the biphenyl BITZ inhibitor compound series. Compound 5 (Figure 3.3 and Table 3.1) has been identified at the most potent BITZ inhibitor against the PfispD enzyme itself, demonstrating an impressive IC$_{50}$ value of 73 ± 20 nM which is six-times more potent than our original HTS hit, DT2009-0168020 (Figure 3.15). BITZ compounds 4 and 6 can be described, not only as potent PfispD inhibitors, but also as the most effective antimalarial compounds identified within this research. Compounds 4 and 6 display potent inhibition against the PfispD enzyme with IC$_{50}$ values of 206 nM and 273 nM respectively; we also observe that this translates into improved inhibition of P. falciparum parasite growth for these two compounds, compared to that achieved with the most potent PfispD enzyme inhibitor 5 [EC$_{50}$ values for 4 and 6 are 920 nM and 646 nM respectively].

Since our most potent BITZ inhibitors belong to the same structural family of biphenyl derived compounds, this suggests that this inhibitor series fits well within the active site of the PfispD enzyme, enabling efficient protein-ligand contacts to be formed which translates to potent enzyme inhibition. To further address the alignment and behaviour of BITZ compounds at PfispD, molecular modelling studies will be undertaken to evaluate these BITZ inhibitors at the enzyme target, as well as explore the nature of the PfispD active site itself. Molecular modelling studies have the potential to inform further compound design and shed light onto the possible mechanism of action by which BITZ compounds are capable of achieving PfispD enzyme inhibition.

Also of note, BITZ inhibitor 15 shows potent activity of the PfispD enzyme which translates favourably into inhibition of P. falciparum parasite growth at a similar, and slightly enhanced order of magnitude: PfispD IC$_{50}$ value = 446 nM alongside a parasite growth EC$_{50}$ value of 416 nM (Figure 3.5 and Table 3.2). This result gives confidence in that the biphenyl BITZ analogue series doesn’t represent the only
BITZ series which is capable of displaying potent inhibition of both the PfIspD enzyme and *P. falciparum* parasite growth.

Using some of our most promising BITZ inhibitors, it has been possible to conduct a number of biological studies evaluating BITZ compound activity at the IspD enzyme and in malaria-causing *Plasmodium* parasites. We have shown that BITZ compounds offer potential as a broad spectrum antimalarial agents as they are able to inhibit the activity of the *P. vivax* IspD enzyme homologue. Several BITZ compounds demonstrate low micromolar to nanomolar activity against PvIspD (Table 3.9), highlighting the efficacy of these compounds across *Plasmodium* enzyme species and therefore their potential to act as broad spectrum antimalarial agents. Importantly, this meets a key developmental requirement in antimalarial drug design since there is an increasing necessity for new therapies to show both potent and broad-spectrum antimalarial activity.\(^67\)

In addition, it has been demonstrated that BITZ derived compounds express inhibitory activity against *P. falciparum* parasite strains which show drug resistance to existing antimalarial therapies (Table 3.11). This finding is of significant importance given the current malaria climate and the widespread drug resistance shown by the *P. falciparum* parasite species. It is crucial that existing drug-resistant parasites show sensitivity to new and novel antimalarial therapies and do not risk pre-determined resistance against them. Encouragingly, our data is in line with this requirement as BITZ compounds display inhibitory activity against existing drug-resistant parasite strains.

Considering more specifically the effects of BITZ inhibitors at the PfIspD enzyme target and consequent effects on *P. falciparum* parasite growth; selected BITZ inhibitors 4-6 and 13-15 have demonstrated good correlation between PfIspD enzyme inhibition and inhibition of parasite growth (Figure 3.9), which indicates that inhibition of the MEP pathway, through targeted inhibition of PfIspD, is able to cause parasite death. In addition, metabolic profiling of MEP pathway intermediates, following treatment of *P. falciparum* cultures with growth-inhibitory concentrations of BITZ compounds, have established that observed anti-parasitic activity is the result of MEP pathway inhibition which is sufficient to disrupt *de novo* production of isoprenoid precursors. Taken together, these findings strongly indicate that BITZ compounds are able to inhibit *Plasmodium* parasite growth and do so, at least in part, through the inhibition of cellular IspD.

In contrast to these lines of evidence, highlighting effective inhibition of PfIspD by the BITZ chemotype which translates into the disruption of *Plasmodium* parasite growth, several series of BITZ compounds indicate slightly differing behaviour towards the PfIspD enzyme and parasite growth. In particular, the biphenyl-pyridinyl BITZ series, alkyl-amino biphenyl BITZ series and aminoquinoline-BITZ hybrid
compounds (Sections 3.5, 3.8 and 3.9 respectively) suggest that BITZ derived compounds may also be capable of eliciting off-target inhibitory effects that may contribute to the observed inhibition of \textit{Plasmodium} parasite growth.

A loss of \textit{Pf}IspD enzymatic activity is observed across the biphenyl-pyridinyl BITZ compound series, with enzymatic IC\textsubscript{50} values consistently outside the required threshold of 5 µM; however, a notable level of anti-parasitic activity is maintained, where low micromolar efficacy is displayed against parasite growth with all derivatives in the series (Section 3.5). This consistent activity trend is indicative of off-target interactions which are capable of inhibiting \textit{Plasmodium} parasite growth, despite a lack of activity against the selected enzyme target. Similarly, this effect is also observed in the alkyl-amino biphenyl BITZ compound series (Section 3.8). Whilst BITZ derivatives in this series are classified as active against the \textit{Pf}IspD enzyme, an increase in efficacy against parasite growth is also observed, with a 3.5-5-fold potency increase against parasite growth, compared to activity against \textit{Pf}IspD.

A less distinct pattern of activity was observed with the aminoquinoline-BITZ hybrid compounds (Section 3.9). Inhibitory activity against the \textit{Pf}IspD enzyme was abolished with over half the compounds in the series and consequently, the extent of inhibitory activity against parasite growth was not tested for these compounds. However, two hybrid compounds within the series displayed moderate activity against \textit{Pf}IspD but highly potent activity (in the low nanomolar region) against \textit{Plasmodium} parasite growth. Indeed, it is worth noting that the aminoquinoline-BITZ hybrid compounds were designed with the purpose of achieving dual-target activity. However, the heightened activity observed against \textit{Plasmodium} parasite growth, with up to a 245-fold potency increase compared to \textit{Pf}IspD inhibition for one analogue (41), far outweighs that expected from the previous distinctions between enzymatic and phenotypic activity which has been observed with other BITZ analogues. Consequently, this result may alternatively suggest that the BITZ chemotype is capable of off-target activity which may contribute to overall inhibition of parasite growth.

In addition to the highlighted potential for off-target BITZ reactivity, exemplified by enzymatic and phenotypic activity data, IPP rescue experiments also suggest that parasite growth inhibition by BITZ derived compounds does not solely result from activity against the IspD enzyme. As a consequence, further studies will be conducted with the aim of identifying any additional molecular targets that the BITZ chemotype and derived compounds may be capable of inhibiting.

Despite the suggestion of off-target BITZ reactivity, it has also been clearly demonstrated that the BITZ chemotype potently inhibits the \textit{Pf}IspD enzyme, which translates into disruption of isoprenoid
precursor synthesis and, as a result, causes *Plasmodium* parasite death. Indeed, the structurally divergent BITZ analogues generated demonstrate a wide range of inhibitory activity against the *Pf*IspD enzyme. Considering requirements for future work, a necessary task is to determine the mechanism of action by which this chemical construct achieves inhibition of the *Pf*IspD enzyme. In addition, the question of BITZ compound metabolism should also be addressed to provide a measure of BITZ compound stability, half-life ($t_{1/2}$) and clearance when introduced to a biological system. This will involve assessing the metabolism profiles of existing BITZ compounds, followed by further SAR studies to facilitate any appropriate chemical modifications to enable movement towards an optimised lead structure.
3.13 Experimental

For general chemical and biological methodologies see Chapter 2, Section 2.7.1.

3.13.1 Organic Synthesis

Preparation of \( N\)\-(3-iodophenyl)\-2\-(methylthio)benzamide 1

To a stirring solution of 2\-(methylthio)benzoic acid (13.8 mmol) in anhydrous toluene (25 ml) under a \( N_2 \) atmosphere, thionyl chloride (41 mmol, 3.0 eq) was added followed by 3 drops of \( N, N\)-dimethylformamide: the resulting solution was refluxed for 2-3 hours. Solvent and thionyl chloride were removed under reduced pressure to give the acid chloride intermediate as a white solid. 3-iodoaniline (16.4 mmol, 1.2 eq) was dissolved in anhydrous THF (10 ml/mmol) to which triethylamine (\( \text{Et}_3\text{N} \)) (27.4 mmol, 2.0 eq) was added. The crude acid chloride was re-dissolved in anhydrous THF (10 ml/mmol) and added drop-wise to the amine and base solution over 10 minutes at 0°C. After a further 10 minutes, the solution was warmed to room temperature and allowed to stir for 20 hours. The solvent was removed under reduced pressure; the resulting crude material diluted with water and extracted into EtOAc (3 x 30 ml). The combined organic extracts were washed with 5% HCl (50 ml), saturated aq. NaHCO\(_3\) (50 ml) and brine (50 ml), dried over MgSO\(_4\) and concentrated under reduced pressure to give 1 in 97.7% yield. \( \text{Rf} = 0.62 \) (40:60 EtOAc:hexane). \( \text{Rf} = 0.62 \) (4:6 EtOAc:hexane). \(^1\text{H}\) NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.69 (s, 1H), 8.08 (s, 1H), 7.70 – 7.61 (m, 2H), 7.51 – 7.46 (m, 1H), 7.46 – 7.41 (m, 1H), 7.40 – 7.35 (m, 1H), 7.29 – 7.24 (m, 1H), 7.09 (t, \( J = 8.1 \text{ Hz}, 1\text{H} \)) and 2.50 (s, 3H); \(^{13}\text{C}\) NMR (101 MHz, CDCl\(_3\)) \( \delta \) 139.4, 137.3, 135.2, 133.9, 131.6, 130.9, 129.3, 120.0, 128.2, 126.0, 119.6, 115.5, 94.6 and 17.3; ES+HRMS \( m/z \) 391.9570 [M+Na]\(^+\). \( C_{14}H_{12}INOS \) requires 368.97.

Preparation of \( N\)\-(3-iodophenyl)\-2\-(methylsulfinyl)benzamide 2

A solution of sodium periodate (11.68 mmol, 1.6 eq) in water (30 ml) was added dropwise to 1 (13.4 mmol, 1.0 eq) in MeOH (10 ml/mmol) at room temperature. The biphasic solution was heated to 50°C for 4-5 hours before the solvent was removed under reduced pressure. The crude precipitate
was diluted with water, filtered through a sinter and washed with water before drying for 12 hours in a desiccator to give 2 as a fine brown powder in 96.3% yield. \( \text{RF} = 0.14 \) (100% EtOAc).\(^{1} \)\text{H NMR} (500 MHz, DMSO) \( \delta \) 10.67 (s, 1H, NH), 8.21 (t, \( J = 1.8 \) Hz, 1H), 8.16 (dd, \( J = 7.8, 1.3 \) Hz 1H), 7.99 (dd, \( J = 7.8, 1.0 \) Hz, 1H), 7.88 (td, \( J = 7.4, 1.3 \) Hz, 1H), 7.72 (ddd, \( J = 4.5, 2.8, 1.1 \) Hz, 2H), 7.51 (ddd, \( J = 7.8, 1.7, 1.0 \) Hz, 1H), 7.19 (t, \( J = 8.1 \) Hz, 1H), 2.83 (s, 3H, CH\(_3\)); \(^{13} \)\text{C NMR} (101 MHz, DMSO) \( \delta \) 165.1, 148.9, 140.5, 133.2, 132.9, 132.3, 131.3, 130.9, 128.9, 128.5, 124.2, 120.0, 94.9, 45.1; \text{ES+HRMS} m/z 407.9529 [M+Na]\(^{+} \) \( \text{C}_{26}\text{H}_{22}\text{NO}_{2}\text{S} \) requires 384.96.

**Preparation of biphenyl methylsulfonyl benzamides 3a-h**

**General procedure (1)**

To a solution of \( \text{K}_2\text{CO}_3 \) (3.3 eq), 2 (1.0 eq), and \( \text{Pd(PPh}_3\text{)}_4 \) (0.0 25eq) in water (5 ml), the required phenyl boronic acid (1.2 eq) was added. The stirring solution was evacuated and purged with \( \text{N}_2 \) before adding anhydrous \( \text{THF} \) (10 ml) and heating to 80°C for 18-22 hours. The solution was quenched with 2N HCl (15 ml) and extracted into EtOAc (3 x 30 ml). The combined organic extracts were washed with brine (30 ml), dried over \( \text{MgSO}_4 \) and concentrated under reduced pressure. The crude products were purified by flash column chromatography.

**Preparation of** \( N\text{-}[4'\text{-methoxy-[1,1'-biphenyl]-3-yl]-2-(methylsulfinyl)benzamide 3a} \)

The general procedure (1) was followed using 4-(methoxy) phenyl boronic acid to give 3a as a cream-brown crystalline solid in 85.9% yield. \( \text{RF} = 0.59 \) (5:95 MeOH:EtOAc).\(^{1} \)\text{H NMR} (500 MHz, DMSO) \( \delta \) 10.66 (s, 1H, NH), 8.17 (d, \( J = 7.8 \) Hz, 1H), 8.02 (d, \( J = 7.6 \) Hz, 1H), 7.98 (s, 1H), 7.89 (t, \( J = 7.6 \) Hz, 1H), 7.77 – 7.68 (m, 2H), 7.59 (d, \( J = 8.6 \) Hz, 2H), 7.44 (t, \( J = 7.8 \) Hz, 1H), 7.39 (d, \( J = 7.7 \) Hz, 1H), 7.06 (d, \( J = 8.6 \) Hz, 2H), 3.81 (s, 3H, CH\(_3\)), 2.84 (s, 3H, CH\(_3\)); \(^{13} \)\text{C NMR} (101 MHz, DMSO) \( \delta \) 165.2, 159.5, 148.8, 140.9, 139.6, 132.8, 132.7, 130.9, 129.7, 128.5, 128.2 (2C), 124.12, 122.6, 119.2, 118.7, 114.9 (2C), 55.7, 45.1; \text{ES+HRMS} m/z 388.0974 [M+Na]\(^{+} \) \( \text{C}_{22}\text{H}_{19}\text{NO}_{3}\text{S} \) requires 365.11.
Preparation of **N-(4'-chloro-[1,1'-biphenyl]-3-yl)-2-(methylsulfinyl)benzamide 3b**

The general procedure (1) was followed using 4-(chloro)phenyl boronic acid to give 3b as a white solid in 83.4% yield. **Rf** = 0.15 (8:2 EtOAc:hexane). ¹H NMR (400 MHz, MeOD) δ 8.23 (dd, 8 = 8.0, 1.0 Hz, 1H), 8.05 – 8.00 (m, 1H), 7.98 (d, 8 = 1.7 Hz, 1H), 7.88 (td, 8 = 7.7, 1.2 Hz, 1H), 7.73 (td, 8 = 7.6, 1.2 Hz, 1H), 7.69 – 7.65 (m, 1H), 7.65 – 7.61 (m, 2H), 7.49 – 7.41 (m, 4H) and 2.98 (s, 3H); ¹³C NMR (101 MHz, DMSO) δ 165.1, 148.7, 139.8, 139.6, 130.8, 129.9, 129.4, 128.8, 128.4, 124.0, 122.9, 120.1, 118.9 and 45.0; ES+HRMS m/z 392.0474 [M+Na]⁺. C_{20}H_{16}ClNO₂S requires 369.06.

Preparation of **2-(methylsulfinyl)-N-(4'-trifluoromethyl-[1,1'-biphenyl]-3-yl) benzamide 3c**

The general procedure (1) was followed using 4-(trifluoromethyl)phenyl boronic acid to give 3c as a cream solid in 86.8% yield. **Rf** = 0.23 (8:2 EtOAc:hexane). ¹H NMR (500 MHz, DMSO) δ 10.75 (s, 1H, NH), 8.18 (dd, 8 = 7.7, 1.0 Hz 1H), 8.12 – 8.10 (m, 1H), 8.03 (dd, 8 = 7.7, 1.0 Hz, 1H), 7.89 (ddd, 8 = 7.9, 7.4, 1.3 Hz, 1H), 7.87 (s, 4H), 7.84 – 7.79 (m, 1H), 7.74 (td, 8 = 7.5, 1.3 Hz, 1H), 7.54 – 7.52 (m, 2H), 2.84 (s, 3H, CH₃); ¹³C NMR (101 MHz, DMSO) δ 165.3, 148.9, 144.5, 139.8, 139.7, 132.8, 132.6, 130.9, 130.1, 128.5 (d, 3J_{CF} = 31.8 Hz, 1C), 128.5, 127.9 (2C), 126.4 (d, 3J_{CF} = 3.9 Hz, 2C), 124.8 (d, 1J_{CF} = 271.9 Hz, 1C), 124.2, 123.4, 120.8, 119.4, 45.1; ES+HRMS m/z 426.0750 [M+Na]⁺. C_{21}H_{16}F₃NO₂S requires 403.09.

Preparation of **N-(4'-(4-methylpiperazin-1-yl)methyl-[1,1'-biphenyl]-3-yl)-2-(methylsulfinyl) benzamide 3d**

The general procedure (1) was followed using 1-methyl-4-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl]piperazine to give 3d as a clear pale yellow viscous oil in 85.9% yield. **Rf** = 0.14 (5:95 MeOH:EtOAc). ¹H NMR (400 MHz, MeOD) δ 8.25 (d, 8 = 7.6 Hz, 1H), 8.05 (d, 8 = 7.6 Hz, 1H), 8.01 (s, 1H), 7.90 (t, 8 = 7.4 Hz, 1H), 7.75 (t, 8 = 7.4 Hz, 1H), 7.68-7.63 (m, 3H), 7.47-7.44(m, 4H), 3.62 (s, 2H, CH₂), 2.99 (s, 3H, CH₃), 2.69 (br s, 8H), 2.82-2.48 (s, 3H, CH₃); ¹³C NMR (101 MHz, MeOD) δ 165.3, 147.0, 141.5, 139.7, 138.6, 136.3, 132.4, 132.4, 130.7, 129.8 (2C), 129.0, 127.9, 126.6 (2C), 123.6, 123.1, 119.5, 119.1, 61.9, 54.2 (2C), 51.9 (2C), 44.4, 43.8. **Elemental analysis (%) found:** C, 68.19; H, 6.57; N, 9.21; S, 6.26%. C_{26}H_{29}N₃O₂S requires C, 69.77; H, 6.53; N, 9.39; S, 7.16%; ES+HRMS m/z 448.2063 [M+H]⁺. C_{26}H_{29}N₃O₂S requires 447.20.

Preparation of **N-(4'-(4-fluoropiperidin-1-yl)methyl)-[1,1'-biphenyl]-3-yl)-2-(methylsulfinyl) benzamide 3e**

The general procedure (1) was followed using (4-((4-fluoropipieridine-1-yl)methyl)phenyl boronic acid to give 3e as a white solid in 79.7% yield. **Rf** = 0.16 (5:95 MeOH:EtOAc). ¹H NMR (400 MHz, MeOD) δ 8.25 (dd, 8 = 7.9, 1.0 Hz, 1H), 8.05 (d, 8 = 7.1 Hz, 1H), 8.00 (s, 1H), 7.90 (dt, 8 = 7.6, 1.1 Hz, 1H), 7.75 (dt,
$J = 7.6, 1.0 \text{ Hz}, 1\text{H}), 7.67 (\text{m}, 3\text{H}), 7.46 (\text{m}, 4\text{H}), 4.68 (\text{m}, 1\text{H}), 3.60 (\text{s}, 2\text{H}), 2.99 (\text{s}, 3\text{H}), 2.56 (\text{m}, 4\text{H}), 1.90 (\text{m}, 4\text{H})$. \textbf{13C NMR} (101 MHz, MeOD) $\delta$ 165.3, 147.0, 141.6, 139.8, 138.6, 136.6, 132.4, 132.4, 130.7, 129.9 (2C), 129.0, 127.5(2C), 126.6, 123.6, 123.1, 119.5, 119.2, 88.8. (d, $^1J_{CF} = 179.8$ Hz, 1C), 62.1, 49.0 (d, $^3J_{CF} = 5.4$ Hz, 2C), 43.8, 30.8 (d, $^2J_{CF} = 19.8$ Hz, 2C).

\textbf{Elemental analysis} (%) found: C, 68.12; H, 5.99; N, 5.98%. \(\text{C}_{26}\text{H}_{27}\text{FN}_{2}\text{O}_{2}\text{S}\) requires C, 69.31; H, 6.03; N, 6.22%; \textbf{ES+HRMS} m/z 451.1844 [M+H]$^+$.

\textbf{Preparation of 2-(methylsulfinyl)-N-(4'-(trifluoromethoxy)-[1,1'-biphenyl]-3-yl) benzamide 3f}

The general procedure (1) was followed using 4-(trifluoromethoxy)-benzene boronic acid to give $3f$ as a cream-brown crystalline solid in 83.6% yield. \textbf{1H NMR} (400 MHz, MeOD) $\delta$ 8.13 (dd, $J = 8.0, 0.9$ Hz, 1H), 7.93 (dd, $J = 7.7, 0.7$ Hz, 1H), 7.90 (s, 1H), 7.78 (td, $J = 7.8, 1.2$ Hz, 1H), 7.64 (br d, $J = 11.5$, Hz, 3H), 7.59 (dt, $J = 7.5, 1.8$ Hz, 1H), 7.41 – 7.32 (m, 2H), 7.27 (d, $J = 8.0$ Hz, 2H), 2.87 (s, 3H); \textbf{13C NMR} (101 MHz, MeOD) $\delta$ 165.3, 148.7, 147.0, 140.4, 139.8, 138.7, 132.4, 132.4, 130.7, 129.2, 128.33 (2C), 127.6, 123.6, 123.2, 121.0 (2C), 120.6 (q, $^1J_{CF} = 255.4$ Hz, 1C), 112.0, 119.2, 43.8; \textbf{Elemental analysis} (%) found: C, 59.58; H, 3.81; N, 3.13%. \(\text{C}_{21}\text{H}_{16}\text{F}_{3}\text{NO}_{3}\text{S}\) requires C, 60.14; H, 3.85; N, 3.34%; \textbf{ES+HRMS} m/z 442.0710 [M+Na]$^+$.

\textbf{Preparation of 2-(methylsulfinyl)-N-(3-(thiophen-3-yl)phenyl)benzamide 3g}

The general procedure (1) was followed using 3-thienylboronic acid to give $3g$ as a fine orange-brown solid in 88.7% yield. \textbf{RF} = 0.17 (8:2 EtOAc:hexane). \textbf{1H NMR} (400 MHz, MeOD) $\delta$ 8.26 (dd, $J = 8.0, 0.9$ Hz, 1H), 8.05 (dd, $J = 7.7, 0.8$ Hz, 1H), 8.02 (t, $J = 1.7$ Hz, 1H), 7.91 (td, $J = 7.5, 1.2$ Hz, 1H), 7.76 (td, $J = 7.6, 1.2$ Hz, 1H), 7.69 – 7.67 (m, 1H), 7.64 (dd, $J = 7.9, 1.3$ Hz, 1H), 7.55 – 7.48 (m, 3H), 7.44 (t, $J = 7.9$ Hz, 1H), 3.01 (s, 3H); \textbf{13C NMR} (101 MHz, MeOD) $\delta$ 165.3, 147.0, 141.7, 138.6, 136.6, 132.4, 132.4, 130.7, 129.0, 127.6, 126.0, 125.8, 123.6, 122.5, 120.3, 119.3, 118.5, 43.7; \textbf{ES+HRMS} m/z 364.0435 [M+Na]$^+$.

\textbf{Preparation of methyl 3'- (2-(methylsulfinyl) benzamido) -[1,1'-biphenyl]-4-carboxylate 3h}

The general procedure (1) was followed using 4-(methoxycarbonyl)phenyl boronic acid and an extended reaction time of 2 days to give $3h$ as a cream solid in 80.7% yield. \textbf{RF} = 0.31 (8:2
$^1$H NMR (400 MHz, DMSO) δ 10.69 (br s, 1H, NH), 8.21 (t, J = 1.7 Hz, 1H), 8.19 – 8.13 (m, 1H), 8.09 (d, J = 8.5 Hz, 1H), 7.98 (dd, J = 7.6, 0.7 Hz, 1H), 7.92 – 7.85 (m, 1H), 7.72 (d, J = 8.5 Hz, 1H), 7.72 (t, J = 7.5 Hz, 3H), 7.54 – 7.47 (m, 2H), 7.18 (t, J = 8.0 Hz, 1H), 3.89 (s, 3H), 2.84 (s, 3H); ES+MS m/z 416.1 [M+Na]$^+$ C$_{22}$H$_{19}$NO$_4$S requires 393.10.

**Preparation of benzo[d]isothiazol-3(2H)-ones 8-12**

**General procedure (2)**

<table>
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<th>4</th>
<th>R$^1$ = OCH$_3$</th>
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<tbody>
<tr>
<td>5</td>
<td>R$^1$ = Cl</td>
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<tr>
<td>6</td>
<td>R$^1$ = CF$_3$</td>
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<tr>
<td>15</td>
<td>R$^1$ = NCH$_3$</td>
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<tr>
<td>14</td>
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<tr>
<td>7</td>
<td>R$^1$ = OCF$_3$</td>
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<tr>
<td>10</td>
<td>R$^1$ = CO$_2$CH$_3$</td>
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</tbody>
</table>

Thionyl chloride (1.3 eq) was added dropwise to a stirring solution of the required biphenyl benzamide (3a-h, 1.0 eq) in dichloromethane (DCM) (15-20 ml) under a N$_2$ atmosphere. The solution was heated to reflux at 50°C for 1.5 hours. Solvent and excess thionyl chloride were removed under reduced pressure and the crude products purified by flash column chromatography.

**Preparation of 2-(4'-methoxy-[1,1'-biphenyl]-3-yl)benzo[d]isothiazol-3(2H)-one 4**

The general procedure (2) was followed using 3a (0.56 mmol) to give 4 as a white-yellow crystalline solid in 58.4% yield. RF = 0.64 (55:45 EtOAc:hexane).$^1$H NMR (400 MHz, DMSO) δ 8.08 (d, J = 8.1 Hz, 1H), 7.98 (d, J = 7.7 Hz, 1H), 7.95 (d, J = 1.7 Hz, 1H), 7.78 (dt, J = 8.2, 1.1 Hz, 1H), 7.67 (d, J = 8.8 Hz, 2H), 7.65 – 7.56 (m, 3H), 7.53 (t, J = 7.8 Hz, 1H), 7.07 (d, J = 8.8 Hz, 2H) and 3.82 (s, 3H); $^{13}$C NMR (101 MHz, DMSO) δ 163.9, 159.8, 141.6, 140.6, 138.2, 133.1, 132.0, 130.5, 128.4 (2C), 126.6, 126.5, 125.3, 124.3, 123.1, 122.7, 122.4, 115.0 (2C) and 55.7; **Elemental analysis** (%) found: C, 71.95; H, 4.47; N, 4.41; S, 9.77%. C$_{20}$H$_{15}$NO$_2$S requires C, 72.05; H, 4.54; N, 4.20; S, 9.62%; ES+HRMS m/z 356.0712 [M+Na]$^+$ C$_{20}$H$_{15}$NO$_2$S requires 333.08.
Preparation of 2-(4′-chloro-[1,1′-biphenyl]-3-yl)benzo[d]isothiazol-3(2H)-one 5

The general procedure (2) was followed using 3b (0.46 mmol) to give 5 as a white crystalline solid in 52.2% yield. 

\[
\text{RF} = 0.06 \text{ (8:2 EtOAc:hexane).} \]

\[
^1H\;\text{NMR} \; (500\;\text{MHz, DMSO}) \; \delta \; 8.08 \; (dt, \; J = 8.1, 0.8\; Hz, 1H), \; 8.02 \; (t, \; J = 2.1, 1H), \; 7.98 \; (ddd, \; J = 7.8, 1.3, 0.7\; Hz, 1H), \; 7.79 \; (dt, \; J = 8.4, 1.3\; Hz, 1H), \; 7.75 \; (dt, \; J = 8.7, 2.1\; Hz, 2H), \; 7.71 \; - \; 7.67 \; (m, 2H), \; 7.64 \; (t, \; J = 7.9\; Hz, 1H), \; 7.57 \; (dt, \; J = 8.7, 2.4\; Hz, 2H), \; 7.53 \; (dt, \; J = 8.0, 1.0\; Hz, 1H); \n\]

\[
^{13}C\;\text{NMR} \; (101\;\text{MHz, DMSO}) \; \delta \; 163.9, \; 140.6, \; 140.6, \; 138.5, \; 138.3, \; 133.4, \; 133.2, \; 130.7, \; 129.5 \; (2C), \; 129.1 \; (2C), \; 126.7, \; 126.6, \; 125.8, \; 124.7, \; 124.2, \; 123.2, \; 122.4. \n\]

Elemental analysis (%) found: C, 67.03; H, 3.62; N, 4.13; S, 8.91%. C_{19}H_{12}ClNOS requires C, 67.55; H, 3.58; N, 4.15; S, 9.49%; ES+HRMS m/z 360.0215 [M+Na]^+ C_{19}H_{12}ClNOS requires 337.03.

Preparation of 2-(4′-(trifluoromethyl)-[1,1′-biphenyl]-3-yl)benzo[d]isothiazol-3(2H)-one 6

The general procedure (2) was followed using 3c (0.43 mmol) to give 6 as a white crystalline solid in 73.6% yield. 

\[
\text{RF} = 0.06 \text{ (8:2 EtOAc:hexane).} \]

\[
^1H\;\text{NMR} \; (500\;\text{MHz, DMSO}) \; \delta \; 8.11 \; - \; 8.07 \; (m, 2H), \; 7.99 \; (ddd, \; J = 7.8, 1.3, 0.7\; Hz, 1H), \; 7.97 \; (d, \; J = 8.2\; Hz, 2H), \; 7.87 \; (d, \; J = 8.2\; Hz, 2H), \; 7.79 \; (dt, \; J = 8.3, 1.3\; Hz, 1H), \; 7.78 \; - \; 7.74 \; (m, 2H), \; 7.69 \; (t, \; J = 8.0\; Hz, 1H), \; 7.54 \; (ddd, \; J = 8.0, 1.0\; Hz, 1H); \n\]

\[
^{13}C\;\text{NMR} \; (101\;\text{MHz, DMSO}) \; \delta \; 163.5, \; 143.7, \; 140.6, \; 140.3, \; 138.4, \; 133.2, \; 130.8, \; 128.8 \; (d, \; J_{CF} = 31.7\; Hz, 1C), \; 128.2 \; (2C), \; 126.7, \; 126.6, \; 126.4 \; (d, \; J_{CF} = 3.7\; Hz, 2C), \; 126.1, \; 124.8 \; (d, \; J_{CF} = 272.0\; Hz, 1C), \; 124.8, \; 124.7, \; 123.6, \; 122.5; \n\]

Elemental analysis (%) found: C, 62.68; H, 3.22; N, 3.19%. C_{20}H_{12}F_{3}NOS requires C, 64.68; H, 3.26; N, 3.77; ES+HRMS m/z 394.0492 [M+Na]^+ C_{20}H_{12}F_{3}NOS requires 371.06.

Preparation of 2-(4′-((4-methylpiperazin-1-yl)methyl)-[1,1′-biphenyl]-3-yl)benzo[d]isothiazol-3(2H)-one 15

The general procedure (2) was followed using 3d (0.549 mmol) to give 15 as an off-white solid in 58.0% yield. 

\[
\text{RF} = 0.12 \text{ (10:90 MeOH:EtOAc).} \]

\[
^1H\;\text{NMR} \; (400\;\text{MHz, MeOD}) \; \delta \; 8.07 \; (d, \; J = 7.9\; Hz, 1H), \; 7.96 \; (s, 1H), \; 7.89 \; (d, \; J = 8.1\; Hz, 1H), \; 7.79 \; (t, \; J = 7.5\; Hz, 1H), \; 7.69-7.62 \; (m, 5H), \; 7.55 \; (t, \; J = 7.6\; Hz, 1H), \; 7.47 \; (d, \; J = 7.6\; Hz, 2H), \; 7.31 \; (s, 2H, \; CH_{3}), \; 2.90-2.22 \; (br s, 8H), \; 2.33 \; (s, 3H, \; CH_{3}); \n\]

\[
^{13}C\;\text{NMR} \; (101\;\text{MHz, MeOD}) \; \delta \; 164.8, \; 142.3, \; 140.8, \; 139.0, \; 137.4, \; 136.9, \; 132.6, \; 129.9 \; (2C), \; 129.7, \; 126.7 \; (2C), \; 126.1, \; 125.9, \; 125.9, \; 124.2, \; 123.6, \; 123.3, \; 120.8, \; 61.9, \; 54.2 \; (2C), \; 52.0 \; (2C), \; 44.5; \n\]

ES+HRMS m/z 416.1810 [M+H]^+ C_{20}H_{12}N_{3}OS requires 415.17.

Preparation of 2-(4′-((4-fluoropiperidin-1-yl)methyl)-[1,1′-biphenyl]-3-yl)benzo[d]isothiazol-3(2H)-one 14

The general procedure (2) was followed using 3e (0.44 mmol) to give 14 as an off-white fine crystalline solid in 70.0% yield. 

\[
\text{RF} = 0.49 \text{ (5:95 MeOH:EtOAc).} \]

\[
^1H\;\text{NMR} \; (400\;\text{MHz, DMSO}) \; \delta \; 8.08 \; (d, \; J = 8.1\; Hz, 1H), \; \]
Chapter 3

8.02-7.95 (m, 2H), 7.78 (t, J = 7.7 Hz, 1H), 7.71-7.59 (m, 5H), 7.53 (t, J = 7.6 Hz, 1H), 7.43 (d, J = 7.8 Hz), 4.69 (d, J = 50.9 Hz, 1H), 3.52 (s, 2H), 2.60-2.25 (m, 4H), 1.95-1.85 (m, 4H); 13C NMR (101 MHz, DMSO) δ 163.9, 141.7, 140.6, 138.8, 138.3, 138.2, 136.3, 133.2, 130.6, 130.0 (2C), 127.1 (2C), 126.7, 126.5, 125.7, 124.8, 123.7, 123.1, 122.4, 89.0 (d, 1JC = 169.8 Hz, 1C), 61.91, 49.53 (d, 3JC = 6.0 Hz, 2C), 31.63 (d, 2JC = 19.0 Hz, 2C);

Elemental analysis (%) found: C, 71.47; H, 5.56; N, 6.62%. C25H23FN2O requires C, 71.75; H, 5.54; N, 6.69; S, 7.66%; ES+HRMS m/z 419.1603 [M+H]+ C25H23FN2O requires 418.15.

Preparation of 2-(4′-(trifluoromethoxy)-[1,1′-biphenyl]-3-yl)benzo[d]isothiazol-3(2H)-one 7

The general procedure (2) was followed using 3f (0.43 mmol) to give 7 as an off-white crystalline solid in 87.2% yield. Rf = 0.05 (8:2 EtOAc:hexane). 1H NMR (400 MHz, MeOD) δ 8.06 (d, J = 7.9 Hz, 1H), 7.98 (t, J = 1.7 Hz, 1H), 7.88 (d, J = 8.2 Hz, 1H), 7.83 – 7.74 (m, 3H), 7.71 – 7.67 (m, 2H), 7.63 (t, J = 7.73 Hz, 1H), 7.55 (td, J = 8.0, 0.9 Hz, 1H), 7.40 (d, J = 8.0 Hz, 2H); 13C NMR (101 MHz, MeOD) δ 164.8, 149.0, 141.1, 140.7, 138.9, 137.5, 132.6, 129.8, 128.5 (2C), 126.1, 126.0, 125.9, 124.2, 124.0, 123.5, 121.1 (2C), 120.74, 120.6 (q, 1JC = 255.7 Hz, 1C); Elemental analysis (%) found: C, 60.75; H, 3.37; N, 3.33%. C20H12F3NO2S requires C, 62.01; H, 3.12; N, 3.62%; CI+HRMS m/z 388.0606 [M+H]+ C20H12F3NO2S requires 387.05.

Preparation of 2-(3-(thiophen-3-yl)phenyl)benzo[d]isothiazol-3(2H)-one 10

The general procedure (2) was followed using 3g (0.49 mmol) to give 10 as a yellow-cream solid in 85.7% yield. Rf = 0.63 (55:45 EtOAc:hexane). 1H NMR (400 MHz, DMSO) δ 8.07 (d, J = 8.1 Hz, 1H), 8.04 – 8.00 (m, 1H), 8.00 – 7.96 (m, 2H), 7.79 (dd, J = 8.0, 7.2, 1.2 Hz, 1H), 7.76 – 7.72 (m, 1H), 7.69 (dd, J = 5.0, 2.9 Hz, 1H), 7.61 (dd, J = 5.1, 1.3 Hz, 1H), 7.60 – 7.57 (m, 2H), 7.56 – 7.50 (td, J = 8.0, 0.9 Hz, 1H); 13C NMR (101 MHz, DMSO) δ 163.8, 140.8, 140.6, 138.0, 136.7, 133.0, 130.4, 127.8, 126.6, 126.5, 126.4, 125.2, 124.6, 123.8, 122.8, 122.4, 122.3; ES+HRMS m/z 332.0170 [M+Na]+ C17H11NOS2 requires 309.03; HPLC (purity analysis %) found: 96.73%; RT: 7.90 min/ 40 min (25/75, 0.5 ml/min IPA:HEXANE, Signal = 230 nm, SYNCHROSILICA).
Preparation of methyl 3’-(3-oxobenzo[d]isothiazol-2(3H)-yl)[1,1’-biphenyl]-4-carboxylate 8

The general procedure (2) was followed using 3h (0.49 mmol) to give 8 as a yellow-cream solid in 31.0% yield. \( R_f = 0.32 \) (30:70 EtOAc:hexane). \(^1\)H NMR (400 MHz, DMSO) \( \delta \) 8.08 (br d, \( J = 8.5 \) Hz, 4H), 7.98 (d, \( J = 7.8 \) Hz, 1H), 7.89 (d, \( J = 7.8 \) Hz, 2H), 7.82 – 7.71 (m, 3H), 7.67 (t, \( J = 7.7 \) Hz, 1H), 7.53 (t, \( J = 7.5 \) Hz, 1H), 3.89 (s, 3H); \(^{13}\)C NMR (101 MHz, DMSO) \( \delta \) 166.5, 163.9, 144.1, 140.6, 140.5, 138.4, 133.2, 130.8, 130.4 (2C), 129.4, 127.7 (2C), 126.7, 126.6, 126.0, 124.7 (2C), 123.5, 122.5, 52.7; Elemental analysis (%) found: C, 69.44; H, 4.21; N, 3.87; S, 8.90%. \( C_{21}H_{15}NO_3S \) requires C, 69.79; H, 4.18; N, 3.88; S, 8.87%.; \( \text{Cl+HRMS} \) m/z 362.0838 [M+H]^+. \( C_{21}H_{15}NO_3S \) requires 361.08.

Preparation of 2-(chlorothio)-benzoyl chloride X

Thionyl chloride (22.8 mmol, 7 eq) and N,N-dimethylformamide (3 drops) were added to a solution of 2,2’-dithiobenzoic acid (3.26 mmol) in anhydrous dichloroethane (25 ml) under a \( \text{N}_2 \) atmosphere at room temperature. The stirring solution was heated to reflux at 80°C for 1.5 hours. Sulfuryl chloride (4.24 mmol, 1.3 eq) was added to the cooled solution at 25°C before heating the solution to 50°C for 45 minutes. Solvent, excess thionyl chloride and excess sulfuryl chloride were removed under reduced pressure to generate X, a yellow-green solid as pure product in quantitative yield. \( R_f = 0.79 \) (50:50 EtOAc:hexane). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.34 (dd, \( J = 8.0, 1.2 \) Hz, 1H), 7.93 (dd, \( J = 8.2, 0.4 \) Hz, 1H), 7.77 (dt, \( J = 8.5, 1.3 \) Hz, 1H), 7.42 (dt, \( J = 8.0, 0.8 \) Hz, 1H); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 169.5, 146.1, 135.8, 134.7, 127.4, 125.8, 124.4.

Preparation of (4-(morpholinomethyl)phenyl)boronic acid 11a

Morpholine (5.56 mmol, 2.0 eq) and N,N-diisopropylethylamine (6.98 mmol, 2.5 eq) were added to a stirring solution of 4-methylbromophenyl boronic acid (2.79 mmol) in anhydrous THF (25 ml) under a \( \text{N}_2 \) atmosphere. The solution was stirred at room temperature for 18 hours before being quenched with brine (30 ml) and extracted into EtOAc (3 x 30 ml). The combined organic extracts were dried over MgSO\(_4\), filtered and concentrated under reduced pressure to give 11a as a pale orange solid in 87.0% yield which was used as crude in the following step. \( R_f = 0.08 \) (100% EtOAc and 1%)
triethylamine). \(^1\)H NMR (400 MHz, MeOD) \(\delta\) 7.50 (s, 2H), 7.21 (d, \(J = 7.9\) Hz, 2H), 3.58 (t, \(J = 4.6\) Hz, 4H), 3.43 (s, 2H, CH\(_2\)), 2.37 (br s, 4H).

Preparation of 4'-{(morpholinomethyl)-[1,1'-biphenyl]-3-amine 11b

\[
\text{H}_2\text{N} \quad \text{H} \quad \text{H} \quad \text{H} \quad \text{N} \quad \text{O} \\
\text{H}_2\text{N} \quad \text{H} \quad \text{H} \quad \text{H} \quad \text{N} \quad \text{O}
\]

To a solution of K\(_2\)CO\(_3\) (6.20 mmol, 3.3 eq), 11a (2.25 mmol, 1.2 eq) and Pd(PPh\(_3\))\(_4\) (0.0047 mmol, 0.025 eq) in water (5 ml), 3-bromoaniline (1.88 mmol, 1.0 eq) was added. The stirring reaction was evacuated and purged with N\(_2\) before the addition of anhydrous THF (10 ml) and heating to 80°C for 22 hours. The solution was quenched with brine (50 ml), extracted into EtOAc (3 x 35 ml) and the combined organic extracts dried over MgSO\(_4\) before concentrating under reduced pressure. The crude product was purified by flash column chromatography to give 11b as a pale yellow-brown oil in 91.0% yield. \(\text{RF} = 0.22\) (100% EtOAc and 1% triethylamine). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.52 (d, \(J = 8.2\) Hz, 2H), 7.37 (d, \(J = 8.2\) Hz, 2H), 7.22 (t, \(J = 7.8\) Hz, 1H), 6.99 (d, \(J = 8.0\) Hz, 1H), 6.90 (t, \(J = 2.0\) Hz, 1H), 6.68 (ddd, \(J = 7.9\), 2.3, 0.8 Hz, 1H), 3.73 (t, \(J = 4.6\) Hz, 4H), 3.53 (s, 2H), 2.48 (m, \(J = 4.2\) Hz, 4H). \(^{13}\)C NMR (101 MHz, MeOD) \(\delta\) 147.7, 141.6, 140.7, 135.7, 129.6 (2C), 129.1, 126.4 (2C), 116.6, 114.3, 113.6, 66.3 (2C), 62.6, 53.2 (2C); CI+HRMS m/z 269.1650 [M+H]+ C\(_{17}\)H\(_{20}\)N\(_2\)O requires 268.16.

Preparation of 2-{(4'-{(morpholinomethyl)-[1,1'-biphenyl]-3-yl)benzo[d]isothiazol-3(2H)-one 13

\[
\text{H}_2\text{N} \quad \text{H} \quad \text{H} \quad \text{H} \quad \text{N} \quad \text{O} \\
\text{H}_2\text{N} \quad \text{H} \quad \text{H} \quad \text{H} \quad \text{N} \quad \text{O}
\]

To a solution of 11b (1.59 mmol, 1.0 eq) in DCM, 2-(chlorothio)-benzoyl chloride (1.9 mmol, 1.2 eq) was added at 0°C under a N\(_2\) atmosphere. Et\(_3\)N (5.24 mmol, 3.3 eq) was added to the solution which was allowed to warm to room temperature and stirred for 20 hours. The solution was quenched with brine (40 ml), extracted into EtOAc (3 x 50 ml) and the combined organic extracts dried over MgSO\(_4\) before concentrating under reduced pressure. The crude product was purified by flash column chromatography to give 13 as a yellow crystalline solid in 79.0% yield. \(\text{RF} = 0.15\) (80:20 EtOAc:hexane and 1% triethylamine). \(^1\)H NMR (400 MHz, MeOD) \(\delta\) 8.05 (d, \(J = 7.9\) Hz, 1H), 7.94 (t, \(J = 1.7\) Hz, 1H), 7.87 (d, \(J = 8.2\) Hz, 1H), 7.77 (dt, \(J = 7.2, 1.1\) Hz, 1H), 7.68-7.62 (m, 4H), 7.59 (d, \(J = 7.6\) Hz, 1H), 7.54 (dt,
$J = 7.9, 0.8$ Hz, 1H), 7.46 (d, $J = 8.2$ Hz, 2H), 3.71 (t, $J = 4.7$ Hz, 4H), 3.57 (s, 2H), 2.49 (br s, 4H); $^{13}$C NMR (101 MHz, MeOD) δ 164.8, 142.2, 140.7, 138.9, 137.4, 136.8, 132.6, 129.9 (2C), 129.7, 126.7 (2C), 126.1, 125.8, 125.8, 124.2, 123.5, 123.3, 120.7, 66.3 (2C), 62.5, 53.2 (2C).

Elemental analysis (%)
found: C, 71.34; H, 5.57; N, 6.78; S, 7.64%. \( \text{C}_{24}\text{H}_{22}\text{N}_{2}\text{O}_{2}\text{S} \) requires C, 71.62; H, 5.51; N, 6.96; S, 7.97%;

ES+HRMS m/z 403.1491 [M+H]$^+$

\( \text{C}_{24}\text{H}_{22}\text{N}_{2}\text{O}_{2}\text{S} \) requires 402.14.

Preparation of \((4\text{-}((4\text{-}(\text{tert}\text{-}butoxycarbonyl)piperazin-1-yl)methyl)phenyl)boronic acid} \text{12a}

To a solution of 4-bromomethyl benzene boronic acid (1.0 g, 4.65 mmol) in a solution of anhydrous THF (20 ml) under a N\(_2\) atmosphere, 1-Boc-piperazine (2.0 eq, 9.31 mmol) was added, followed by iPr\(_2\)EtN (2.5 eq, 2.1 ml). The N\(_2\) atmosphere was restored and the resulting solution allowed to stir overnight at room temperature. The solution was quenched with brine (50 ml) and the organic layer extracted into EtOAc (3 x 40 ml). The combined organic extracts were then dried with MgSO\(_4\) and concentrated \text{in vacuo}. The crude product was purified by flash column chromatography to give 12a as a clear viscous oil in a 53.0% yield. \( \text{Rf} = 0.68 \) (5:95 MeOH:EtOAc and 1% triethylamine with permanganate stain).

\( ^{1}H\text{NMR} \) (400 MHz, MeOD) δ 7.68 (br s, 2H), 7.30 (d, $J = 7.8$ Hz, 2H), 3.54 (s, 2H), 3.43 (t, $J = 4.4$ Hz, 4H), 2.42 (t, $J = 5.0$ Hz, 4H), 1.46 (s, 9H); \( ^{13}C\text{NMR} \) (101 MHz, MeOD) δ 155.0, 133.4 (2C), 128.3 (4C), 79.9, 62.6, 46.2 (2C), 43.5, 42.6, 27.2 (3C).

Preparation of tert-butyl 4-\((3\text{-}amino\text{-}[1,1\text{-}\text{biphenyl}]\text{-}4\text{-}yl)methyl)piperazine-1-carboxylate \text{12b}

To a solution of Na\(_2\)CO\(_3\) (0.53 g, 5.02 mmol, 2.1 eq), 12a (0.77g, 2.39 mmol) and Pd(PPh\(_3\))\(_4\) (0.069 g, 0.060 mmol, 0.025 eq) in DME:water (15:7.5 ml), 3-bromoaniline (0.27 ml, 2.63 mmol, 1.1 eq) was added. The stirring solution was evacuated and purged with N\(_2\) before heating to 90°C for 18 hours. The solution was concentrated under reduced pressure to remove DME. The crude material was then quenched with brine (20 ml) and organic layer extracted into EtOAc (2 x 30 ml), dried over MgSO\(_4\) and concentrated under reduced pressure. The crude product was purified by flash column chromatography to give 12b as a yellow-brown viscos oil in a 96.7% yield. \( \text{Rf} = 0.51 \) (100% EtOAc and 1% triethylamine).

\( ^{1}H\text{NMR} \) (400 MHz, CDC\(_3\)) δ 7.52 (d, $J = 8.1$ Hz, 2H), 7.36 (d, $J = 8.1$ Hz, 2H), 7.22 (t,
J = 7.8 Hz, 1H), 6.99 (d, J = 7.7 Hz, 1H), 6.90 (s, 1H), 6.68 (dd, J = 7.9, 1.7 Hz, 1H), 3.54 (s, 2H), 3.44 (t, J = 4.6 Hz, 4H), 2.42 (s, 4H), 1.46 (s, 9H); 13C NMR (101 MHz, CDCl3) δ 154.8, 146.7, 142.1, 140.3, 136.8, 129.7, 129.5 (2C), 127.0 (2C), 117.6, 114.1, 113.8, 79.6, 62.8, 52.9 (2C), 44.1 (2C), 28.4 (3C); ES+HRMS m/z 368.2327 [M+H]+ C22H29N3O2 requires 367.23.

Preparation of tert-butyl 4-((3'-((3-oxobenzo[d]isothiazol-2(3H)-yl)-[1,1'-biphenyl]-4-yl)methyl)piperazine-1-carboxylate 12c

To a solution of 12b (0.85 g, 2.31 mmol) in DCM, X (1.2 eq, 2.77 mmol) was added at 0°C under a N₂ atmosphere. Et₃N (4.5 eq, 9.24 mmol) was added to the solution which was allowed to gradually warm to room temperature and stir for 20 hours. The solution was quenched with NaHCO₃ (30 ml) and the organic layer extracted into DCM (3 x 30 ml). The combined organic extracts were then washed with brine (30 ml), dried over MgSO₄ and concentrated under reduced pressure. The crude product was purified by flash column chromatography to give 12c as a yellow-orange foam in a 61.7% yield. Rf = 0.68 (100% EtOAc and 1% triethylamine). 1H NMR (400 MHz, DMSO) δ 8.09 (d, J = 8.1 Hz, 1H), 8.00 (t, J = 1.8 Hz, 1H), 7.98 (d, J = 7.7 Hz, 1H), 7.79 (td, J = 8.3, 1.2 Hz, 1H), 7.71 – 7.65 (m, 4H), 7.62 (t, J = 7.5 Hz, 1H), 7.53 (td, J = 7.9, 0.7 Hz, 1H), 7.43 (d, J = 8.2 Hz, 2H), 3.53 (s, 2H), 3.33 (br s, 4H), 2.34 (d, J = 4.8 Hz, 4H), 1.39 (s, 9H); 13C NMR (101 MHz, DMSO) δ 163.9, 154.3, 141.7, 140.6, 138.4, 138.3, 138.2, 133.1, 130.6, 130.1 (2C), 127.2 (2C), 126.7, 126.5, 125.7, 124.8, 123.7, 123.1, 122.5, 79.2, 65.4, 52.9 (2C), 44.1 (2C), 28.5 (3C); ES+HRMS m/z 524.1982 [M+Na]+ C29H31N3O2S requires 501.21.
Preparation of 2-(4’-(piperazin-1-ylmethyl)-[1,1’-biphenyl]-3-yl)benzo[d]isothiazol-3(2H)-one 16

To a solution of 12c (0.69 g, 1.38 mmol) in DCM (8 ml) under a N₂ atmosphere, trifluoroacetic acid (TFA) (1.6 ml, 20.7 mmol, 15.0 eq) was added and allowed to stir at room temperature overnight. After this time, DCM and excess TFA were removed under reduced pressure. The crude material was quenched with NaHCO₃ and the organic layer extracted into DCM (3 x 40 ml), washed with brine (40 ml), dried with MgSO₄ and concentrated in vacuo to give 16 as a yellow solid in a 89.4% yield. Rf = 0.03 (5:95 MeOH:EtOAc and 1% triethylamine). 1H NMR (400 MHz, MeOD) δ 8.07 (d, J = 7.9 Hz, 1H), 7.97 (t, J = 1.8 Hz, 1H), 7.89 (d, J = 8.2 Hz, 1H), 7.79 (td, J = 7.2, 1.2 Hz, 1H), 7.68 (d, J = 8.2 Hz, 3H), 7.64 (t, J = 7.6 Hz, 2H), 7.56 (td, J = 8.0, 0.9 Hz, 1H), 7.47 (d, J = 8.2 Hz, 2H), 3.62 (s, 2H), 2.96 (t, J = 5.0 Hz, 4H), 2.56 (br s, 4H); 13C NMR (101 MHz, CDCl₃) δ 164.2, 142.3, 139.9, 139.1, 137.7, 137.5, 132.5, 129.8, 129.7 (2C), 127.2, 127.2 (2C), 125.9, 125.7, 124.8, 123.3, 123.3, 120.1, 63.0, 53.1 (2C), 45.3 (2C); ES+HRMS m/z 402.1629 [M+H]⁺ C₂₄H₂₃N₃OS requires 401.16.

Preparation of 2-(4’-(4-(methylsulfonyl)piperazin-1-yl)methyl)-[1,1’-biphenyl]-3-yl)benzo[d]isothiazol-3(2H)-one 17

To a solution of 16 (0.10 g, 0.249 mmol) in DCM (8 ml) under a N₂ atmosphere at 0°C, methanesulfonyl chloride (0.03 ml, 0.299 mmol, 1.2 eq) and Et₃N (0.07 ml, 0.498 mmol, 2.0 eq) were added. The resulting solution was allowed to stir at 0°C for 4.5 hours. After this time, the crude material was diluted with brine and the organic layer extracted into DCM (2 x 40 ml), dried with MgSO₄ and concentrated in vacuo. The crude product was purified by flash column chromatography to give 17 as an off-white solid in a 65.7% yield. Rf = 0.73 (15:85 MeOH:EtOAc and 1% triethylamine). 1H NMR (400 MHz, MeOD) δ 8.07 (d, J = 7.8 Hz, 1H), 7.97 (t, J = 1.8 Hz, 1H), 7.89 (d, J = 8.2 Hz, 1H), 7.79 (td, J = 7.2, 1.2 Hz, 1H), 7.47 (d, J = 8.2 Hz, 2H), 3.62 (s, 2H), 2.96 (t, J = 5.0 Hz, 4H), 2.56 (br s, 4H); 13C NMR (101 MHz, CDCl₃) δ 164.2, 142.3, 139.9, 139.1, 137.7, 137.5, 132.5, 129.8, 129.7 (2C), 127.2, 127.2 (2C), 125.9, 125.7, 124.8, 123.3, 123.3, 120.1, 63.0, 53.1 (2C), 45.3 (2C); ES+HRMS m/z 402.1629 [M+H]⁺ C₂₄H₂₃N₃OS requires 401.16.
1.1 Hz, 1H), 7.72 – 7.60 (m, 5H), 7.57 (td, J = 7.9, 0.8 Hz, 1H), 7.48 (d, J = 8.2 Hz, 2H), 3.65 (s, 2H), 3.26 (t, J = 4.7 Hz, 4H), 2.86 (s, 3H), 2.61 (t, J = 4.8 Hz, 4H); 13C NMR (101 MHz, DMSO) δ 163.9, 141.7, 140.6, 138.5, 138.3, 138.2, 133.1, 130.6, 130.0 (2C), 127.2 (2C), 126.7, 126.5, 125.7, 124.8, 123.8, 123.1, 122.5, 61.6, 52.3 (2C), 46.0 (2C), 34.0; Elemental analysis (%) found: C, 61.05; H, 5.27; N, 8.51; S, 13.01%. C25H25N3O3S2 requires C, 62.61; H, 5.25; N, 8.76; S, 13.37%; ES+MS m/z 502.1

Preparation of (3-nitrobenzyl) heterocycles 18a-i, 18a-ii

**General procedure (3)**

To a solution of 3-nitrobenzyl bromine (0.8 g, 3.70 mmol) in a solution of anhydrous THF (15 ml) under a N2 atmosphere, the appropriate heterocycle (2.0 eq, 7.41 mmol) was added, followed by iPr2EtN (2.2 - 3.2 eq). The N2 atmosphere was restored and the resulting solution allowed to stir overnight at room temperature. The solution was quenched with brine (50 ml) and the organic layer extracted into EtOAc (2 x 40 ml). The combined organic extracts were then dried with MgSO4 and concentrated in vacuo to afford the crude product.

Preparation of 4-fluoro-1-(3-nitrobenzyl)piperidine 18a-i

The general procedure (3) was followed using 4-fluoropiperidine hydrochloride. The resulting crude product was purified by flash column chromatography to give 18a-i as a clear yellow oil in 72.7% yield. RF = 0.66 (2:98 MeOH:EtOAc and 1% triethylamine). 1H NMR (400 MHz, CDCl3) δ 8.21 (s, 1H), 8.11 (d, J = 8.2, 1.3 Hz, 1H), 7.67 (d, J = 7.6 Hz, 1H), 7.49 (t, J = 8.3 Hz, 1H), 4.84 – 4.59 (dm, JHF = 48.6 Hz, 1H), 2.67 – 2.50 (m, 2H), 2.50 – 2.31 (m, 2H), 2.01 – 1.80 (dm, JHF = 24.4 Hz, 4H); 13C NMR (101 MHz, CDCl3) δ 148.4, 141.1, 134.8, 129.2, 123.6, 122.2, 88.2 (d, J = 170.8 Hz, 1C), 62.0, 49.5 (d, J = 5.5 Hz, 2C), 31.4 (d, J = 19.6 Hz, 2C); CI+HRMS m/z 239.1192 [M+H]+ C12H15FN2O2 requires 238.11.

Preparation of 1-methyl-4-(3-nitrobenzyl)piperazine 18a-ii

The general procedure (3) was followed using N-methylpiperazine. The resulting crude product was purified by trituration with Et2O to give 18a-ii as an off-white solid in 88.7% yield. RF = 0.10 (5:95 MeOH:EtOAc and 1% triethylamine). 1H NMR (400 MHz, CDCl3) δ 8.21 (s, 1H), 8.11 (d, J = 8.2, 1.5 Hz, 1H), 7.67 (d, J = 7.6 Hz, 1H), 7.48 (t, J = 7.9 Hz, 1H), 3.60 (s, 2H), 2.49 (br s, 8H), 2.30 (s, 3H); 13C NMR
(101 MHz, CDCl$_3$) δ 148.4, 140.8, 135.0, 129.1, 123.7, 122.2, 62.0, 55.1 (2C), 53.1 (2C), 46.0; CI+HRMS m/z 236.1393 [M+H]$^+$ requires 235.13.

**Preparation of (3-aminobenzyl) heterocycles 18b-i, 18b-ii, 18b-iii**

**General procedure (4)**

To a solution of the appropriate (3-nitrobenzyl) heterocycle (4i-5i) in 50:50 EtOAc-MeOH (10 ml/mmol) 10% Pd/C (10% by mass) was added. The solution was placed under a H$_2$ atmosphere and allowed to stir overnight at room temperature. After this time, the solution was filtered through Celite under vacuum, the resulting material washed with 50:50 EtOAc-hexane and concentrated under reduced pressure. The crude products were purified by flash column chromatography.

**Preparation of 3-((4-fluoropiperidin-1-yl)methyl)aniline 18b-ii**

The general procedure (4) was followed using 18a-i (0.6 g, 2.52 mmol) to give 18b-ii as a white solid in 49.8% yield. Rf = 0.15 (EtOAc and 1% triethylamine). $^1$H NMR (400 MHz, MeOD) δ 7.07 (t, J = 7.7 Hz, 1H), 6.72 (t, J = 1.7 Hz, 1H), 6.69 – 6.63 (m, 2H), 4.75 – 4.54 (dm, $^3$J$_{HF}$ = 48.9 Hz, 1H), 3.43 (s, 2H), 2.68 – 2.50 (m, 2H), 2.52 – 2.32 (m, 2H), 2.00 – 1.73 (dm, $^3$J$_{HF}$ = 25.1 Hz, 4H); $^{13}$C NMR (101 MHz, MeOD) δ 148.7, 139.3, 130.0, 120.6, 117.8, 115.7, 89.2 (d, J = 170.8 Hz, 1C), 64.1, 50.5 (d, J = 5.8 Hz, 2C), 32.17 (d, J = 19.8 Hz, 2C); CI+HRMS m/z 209.1450 [M+H]$^+$ requires 208.14.

**Preparation of 3-((4-methylpiperazin-1-yl)methyl)aniline 18b-iii**

The general procedure (4) was followed using 18a-ii (0.77 g, 3.29 mmol) to give 18b-iii as a whitish-yellow solid in 62.1% yield. Rf = 0.14 (1:5 MeOH:EtOAc and 1% triethylamine). $^1$H NMR (400 MHz, MeOD) δ 7.06 (t, J = 7.7 Hz, 1H), 6.72 (t, J = 1.6 Hz, 1H), 6.69 – 6.62 (m, 2H), 3.44 (s, 2H), 2.52 (br s, 8H), 2.30 (s, 3H); $^{13}$C NMR (101 MHz, MeOD) δ 148.8, 139.0, 130.0, 120.6, 117.8, 115.7, 64.0, 55.6 (2C), 53.5 (2C), 45.9; CI+HRMS m/z 206.1649 [M+H]$^+$ requires 205.16.
Preparation of Mono-phenyl, methylene-linked saturated heterocyclic benzo[d]isothiazol-3(2H)-ones 19-21

General procedure (5)

To a solution of the appropriate (3-aminobenzyl) heterocycle (18b-i - 18b-ii) (1.2 eq, 1.2 mmol) in DCM, 2-(chlorothio)-benzoyl chloride (X) (1.0 eq, 1.00 mmol) was added at 0°C under a N₂ atmosphere. Et₃N (3.3 eq, 3.3 mmol) was added to the solution which was allowed to gradually warm to room temperature and stir for 20 hours. The solution was quenched with NaHCO₃ (30 ml) and the organic layer extracted into DCM (3 x 30 ml). The combined organic extracts were then washed with brine (30 ml), dried over MgSO₄ and concentrated under reduced pressure. The crude products were purified by flash column chromatography.

Preparation of 2-(3-(morpholinomethyl)phenyl)benzo[d]isothiazol-3(2H)-one 19

The general procedure (5) was followed using 3-morpholine-4-yl methyl-phenylamine (18b-i) (purchased from Sigma Aldrich) to give 19 as a bright yellow solid in 56.0% yield. Rf = 0.35 (2:98 MeOH:EtOAc and 1% triethylamine). ¹H NMR (400 MHz, CDCl₃) δ 8.11 (d, J = 7.9, 0.8 Hz, 1H), 7.71 (s, 1H), 7.67–7.57 (m, 2H), 7.45 (td, J = 8.0, 1.0 Hz, 1H), 7.42 (t, J = 7.9 Hz, 1H), 7.29 (d, J = 7.7 Hz, 1H), 7.20 (t, J = 4.6 Hz, 4H), 3.56 (s, 2H), 3.72 (t, J = 4.6 Hz, 4H); ¹³C NMR (101 MHz, CDCl₃) δ 164.1, 139.9, 139.6, 137.3, 132.4, 129.2, 127.7, 127.2, 125.8, 125.1, 124.9, 123.3, 120.1, 67.0 (2C), 63.0, 53.6 (2C); Elemental analysis (%) found: C, 65.89; H, 5.63; N, 8.47; S, 9.65%; C₁₈H₁₈N₂O₂S requires C, 66.23; H, 5.66; N, 8.58; S, 9.82%; CI+HRMS m/z 327.1165 [M+H]⁺ C₁₈H₁₈N₂O₂S requires 326.11.

Preparation of 2-(3-((4-fluoropiperidin-1-yl)methyl)phenyl)benzo[d]isothiazol-3(2H)-one 20

The general procedure (5) was followed using 18b-ii to give 20 as a yellow crystalline solid in 68.2% yield. Rf = 0.28 (100% EtOAc). ¹H NMR (400 MHz, MeOD) δ 8.04 (d, J = 7.9 Hz, 1H), 7.87 (d, J = 8.2 Hz, 1H), 7.77 (td, J = 7.2, 1.1 Hz, 1H), 7.70 (s, 1H), 7.62 (d, J = 8.0 Hz, 1H), 7.54 (td, J = 7.9, 0.7 Hz, 1H), 7.51 (t, J = 7.9 Hz, 1H), 7.39 (d, J = 7.6 Hz, 1H), 4.77–4.58 (dm, 2JHF = 48.8 Hz, 1H), 3.62 (s, 2H), 2.79–2.56 (m, 2H), 2.57–2.33 (m, 2H), 2.02–1.78 (dm, 3JHF = 25.5 Hz, 4H); ¹³C NMR (101 MHz, MeOD) δ 164.7, 140.7, 139.4, 136.9, 132.5, 129.1, 128.4, 126.1, 125.8, 125.7, 124.2, 123.8, 120.7, 87.7 (d, J = 171.3 Hz, 63.0, 53.6 (2C);
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1C, 61.9, 49.1 (d, J = 5.9 Hz, 2C), 30.9 (d, J = 19.7 Hz, 2C). **Elemental analysis** (%) found: C, 66.39; H, 5.63; N, 8.13; S, 9.52%. \( \text{C}_{19}\text{H}_{19}\text{FN}_{2}\text{OS} \) requires C, 66.64; H, 5.59; N, 8.18; S, 9.36%; \( \text{ES}+\text{HRMS} \) \( m/z \) 343.1283 \([\text{M+H}]^+\). \( \text{C}_{19}\text{H}_{19}\text{FN}_{2}\text{OS} \) requires 342.12.

**Preparation of 2-((4-methylpiperazin-1-yl)methyl)phenyl)benzo[d]isothiazol-3(2H)-one 21**

The general procedure (5) was followed using 18b-iii to give 21 as a yellow-brown solid in 64.5% yield. \( R_f = 0.10 \) (1:5 MeOH:EtOAc and 1% triethylamine). \( ^1\text{H NMR} \) (400 MHz, MeOD) \( \delta \) 8.05 (d, J = 7.9 Hz, 1H), 7.88 (d, J = 8.2 Hz, 1H), 7.78 (td, J = 7.2, 1.2 Hz, 1H), 7.71 (s, 1H), 7.62 (d, J = 8.0 Hz, 1H), 7.58 – 7.48 (m, 2H), 7.40 (d, J = 7.6 Hz, 1H), 3.64 (s, 2H), 2.56 (br s, 8H), 2.31 (s, 3H); \( ^{13}\text{C NMR} \) (101 MHz, MeOD) \( \delta \) 164.7, 140.7, 139.2, 136.9, 129.1, 128.3, 126.1, 125.8, 125.6, 124.2, 123.8, 120.8, 61.7, 54.3 (2C), 52.0 (2C), 44.5; **Elemental analysis** (%) found: C, 66.60; H, 6.27; N, 12.27; S, 9.12%. \( \text{C}_{19}\text{H}_{21}\text{N}_3\text{OS} \) requires C, 67.23; H, 6.24; N, 12.38; S, 9.45%. \( \text{Cl}+\text{HRMS} \) \( m/z \) 340.1480 \([\text{M+H}]^+\). \( \text{C}_{19}\text{H}_{21}\text{N}_3\text{OS} \) requires 339.14.

**Preparation of 3-pyridinyl anilines 22a-d**

**General procedure (6)**

The appropriate pyridinyl boronic acid (1.5 eq) was combined with 3-bromo aniline in a solution of DME (15 ml) at room temperature. \( \text{Na}_2\text{CO}_3 \) (2.1 eq) and \( \text{Pd(PPh}_3)_4 \) (0.03 eq) in water (3 ml) (giving a 1:5 \( \text{H}_2\text{O}:\text{DME} \) ratio) were added to the stirring solution which was evacuated and purged with \( \text{N}_2 \) before refluxing at 90°C for 18 hours. Solvent was removed under reduced pressure and the crude material re-suspended in DCM (40 ml), dried over \( \text{MgSO}_4 \) and concentrated under reduced pressure. The crude products were purified by flash column chromatography.

**Preparation of 3-(pyridin-4-yl)aniline 22a**

The general procedure (6) was followed using 4-pyridinyl phenyl boronic acid (0.4 g, 3.25 mmol) to give 22a as a white solid in 97.3% yield. \( R_f = 0.14 \) (7:3 EtOAc:hexane). \( ^1\text{H NMR} \) (400 MHz, MeOD) \( \delta \) 8.55 (d, J = 4.6 Hz, 1H), 8.55 (d, J = 4.6 Hz, 1H), 7.68 (d, J = 4.6 Hz, 1H), 7.67 (d, J = 4.6 Hz, 1H), 7.25 (t, J = 7.8 Hz, 1H), 7.09 (t, J = 1.9 Hz, 1H), 7.05 (ddd, J = 7.6, 1.5, 0.9 Hz, 1H), 6.83 (ddd, J = 8.0, 2.2, 0.9 Hz,
1H; \(^{13}\text{C NMR}\) (101 MHz, MeOD) δ 150.0, 148.9 (2C), 148.5, 138.2, 129.6, 121.7 (2C), 116.0, 116.0, 116.0, 113.1; CI+HRMS m/z 171.0920 \([\text{M+H}]^+\) \(\text{C}_{11}\text{H}_{10}\text{N}_2\) requires 170.08.

Preparation of 3-(6-methoxypyridin-3-yl)aniline 22b

The general procedure (6) was followed using 6-methoxy-3-pyridinyl phenyl boronic acid (0.5 g, 3.27 mmol) to give 22b as a yellow-brown oil solid in 70.5% yield. \(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)) δ 8.36 (d, J = 2.5 Hz, 1H), 7.76 (dd, J = 8.6, 2.5 Hz, 1H), 7.23 (t, J = 7.8 Hz, 1H), 6.92 (d, J = 7.7 Hz, 1H), 6.83 (t, J = 2.0 Hz, 1H), 6.80 (d, J = 8.6 Hz, 1H), 6.68 (ddd, J = 8.0, 2.3, 0.7 Hz, 1H), 3.98 (s, 3H); \(^{13}\text{C NMR}\) (101 MHz, CDCl\(_3\)) δ 163.6, 146.9, 144.9, 139.1, 137.5, 130.3, 129.9, 117.2, 114.1, 113.3, 110.7, 53.6; CI+HRMS m/z 201.1027 \([\text{M+H}]^+\) \(\text{C}_{12}\text{H}_{12}\text{N}_2\text{O}\) requires 200.09.

Preparation of 3-(6-chloropyridin-3-yl)aniline 22c

The general procedure (6) was followed using (6-chloro-pyridin-3-yl)boronic acid (0.2 g, 1.27 mmol) to give 22c as an orange oil in 89.5% yield. \(\text{RF} = 0.68\) (8:2 EtOAc:hexane and 1% triethylamine). \(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)) δ 8.57 (dd, J = 2.5, 0.4 Hz, 1H), 7.81 (dd, J = 8.3, 2.6 Hz, 1H), 7.38 (dd, J = 8.3, 0.6 Hz, 1H), 7.26 (t, J = 7.7 Hz, 1H), 6.92 (ddd, J = 7.6, 1.6, 0.9 Hz, 1H), 6.84 (t, J = 2.0 Hz, 1H), 6.74 (ddd, J = 8.0, 2.3, 0.8 Hz, 1H), 3.82 (s, 2H, NH\(_2\)); \(^{13}\text{C NMR}\) (101 MHz, CDCl\(_3\)) δ 150.2, 147.9, 147.1, 137.6, 137.2, 135.9, 130.2, 124.2, 117.4, 115.1, 113.4; CI+HRMS m/z 205.0635 \([\text{M+H}]^+\) \(\text{C}_{11}\text{H}_{9}\text{ClN}_2\) requires 204.05.

Preparation of 3-(6-(trifluoromethyl)pyridin-3-yl)aniline 22d

The general procedure (6) was followed using 2-(trifluoromethyl)pyridine-5-boronic acid (0.4 g, 2.10 mmol) to give 22d as an off-white crystalline solid in quantitative yield. \(\text{RF} = 0.72\) (7:3 EtOAc:hexane and 1% triethylamine). \(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)) δ 8.91 (d, J = 1.9 Hz, 1H), 8.00 (dd, J = 8.1, 1.7 Hz, 1H), 7.73 (d, J = 8.1 Hz, 1H), 7.29 (t, J = 7.8 Hz, 1H), 6.97 (ddd, J = 7.6, 1.5, 0.8 Hz, 1H), 6.89 (t, J = 2.0 Hz, 1H), 6.78 (ddd, J = 8.0, 2.3, 0.7 Hz, 1H), 3.85 (s, 2H, NH\(_2\)); \(^{13}\text{C NMR}\) (101 MHz, CDCl\(_3\)) δ 148.5, 147.2, 146.69 (q, \(^3\text{J}_{\text{CF}} = 34.8\text{Hz}, 1\text{C}\)), 139.7, 137.5, 135.5, 130.3, 121.73 (q, \(^3\text{J}_{\text{CF}} = 273.8\text{Hz}, 1\text{C}\)), 120.37 (q, \(^3\text{J}_{\text{CF}} = 2.6\text{Hz}, 1\text{C}\)), 117.64, 115.6, 113.6; CI+HRMS m/z 239.0790 \([\text{M+H}]^+\) \(\text{C}_{12}\text{H}_{9}\text{F}_3\text{N}_2\) requires 238.07.
Preparation of biphenyl-pyridinyl benzo[d]isothiazol-3(2H)-ones 23-26

**General procedure (7)**

To a solution of the appropriate 3-pyridinyl aniline 22a-d in DCM (10 ml/mmol), X (1.2 eq) was added at 0°C under a N₂ atmosphere. Et₃N (3.3 eq) was added to the solution which was allowed to gradually warm to room temperature and stir for 20 hours. The solution was quenched with NaHCO₃ (30 ml) and the organic layer extracted into DCM (3 x 30 ml). The combined organic extracts were then washed with brine (30 ml), dried over MgSO₄ and concentrated under reduced pressure. The crude products were purified by trituration using Et₂O and EtOAc.

**Preparation of 2-(3-(pyridin-4-yl)phenyl)benzo[d]isothiazol-3(2H)-one 23**

The general procedure (7) was followed using 22a (0.2 g, 1.18 mmol) to give 23 as an off-white solid in 21.1% yield. **Rf** = 0.25 (8:2 EtOAc:hexane). **¹H NMR** (400 MHz, MeOD) δ 8.64 (br s, 2H), 8.12 (t, J = 1.8 Hz, 1H), 8.05 (d, J = 7.9 Hz, 1H), 7.87 (d, J = 8.2 Hz, 1H), 7.82 – 7.72 (m, 5H), 7.68 (t, J = 7.9 Hz, 1H), 7.54 (td, J = 7.1, 0.8 Hz, 1H); **¹³C NMR** (101 MHz, MeOD) δ 164.8, 149.3 (2C), 148.1, 140.7, 139.0, 137.9, 132.7 (2C), 130.1, 126.2, 125.9, 125.8, 125.4, 124.1, 123.4, 120.8; **Elemental analysis (%)** found: C, 70.42; H, 4.07; N, 9.27; S, 10.54%. **Cl+HRMS** m/z 305.0749 [M+H]⁺. C₁₈H₁₂N₂O requires C, 71.03; H, 3.97; N, 9.20; S, 10.54%.

**Preparation of 2-(3-(6-methoxypyridin-3-yl)phenyl)benzo[d]isothiazol-3(2H)-one 24**

The general procedure (7) was followed using 22b (0.13 g, 0.67 mmol) to give 24 as an off-white solid in 33.9% yield. **Rf** = 0.65 (6:4 EtOAc:hexane). **¹H NMR** (400 MHz, DMSO) δ 8.54 (d, J = 2.5 Hz, 1H), 8.12 – 8.04 (m, 2H), 8.01 – 7.94 (m, 2H), 7.79 (td, J = 8.3, 1.2 Hz, 1H), 7.72 – 7.58 (m, 3H), 7.53 (td, J = 7.9, 0.7 Hz, 1H), 6.96 (d, J = 8.6 Hz, 1H), 3.92 (s, 3H); **¹³C NMR** (101 MHz, DMSO) δ 163.9, 163.9, 145.4, 140.6, 138.8, 138.3, 138.2, 133.1, 130.7, 128.9, 126.7, 126.5, 125.5, 124.7, 123.9, 122.9, 122.4, 111.2, 53.8; **Elemental analysis (%)** found: C, 65.50; H, 4.44; N, 8.18; S, 9.38%. **Cl+HRMS** m/z 335.0581 [M+H]⁺. C₁₉H₁₄N₂O₂S requires C, 68.24; H, 4.22; N, 8.38; S, 9.59%. **Cl+HRMS** m/z 335.0581 [M+H]⁺. C₁₉H₁₄N₂O₂S requires 334.08.
Preparation of 2-(3-(6-chloropyridin-3-yl)phenyl)benzo[d]isothiazol-3(2H)-one 25

The general procedure (7) was followed using 22C (0.05 g, 0.26 mmol) to give 25 as an off-white solid in 49.3% yield. \( \text{Rf} = 0.65 \) (6:4 EtOAc:hexane). \( ^1H \text{NMR} \) (400 MHz, DMSO) \( \delta 8.80 \) (d, \( J = 2.6 \) Hz, 1H), 8.23 (dd, \( J = 8.4, 2.7 \) Hz, 1H), 8.82 – 8.76 (m, 2H), 7.98 (d, \( J = 7.8 \) Hz, 1H), 7.82 – 7.72 (m, 3H), 7.71 – 7.62 (m, 2H), 7.53 (td, \( J = 7.9, 0.7 \) Hz, 1H); \( ^{13}C \text{NMR} \) (101 MHz, DMSO) \( \delta 163.9, 150.2, 148.5, 140.7, 138.5, 138.4, 137.4, 134.6, 133.2, 130.8, 126.7, 126.6, 126.1, 125.1, 125.0, 124.6, 123.6, 122.5); \(+\text{HRMS} \) m/z 339.0363 [M+H] \(^+\) \( C_{18}H_{11}ClN_2O \) requires 338.03; \(+\text{HPLC} \) (purity analysis %) found: 99.10%; RT: 9.11 min/40 min (25/75, 0.5 ml/min IPA:HEXANE, Signal = 230 nm, SYNCHROSILICA).

Preparation of 2-(3-(6-(trifluoromethyl)pyridin-3-yl)phenyl)benzo[d]isothiazol-3(2H)-one 26

The general procedure (7) was followed using 22d (0.19 g, 0.81 mmol) to give 26 as an off-white solid in 62.5% yield. \( \text{Rf} = 0.71 \) (6:4 EtOAc:hexane). \( ^1H \text{NMR} \) (400 MHz, DMSO) \( \delta 9.15 \) (d, \( J = 1.9 \) Hz, 1H), 8.43 (dd, \( J = 8.1, 1.9 \) Hz, 1H), 8.17 (t, \( J = 1.8 \) Hz, 1H), 8.09 (d, \( J = 8.1 \) Hz, 1H), 8.04 (d, \( J = 8.2 \) Hz, 1H), 7.99 (d, \( J = 7.9 \) Hz, 1H), 7.87 – 7.76 (m, 3H), 7.72 (t, \( J = 7.9 \) Hz, 1H), 7.54 (td, \( J = 7.9, 0.8 \) Hz, 1H); \( ^{13}C \text{NMR} \) (101 MHz, DMSO) \( \delta 164.0, 148.9, 146.1 \) (q, \( ^2J_CF = 34.0 \) Hz, 1C), 140.7, 138.5, 137.3, 136.9, 133.2, 130.9, 126.7, 126.6, 126.5, 126.3, 125.6, 124.6, 124.0, 122.5, 122.2 (q, \( ^1J_CF = 274.2 \) Hz, 1C), 121.4 (q, \( ^3J_CF = 2.8 \) Hz, 1C); \(+\text{HRMS} \) m/z 373.0617 [M+H] \(^+\) \( C_{19}H_{11}F_3N_2O_S \) requires 372.05; \(+\text{HPLC} \) (purity analysis %) found: 99.47%; RT: 8.71 min/40 min (25/75, 0.5 ml/min IPA:HEXANE, Signal = 230 nm, SYNCHROSILICA).

Preparation of 4-(2-(3-nitrophenoxy)ethyl)morpholine 27a

![Chemical Structure](image)

3-nitrophenol (1.0 g, 7.19 mmol), \( K_2CO_3 \) (2.09 g, 15.1 mmol, 2.1 eq) and 4-(2-chloroethyl)morpholine hydrochloride (1.47 g, 7.91 mmol, 1.1 eq) were dissolved DMF (10 ml) and placed under a \( N_2 \) atmosphere and the solution heated to 50°C overnight. After 16 hours the reaction was complete and DMF was removed under reduced pressure to produce an orange/red crude solid which was partitioned between EtOAc (50 ml) and \( H_2O \) (50 ml). The organic layer was extracted into EtOAc (2x40 ml), dried with \( Na_2CO_3 \) and concentrated under reduced pressure. The crude product was purified by column chromatography to give 27a as clear brown oil in a 90.0% yield. \( \text{Rf} = 0.10 \) (8:2 EtOAc:hexane). \( ^1H \text{NMR} \) (400 MHz, CDCl\(_3\)) \( \delta 7.83 \) (ddd, \( J = 8.1, 1.3, 0.6 \) Hz, 1H), 7.75 (t, \( J = 2.3 \) Hz, 1H), 7.43 (t, \( J = 8.2 \) Hz, 1H), 7.24 (ddd, \( J = 7.7, 1.9, 0.6 \) Hz, 1H), 4.19 (t, \( J = 5.6 \) Hz, 2H), 3.74 (apparent t, \( J = 4.7 \) Hz, 4H), 2.84 (t, \( J = 5.6 \) Hz, 2H), 2.59 (J = 4.6 Hz, 4H); \( ^{13}C \text{NMR} \) (101 MHz, CDCl\(_3\)) \( \delta 159.9, 149.2, 130.0, 121.8, 115.9, 108.9, 66.9 \) (2C), 66.8, 57.4, 54.1 (2C); \(+\text{HRMS} \) m/z 253.1181 [M+H] \(^+\) \( C_{12}H_{16}N_2O_4 \) requires 252.1
Preparation of 3-(2-morpholinoethoxy)aniline 27b

10% Pd/C (0.05g, 10% by mass) was added to a solution of 27a (0.5 g, 1.98 mmol) in 50:50 EtOAc-MeOH (30 ml). The solution was put under a hydrogen atmosphere at room temperature overnight after which the reaction was complete. The solution was filtered through Celite under vacuum and resulting material was washed with 50:50 EtOAc-hexane and concentrated under reduced pressure to give 27b as the pure product, a red/orange viscous oil in a 80.0% yield. \textit{Rf} = 0.10 (EtOAc and 1% triethylamine). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.05 (t, $J = 7.7$ Hz, 1H), 6.31 (apparent t, $J = 8.9$ Hz, 2H), 6.25 (s, 1H), 4.07 (br s, 2H), 3.73 (s, 4H), 3.64 (s, 2H, NH), 2.78 (s, 2H), 2.57 (s, 4H); $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 159.9, 147.8, 130.1, 108.1, 104.6, 101.8, 70.0 (2C), 65.6, 57.7, 54.1 (2C); CI+HRMS m/z 223.1449 [M+H]$^+$ C$_{12}$H$_{18}$N$_2$O$_2$ requires 222.1.

Preparation of 2-(3-(2-morpholinoethoxy)phenyl)benzo[d]isothiazol-3(2H)-one 28

A solution of 27b (0.388 g, 1.75 mmol) in DCM (12 ml) was added to a solution of X (0.434 g, 2.09 mmol, 1.2 eq) in DCM (12 ml) at 0°C under a N$_2$ atmosphere. Et$_3$N (0.80 ml, 5.76 mmol, 3.3 eq) was added to the solution, the flask evacuated and purged with N$_2$ before gradually warming to room temperature and allowed to stir overnight. The solution was quenched with brine (30 ml) and the organic layer extracted into DCM (2 x 30 ml), washed with NaHCO$_3$ (30 ml), dried with MgSO$_4$ and concentrated \textit{in vacuo}. The crude product was purified by column chromatography to give 28 as a yellow solid in a 28.4% yield. \textit{Rf} = 0.30 (5:95 MeOH:EtOAc and 1% triethylamine). $^1$H NMR (250 MHz, MeOD) $\delta$ 8.02 (dt, $J = 7.6$, 1.1 Hz, 1H), 7.84 (dt, $J = 8.1$, 0.9 Hz, 1H), 7.75 (td, $J = 8.3$, 1.2 Hz, 1H), 7.52 (td, $J = 8.0$, 1.1 Hz, 1H), 7.43 (t, $J = 8.1$ Hz, 1H), 7.35 (td, $J = 2.2$ Hz, 1H), 7.23 (ddd, $J = 8.0$, 2.0, 0.9 Hz, 1H), 6.99 (ddd, $J = 8.3$, 2.5, 0.9 Hz, 1H), 4.21 (t, $J = 5.5$ Hz, 2H), 3.72 (t, $J = 4.7$ Hz, 4H), 2.84 (t, $J = 5.5$ Hz, 2H), 2.61 (t, $J = 4.7$ Hz, 4H); $^{13}$C NMR (63MHz, MeOD) $\delta$ 164.7, 159.5, 140.7, 137.9, 132.6, 130.0, 126.1, 125.8, 124.3, 120.7, 116.9, 113.6, 111.2, 66.2 (2C), 65.3, 57.2, 53.7 (2C); \textit{Elemental analysis} (%) found: C, 63.92; H, 5.56; N, 7.95; S, 8.48%. C$_{19}$H$_{20}$N$_2$O$_3$S requires C, 64.02; H, 5.66; N, 7.86; S, 9.00%; ES+HRMS m/z 357.1270 [M+H]$^+$ C$_{19}$H$_{20}$N$_2$O$_3$S requires 356.12.
Preparation of **tert-butyl 4-(4-(3-aminophenyl)-1H-pyrazol-1-yl)piperidine-1-carboxylate 29a**

![Chemical Structure](image)

To a solution of Na$_2$CO$_3$ (0.295 g, 2.78 mmol, 2.1 eq), 1-(1-Boc-4-piperidyl)pyrazole-4-boronic acid pinacol ester (0.26 g, 0.82 mmol) and Pd(PPh$_3$)$_4$ (0.038 g, 0.033 mmol, 0.025 eq) in DME:water (10:5 ml), 3-bromoaniline (1.6 ml, 1.46 mmol, 1.1 eq) was added. The stirring solution was evacuated and purged with N$_2$ before heating to 91°C for 18 hours. The solution was then concentrated under reduced pressure to remove DME and the crude material quenched with brine (20 ml) and organic layer extracted into EtOAc (2 x 30 ml), dried over MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by column chromatography to give 29a as a yellow-brown oil in a 97.5% yield. **Rf** = 0.60 (EtOAc and 1% triethylamine). **$^1$H NMR** (400 MHz, CDCl$_3$) δ 7.74 (s, 1H), 7.62 (s, 1H), 7.14 (t, J = 7.8 Hz, 1H), 6.87 (d, J = 7.7 Hz, 1H), 6.80 (t, J = 1.9 Hz, 1H), 6.56 (dd, J = 7.9, 1.6 Hz, 1H), 4.33 – 4.21 (m, 3H), 3.69 (br s, NH$_2$), 2.90 (t, J = 11.7 Hz, 2H), 2.15 (dd, J = 12.6, 1.8 Hz, 2H), 1.94 (dd, J = 24.4, 12.2, 4.2 Hz, 2H), 1.48 (s, 9H); **$^{13}$C NMR** (101 MHz, CDCl$_3$) δ 154.6, 146.8, 136.5, 133.6, 129.8, 123.6, 122.9, 116.0, 113.4, 112.1, 79.9, 59.4, 42.7 (2C), 32.4 (2C), 28.4 (3C); **ES+HRMS** m/z 365.1952 [M+Na]$^+$ requires 342.21.

Preparation of **tert-butyl 4-(4-(3-oxobenzo[d]isothiazol-2(3H)-yl)phenyl)-1H-pyrazol-1-yl)piperidine-1-carboxylate 29b**

A solution of 29a (0.422 g, 1.23 mmol) in DCM (12 ml) was added to a solution of X (0.31 g, 1.48 mmol, 1.2 eq) in DCM (12 ml) at 0°C under a N$_2$ atmosphere. Et$_3$N (0.60 ml, 4.07 mmol, 3.3 eq) was added to the solution, the flask evacuated and purged with N$_2$ before gradually warming to room temperature and allowed to stir overnight. The solution was diluted further with DCM (15 ml) and washed with
brine (30 ml). The organic layer was then extracted into DCM (2 x 30 ml), washed with NaHCO₃ (30 ml), dried with MgSO₄ and concentrated in vacuo. The crude product was purified by trituration with Et₂O to give 29b as a yellow viscous liquid in a 96.5% yield. \( \text{RF} = 0.31 \) (7:3 EtOAc:hexane and 1% triethylamine). \(^1\text{H NMR}\) (400 MHz, CDCl₃) \( \delta \): 8.12 (d, \( J = 7.9 \) Hz, 1H), 7.89 (t, \( J = 1.5 \) Hz, 1H), 7.82 (s, 1H), 7.73 (s, 1H), 7.68 (dt, \( J = 8.2, 1.2 \) Hz , 1H), 7.60 (d, \( J = 8.1 \) Hz, 1H), 7.53 – 7.40 (m, 4H), 4.37 – 4.21 (m, 3H), 3.08 – 2.85 (m, 2H), 2.17 (d, \( J = 10.4 \) Hz, 2H), 1.95 (ddd, \( J = 24.5, 12.3, 4.4 \) Hz, 3H), 1.49 (s, 9H); \(^{13}\text{C NMR}\) (101 MHz, CDCl₃) \( \delta \): 164.2, 154.6, 139.9, 137.8, 136.6, 134.1, 132.5, 129.86, 127.2, 125.9, 124.8, 124.1, 124.0, 122.2, 122.0, 121.7, 120.172, 80.0, 59.6, 43.2 (2C), 32.5 (2C), 28.4 (3C); \( \text{ES+HRMS} \) m/z 499.1790 [M+Na]⁺ \( \text{C}_{26}\text{H}_{28}\text{N}_{4}\text{O}_{3}\text{S} \) requires 476.19.

Preparation of 2-(3-{1-(piperidin-4-yl)-1H-pyrazol-4-yl)phenyl}benzo[d]isothiazol3(2H)-one 30

![Preparation of 2-(3-{1-(piperidin-4-yl)-1H-pyrazol-4-yl)phenyl}benzo[d]isothiazol3(2H)-one 30](image)

To a solution of 29b (0.588 g, 1.23 mmol) in DCM (7 ml) under a N₂ atmosphere, trifluoroacetic acid (TFA) (1.5 ml, 18.5 mmol, 15.0 eq) was added and allowed to stir at room temperature overnight. After this time, DCM and excess TFA were removed under reduced pressure. The crude material was quenched with NaHCO₃ and the organic layer extracted into DCM (additional drops of MeOH to add solubility) (3 x 40 ml), washed with brine (40 ml), dried with MgSO₄ and concentrated in vacuo. The crude product was purified by trituration with Et₂O to give 30 as an off-white solid in a 58.7% yield. \( \text{RF} = 0.02 \) (8:2 EtOAc:hexane and 1% triethylamine). \(^1\text{H NMR}\) (400 MHz, MeOD) \( \delta \): 8.19 (s, 1H), 8.06 (d, \( J = 7.9 \) Hz, 1H), 7.94 (s, 1H), 7.91 (t, \( J = 1.7 \) Hz, 1H), 7.89 (d, \( J = 8.2 \) Hz, 1H), 7.79 (td, \( J = 8.2, 1.1 \) Hz, 1H), 7.64 (dt, \( J = 7.3, 1.5 \) Hz, 1H), 7.58 – 7.48 (m, 3H), 4.37 (tt, \( J = 11.5, 3.9 \) Hz, 1H), 3.25 (d, \( J = 13.0 \) Hz, 2H), 2.83 (td, \( J = 12.8, 2.4 \) Hz, 2H), 2.18 (d, \( J = 12.3 \) Hz, 2H), 1.95 (ddd, \( J = 24.9, 12.6, 4.1 \) Hz, 2H); \(^{13}\text{C NMR}\) (101 MHz, MeOD) \( \delta \): 164.8, 140.8, 137.4, 136.4, 134.1, 132.6, 129.7, 126.1, 125.9, 125.7, 124.4, 124.1, 122.7, 121.9, 121.7, 120.7, 57.2, 43.5 (2C), 30.5 (2C); \( \text{ES+HRMS} \) m/z 377.1430 [M+H]⁺ \( \text{C}_{21}\text{H}_{20}\text{N}_{4}\text{OS} \) requires 376.14.
Preparation of 2-(1-benzylpiperidin-4-yl)-linked benzo[d]isothiazol-3(2H)-ones 33-35

**General Procedure (8)**

![Chemical structure of 33, 34, and 35 with n = 0, 1, and 2](image)

To a solution of X (1.00 mmol) in DCM (15 ml), the appropriate amino benzyl piperidine (1.2 mmol, 1.2 eq) was added at 0°C under a N₂ atmosphere. Et₃N (3.3 mmol, 3.3 eq) was then added to the solution which was allowed to warm gradually to room temperature and stir overnight. The solution was diluted with DCM (30 ml), washed with brine (30 ml) and NaHCO₃ (30 ml), extracted into DCM (3 x 30 ml) and the combined organic extracts dried over MgSO₄ before concentrating under reduced pressure. The crude products were purified by flash column chromatography.

**Preparation of 2-(1-benzylpiperidin-4-yl)benzo[d]isothiazol-3(2H)-one 33**

The general procedure (8) was followed using 4-amino-1-benzylpiperidine (1.2 mmol) to give 33 as an off-white solid in 71.0% yield. Rf = 0.36 (8:2 EtOAc:hexane). ¹H NMR (400 MHz, CDCl₃) δ 8.03 (d, J = 7.9 Hz, 1H), 7.62 – 7.53 (m, 2H), 7.39 (dt, J = 8.0, 1.4 Hz, 1H), 7.33 (apparent d, J = 4.4 Hz, 4H), 7.31 – 7.26 (m, 1H), 4.64 (tt, J = 11.5, 4.7 Hz, 1H), 3.55 (s, 2H), 3.01 (d, J = 11.9 Hz, 2H), 2.19 (td, J = 11.7, 2.6 Hz, 2H), 2.05 – 1.85 (m, 4H); ¹³C NMR (101 MHz, CDCl₃) δ 165.0, 140.3, 138.2, 131.5, 129.2 (2C), 128.3 (2C), 127.2, 126.5, 125.4, 125.3, 120.4, 62.9, 52.8 (2C), 51.6, 32.0 (2C); Elemental analysis (%) found: C, 70.13; H, 6.22; N, 8.52; S, 9.86%. C₁₉H₂₀N₂O requires C, 70.34; H, 6.21; N, 8.63; S, 9.88%; ES+HRMS m/z 325.1375 [M+H]⁺ C₁₉H₂₀N₂O requires 324.13.

**Preparation of 2-((1-benzylpiperidin-4-yl)methyl)benzo[d]isothiazol-3(2H)-one 34**

The general procedure (8) was followed using C-(1-benzyl)-piperidin-4-yl methyamine (1.2 mmol) to give 34 as a yellow viscous oil in 63.0% yield. Rf = 0.07 (100% EtOAc). ¹H NMR (400 MHz, CDCl₃) δ 8.03 (d, J = 7.9 Hz, 1H), 7.60 (td, J = 8.1, 1.1 Hz, 1H), 7.53 (d, J = 8.1 Hz, 1H), 7.39 (td, J = 7.9, 0.9 Hz, 1H), 7.30 (apparent d, J = 4.4 Hz, 4H), 7.24 (m, 1H), 3.78 (d, J = 7.2 Hz, 2H), 3.49 (s, 2H), 2.88 (d, J = 11.8 Hz, 2H), 1.96 (td, J = 11.7, 2.3 Hz, 2H), 1.91 – 1.79 (m, 1H), 1.69 (apparent d, J = 15.9 Hz, 2H), 1.42 (qd, J = 11.9, 3.7 Hz, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 165.5, 140.2, 138.5, 131.7, 129.1 (2C), 128.2 (2C), 126.9, 126.7, 125.4, 124.6, 120.2, 63.2, 53.1 (2C), 49.5, 36.3, 29.8 (2C); Elemental analysis (%) found: C, 70.53; H, 6.46; N, 8.19; S, 9.36%. C₂₀H₂₂N₂O requires C, 70.97; H, 6.55; N, 8.28; S, 9.47%; ES+HRMS m/z 339.1530 [M+H]⁺ C₂₀H₂₂N₂O requires 338.15.
Preparation of 2-(2-(1-benzylpiperidin-4-yl)ethyl)benzo[d]isothiazol-3(2H)-one 35

The general procedure (8) was followed using 2-(1-benzyl)-4-piperidinyl ethanamine (1.2 mmol) to give 35 as a clear yellow-brown oil in 73.6% yield. \( \text{Rf} = 0.15 \) (5:95 MeOH: EtOAc). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.03 (d, \( J = 7.9 \) Hz, 1H), 7.60 (d, \( J = 8.1, 1.2 \) Hz, 1H), 7.54 (d, \( J = 8.0 \) Hz, 1H), 7.40 (td, \( J = 8.0, 1.0 \) Hz, 1H), 7.30 (apparent d, \( J = 4.3 \) Hz, 4H), 7.26 – 7.22 (m, 1H), 3.93 (t, \( J = 7.1 \) Hz, 2H), 3.48 (s, 2H), 2.87 (d, \( J = 11.7 \) Hz, 2H), 1.94 (t, \( J = 11.1 \) Hz, 2H), 1.80 – 1.61 (m, 5H), 1.40 – 1.31 (m, 2H); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 165.3, 140.1, 138.4, 131.7, 129.2 (2C), 128.1 (2C), 127.0, 126.7, 125.5, 124.8, 120.3, 63.4, 53.6 (2C), 41.7, 36.1, 33.1, 32.1 (2C); Elemental analysis (%) found: C, 71.46; H, 6.85; N, 7.94; S, 9.18%. \( C_{21}H_{24}N_2OS \) requires C, 71.55; H, 6.86; N, 7.95; S, 9.10%; \( \text{ES+HRMS} \) \text{m/z } 353.1689 \[ M+H \]^+ \( C_{21}H_{24}N_2OS \) requires 352.16.

Preparation of 4'-methoxy-[1,1'-biphenyl]-3-amine 36

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{OCH}_3 \\
& \\
& \\
& \\
& \\
&
\end{align*}
\]

To a solution of Na\(_2\)CO\(_3\) (1.22 g, 11.52 mmol, 2.1 eq), 4-(methoxy)phenyl boronic acid (1.0 g, 6.58 mmol, 1.2 eq) and Pd(PPh\(_3\))\(_4\) (0.16 g, 0.137 mmol, 0.025 eq) in DME:water (20:10 ml), 3-bromoaniline (0.60 ml, 5.48 mmol) was added. The stirring solution was evacuated and purged with N\(_2\) before heating to 90°C for 18 hours. The solution was concentrated under reduced pressure to remove DME. The crude material was then quenched with brine (40 ml) and organic layer extracted into EtOAc (2 x 40 ml), dried over MgSO\(_4\) and concentrated under reduced pressure. The crude product was purified by flash column chromatography to give 36 as an off-white solid in a 80.9% yield. \( \text{Rf} = 0.54 \) (45:55 EtOAc:hexane). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 7.50 (dt, \( J = 8.8, 2.1 \) Hz, 2H), 7.21 (t, \( J = 7.8 \) Hz, 1H), 7.01 – 6.92 (m, 3H), 6.87 (t, \( J = 2.0 \) Hz, 1H), 6.64 (ddd, \( J = 7.9, 2.3, 0.9 \) Hz, 1H), 3.85 (s, 3H), 3.72 (s, 2H, NH\(_2\)); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 159.1, 146.7, 142.1, 134.0, 129.7, 128.1 (2C), 117.3, 114.1 (2C), 113.6, 113.5, 55.3; \( \text{CI+HRMS} \) \text{m/z } 200.1069 \[ M+H \]^+ \( C_{13}H_{13}NO \) requires 119.10. 

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Preparation of \(2-(4'\text{-methoxy-}[1,1'\text{-biphenyl}]3\text{-yl})\)benzo[\(d\)]isothiazol-3(2\(H\))\)-one 4

To a solution of 36 (0.87 g, 4.35 mmol) in DCM (20 ml), \(X\) (1.08 g, 5.22 mmol, 1.2 eq) was added at 0°C under a N\(_2\) atmosphere. Et\(_3\)N (2.0 ml, 14.3 mmol, 3.3 eq) was added to the solution which was allowed to gradually warm to room temperature and stir for 20 hours. The solution was quenched with NaHCO\(_3\) (30 ml) and the organic layer extracted into DCM (3 x 30 ml). The combined organic extracts were then washed with brine (30 ml), dried over MgSO\(_4\) and concentrated under reduced pressure to give the pure product 4 as a cream solid in quantiative yield. \(R_f = 0.65\) (50:50 EtOAc:hexane). \(^1\)H NMR (400 MHz, DMSO) \(\delta\) 8.07 (d, \(J = 8.1\) Hz, 1H), 7.97 (d, \(J = 7.8\) Hz, 1H), 7.95 (t, \(J = 1.8\) Hz, 1H), 7.77 (td, \(J = 7.1, 1.2\) Hz, 1H), 7.66 (dt, \(J = 8.8, 2.0\) Hz, 2H), 7.64 – 7.55 (m, 3H), 7.52 (td, \(J = 7.9, 0.7\) Hz, 1H), 7.06 (d, \(J = 8.8, 2.0\) Hz, 2H), 3.81 (s, 3H); \(^{13}\)C NMR (101 MHz, DMSO) \(\delta\) 163.7, 159.8, 141.6, 140.6, 138.2, 133.1, 132.0, 130.5, 128.4(2C), 126.7, 126.5, 125.36, 124.8, 123.1, 122.7, 122.4, 115.0 (2C), 55.7; ES+HRMS m/z 356.0720 [M+Na]\(^+\) \(C_{20}H_{15}NO_2\) requires 333.08.

Preparation of \(2-(4'\text{-hydroxy-}[1,1'\text{-biphenyl}]3\text{-yl})\)benzo[\(d\)]isothiazol-3(2\(H\))\)-one 9

To a solution of 4 (1.49 g, 4.47 mmol) in DCM (20 ml), BBr\(_3\) (1.0 M in DCM, 9.0 ml, 8.95 mmol, 2.0 eq) was added under a N\(_2\) atmosphere at 0°C. The resulting solution was maintained at 0°C for 2 hours, after which time the reaction was complete. The solution was quenched with MeOH (6 ml), extracted into DCM (3 x 50 ml), washed with NaHCO\(_3\) (30 ml) and brine (40 ml), dried with MgSO\(_4\) and concentrated in vacuo to give 9 as the pure product, an off-white solid in a 36.9% yield. \(R_f = 0.46\) (50:50 EtOAc:hexane). \(^1\)H NMR (400 MHz, DMSO) \(\delta\) 9.65 (s, 1H, OH), 8.07 (d, \(J = 8.1\) Hz, 1H), 7.97 (d, \(J = 7.5\) Hz, 1H), 7.90 (t, \(J = 1.5\) Hz, 1H), 7.77 (td, \(J = 7.1, 1.2\) Hz, 1H), 7.62 – 7.48 (m, 6H), 6.88 (dt, \(J = 8.7, 2.0\) Hz, 2H); \(^{13}\)C NMR (101 MHz, DMSO) \(\delta\) 163.8, 158.1, 142.0, 140.6, 138.2, 133.1, 130.4, 128.4 (2C), 126.6, 126.5, 125.0, 124.8, 122.8, 122.5, 122.4, 116.3 (2C); Elemental analysis (%) found: C,
Preparation of 2-methylene-amino-(4-hydroxy)-[1,1'-biphenyl]-3-yl]benzo[d]isothiazol-3(2H)-ones 37-39

**General Procedure (9)**

To a solution of 9 (0.15 g, 0.469 mmol) in 7:3 EtOH:H$_2$O (15ml), the appropriate 2° amine (3.0 – 5.0 eq) and formaldehyde 37% wt in H$_2$O (3.0 – 5.0 eq) were added at room temperature. The resulting solution was heated to reflux at 95°C overnight under normal atmospheric conditions. After this time, the crude solution was diluted in EtOAc (20 ml), washed with H$_2$O (25 ml) and extracted into EtOAc (3 x 30 ml). The combined organic extracts were then washed with brine (30 ml), dried with MgSO$_4$ and concentrated under reduced pressure. The crude products were purified by flash column chromatography.

Preparation of 2-(4'-hydroxy-3'-(morpholinomethyl)-[1,1'-biphenyl]-3-yl]benzo[d]-isothiazol-3(2H)-one 37

The general procedure (9) was followed using the 2° amine morpholine (1.41 mmol, 3.0 eq) and 3.0 eq of formaldehyde 37% wt in H$_2$O to give 37 as an off-white crystalline solid in 39.7% yield. $R_f = 0.24$ (50:50 EtOAc:hexane). $^1$H NMR (400 MHz, MeOD) $\delta$ 8.07 (d, $J = 7.9$ Hz, 1H), 7.93 – 7.86 (m, 2H), 7.79 (td, $J = 8.2, 1.0$ Hz, 1H), 7.65 – 7.53 (m, 4H), 7.51 (dd, $J = 8.4, 2.3$ Hz, 1H), 7.46 (d, $J = 2.2$ Hz, 1H), 6.89 (d, $J = 8.4$ Hz, 1H), 3.81 (s, 2H), 3.76 (t, $J = 4.6$ Hz, 4H), 2.61 (br s, 4H); $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 164.2, 157.6, 142.3, 139.9, 137.6, 132.4, 131.4, 129.7, 127.8, 127.7, 127.2, 125.9, 125.3, 124.9, 122.9, 122.6, 121.1, 120.1, 116.6, 66.8 (2C), 61.9, 53.0 (2C); **Elemental analysis (%) found:** C, 76.40; H, 5.32; N, 6.39; S, 7.28%. C$_{24}$H$_{22}$N$_2$O$_3$S requires C, 68.88; H, 5.30; N, 6.69; S, 7.66%; **ES+HRMS** m/z 419.1422 [M+H]$^+$ C$_{24}$H$_{22}$N$_2$O$_3$S requires 418.14.
Preparation of 2-(3'-((diethylamino)methyl)-4'-hydroxy-[1,1'-biphenyl]-3-yl)benzo[d]-isothiazol-3(2H)-one 38

The general procedure (9) was followed using the 2° amine diethylamine (2.41 mmol, 5.0 eq) and 5.0 eq of formaldehyde 37% wt in H₂O to give 38 as a yellow/cream crystalline solid in 22.8% yield. $R_f = 0.22$ (50:50 EtOAc:hexane and 1% triethylamine). $^1$H NMR (400 MHz, CDCl₃) δ 8.13 (d, J = 7.9 Hz, 1H), 7.89 (s, 1H), 7.68 (td, J = 8.0, 0.9 Hz, 1H), 7.63 – 7.56 (m, 2H), 7.52 – 7.38 (m, 4H), 7.29 – 7.23 (m, 1H, under solvent peak), 6.88 (d, J = 8.4 Hz, 1H), 3.84 (s, 2H), 2.66 (q, J = 7.2 Hz, 4H), 1.13 (t, J = 7.2 Hz, 6H); $^{13}$C NMR (101 MHz, CDCl₃) δ 164.2, 158.5, 142.5, 140.0, 137.6, 132.4, 130.8, 129.6, 127.3, 127.2, 125.8, 125.3, 124.9, 122.9, 122.4, 120.1, 116.6, 57.0, 46.3 (2C), 11.2 (2C); ES+HRMS m/z 405.1631 [M+H]⁺ C₂₄H₂₄N₂O₂S requires 404.16.

Preparation of 2-(4'-hydroxy-3'-pyrrolidin-1-ylmethyl)-[1,1'-biphenyl]-3-yl]benzo[d]-isothiazol-3(2H)-one 39

The general procedure (9) was followed using the 2° amine pyrrolidine (1.34 mmol, 3.0 eq) and 3.0 eq of formaldehyde 37% wt in H₂O to give 39 as a translucent yellow-cream film in 5.9% yield. $R_f = 0.18$ (50:50 EtOAc:hexane and 1% triethylamine). $^1$H NMR (400 MHz, CDCl₃) δ 8.13 (d, J = 7.9 Hz, 1H), 7.89 (s, 1H), 7.68 (td, J = 7.2, 0.8 Hz, 1H), 7.64 – 7.55 (m, 2H), 7.49 (d, J = 5.1 Hz, 2H), 7.47 – 7.38 (m, 2H), 7.28 – 7.26 (m, 1H, under solvent peak), 6.91 (d, J = 8.4 Hz, 1H), 3.95 (s, 2H), 2.82 – 2.61 (m, 4H), 1.92 – 1.84 (m, 4H); $^{13}$C NMR (101 MHz, CDCl₃) δ 164.3, 158.2, 142.5, 134.0, 137.5, 132.4, 130.8, 129.7, 127.5, 127.2, 126.9, 125.9, 125.3, 124.9, 122.9, 122.6, 122.5, 120.1, 116.6, 58.7, 53.6 (2C), 23.6 (2C); ES+HRMS m/z 403.1477 [M+H]⁺ C₂₄H₂₂N₂O₂S requires 402.14.

Preparation of tert-butyl-(benzo[d]isothiazol-2(3H)-yl) carbamates 40a-f

General Procedure (10)

40a $R^3 = $ Ethyl
40b $R^3 = $ 1,5-Phenyl
40c $R^3 = $ 2-Methyl-1,5-phenyl
40d $R^3 = $ 1,4-Cyclohexane
40e $R^3 = $ 1,6-Cyclohexane
40f $R^3 = $ 2-Dimethyl-propane

To a solution of X (1.2 eq) in DCM (20 ml), the appropriate N-Boc-(Y)-amine (1.0 eq) was added at 0°C under a N₂ atmosphere. Et₃N (3.3 eq) was then added to the solution which was allowed to warm.
gradually to room temperature and stir overnight. The solution was diluted with DCM, washed with NaHCO₃ (30 ml) and brine (30 ml), extracted into DCM (3 x 40 ml) and the combined organic extracts dried over MgSO₄ before concentrating under reduced pressure to give the crude products.

Preparation of tert-butyl (2-(3-oxobenzod[d]isothiazol-2(3H)-yl)ethyl)carbamate 40a

The general procedure (10) was followed using N-Boc-ethylenediamine (0.5 g, 3.12 mmol) to give 40a as the pure product, a yellow-brown solid, in quantitative yield. \( R_f = 0.42 \) (60:40 EtOAc:hexane and 1% triethylamine). \( ^1H \text{NMR} \) (400 MHz, MeOD) \( \delta \) 7.98 (d, \( J = 7.9 \) Hz, 1H), 7.80 (d, \( J = 8.1 \) Hz, 1H), 7.70 (t, \( J = 7.4 \) Hz, 1H), 7.47 (t, \( J = 7.5 \) Hz, 1H), 3.99 (t, \( J = 5.7 \) Hz, 2H), 3.43 (t, \( J = 5.7 \) Hz, 2H), 1.40 (s, 9H); \( ^{13}C \text{NMR} \) (101 MHz, MeOD) \( \delta \) 166.1, 157.0, 141.3, 131.8, 125.6, 125.3, 123.9, 120.7, 78.9, 43.6, 39.2, 27.3 (3C); \( \text{ES}^+\text{HRMS} \) m/z 317.0931 [M+Na]\(^+\) \( C_{14}H_{18}N_2O_3S \) requires 294.10.

Preparation of tert-butyl(3-(3-oxobenzod[d]isothiazol-2(3H)-yl)phenyl)carbamate 40b

The general procedure (10) was followed using N-Boc-m-phenylenediamine (1.1 g, 5.26 mmol). The crude product was purified by flash column chromatography to give 40b as a yellow oil in a 27.2% yield. \( R_f = 0.37 \) (30:70 EtOAc:hexane and 1% triethylamine). \( ^1H \text{NMR} \) (400 MHz, CDCl₃) \( \delta \) 8.10 (d, \( J = 7.9 \) Hz, 1H), 7.87 (s, 1H), 7.66 (td, \( J = 8.2, 1.2 \) Hz, 1H), 7.57 (d, \( J = 8.1 \) Hz, 1H), 7.44 (td, \( J = 8.0, 0.9 \) Hz, 1H), 7.41 – 7.27 (m, 3H), 6.66 (s, 1H, NH), 1.52 (s, 9H); \( ^{13}C \text{NMR} \) (101 MHz, CDCl₃) \( \delta \) 164.1, 152.5, 139.9, 139.5, 137.9, 132.4, 129.8, 127.2, 125.8, 124.9, 120.0, 118.7, 116.8, 114.4, 80.9, 28.3 (3C); \( \text{ES}^+\text{HRMS} \) m/z 365.0934 [M+Na]\(^+\) \( C_{18}H_{18}N_2O_3S \) requires 342.10.

Preparation of tert-butyl(4-(methyl-3-(3-oxobenzod[d]isothiazol-2(3H)-yl)phenyl)carbamate 40c

The general procedure (10) was followed using 5-(Boc-amino)-2-methylaniline (0.40 g, 1.79 mmol). The crude product was purified by flash column chromatography to give 40c as a cream solid in a 49.8% yield. \( R_f = 0.62 \) (60:40 EtOAc:hexane and 1% triethylamine). \( ^1H \text{NMR} \) (400 MHz, MeOD) \( \delta \) 8.03 (d, \( J = 7.9 \) Hz, 1H), 7.87 (d, \( J = 8.2 \) Hz, 1H), 7.77 (td, \( J = 8.2, 1.0 \) Hz, 1H), 7.54 (m, 2H), 7.40 (dd, \( J = 8.4, 2.0 \) Hz, 1H), 7.29 (d, \( J = 8.4 \) Hz, 1H), 2.13 (s, 3H), 1.51 (s, 9H); \( ^{13}C \text{NMR} \) (101 MHz, MeOD) \( \delta \) 165.0, 153.6, 141.9, 138.5, 134.5, 132.4, 131.0, 130.8, 126.1, 125.8, 123.4, 120.9, 119.5, 118.3, 79.7, 27.2 (3C), 15.8; \( \text{ES}^+\text{HRMS} \) m/z 379.1081 [M+Na]\(^+\) \( C_{19}H_{20}N_2O_3S \) requires 356.12.

Preparation of tert-butyl(1R,4R)-4-(3-oxobenzod[d]isothiazol-2(3H)-yl)cyclohexyl)carbamate 40d

The general procedure (10) was followed using N-Boc-trans-1,4-cyclohexane-diamine (0.40 g, 1.87 mmol). The crude product was purified by flash column chromatography to give 40d as a yellow/off-white fine crystalline solid in a 58.9% yield. \( R_f = 0.40 \) (60:40 EtOAc:hexane and 1% triethylamine). \( ^1H \)
**Chapter 3**

**NMR** (400 MHz, MeOD) δ 7.96 (d, J = 7.9 Hz, 1H), 7.81 (d, J = 8.2 Hz, 1H), 7.68 (td, J = 8.2, 1.0 Hz, 1H), 7.47 (td, J = 7.8, 0.5 Hz, 1H), 4.53 (tt, J = 12.0, 3.5 Hz, 1H), 3.41 (tt, J = 15.1, 4.8 Hz, 1H), 2.06 (bd, J = 10.6 Hz, 4H), 1.81 (qd, J = 12.2, 3.1 Hz, 2H), 1.55 – 1.39 (m, 11H); **13C NMR** (101 MHz, MeOD) δ 165.4, 156.4, 140.9, 131.8, 125.4, 125.4, 124.6, 120.8, 78.6, 52.8, 48.4, 31.5 (2C), 30.9 (2C), 27.4 (3C); **ES+HRMS** m/z 371.1412 [M+Na]⁺ require 348.15.

**Preparation of tert-butyl((1R,2R)-2-(3-oxobenzo[d]isothiazol-2(3H)-yl)cyclohexyl) carbamate 40e**

The general procedure (10) was followed using tert-butyl(trans-2-aminocyclohexyl) carbamate (0.45 g, 2.10 mmol). The crude product was purified by flash column chromatography to give 40e as off-white solid crystals in a 53.5% yield. **Rf = 0.51 (60:40 EtOAc:hexane and 1% triethylamine). ¹H NMR** (400 MHz, MeOD) δ 7.96 (d, J = 8.0 Hz, 1H), 7.78 (d, J = 8.2 Hz, 1H), 7.67 (t, J = 7.67 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 4.45 (td, J = 11.5, 4.0 Hz, 1H), 3.71 (td, J = 10.5, 4.2 Hz, 1H), 2.12 – 2.00 (m, 2H), 1.93 – 1.75 (m, 3H), 1.52 – 1.40 (m, 3H), 1.15 (s, 9H); **13C NMR** (101 MHz, MeOD) δ 166.4, 156.0, 141.2, 131.8, 125.5, 125.2, 124.4, 120.7, 78.6, 57.4, 53.3, 32.7, 32.0, 27.0 (3C), 24.9, 24.6; **ES+MS** m/z 371.1398 [M+Na]⁺ require 348.15.

**Preparation of tert-butyl(2,2-dimethyl-3-(3-oxobenzo[d]isothiazol-2(3H)-yl)propyl) carbamate 40f**

The general procedure (10) was followed using tert-butyl-3-amino-2,2-dimethylpropyl carbamate (0.25 g, 1.24 mmol). The crude product was filtered through a pad of silica and washed with 50:50 EtOAc:hexane to give 40f as a brown solid in a 92.6% yield. **Rf = 0.21 (20:80 EtOAc:hexane and 1% triethylamine). ¹H NMR** (400 MHz, MeOD) δ 7.97 (d, J = 7.9 Hz, 1H), 7.79 (d, J = 8.2 Hz, 1H), 7.71 (td, J = 7.1, 1.2 Hz, 1H), 7.48 (td, J = 8.0, 0.9 Hz, 1H), 3.75 (s, 2H), 2.95 (s, 2H), 1.46 (s, 9H), 1.02 (s, 6H); **13C NMR** (101 MHz, MeOD) δ 167.0, 157.2, 141.3, 132.1, 125.7, 125.7, 125.5, 125.3, 120.5, 78.7, 51.9, 47.3, 37.3, 27.4 (3C), 22.7 (2C); **ES+HRMS** m/z 359.1401 [M+Na]⁺ require 336.15.
Preparation of \(2-(R^3)-(\text{benzo}[d]\text{isothiazol}-2(3H)-yl)\) amines 41a-f

**General Procedure (11)**

To a solution of the appropriate \(\text{tert-buty}-\text{[benzo}[d]\text{isothiazol}-2(3H)-yl] \text{carbamate} \ 40a-f\) in DCM (15-25 ml) under a \(\text{N}_2\) atmosphere, trifluoroacetic acid (TFA) (15.0 eq) was added and allowed to stir at room temperature overnight. After this time, DCM and excess TFA were removed under reduced pressure. The crude material was redissolved in DCM (30 ml), quenched with NaHCO\(_3\) (30 ml) and the organic layer extracted into DCM (3 x 40 ml). The combined organic layers were then washed with brine (40 ml), dried with MgSO\(_4\) and concentrated in vacuo to isolate the pure product.

Preparation of \(2-(2\text{-aminoethyl})\text{benzo}[d]\text{isothiazol}-3(2H)\)-one 41a

The general procedure (11) was followed using \(40a\) (0.92 g, 3.13 mmol) to give \(41a\) as a yellow-brown viscous oil in a 35.3% yield. \(R_f = 0.14\ (10:90\ \text{MeOH}:\text{EtOAc}\) and 1% triethylamine). \(^1\text{H NMR} (400 MHz, CDCl\(_3\)) \delta 8.04 (d, \(J = 7.9\ Hz, 1H\), 7.61 (t, \(J = 7.4\ Hz, 1H\)), 7.55 (d, \(J = 8.0\ Hz, 1H\)), 7.40 (t, \(J = 7.4\ Hz, 1H\)). \(3.97 (t, \ J = 5.9\ Hz, 2H), 3.10 (t, J = 5.5\ Hz, 2H); \(^{13}\text{C NMR} (101 MHz, CDCl\(_3\)) \delta 165.9, 140.4, 143.0, 131.9, 126.7, 125.6, 124.5, 120.3, 47.0, 41.5; \text{Cl+HRMS} \ m/z 195.0590 \ [M+H]^+ \text{C}_9\text{H}_{10}\text{N}_2\text{OS} \text{requires 194.05.}

Preparation of \(2-(3\text{-aminophenyl})\text{benzo}[d]\text{isothiazol}-3(2H)\)-one 41b

The general procedure (11) was followed using \(40a\) (0.14 g, 0.414 mmol) to give \(41b\) as a yellow-orange solid in a 97.2% yield. \(R_f = 0.31\ (65:35\ \text{EtOAc}:\text{hexane}\) and 1% triethylamine). \(^1\text{H NMR} (400 MHz, CDCl\(_3\)) \delta 8.09 (d, \(J = 7.9\ Hz, 1H\)), 7.65 (Td, \(J = 8.1, 1.1\ Hz, 1H\)), 7.57 (d, \(J = 8.0\ Hz, 1H\)), 7.44 (t, \(J = 7.5\ Hz, 1H\)), 7.22 (t, \(J = 8.0\ Hz, 1H\)), 7.15 (t, \(J = 2.1\ Hz, 1H\)), 7.01 (dd, \(J = 8.0, 1.4\ Hz, 1H\)), 6.62 (dd, \(J = 8.0, 1.8\ Hz, 1H\)), 3.82 (s, 2H, NH\(_2\)). \(^{13}\text{C NMR} (101 MHz, CDCl\(_3\)) \delta 164.1, 147.4, 139.9, 138.2, 132.3, 130.1, 127.1, 125.7, 125.1, 120.0, 114.2, 113.8, 111.1; \text{Cl+HRMS} \ m/z 243.0590 \ [M+H]^+ \text{C}_{13}\text{H}_{10}\text{N}_2\text{OS} \text{requires 242.05.} \)
Preparation of 2-((2-amino-2-methylphenyl)benzo[d]isothiazol-3(2H)-one 41c

The general procedure (11) was followed using 40c (0.31 g, 0.868 mmol) to give 41c as a yellow solid in a 98.2% yield. \( R_f = 0.33 \) (60:40 EtOAc:hexane and 1% triethylamine). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.11 (d, \( J = 7.9 \) Hz, 1H), 7.67 (td, \( J = 8.2, 1.1 \) Hz, 1H), 7.59 (d, \( J = 8.1 \) Hz, 1H), 7.45 (td, \( J = 7.9, 0.8 \) Hz, 1H), 7.10 (d, \( J = 7.8 \) Hz, 1H), 6.74 – 6.65 (m, 2H), 3.43 (s, 2H, NH\(_2\)), 2.12 (s, 3H); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 164.5, 145.3, 141.2, 135.2, 132.1, 131.9, 127.2, 127.1, 125.6, 124.0, 120.2, 116.8, 115.5, 17.0; CI+HRMS m/z 257.0753 [M+H]\(^+\) C\(_{14}\)H\(_{12}\)N\(_2\)OS requires 256.07.

Preparation of 2-((1R,4R)-4-aminocyclohexyl)benzo[d]isothiazol-3(2H)-one 41d

The general procedure (11) was followed using 40d (0.37 g, 1.07 mmol) to give 41d as a yellow solid in a 75.7% yield. \( R_f = 0.00 \) (60:40 EtOAc:hexane and 1% triethylamine). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.04 (d, \( J = 7.9 \) Hz, 1H), 7.60 (td, \( J = 8.0, 1.1 \) Hz, 1H), 7.56 (d, \( J = 7.6 \) Hz, 1H), 7.40 (td, \( J = 8.0, 1.3 \) Hz, 1H), 4.60 (tt, \( J = 11.9, 3.9 \) Hz, 1H), 2.79 (t, \( J = 11.0 \) Hz, 1H), 2.13 – 1.95 (m, 4H), 1.83 – 1.60 (m, 4H), 1.46 – 1.30 (m, 2H, NH\(_2\)); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 165.0, 140.2, 131.6, 126.5, 125.4, 120.4, 52.4, 49.7, 35.4 (2C), 31.3 (2C); ES+HRMS m/z 249.1056 [M+H]\(^+\) C\(_{13}\)H\(_{16}\)N\(_2\)OS requires 248.10.

Preparation of 2-((1R,2R)-2-aminocyclohexyl)benzo[d]isothiazol-3(2H)-one 41e

The general procedure (11) was followed using 40e (0.37 g, 1.07 mmol) to give 41e as an opaque, off-white oil in quantitative yield. \( R_f = 0.00 \) (EtOAc and 1% triethylamine). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.06 (d, \( J = 7.9 \) Hz, 1H), 7.62 (td, \( J = 8.0, 0.9 \) Hz, 1H), 7.58 (d, \( J = 7.4 \) Hz, 1H), 7.42 (td, \( J = 7.9, 1.3 \) Hz, 1H), 4.37 (ddd, \( J = 10.3, 4.0, 1.3 \) Hz, 1H), 2.85 (t, \( J = 4.0 \) Hz, 1H), 2.19 – 1.96 (m, 2H), 1.85 (m, 2H), 1.62 (qd, \( J = 12.3, 3.3 \) Hz, 1H, NH), 1.54 – 1.23 (m, 4H and NH); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 166.0, 140.2, 131.6, 126.5, 125.4, 125.4, 120.4, 52.4, 49.7, 35.4 (2C), 31.3 (2C); CI+HRMS m/z 249.1056 [M+H]\(^+\) C\(_{13}\)H\(_{16}\)N\(_2\)OS requires 248.10.

Preparation of 2-((3-amino-2,2-dimethylpropyl)benzo[d]isothiazol-3(2H)-one 41f

The general procedure (11) was followed using 40f (0.38 g, 1.12 mmol) to give 41f as a pale-yellow, cloudy viscous oil in a 80.0% yield. \( R_f = 0.00 \) (50:50 EtOAc:hexane and 1% triethylamine). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 8.04 (d, \( J = 7.9 \) Hz, 1H), 7.62 (td, \( J = 8.1, 1.2 \) Hz, 1H), 7.53 (d, \( J = 8.1 \) Hz, 1H), 7.41 (td, \( J = 7.9, 0.9 \) Hz, 1H), 3.76 (s, 2H), 2.55 (s, 2H), 1.84 (br s, 2H, NH\(_2\)), 1.04 (s, 6H); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \( \delta \) 166.5, 140.7, 131.9, 126.8, 125.4, 124.0, 120.0, 51.7, 49.7, 29.7, 24.0 (2C); ES+HRMS m/z 237.1058 [M+H]\(^+\) C\(_{13}\)H\(_{16}\)N\(_2\)OS requires 236.10.
Preparation of 7-chloroquinolin-4-yl-amine-linked benzo[d]isothiazol-3(2H)-ones 42-43

General Procedure (12)

To a solution of the appropriate 2-(R^3)-(benzo[d]isothiazol-2(3H)-yl) amine (41a-b) in phenol (5-10 ml) under atmospheric conditions, 4,7-dichloroquinoline (1.1 eq) was added. The resulting solution was heated to 140°C in a sealed tube for 12 hours. Excess phenol was removed under reduced pressure (as an azeotrope with toluene) and the crude products were purified by flash column chromatography.

Preparation of 2-(2-((7-chloroquinolin-4-yl)amino)ethyl)benzo[d]isothiazol-3(2H)-one 42

The general procedure (12) was followed using 41a (0.195 g, 1.00 mmol) to give 42 as an off-white solid in a 61.9% yield. Rf = 0.54 (5:95 MeOH:EtOAc and 1% triethylamine). 1H NMR (400 MHz, DMSO) δ 8.41 (d, J = 5.4 Hz, 1H), 8.19 (d, J = 9.1 Hz, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.89 (d, J = 7.8 Hz, 1H), 7.81 (d, J = 2.2 Hz, 1H), 7.67 (td, J = 8.2, 1.0 Hz, 1H), 7.56 (t, J = 5.6 Hz, 1H), 7.50–7.41 (m, 2H), 6.64 (d, J = 5.5 Hz, 1H), 4.11 (m, 2H), 3.66 (q, J = 5.8 Hz, 2H), 3.18 (d, J = 5.2 Hz, 2H); 13C NMR (101 MHz, DMSO) δ 165.2, 152.4, 150.2, 149.6, 141.5, 134.0, 132.3, 128.0, 126.0, 125.9, 124.8, 124.4, 124.3, 122.3, 118.1, 99.2, 49.1, 42.2; Elemental analysis (%) found: C, 60.14; H, 4.07; N, 11.63%; C_{18}H_{14}ClN_{3}OS requires C, 60.76; H, 3.97; N, 11.81%; ES+MS m/z 356.0622 [M+H]^+ C_{18}H_{14}ClN_{3}OS requires 355.05.

Preparation of 2-(3-((7-chloroquinolin-4-yl)amino)phenyl)benzo[d]isothiazol-3(2H)-one 43

The general procedure (12) was followed using 41b (0.089 g, 0.367 mmol) to give 43 as an off-white/yellow solid in a 21.8% yield. Rf = 0.46 (65:35 EtOAc:hexane and 1% triethylamine). 1H NMR (400 MHz, DMSO) δ 9.31 (s, 1H), 8.55 (d, J = 5.3 Hz, 1H), 8.45 (d, J = 9.1 Hz, 1H), 8.08 (d, J = 8.2 Hz, 1H), 7.98 (d, J = 7.8 Hz, 1H), 7.94 (d, J = 2.1 Hz, 1H), 7.82 (s, 1H), 7.78 (t, J = 7.3 Hz, 1H), 7.62 (dd, J = 9.0, 2.0 Hz, 1H), 7.60–7.49 (m, 2H), 7.45 (dd, J = 7.9, 1.1 Hz, 1H), 7.38 (dd, J = 7.9, 0.9 Hz, 1H), 7.17 (d, J = 5.3 Hz, 1H), 4.05 (br s, NH); Elemental analysis (%) found: C, 64.35; H, 3.81; N, 9.81; S, 7.17%. C_{22}H_{14}ClN_{3}OS requires C, 65.42; H, 3.49; N, 10.40; S, 7.94%; ES+HRMS m/z 404.0612 [M+H]^+ C_{22}H_{14}ClN_{3}OS requires 403.05.
Preparation of 2-(5-((7-chloroquinolin-4-yl)amino)-2-methylphenyl)benzo[d]isothiazol-3(2H)-one 44

To a solution of 41c (0.196 g, 0.766 mmol) in anhydrous ethanol (5.5 ml), 4,7-dichloroquinoline (1.1 eq) was added. The solution was placed under a N\textsubscript{2} atmosphere and heated to 100°C for 12 hours in a sealed tube. Excess ethanol was removed under reduced pressure to give a crude product which was insoluble in EtOAc. The crude product was purified by trituration with EtOAc and recrystallization with DCM-EtOAc at room temperature to give 44 as a pale yellow solid in a 75.8% yield. \(R_f = 0.13 \) (60:40 EtOAc:hexane and 1% triethylamine). \textbf{1H NMR} (400 MHz, MeOD) \(\delta\) 8.57 (d, \(J = 9.1\) Hz, 1H), 8.43 (d, \(J = 7.1\) Hz, 1H), 8.06 (d, \(J = 7.9\) Hz, 1H), 7.97 (d, \(J = 1.9\) Hz, 1H), 7.90 (d, \(J = 8.2\) Hz, 1H), 7.83 – 7.76 (m, 2H), 7.67 – 7.62 (m, 1H), 7.60 – 7.53 (m, 3H), 7.04 (d, \(J = 7.1\) Hz, 1H), 2.31 (s, 3H); \textbf{13C NMR} (101 MHz, MeOD) \(\delta\) 165.3, 156.0, 143.1, 141.9, 140.2, 139.2, 137.7, 136.0, 135.7, 132.7, 132.7, 128.0, 126.7, 126.2, 126.1, 126.0, 124.9, 123.1, 121.1, 119.1, 116.1, 100.4, 16.4; \textbf{Elemental analysis} (%) found: C, 64.35; H, 3.81; N, 9.81; S, 7.17%. \(C_{23}H_{16}ClN_3OS\) requires C, 66.10; H, 3.86; N, 10.05; S, 7.67%; \textbf{ES+HRMS} m/z 418.0771 [M+H]\(^+\). \(C_{23}H_{16}ClN_3OS\) requires 471.07.

Preparation of 2-((1R,4R)-4-((7-chloroquinolin-4-yl)amino)cyclohexyl)benzo[d]isothiazol -3(2H)-one 45

To a solution of 41d (0.168 g, 0.677 mmol) in isopropanol (IPA) (3 ml), 4,7-dichloroquinoline (1.2 eq) was added. The solution was heated to 190°C in a microwave reactor where it was maintained for 30 minutes before cooling slowly to 50°C and then to room temperature. To improve reaction progression, the solution was heated again to 200°C in a microwave reactor where it was maintained for 30 minutes before cooling. IPA was removed under reduced pressure to provide the crude product which was purified by flash column chromatography to give 45 as a fine, yellow solid in a 38.8% yield. \(R_f = 0.23 \) (80:20 EtOAc:hexane and 1% triethylamine). \textbf{1H NMR} (400 MHz, DMSO) \(\delta\) 8.41 (d, \(J = 5.4\) Hz, 1H), 8.39 (d, \(J = 8.3\) Hz, 1H), 7.97 (d, \(J = 8.2\) Hz, 1H), 7.84 – 7.76 (m, 2H), 7.67 – 7.62 (m, 1H), 7.60 – 7.53 (m, 3H), 7.04 (d, \(J = 7.1\) Hz, 1H), 2.31 (s, 3H); \textbf{13C NMR} (101 MHz, MeOD) \(\delta\) 165.3, 156.0, 143.1, 141.9, 140.2, 139.2, 137.7, 136.0, 135.7, 132.7, 132.7, 128.0, 126.7, 126.2, 126.1, 126.0, 124.9, 123.1, 121.1, 119.1, 116.1, 100.4, 16.4; \textbf{Elemental analysis} (%) found: C, 64.35; H, 3.81; N, 9.81; S, 7.17%. \(C_{23}H_{16}ClN_3OS\) requires C, 66.10; H, 3.86; N, 10.05; S, 7.67%; \textbf{ES+HRMS} m/z 418.0771 [M+H]\(^+\). \(C_{23}H_{16}ClN_3OS\) requires 471.07.
1H), 8.36 (d, J = 9.1 Hz, 1H), 8.02 (d, J = 8.1 Hz, 1H), 7.89 (d, J = 7.7 Hz, 1H), 7.79 (d, J = 2.2 Hz, 1H), 7.70 (td, J = 8.1, 1.0 Hz, 1H), 7.50 – 7.41 (m, 2H), 7.02 (d, J = 7.7 Hz, 1H, NH), 6.66 (d, J = 5.6 Hz, 1H), 4.51 (tt, J = 14.8, 3.9 Hz, 1H), 3.75 – 3.63 (m, 1H), 2.22 - 2.11 (m, 2H), 2.01 – 1.99 (m, 2H), 1.95 – 1.81 (m, 2H), 1.71 – 1.56 (m, 2H); \(^{13}\)C NMR (101 MHz, DMSO) δ 164.4, 152.4, 149.8, 149.7, 140.8, 133.8, 132.2, 127.9, 126.0, 125.2, 124.9, 124.4, 122.4, 118.0, 99.7, 52.4, 50.2, 31.1 (2C), 31.0 (2C); **Elemental analysis (%)** found: C, 64.21; H, 4.96; N, 10.15; S, 7.56%; \(\text{C}_{22}\text{H}_{20}\text{ClN}_{3}\text{OS}\) requires C, 64.46; H, 4.92; N, 10.25; S, 7.82%; **ES+MS m/z** 410.1084 [M+H]⁺; **C_{22}H_{20}ClN_{3}OS** requires 409.10.

**Preparation of 2-((1R,2R)-2-((7-chloroquinolin-4-yl)amino)cyclohexyl)benzo[d]isothiazol-3(2H)-one 46**

![Structure of 46](image)

To a solution of 41e (0.250 g, 1.01 mmol) in isopropanol (IPA) (3 ml), 4,7-dichloroquinoline (1.2 eq) was added. The solution was heated to 190°C in a microwave reactor where it was maintained for 60 minutes before cooling slowly to 55°C and then to room temperature at which point the reaction was complete. IPA was removed under reduced pressure to provide the crude product which was purified by flash column chromatography to give 46 as a pale yellow, powdery solid in a 53.9% yield. \(R_f = 0.16\) (80:20 EtOAc:hexane and 1% triethylamine). \(^1\)H NMR (400 MHz, DMSO) δ 8.27 (d, J = 5.5 Hz, 1H), 8.10 (d, J = 9.1 Hz, 1H), 7.85 (d, J = 8.1 Hz, 1H), 7.74 (d, J = 7.8 Hz, 1H), 7.68 (d, J = 2.2 Hz, 1H), 7.57 (dt, J = 8.2, 1.1 Hz, 1H), 7.39 (dd, J = 9.0, 2.2 Hz, 1H), 7.33 (t, J = 7.4 Hz, 1H), 7.05 (d, J = 9.0 Hz, 1H, NH), 6.65 (d, J = 5.6 Hz, 1H), 4.70 (app td, J = 11.3, 3.7 Hz, 1H), 4.11 – 4.05 (m, 1H), 2.20 – 2.02 (m, 2H), 1.92 – 1.70 (m, 3H), 1.64 – 1.38 (m, 3H); \(^{13}\)C NMR (101 MHz, DMSO) δ 165.3, 152.2, 149.7, 149.6, 140.8, 133.8, 132.1, 127.8, 125.8, 125.8, 125.0, 124.4 (2C), 122.2, 117.8, 99.0, 60.2, 57.1, 32.0, 25.3, 24.4, 21.2; **ES+HRMS m/z** 410.1078 [M+H]⁺; \(\text{C}_{22}\text{H}_{20}\text{ClN}_{3}\text{OS}\) requires 409.10; **HPLC** (purity analysis %) found: 99.41%; RT: 10.81 min/ 40 min (25/75, 1.0 ml/min IPA:HEXANE, Signal = 230 nm, SYNCHROSILICA).
Preparation of 2-(3-((7-chloroquinolin-4-yl)amino)-2,2-dimethylpropyl)benzo[d]-isothiazol-3(2H)-one 47

To a solution of 41f (0.291 g, 0.93 mmol) in isopropanol (IPA) (3 ml), 4,7-dichloroquinoline (1.2 eq) was added. The solution was heated to 190°C in a microwave reactor where it was maintained for 45 minutes before cooling slowly to 55°C and then to room temperature. IPA was removed under reduced pressure to provide the crude product which was purified by flash column chromatography and trituration with Et₂O to give 47 as a cream, powdery solid in a 64.1% yield. Rf = 0.19 (70:30 EtOAc:hexane and 1% triethylamine). 1H NMR (400 MHz, MeOD) δ 8.32 (d, J = 5.7 Hz, 1H), 8.26 (d, J = 9.0 Hz, 1H), 8.05 (d, J = 7.9 Hz, 1H), 7.81 (d, J = 8.2 Hz, 1H), 7.78 (d, J = 2.1 Hz, 1H), 7.72 (td, J = 8.0, 0.8 Hz, 1H), 7.49 (t, J = 7.8 Hz, 1H), 7.46 (dd, J = 8.9, 2.1 Hz, 1H), 6.64 (d, J = 5.8 Hz, 1H), 3.88 (s, 2H), 3.25 (s, 2H), 1.13 (s, 6H); 13C NMR (101 MHz, MeOD) δ 167.4, 151.9, 151.0, 148.5, 141.5, 135.1, 132.3, 126.3, 125.8, 125.6, 124.8, 123.4, 122.9, 120.6, 117.3, 98.3, 52.1, 48.4, 38.3, 23.1 (2C); Elemental analysis (%) found: C, 63.51; H, 5.18; N, 10.59; S, 7.75%; C₂₁H₂₀ClN₃OS requires C, 63.39; H, 5.07; N, 10.56; S, 8.06%; ES+MS m/z 398.1083 [M+H]+ C₂₁H₂₀ClN₃OS requires 397.10.
3.13.2 Biological Methods

 MEP Metabolite Profiling

Prior to treatment, parasites were synchronized by 1-2 treatments with 5% sorbitol until > 8% parasitemia and > 75% ring-stage cultures were achieved. At this point, cultures were treated 10 hours with 5 µM compound 4, 5 µM fosmidomycin, or left untreated. Following treatment, parasite-infected erythrocytes were lysed with 0.1% saponin, washed in phosphate-buffered saline, and stored at -80°C until extraction and quantitative LC-MS/MS measurement of DOXP and MEcPP, as previously described. Values reflect the mean and standard error from ≥ 3 independent experiments and were compared using the Student’s t-test (two-tailed).

Cloning of PfISP and Orthologues

*P. falciparum:* The construct used for expression of the codon-optimized PfISP protein has been described previously.[73] To generate the PfISP variants containing the C202A mutation, N- and C-terminal fragments of the optimized PfISP gene were amplified from the above vector using the following primers. C202A N-terminal fragment: T7 Fwd primer, 5'-TAATACGACTCACTATAGGG-3', with C202A Rev primer, 5'-GTTTGGCAATACGGCGCCTAGCAGGATATGAAT-3'; C202 C-terminal fragment, C202A Fwd primer, 5'-CATTCATAGTATCCTGCTAGCCGCGGTATTGCCAAC-3', with T7 Rev primer, 5'-GCTAGTTATGCTCAGCGG-3'. Amplicons containing the entire PfISP gene were generated using PfISP Fwd and Rev primers, which introduce a 6-His tag, with N- and C-terminal fragments as template (PfISP Fwd primer: 5'-CTACACCACCACCACTGATGCACATCTACGATAATAAC-3'; PfISP Rev: 5'-ATCTTACCTCTACTTTTATTTGGAGGATAGATAAT-3'). Following amplification, PfISP variants were cloned into pBG1861 by ligation-independent cloning as previously described,[73,82] and verified by Sanger sequencing.

*P. vivax:* The construct used for expression of codon-optimized PvISP (P. vivax Sal-1; PVX_081425) has been previously described.[73]

*E. coli:* The construct used for expression of EcISP-Dwt has been previously described.[70] The A14C mutation was introduced by first generating N- and C-terminal gene fragments containing the mutation. These fragments were generated using the following primers: A14C N-terminal fragment: T7 Fwd primer, 5'-TAATACGACTCACTATAGGG-3', with A14C Rev primer, 5'-CATTCAGACGGCCAAATCCCGGCACACAGGAACCACCGGCAAACAT-3'; A14C C-terminal fragment, A14C Fwd primer, 5'-GATGTTTCGCGCGTGGTATCCGGGTGCTTGGCGCGTCAGAT-3', with T7 Rev.
primer, 5’-GCTAGTTATTGCTCAGCGG-3’. Amplicons containing the entire *E. coli* *ispD* gene were generated using *Ec* *ispD* Fwd and Rev primers, which introduce a 6-His tag, with N- and C-terminal fragments as template (*Ec* *ispD* Fwd primer: 5’-CTCACCACCACCACCACCATATGGGCAACCACTCATT-3’; *Ec* *ispD* Rev primer: 5’-ATCCTATCTTAATCACTTATATTGTATTCTCCTGATGG-3’). Following amplification, *E. coli* *A14C-IspD* was cloned into pBG1861 by ligation-independent cloning as previously described,[82] and verified by Sanger sequencing.

**Parasite Sensitivity Assays**

Asynchronous cultures of *P. falciparum* strain 3D7 were diluted to 1% parasitemia and cultured at indicated concentrations of inhibitor compounds in 100 µL culture volumes. During rescue experiments, parasite growth medium was supplemented with 200 µM isopentenyl pyrophosphate (IPP; Echelon Biosciences). After 72 hours, parasite growth was quantified using PicoGreen dye (Invitrogen) to measure DNA content as previously described.[70] PicoGreen fluorescence was measured (485 nm excitation/ 528 nm emission) in a POLARStar Omega microplate reader (BMG Labtech). IC$_{50}$ values were calculated by nonlinear regression analysis using GraphPad Prism software, and reflect the mean and standard error from ≥ 3 independent experiments. Values were compared using the Student’s t-test (two-tailed).
3.14 References


Chapter 4
Determining the Mechanism of Action of the BITZ Chemotype:
A Computational and Biological Study
4.1 Computational Modelling

In addition to in vitro enzymatic and cellular analysis of 1,2-benzo[d]isothiazol-3(2H)-one (BITZ) compound inhibition, molecular modelling studies were undertaken to provide insight as to how BITZ compounds may bind to the PfIspD enzymatic target. Three-dimensional modelling of the IspD active site, using natural substrates and products of the enzyme, as well as potential inhibitor structures has been used to refine compound selection, guide rational inhibitor design and inform mechanism of action studies.

Ligand-protein docking is a computational approach that attempts to predict how small molecules bind to the structure of macromolecular targets and ‘scores’ their complementarity to the binding site. This computational technique, when used for virtual screening, can be used as a tool of enrichment increasing the potential of identifying compounds likely to show activity, potency and selectivity at an enzyme, making it widely used in both hit identification and lead optimisation stages of drug discovery.\[1\]

The prediction of a compounds inhibitory activity can be made through the assessment of a molecule’s binding affinity at a given binding site; this assessment determines how strongly a ligand interacts with enzyme protein which can be quantified by a measure known as a fitness score.\[2\] Molecular docking involves an exploration of “docking space” where molecules are computationally placed in different conformations, orientations and locations within an enzyme active site to identify the most favourable binding “pose”. The predicted binding energies of compounds and these identified binding “poses” are evaluated using scoring functions.\[1\] Broadly, two docking methods can be adopted to explore “docking space”: rigid-docking which treats both the ligand and protein as rigid molecules, or flexible-docking where the ligand, receptor or indeed both are treated as flexible entities. However, the two consistent features of all docking strategies are a search algorithm and a scoring function.\[2\]

Docking algorithms generate “poses” of molecules at a docking site, sampling the “docking space” of a molecule to determine whether a given conformer or ligand orientation fits into a specified enzyme site.\[1\] These docking algorithms consequently identify a molecular conformation that best complements the receptor structure. Genetic algorithms (GA) represent a stochastic search method, based on various computational models of evolution, which aim to mimic the natural process of evolution in determining an optimal combination of binding features.\[3,4\] GAs represent a way of searching conformational space for an optimal docking solution that considers the number of degrees of freedom of the docked ligand and how each proposed ligand conformation may fit into the defined binding site of a macromolecule. GAs do not just search for a single solution but combine the best
aspects of good solutions, considering both ligand orientation and receptor alignment, therefore increasing the fitness of identified solutions to determine optimal binding poses.

Scoring functions predict the biological activity of compounds through the evaluation of interactions between the docked compound and biological target in an identified “pose”; this allows a fitness score to be assigned to the “pose” and the docking algorithm to be completed. The evaluation and ranking of predicted ligand conformations is a therefore crucial aspect of molecular modelling and computational screening approaches. The scoring functions used within docking programs often make various assumptions and simplifications in the evaluation of modelled protein-ligand complexes where three main types of scoring function are commonly used: force-field-based, empirical and knowledge-based scoring functions. Each of these categories classifies a number of distinct scoring functions, where each has unique subtleties that may be more suited to a specific modelling scenario. It is therefore unsurprising that much time and research has been invested in the optimisation and refinement of scoring functions, as well as determining specific scoring functions that may be most suited to a particular research question. Detailed analysis and evaluations of docking programs and scoring functions are well reviewed elsewhere.

Force-field-based scoring functions use molecular mechanic force fields to quantify the sum of two energies: the receptor–ligand interaction energy and internal ligand energy. With examples including GOLD and AutoDock, most force-field scoring functions consider only a single protein conformation, making it possible to eliminate the calculation of internal protein energy which greatly simplifies scoring. Empirical scoring functions, such as ChemScore or F-Score, are able to reproduce experimental data and are based on the idea that binding energies can be approximated by a summation of individual uncorrelated terms. Finally, knowledge-based scoring functions, such as DrugScore, are designed to reproduce experimental structures rather than binding energies.

This chapter will discuss the use of such molecular modelling techniques and described docking methods for the purpose of assessing BITZ compound alignment at the PfIspD enzyme active site and subsequent deduction of protein-ligand interactions and a proposed mechanism of BITZ compound inhibition.

4.2 Utilising IspD Enzyme Homologues

Due to difficulties crystallising the P. falciparum IspD (PfIspD) enzyme, we have not had access to the crystal structure of PfIspD within our studies to date. Therefore, to facilitate docking studies of small molecules at the IspD enzyme, it was necessary to identify a IspD protein homologue with sequence
similarity to PfISP that could be used as a protein model to predict how small molecules are likely to bind to PfISP.

Comparison of E. coli and P. falciparum IspD enzyme homologues (enzyme active site defined as residues within 5 Å of the proposed binding site) indicates that 13 out of the 21 active site protein residues are identical between the two species, giving a 62% identity between E. coli and P. falciparum IspD at the active site. Considering the entire proteins of the two IspD homologues, 14% of protein residues show similarity, as opposed to exact identity, which puts the overall structure similarity of E. coli IspD (EcIspD) and PfIspD close to 23%, but with notably increased sequence and homologue identity at the active site. This heightened degree of active site similarity gives reassurance that EcIspD (Figure 4.1) can be used as a suitable surrogate protein for PfIspD.

**Figure 4.1** Tertiary structure of the E. coli IspD monomer with CDP-ME bound (PDB:1INI[12]); α-helices and β-sheets are shown as cyan and purple cartoons respectively. In the close-up image of the CTP binding site; the cytosine binding region and residues are rendered pink; the ribose binding region and corresponding residues are rendered tan; the phosphate and MEP binding residues are rendered yellow and orange respectively. CDP-ME is coloured by element; carbon is green. The active site Mg²⁺ ion is shown as a grey sphere.¹³,¹⁴

Working on the understanding that EcISP has an acceptable active site similarity to PfISP, it was selected as a surrogate protein for comparative three-dimensional docking studies and will continue to be used within molecular modelling studies until a crystal structure of PfISP is available. EcISP was therefore selected as a protein homologue of PfISP to identify a suitable modelling protocol with which to dock small molecules at the IspD active site. The rationale being that the identified docking protocol could then be transferred and applied to a homology model of the PfISP protein to facilitate
the assessment of small molecule BITZ inhibitors at the *P. falciparum* homologue of the IspD antimalarial enzyme target.

Evaluation of the binding poses of inhibitor structures at the *PfIspD* enzyme not only provides insight into aspects of the mechanism of action of BITZ compounds, but also facilitates application of the molecular design loop (Figure 4.2). The molecular design loop is comprised of three phases, molecular modelling, organic synthesis and biological testing, where knowledge gained from each phase can be fed directly into the next, therefore informing all stages of molecular design and continuing iterations of the cycle. With this depiction of a design and feedback strategy (Figure 4.2), it is easy to see that determining the predicted activities of small molecules at *PfIspD* will facilitate a more informed and predictive approach to inhibitor design, therefore enabling a refined compound selection process and optimised drug design.

![The molecular design loop](image)

**Figure 4.2** The molecular design loop.

### 4.2.1 Comparative Three-Dimensional Modelling at *E. coli* IspD

Due to the aforementioned sequence similarity between *EcIspD* and *PfIspD* (Section 4.1.1), the known crystal structure of *EcIspD* with the natural product, CDP-ME, co-crystallised in the enzyme active site (PDB: 1INI) was selected as the *EcIspD* surrogate protein for comparative three-dimensional modelling studies. The first stage of these studies was to extract co-crystallised CDP-ME from the 1INI structure and re-dock the extracted ligand back into the *E. coli* enzyme. Molecular docking was initially carried out using the docking program GOLD 5.0.1 and the ChemPLP scoring function, aiming to reproduce the crystallographic binding pose of CDP-ME at the *EcIspD* active site of the 1INI structure. The overall aim of this process was to determine a well-defined docking protocol capable of reproducing the crystallographic binding pose of the 1INI protein-ligand complex, as obtained from the Protein Data Bank (PDB) archive. Identification of an accurate docking protocol then
facilitates the docking of unknown (or previously unstudied) small molecules at EcIspD to determine a measure of binding affinity and an initial outlook as to the feasibility of proposed molecular docking studies.

### 4.2.2 Determining a Docking Protocol

The ligand docking program GOLD 5.0.1 (Genetic Optimisation for Ligand Docking), a component of the GOLD suite and work of The Cambridge Crystallographic Data Centre (CCDC),\(^{[16,19]}\) has been used as the docking program for molecular modelling studies throughout this body of research. GOLD software searches for the best ligand-protein interaction (“poses”) using GAs as previously described (Section 4.1).

A number of docking protocols were examined in order to identify an optimal protocol capable of accurately reproducing the crystallographic binding pose of CDP-ME in the EcIspD 1INI active site. A suitable and accurate docking protocol is capable of consistently reproducing the experimental binding mode of a ligand (i.e. the binding mode defined by a crystallographic structure) which can be quantified by root-mean-square deviation (RMSD). RMSD provides a measure of the difference in values predicted by a model or estimator, compared to known values, and is therefore a measure of accuracy since it provides a means of comparing and measuring the 3D similarity of molecular poses. The prediction of a binding mode is considered successful, and therefore the docking protocol accurate, if the calculated RMSD value is below a threshold of 2 Å.\(^{[16]}\)

For each protocol generated, all standard default settings (SDS) in the GOLD Docking Setup remained unaltered, with the exception of the specified changes outlined in the tables below (Tables 4.1, 4.3 and 4.5). The natural enzyme product, CDP-ME, was used to define the ligand binding site as residues within 10 Å of the known crystallographic CDP-ME pose within EcIspD (PDB ID: 1INI\(^{[12]}\)). Early docking termination was not allowed across all protocols. Varying numbers of GA runs were specified (Table 4.2, 4.4, 4.6 and 4.8) and performed for each protocol; this enabled a range of poses to be collected and an assessment made as to the effects of altering the number of GAs requested on the accuracy of resulting docking poses. Tables 4.2, 4.4, 4.6 and 4.8 illustrate the average fitness scores across the poses generated for each specification of GAs (10, 50, 100 and 500 respectively), as well as the associated RMSD values as compared to the native ligand, CDP-ME. ChemPLP was initially used as the scoring function in Protocols 1-3 since it had recently been identified as one of the best performing scoring functions for applications both in pose prediction and virtual screening studies.\(^{[18]}\)

Previous molecular modelling studies have indicated that a specified water molecule (HOH1091) in the active site of EcIspD PDB ID: 1INI\(^{[12]}\) may be involved in mediating protein-ligand interactions.
between the EcIspD protein and native ligands, facilitating a water-mediated hydrogen bonding interaction between EcIspD protein residues and bound ligands. Further consideration and assessment of this water molecule was therefore considered appropriate in determining our docking protocol for application with this protein. It was important to be aware that HOH1091 may retain a role or exert influence in ligand docking and molecular pose alignment in ongoing docking studies.

### Protocol 1

<table>
<thead>
<tr>
<th>Component</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$ (Atom 1717)</td>
<td>Tetrahedral coordination geometry</td>
</tr>
<tr>
<td>Protonation</td>
<td>Hydrogens added</td>
</tr>
<tr>
<td>Waters</td>
<td>Water molecule HOH1091 extracted</td>
</tr>
<tr>
<td></td>
<td>All other waters deleted</td>
</tr>
<tr>
<td>Ligands</td>
<td>‘A’ (CDP-ME) extracted</td>
</tr>
<tr>
<td>Binding site</td>
<td>Defined as 10 Å</td>
</tr>
<tr>
<td>Number of GA Runs</td>
<td>10, 50, 100, 500 (4 docking runs)</td>
</tr>
<tr>
<td>Scoring function</td>
<td>ChemPLP</td>
</tr>
</tbody>
</table>

**Table 4.1** Specified parameters of docking Protocol 1.

<table>
<thead>
<tr>
<th>GA Runs</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Fitness Score</td>
<td>107.58</td>
<td>104.77</td>
<td>105.52</td>
<td>100.99</td>
<td>103.97</td>
</tr>
<tr>
<td>Fitness Score SD</td>
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<td>12.82</td>
<td>15.13</td>
<td>14.13</td>
<td>13.69</td>
</tr>
<tr>
<td>Average RMSD</td>
<td>5.11</td>
<td>4.29</td>
<td>5.23</td>
<td>5.29</td>
<td>4.98</td>
</tr>
</tbody>
</table>

**Table 4.2** Outputs of CDP-ME docking into EcIspD using Protocol 1.

The average RMSD of docking solutions generated using Protocol 1 (Table 4.1) were significantly above the specified threshold of 2 Å (Table 4.2), highlighting that protein-ligand poses with low 3D similarity were being predicted and were not representative of the known crystallographic binding pose. It was therefore clear that Protocol 1 (Table 4.1) could not accurately reproduce the CDP-ME binding pose of 1INI.

In order to improve the accuracy and reproducibility of docking predictions (reducing the RMSD of calculated poses) the specified ligand binding site was reduced from considering residues within 10 Å to those within 6 Å of the crystallographic CDP-ME binding orientation. This enabled the docking algorithm to search a more defined area of space within the EcIspD binding pocket, slightly reducing the range and diversity of possible poses that could be identified, through a reduced number of degrees of freedom, therefore aiming to increase the accuracy of the protein-ligand poses identified.\(^{[18]}\)
Protocol 2

<table>
<thead>
<tr>
<th>Mg$^{2+}$ (Atom 1717)</th>
<th>Tetrahedral coordination geometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protonation</td>
<td>Hydrogens added</td>
</tr>
<tr>
<td>Waters</td>
<td>Water molecule HOH1091 extracted  All other waters deleted</td>
</tr>
<tr>
<td>Ligands</td>
<td>‘A’ (CDP-ME) extracted</td>
</tr>
<tr>
<td>Binding site</td>
<td>Defined as 6 Å</td>
</tr>
<tr>
<td>Number of GA Runs</td>
<td>10, 50, 100, 500 (4 docking runs)</td>
</tr>
<tr>
<td>Scoring function</td>
<td>ChemPLP</td>
</tr>
</tbody>
</table>

Table 4.3 Specified parameters of docking Protocol 2.

<table>
<thead>
<tr>
<th>GA Runs</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Fitness Score</td>
<td>109.03</td>
<td>96.42</td>
<td>99.89</td>
<td>97.37</td>
<td>100.68</td>
</tr>
<tr>
<td>Fitness Score SD</td>
<td>9.81</td>
<td>14.59</td>
<td>15.45</td>
<td>14.95</td>
<td>13.70</td>
</tr>
<tr>
<td>Average RMSD</td>
<td>3.79</td>
<td>5.08</td>
<td>4.86</td>
<td>5.13</td>
<td>4.72</td>
</tr>
</tbody>
</table>

Table 4.4 Outputs of CDP-ME docking into EcIspD using Protocol 2.

The decrease in measured RMSD on reducing the specified binding site around the CDP-ME ligand from 10 Å to 6 Å, denoted an improvement in the accuracy of Protocol 2 (Table 4.3) compared to Protocol 1 (Table 4.1). However, the reduction in RMSD was not deemed sufficient (Table 4.4) and therefore additional changes to the docking protocol were made.

Protocol 3

<table>
<thead>
<tr>
<th>Mg$^{2+}$ (Atom 1717)</th>
<th>Metal ion extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protonation</td>
<td>Hydrogens added</td>
</tr>
<tr>
<td>Waters</td>
<td>Water molecule HOH1091 extracted All other waters deleted</td>
</tr>
<tr>
<td>Ligands</td>
<td>‘A’ (CDP-ME) extracted</td>
</tr>
<tr>
<td>Binding site</td>
<td>Defined as 6 Å</td>
</tr>
<tr>
<td>Number of GA Runs</td>
<td>10, 50, 100, 500 (4 docking runs)</td>
</tr>
<tr>
<td>Scoring function</td>
<td>ChemPLP</td>
</tr>
</tbody>
</table>

Table 4.5 Specified parameters of docking Protocol 3.
Table 4.6 Outputs of CDP-ME docking into EcIspD using Protocol 3.

Extraction of the Mg²⁺ ion in the application of Protocol 3 (Table 4.5) was seen to slightly reduce the RMSD of generated poses; however, the reduction in RMSD still did not meet the required 2 Å value and, in addition, an undesirable reduction in the average fitness scores of poses was now also seen. This is perhaps unsurprising as the Mg²⁺ divalent cation is a known feature of the EcIspD binding site and should therefore be retained during the development of a docking protocol. Comparison of the outputs from Protocols 1-3 highlight that the measured fitness scores were generally higher when the Mg²⁺ ion was specified as part of the docking procedure.

Overall, Protocols 1-3, (Tables 4.1, 4.3 and 4.5) were unable to accurately reproduce the crystallographic binding pose of CDP-ME as defined by the 1INI crystal structure, as highlighted by consistent RMSD values of > 2 Å, indicating inaccuracy in the protocol. It was therefore decided that the scoring function specified in the docking protocol should be replaced in an attempt to correct this problem. GoldScore was selected as the scoring function to replace ChemPLP in Protocol 4 (Table 4.7) due to previous successes with this scoring function in other molecular modelling studies conducted within the group.[20]

<table>
<thead>
<tr>
<th>GA Runs</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Fitness Score</td>
<td>94.84</td>
<td>90.60</td>
<td>84.24</td>
<td>85.45</td>
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<tr>
<td>Fitness Score SD</td>
<td>6.44</td>
<td>16.35</td>
<td>16.35</td>
<td>14.95</td>
<td>13.52</td>
</tr>
<tr>
<td>Average RMSD</td>
<td>2.72</td>
<td>3.97</td>
<td>4.31</td>
<td>4.30</td>
<td>3.83</td>
</tr>
</tbody>
</table>

Table 4.7 Specified parameters of docking Protocol 4.
Table 4.8 Outputs of CDP-ME docking into EcIspD using Protocol 4.

<table>
<thead>
<tr>
<th>GA Runs</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>Average</th>
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<td>Average Fitness Score</td>
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<td>89.36</td>
<td>93.26</td>
<td>92.91</td>
<td>92.79</td>
</tr>
<tr>
<td>Fitness Score SD</td>
<td>12.97</td>
<td>11.78</td>
<td>12.90</td>
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<td>12.56</td>
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<tr>
<td>Average RMSD</td>
<td>2.56</td>
<td>2.09</td>
<td>1.79</td>
<td>1.96</td>
<td>2.10</td>
</tr>
</tbody>
</table>

The docking outputs from Protocol 4 (Table 4.7) showed improved levels of accuracy, with RMSD values now < 2 Å (Table 4.8) when ≥ 100 GA docking poses were requested. The improvement in protocol accuracy, in terms of increased average fitness scores and lower RMSD values, may be due to a combination of both reducing the specified binding site around the CDP-ME ligand and altering the scoring function used to predict the strength of binding of identified poses. Docking Protocol 4 (Table 4.7) was consequently selected and applied within our EcIspD molecular modelling studies from this point.

4.2.3 Molecular Modelling of the HTS Hit Compounds at EcIspD

Docking Protocol 4 (Table 4.7) was used to model the 76 hit compounds identified from the initial chemoinformatic HTS and virtual screening study conducted using the “Biofocus Combined Library” (Section 2.2.1 and Appendix 1). 50 GA docking poses were modelled for each ligand in order to achieve an acceptable compromise between docking accuracy and computational calculation time. The selected 76 ligands were built manually using ChemDraw[21] and energy minimised using Spartan ’08.[22] Spartan ’08 was used to perform equilibrium geometry calculations using the molecular mechanics MMFF level of theory,[23] prior to docking the built ligands at the EcIspD active site. The GoldScore scoring function was used to evaluate the docking poses of these 76 ligands at EcIspD; where the identified poses were then rescored (a method of pose re-evaluation)[11] using the ChemPLP and ChemScore scoring functions. The process of rescoring identified poses in a separate step, distinct from the initial docking procedure, using alternative scoring functions is a way of certifying that consistent and optimal docking poses are identified by a defined docking protocol and the original scoring function used.[11]

The 76 docked compounds were ranked according to their associated fitness scores for complementarity to the binding site which gave a prediction of inhibitory compound activity at the EcIspD enzyme. A higher assigned fitness score (or higher average fitness score from across the 50 GA poses calculated for each molecule) gave a higher compound ranking, therefore indicating likely enhanced inhibitory activity at EcIspD. Of the 76 HTS hit compounds docked at the EcIspD active site,
GA docking poses were identified for 12 compounds that had measured GoldScore fitness scores of > 70.

Of these 12 compounds, 10 showed consistent levels of complementarity to the EcIspD binding site with average GoldScore fitness scores of > 61 (averaged across the 50 docking poses). Although these values not as high as the average GoldScore score calculated for the native CDP-ME ligand; [CDP-ME GoldScore = 89.36; Fitness Score SD = 11.78; averaged across 50 GA poses using Protocol 4 at EcIspD (Table 4.7)]; comparison of these predicted protein-ligand binding energies provides a general measure of our HTS compound complementarity to EcIspD, compared to the native enzyme product. Significantly, the two most active HTS hit compounds DT2009-0097223 and DT2009-0168020 (Figure 4.3, see also Chapter 2, Section 2.2.4) were present in this small group of 10 compounds that were highlighted as showing satisfactory and consistent complementarity to EcIspD.

![Chemical structure](image)

**Figure 4.3** Chemical structure; PflspD enzyme activity; molecular weight; ligand efficiency, average GoldScore fitness score and the GoldScore fitness score of the highest scored binding pose predicted in EcIspD molecular docking studies of DT2009-0097223 and DT2009-0168020.

An appropriate and functioning docking protocol (Protocol 4, Table 4.7) was therefore identified that is capable of accurately reproducing the known crystallographic binding pose of CDP-ME (PDB ID: 1INI[12]) and generating rational protein-ligand binding poses (since no negative or anomalous binding scores were identified) with a diverse range of small molecules. The latter was highlighted by the successful assessment of the 76 HTS hit compounds (Appendix 1) at EcIspD. These ligands cover a broad range of chemical space that is very much unrelated to the native CDP-ME ligand and therefore confirms that docking Protocol 4 is capable of assessing a broad range of molecules at a defined binding site. Having acquired confidence in Protocol 4, the outlined docking protocol (Table 4.7) was
transferred and applied to the study of the PfISPd enzyme, enabling the evaluation of small molecules at this selected antimalarial target.

### 4.3 PfISPd Homology Model

A homology model of the PfISPd enzyme (Figure 4.4), based on the primary sequence Q8I273 and the 1INI E. coli structure template[12] was created with the PHYRE online homology modelling tool[24] and, after validation using the WHATIF interface, was assessed to have a confidence of 98.2%. [25] The PfISPd homology model and determined protocol (Protocol 4, Table 4.7) were used to dock BITZ compounds into the CTP binding site of PfISPd using GOLD 5.0.1 software.[16]

![Figure 4.4 Tertiary structure of the P. falciparum IspD homology model with the natural ligand, CDP-ME, occupying the enzyme binding pocket. The surface of PfISPd homology model is rendered pink; CDP-ME is shown as a stick representation and coloured by element: carbon atoms are grey; the Mg²⁺ divalent cation is shown as a grey sphere.][26]

As an initial docking study, the CTP enzyme substrate was docked into the PfISPd homology model and found itself to have an average GoldScore of 72.0 ± 4. This enabled a more precise and informed assessment of the predicted binding energies of BITZ derived compounds in subsequent molecular modelling, giving a truer picture and more exact measure of the relative binding affinity of specified BITZ compounds at the PfISPd CTP binding site. The aims of modelling studies from this point were to determine the likely binding mode and alignment of BITZ derived compounds as inhibitors of PfISPd; visualize contacts made between these inhibitors and the defined protein binding site; assess the
strength of inhibitor-protein interactions and therefore estimate the binding affinity and behaviour of BITZ compounds at the PfIspD active site (Figure 4.5).

Figure 4.5 Image of the *P. falciparum* IspD homology model active site with the natural product, CDP-ME, occupying the binding pocket. The surface of PfIspD active site is rendered white; CDP-ME is shown as a stick representation and coloured by element: carbon atoms are light-grey, nitrogen atoms are blue, oxygen atoms are red and phosphorus atoms are orange. Selected PfIspD active site protein residues, Cys-202, Lys-215, Thr-664 and Arg-208, are highlighted and depicted as sticks with their carbon protein backbone coloured orange. The Mg²⁺ divalent cation is shown as a small sphere and rendered dark grey. Image created using PyMOL.

4.3.1 Molecular Modelling of the Focused Ligand Library of 5000 Compounds at PfIspD

To ensure that comparable docking outputs to those achieved with EclspD (PDB ID: 1INI[12]), in terms of rational docking poses and similar docking scores (in the 40 – 70 range), could be achieved using the PfIspD homology model: the original focused ligand library of 5000 compounds, selected from the “Biofocus Combined Library” (Chapter 2, Section 2.2.1), was docked into the PfIspD homology model using Protocol 4 (Table 4.7). 10 GA searches were specified per ligand and GoldScore was used as the scoring function. Compared with earlier EclspD molecular docking (Section 4.2.2), the number of GA searches requested per ligand, now using the PfIspD homology model, was reduced from 50 GA to 10 GA to minimise the computational time expenditure required to dock and assess the large number of compounds within the focused ligand library.

Following docking of these 5000 compounds into the active site of the PfIspD homology model, all assessed ligands were attributed a GoldScore docking score within the range of 20-70. Consequently,
it can be argued that the initial molecular docking outputs using PfISP-D are broadly representative of the varying PfISP-D activity of the compounds identified within the focused ligand library (Chapter 2, Section 2.2.4). The majority of compounds from the focused ligand library were predicted to adopt docking poses that were assessed to have mid-range fitness scores (between 30 and 50). This suggests a lack of complementarity to the PfISP-D active site for the majority of the 5000 compounds and therefore a likely lack of PfISP-D inhibitory activity. This assessment is supported by the findings of the HTS output in which > 4900 of the compounds screened were found to possess suboptimal PfISP-D activity of less than the required activity threshold of 20 μM against the enzyme.

A small number of the 5000 docked compounds were predicted to adopt binding poses of acceptable complementarity to PfISP-D which was reflected in higher attributed binding scores (values ≥ 50) of these predicted poses. The docking outputs therefore suggest that a small proportion of the 5000 compounds are likely to display inhibitory activity against PfISP-D. This general observation is reflected in and supported by the identification of 76 hit compounds (a small compound subset) from the initial HTS study of the “Biofocus Combined Library” (Chapter 2, Section 2.2.4) as being active against PfISP-D.

Further computational analysis has shown that these docking studies, and the resulting binding scores for predicted compound poses, did not provide a good indication of whether a compound would be active or inactive against the PfISP-D enzyme. Average docking scores of compounds categorised as active were highly similar to the average scores of those classified as inactive, showing that the distribution of compound binding scores did not correlate to classifications of enzymatic activity. Despite this, we were able to take confidence from these molecular modelling studies using the ‘Focused Ligand Library’ as attentions were now focused towards docking designed BITZ inhibitors into the PfISP-D homology model.
**4.3.2 Molecular Modelling of BITZ Compounds at PfispD**

To gain insight into the behaviour, alignment and spatial fit of BITZ inhibitors within the PfispD active site, one of the most promising BITZ compounds 1 (Figure 4.6) (see also Chapter 3, Section 3.2, Figure 3.3, Table 3.1: Compound 4) was modelled in the PfispD homology model using docking Protocol 4 (Section 4.2.1, Table 4.7).

BITZ inhibitor 1 (Figure 4.6) was docked into the active site of the PfispD homology model using Protocol 4 (Table 4.7) with a search criteria of 50 GA predictions to assess the alignment and predicted docking pose of 1 at PfispD. The highest scored binding pose and average fitness scores from the 50 calculated binding poses are given below (Figure 4.6), along with the chemical structure of 1.

![Chemical structure of BITZ inhibitor 1](image)

**Figure 4.6** Chemical structure; PfispD enzyme activity; whole cell 3D7 activity; molecular weight; average GoldScore fitness score and the GoldScore fitness of the highest scored binding pose predicted in PfispD molecular docking studies of 1 in the PfispD homology model.

Molecular docking of 1 and the subsequent evaluation of resulting conformational poses has revealed that BITZ compounds target the CTP binding pocket of PfispD and adopt a broadly similar binding alignment to the natural product, CDP-ME (Figure 4.7). BITZ compounds therefore demonstrate good complementarity and shape-fit to the PfispD active site where a number of hydrogen bonding and hydrophobic interactions can be made between BITZ inhibitors and the protein residues in the active site which aid initial non-covalent recognition and protein-ligand interactions.
**Figure 4.7** Inhibitor 1 and CDP-ME modelled in the active site of the PfIspD homology model. PfIspD active site rendered as a white surface. Key active site protein residues are depicted as sticks: carbon atoms coloured orange; inhibitor 1 is shown as a stick representation: carbon atoms are dark grey; CDP-ME shown as a stick representation: carbon atoms are light-grey. Image created using PyMOL.\(^{[26]}\)

Overlaying the two modelled structures of 1 and CDP-ME highlights that they occupy the same area of space within the PfIspD active site and that the BITZ analogue is aligned to make a number of hydrogen bond contacts and hydrophobic interactions with protein residues in the binding site (**Figure 4.8**).

A hydrogen bonding interaction of 2.9 Å is predicted (highlighted in **Figure 4.8**) between the oxygen atom of the methoxy functionality in 1 and the Arg-208 PfIspD active site protein residue. A second binding interaction was also identified between a hydrogen atom on the aromatic phenyl D ring of 1 and the Thr-664 PfIspD active site protein residue: the measured distance between these two entities is 3.4 Å and therefore likely represents a second hydrogen bonding interaction, or potentially a hydrophobic interaction. These two identified molecular interactions represent non-covalent protein-ligand contacts which aid initial compound recognition in the PfIspD active site therefore promoting the alignment and binding of 1.
Figure 4.8 Inhibitor 1 and CDP-ME modelled in the active site of the PfIspD homology model. Key protein residues are depicted as sticks: carbon atoms coloured orange; inhibitor 1 is shown as a stick representation: carbon atoms are dark grey; CDP-ME is shown as a stick representation: carbon atoms are light-grey; hydrogen bonds between 1 and specified protein residues are depicted as black, dotted lines with predicted hydrogen bond distance in Ångstroms. Image created using PyMOL.[26]

Indeed, a number of potential docking poses were predicted for compound 1 which were assigned a range of binding scores (representative example, Figure 4.9) However, since these predicted poses predominately mirror the binding alignment of CDP-ME (Figure 4.5), this supports the assessment that the identified BITZ poses are representative of the true binding orientation and alignment of BITZ inhibitors at the PfIspD active site.
Figure 4.9 Image shows an alternative binding pose predicted for 1 modelled with CDP-ME in the active site of the PfIspD homology model. The depiction of this alternative predicted binding pose highlights that 1 remains aligned to CDP-ME in the PfIspD active site. Image created using PyMOL. 

Visualisation of 1 within the PfIspD binding pocket also provides an assessment of inhibitor shape fit at the active site. Docking images clearly show that the C-ring meta-substitution pattern within BITZ compounds enhances complementarity to the binding site through the presence of this molecular ‘kink’ that mirrors the shape of the active site. Molecular modelling therefore highlights that the PfIspD active site is neither linear nor tube-like in shape which perhaps explains the loss of PfIspD inhibitory activity observed with linear BITZ analogues.

It can therefore be rationalised that the chemical construct of linear BITZ analogues, with para-substitution across the C-ring are likely to have poor complementary to the PfIspD active site, with poor shape-fit and little alignment to active site amino acids (as observed (Chapter 2, Section 2.7)). However, it is also clear from the construct of the PfIspD active site there is room for expansion of the BITZ construct both in the inhibitor side chain and around the BITZ head group itself. Structural expansion of BITZ analogues could enable more complete occupation of the PfIspD active site and therefore increase the likelihood of achieving protein-ligand contacts which may in turn promote stronger compound binding. This, in theory, should produce more active PfIspD BITZ inhibitors.
4.3 Mechanism of Action

In addition to inhibitor alignment and spatial fit with the PfIspD active site, molecular modelling studies also enabled insights to be gained into the proposed mechanism of PfIspD enzyme inhibition. Molecular docking of 1 clearly indicates that the inhibitor binds within the CTP substrate-binding pocket, aligned with the natural product CDP-ME. This identified alignment and orientation places the BITZ warhead proximal to the PfIspD active site cysteine-202 (Cys-202) residue (Figure 4.10).[27]

![Figure 4.10](image1.png)

**Figure 4.10** Inhibitor 1 modelled in the active site of PfIspD homology model with potential interactions between the compound and active site protein residues highlighted. Points of interaction are depicted by black, dotted lines with predicted distances given in Ångstroms. Image created using PyMOL.[26]

It is predicted that the thiol moiety of Cys-202 could react with the sulfur heteroatom of the BITZ warhead, forming a covalent disulfide adduct, while the carbonyl oxygen of the head group would be within hydrogen bonding distance of the backbone nitrogen atoms of the Gly-203 and Gly-204 residues (Figure 4.11). Formation of this covalent adduct would result in the occlusion of the PfIspD active site, preventing natural substrate binding and therefore render the enzyme inactive.[27] Taken all together, a distinctive mechanism of action can be proposed for these novel PfIspD-inhibiting BITZ compounds, which proceeds with initial non-covalent active site recognition, promoted by complimentary shape fit and non-covalent protein-inhibitor interactions; but that ultimately relies on the formation of a covalent enzyme-inhibitor adduct which renders the enzyme inactive.
Figure 4.11 Inhibitor 1 modelled in the active site of PfIspD homology model, covalently bound to the Cys-202 residue. The PfIspD active site is rendered as a white surface. Active site protein residues predicted to interact with BITZ inhibitors (Gly-203 and Gly-204, hydrogen bonds; Cys-202, disulfide adduct) are depicted as sticks with carbon atoms coloured orange; inhibitor 1 is displayed as sticks: carbon atoms are dark grey; hydrogen bonds are depicted as black, dotted lines with predicted hydrogen bond distance in Ångstroms. Image created using PyMOL.\[26\]

The model of proposed covalent PfIspD enzyme inhibition is in line with known mechanistic features of this mode of protein inhibition and a general description of target-specific covalent inhibition (Scheme 4.1) which occurs over at least two steps.\[28\] For covalent inhibition to occur, a compound must first bind non-covalently to its target protein, placing a reactive electrophile in the inhibitor close to a specific nucleophile within the target protein. The resulting non-covalent complex can then undergo targeted and specific bond formation between these two entities, giving rise to the final inhibited complex (Scheme 4.1).

Scheme 4.1 The generic mechanism of action of target-specific covalent inhibition. Scheme adapted from \[28\].
4.3.1 Biomimetic Mechanistic Evaluation

The proposed mechanism of action of BITZ derived inhibitors involves a combination of N-S bond cleavage in the BITZ warhead and cysteine-trapping of the Cys-202 residue in the PfIspD active site. Disulfide bond formation between the BITZ motif and Cys-202 is mediated by nucleophilic attack of the Cys-202 thiol onto the sulfur atom of the N-S bond within the BITZ core (Scheme 4.2). Cleavage of the N-S heteroatom bond results in disulfide bond formation which ultimately blocks the PfIspD active site to entry and binding of natural enzyme substrates: CTP and MEP.

Scheme 4.2 Proposed mechanism of BITZ ring opening mediated by the Cys-202 residue in the PfIspD active site.

To explore the validity of this proposal, ring opening of the benzo[d]isothiazol-3(2H)-one core was assessed by reaction of 2 with the nucleophilic species, sodium ethanethiolate to chemically cleave the N-S bond of the BITZ head group.\(^{[29]}\) Detection of BITZ ring opening was assessed by \(^1\)H NMR and mass spectrometry.

Scheme 4.3 Synthetic strategy for compound 3; cleavage and ring opening of 2 mediated by the nucleophilic species sodium ethanethiolate.\(^{[29]}\)

Despite the rapid synthetic route used to generate 3, which proceeded in moderate yield; analysis of the resulting material by \(^1\)H NMR was unable to confirm generation of required chemical construct of 3. The obtained \(^1\)H NMR spectra identified a broad singlet, with an integration of one, at 10.57 ppm, which likely corresponds to the NH of the amide functionality in 3 and therefore suggests that sodium
Ethanethiolate causes ring opening of 2. However, the characteristic ethyl splitting pattern (a quartet, with an integration of two, and a triplet, with an integration of three) was not present. Analysis of the synthesised material by mass spectrometry identified a mass ion (the base peak of the spectrum) with an associated ES+HRMS m/z of 479.0868 [M+Na]⁺. It is likely that this mass ion corresponds to the proposed dimer structure 4 (Figure 4.12), that would be generated through the self-dimerization of 3 following ring opening and cleavage of 2 by sodium ethanethiolate.

![Figure 4.12 Chemical structure of the proposed dimer 4.](image)

Further analysis of the product material (Scheme 4.3) by infrared (IR) spectroscopy was unable to detect the proposed disulfide bond of 3 due to its weak absorption and low wavenumber at ~ 500 cm⁻¹ which is difficult to detect in the fingerprint region of IR spectra. In addition, no thiol peak, usually detectable as a narrow and weak absorption between 2600 - 2400 cm⁻¹, or a S-CH₂ stretch at ~ 2860 cm⁻¹ could be identified. Together, these results strongly suggest that 4 is the isolatable and detectable product following the nucleophilic ring opening of 2, outlined in Scheme 4.3, due to the self-dimerization of 3 following the initial reaction of 2 with sodium ethanethiolate. This result therefore remains consistent with the nucleophilic ring opening of the BITZ motif, supporting the proposed mechanism of inhibition via disulfide bond formation through attack of the active site cysteine-202 residue on the BITZ core, occluding the PfIspD active site and causing inhibition of the enzyme.
4.3.2 PfispD Enzyme Rescue

In order to provide further evidence that inhibition of PfispD by BITZ compounds is the result of disulfide bond formation, some PfispD enzyme rescue experiments were conducted. The aim of these experiments was to determine if PfispD enzyme activity could be restored (or ‘rescued’) by application of a disulfide reducing agent following inhibition of the PfispD enzyme by treatment with BITZ compounds.

Dithiothritol (DTT) (Figure 4.13), also known as Cleland’s reagent, is a small, highly potent redox reagent that is widely used in the analysis of protein structure and function.[31] This synthetic reagent is able to completely reduce disulfide bonds as well as protect thiol groups (–SH) from oxidation, therefore making it an ideal protecting agent for this chemical functionality.[32] In relation to our study, DTT is able to reduce disulfide bonds between cysteine residues and can therefore be used to specifically implicate these residues in the biological activity of molecules at protein targets.[31]

![Dithiothritol](image)

Figure 4.13 Chemical structure of 1,4-dithiothreitol (DTT).

PfispD enzyme rescue experiments using DTT were performed in a proof-of-concept study to confirm that BITZ compounds induce PfispD enzyme inhibition through formation of a covalent disulfide bond to cysteine residues in the enzyme active site. Since a reducing agent, such as DTT, is capable of reducing and therefore breaking the inhibitory sulfur-Cys-202 bond, relief of PfispD enzyme inhibition should be observed following addition of the reducing agent into the PfispD enzyme assay. Observation of this phenomenon would consequently provide further support for the proposed mechanism of PfispD inhibition through formation of a disulfide bond between the sulfur heteroatom of the BITZ warhead and the Cys-202 residue.

Consequently, a modified PfispD enzyme assay (Chapter 2, Section 2.2.3) was carried out in which the assay was spiked with DTT seven minutes into the usual protocol. Standard PfispD enzyme assays were conducted, with and without 5 μM of 1; after seven minutes of standard conditions, the assay was spiked with DTT to give a final concentration of 5 mM. DTT spiking of the PfispD enzyme assay, first treated with 5 μM of 1, was shown to relieve inhibition of the enzyme (Figure 4.14). The bar graph depicts the level of PfispD enzyme activity, measured by 2-amino-6-mercapto-7-methyl purine release.
at an absorption of 360 nm\textsuperscript{[33]} (Chapter 2, Section 2.2.3), both pre- and post-DTT treatment in separate experiments, that were conducted with and without the presence of 1.

\textbf{Figure 4.14} Bar graph representing the reaction rate of purified recombinant PfIspD, in the presence and absence of 5 µM 1, before and after treatment with 5 mM DTT. All data points are representative of four independent replicates.

The average measured absorption at 360 nm in the PfIspD enzyme assay, without treatment with 1 (represented by black bars, \textbf{Figure 4.14}; and the black line, \textbf{Figure 4.15}), is highly comparable both before and after treatment with DTT: (without 1, pre-DDT: 0.012 ± 0.001; without 1, post-DDT: 0.014 ± 0.0001). Whereas, measured absorption in the PfIspD enzyme assay, when treated with 5 µM 1 (represented by red bars, \textbf{Figure 4.14}; and the red line, \textbf{Figure 4.15}), changes considerably after treatment with DTT: (with 1, pre-DDT: 0.001 ± 0.002; with 1, post-DDT: 0.012 ± 0.002).

Statistical analysis by means of a student t-test showed that, when comparing pre-DDT (abs360) slopes, there is a statistically significant difference in PfIspD enzyme activity comparing the untreated enzyme and following treatment with 5 µM of 1 (p = 0.002). (A student t-test represents a significance test for the mean value of a normal distribution of data\textsuperscript{[34]}). Considering the same data from the perspective of a line graph (\textbf{Figure 4.15}), it is clear that when PfIspD activity is inhibited by the presence of 1, addition of DTT to the PfIspD enzyme assay at a time point of seven minutes, restores PfIspD activity (shown by an increase in absorbance at 360 nm), indicating restored enzyme turnover.\textsuperscript{[33,35]}

In contrast, in the PfIspD enzyme assay that is not treated with 1, an arrest of enzyme activity is not observed at any point, prior to or following treatment with DTT. This highlights that DTT does not affect the PfIspD enzyme assay when BITZ inhibitors are not present.
Figure 4.15 Line graph representing PfIspD enzyme activity in the presence and absence of 5 μM 1, treating with DTT at a seven-minute time point.

Comparing the absorbance slopes at 360 nm following addition of DTT to the PfIspD enzyme assay, there is no statistically significance difference between the activity of the untreated PfIspD enzyme and that treated with 5 μM of 1 (p = 0.402); highlighting that DTT successfully relieves PfIspD inhibition following treatment with BITZ inhibitors.

Alternative analysis of the data shows that the addition of DTT did not cause a statistically significant difference in the slope of the reactions (PfIspD enzyme activity) in assays that were not treated with 1 (p = 0.283). And furthermore, the addition of DTT caused a statistically significant increase in the slope of the reaction (rate of PfIspD enzyme activity) in the assay that was treated with 1, prior to DTT (p = 0.008).

This PfIspD enzyme rescue experiment therefore demonstrates that the addition of DTT following initial incubation of the PfIspD enzyme with 1 produces rapid enzyme rescue, relieving the effects of BITZ compound inhibition and restoring PfIspD enzyme activity. This observation strongly indicates that DTT reduces the sulfur-Cys-202 bond of the proposed enzyme-inhibitor complex, releasing the active site Cys-202 residue and enabling PfIspD enzymatic activity to be restored. This result therefore provides further evidence that BITZ compounds specifically target the PfIspD enzyme and supports our proposed model of PfIspD enzyme inhibition.
4.3.3 Molecular Modelling of BITZ Compounds at PfIspD (2)

To further support our proposed model of BITZ compound activity, comparable molecular modelling studies were carried out with BITZ inhibitors, 5 and 6, which also demonstrate good inhibitory PfIspD activity (Figure 4.16). Compounds 5 and 6 were docked into the active site of the PfIspD homology model using Protocol 4 (Table 4.7) and a search criteria requesting 50 GA pose predictions for each compound. The docking procedure was analogous to that previously conducted with 1 (Figure 4.6).

![Chemical structures of compounds 5 and 6]

**Figure 4.16** Chemical structure; PfIspD enzyme activity; whole cell 3D7 activity; molecular weight; average GoldScore fitness score and GoldScore fitness of the highest scored binding pose predicted in PfIspD molecular docking studies of 5 and 6 using the PfIspD homology model.

Molecular modelling of 5 and 6 produced comparable docking poses and binding scores to those initially identified with 1 (Figure 4.6); three-dimensional evaluation of additional BITZ analogues therefore supports a similar mode of binding to that observed with 1. In these studies, inhibitor binding was again characterised by initial non-covalent recognition within the enzyme active site (Figures 4.17 and 4.18); followed by covalent disulfide bond formation between the BITZ warhead and active site Cys-202 residue, facilitated by their relative alignment and proximity.
**Figure 4.17** Inhibitor 5 modelled in the active site of PfIspD homology model. The PfIspD active site is rendered as a white surface; inhibitor 5 is displayed as sticks and coloured by element: carbon atoms are purple; the selected PfIspD active site protein residues (Cys-202, Lys-215, Thr-664 and Arg-208) are highlighted and depicted as sticks with carbon atoms coloured orange. Image created using PyMOL.[26]

**Figure 4.18** Inhibitor 6 modelled in the active site of PfIspD homology model. The PfIspD active site is rendered as a white surface; inhibitor 6 is displayed as sticks and coloured by element: carbon atoms are yellow; the selected PfIspD active site protein residues (Cys-202, Lys-215, Thr-664 and Arg-208) are highlighted and depicted as sticks with carbon atoms coloured orange. Image created using PyMOL.[26]
As again illustrated from the molecular docking of 5 and 6, initial non-covalent alignment of BITZ inhibitors at PfISPd facilities disulfide bond formation in the active site through attack of Cys-202 on the sulfur heteroatom of the BITZ warhead, resulting in covalent modification of the PfISPd binding site (Figures 4.19 and 4.20). These additional docking studies therefore support the proposed mechanism of covalent modification and inhibition of PfISPd by BITZ derived compounds, as initially proposed following the molecular docking of 1.

![Inhibitor 5 modelled in the PfISPd homology model, covalently bound to the active site Cys-202 residue and occupying the PfISPd binding pocket. The PfISPd active site is rendered as a white surface; inhibitor 5 is displayed as sticks and coloured by element: carbon atoms are purple; the selected PfISPd active site protein residues (Cys-202, Lys-215, Thr-664 and Arg-208) are highlighted and depicted as sticks with carbon atoms coloured orange. Image created using PyMOL.](image)

**Figure 4.19**
Figure 4.20 Inhibitor 6 modelled in the PfIspD homology model, covalently bound to the active site Cys-202 residue and occupying the PfIspD binding pocket. The PfIspD active site is rendered as a white surface; inhibitor 6 is displayed as sticks and coloured by element: carbon atoms are yellow; the selected PfIspD active site protein residues (Cys-202, Lys-215, Thr-664 and Arg-208) are highlighted and depicted as sticks with carbon atoms coloured orange. Image created using PyMOL.\textsuperscript{[26]}

4.4 Biological Evaluation of BITZ Inhibitor Mechanism of Action

Mechanistic evaluations and development of SAR has specifically highlighted that the N-S bond of the BITZ warhead and Cys-202 residue in the PfIspD active site are crucial to achieve PfIspD enzyme inhibition via disulfide bond formation. Small structural manipulations around the core BITZ motif (Chapter 2, Section 2.4) have confirmed the requirement for the specified BITZ chemotype in achieving PfIspD enzyme inhibition. Having demonstrated modelling and chemical evidence for the importance of the N-S bond for this proposed mechanism of action, biological investigations were also undertaken to further support this hypothesis.

4.4.1 Site Directed Mutagenesis

Site directed mutagenesis (SDM) is a technique used to characterise genes and proteins, determining the functions that enable their biology and the interactions which may occur between proteins and compounds that may become associated.\textsuperscript{[36]} SDM involves altering a nucleotide sequence of interest using synthetic oligonucleotides in order to study the effect on the resulting gene function. SDM introduces a mutation at a defined site within a DNA sequence to make specific changes in a portion of the gene which encodes the protein product of interest: this is usually with the purpose of assessing
a specific gene and its functional role in biology.\textsuperscript{36} The methods by which the micromanipulation (or SDM) of genes can be approached are now highly advanced and the subsequent applications are immensely broad. As a result, it is possible to modify a gene so that any amino acid within the protein can be replaced by any other specified amino acid therefore varying the construct, shape, function and activity of the resulting modified protein.\textsuperscript{37}

4.4.1.1  \textit{E. coli} IspD

The IspD active site Cys-202 residue is conserved in \textit{Plasmodium} spp. IspD enzymes but not in bacterial enzyme homologues (\textbf{Figure 4.21}) which have been found to be insensitive to BITZ derived inhibitors. BITZ compounds are ineffective against the \textit{Ecl}ispD enzyme (\textbf{Figure 4.22}) which therefore supports the proposed involvement of and specific requirement for the active site Cys-202 residue which is present in PfispD but not in the \textit{E. coli} enzyme homologue.\textsuperscript{27}

\begin{verbatim}
P. falciparum  192- KKKNIHSILLCGGIGKRETEIGPKQFLKLDIPFIYSFNLFIKCNLIKLSTL -244
P. vivax      187- HKKKNIHALLCGGGIGQGRTLBEASRKQFLKLNDVPLVYFSNFVLFVKCNLIKAITL -239
P. berghei    159- NKKNIHSIFLCGGVGKRTELESRKQFHLNDNIPIFIYSFNLFIKCNYIKSIL -211
E. coli      4-  THLDCAVVPAGFGRMQTECPQYLSIGNQTLHESVYALLAHPRVKRVVI -56
M. tuberculosis 4- EGAGEVVAIVPAAGSGERIALAVGVPKAFYQLDQTLIERAVDGLLDSGVDTVV -56
\end{verbatim}

\textbf{Figure 4.21} Alignment of IspD enzyme homologues highlighting interspecies variation.\textsuperscript{27}

The alignment of IspD enzyme homologues across five different MEP-pathway containing pathogens highlights that the active site Cys residue is conserved in \textit{Plasmodium} spp. but is absent in \textit{E. coli} and \textit{M. tuberculosis} bacterial orthologues; where the corresponding residue in bacteria is alanine (Ala) (\textbf{Figure 4.21}). Both \textit{P. falciparum} and \textit{P. vivax} IspD, possessing the active site Cys-202 residue, have been shown to be sensitive to inhibition by BITZ compounds (\textbf{Chapter 3, Section 3.11}). However, exposure of recombinant EcispD (EcispD-wt) to BITZ compound 1 found the enzyme to be highly resistant to inhibition by the compound (\textbf{Figure 4.22} and \textbf{Table 4.9}) providing a distinct contrast to the inhibitory IspD activity shown by the same compound at \textit{P. falciparum} and \textit{P. vivax} IspD homologues (\textbf{Chapter 3, Section 3.11, Table 3.9}).
Figure 4.22 Dose-dependent inhibition of recombinant EclspD and EclspD-A14C following treatment with 1; representative data shown; n ≥ 3.

To confirm the critical role of active site Cys residues in PfIspD inhibition, a mutant EclspD protein was generated, using SDM, in which the corresponding Ala residue was substituted with a Cys residue by introduction of the A14C mutation. Encouragingly, EclspD-A14C was rendered sensitive to 1 (Table 4.9 and Figure 4.22) highlighting that the E. coli enzyme can be rendered sensitive to BITZ derived compounds through replacement of the specified alanine residue with cysteine.

<table>
<thead>
<tr>
<th>E. coli IspD enzyme variant</th>
<th>Enzyme inhibition IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EclspD-wt</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>EclspD-A14C</td>
<td>440 ± 48</td>
</tr>
</tbody>
</table>

Table 4.9 Inhibitory enzyme activity of 1 against wildtype EclspD and the mutant EclspD-A14C enzyme. Mean IC₅₀ and SEM displayed; representative data shown: n ≥ 3. [27]

This result further validates the requirement for active site Cys residues to achieve PfIspD inhibition and therefore supports the proposed mechanism of enzyme inhibition. In addition, this data also provides an explanation for the marked species-selectivity of BITZ compounds and their selective inhibitory activity amongst IspD homologues, despite the overall sequence homology across this group of enzymes.

4.4.1.2 P. falciparum IspD

Together, PfIspD molecular modelling studies and SDM in EclspD clearly implicate the specific Cys-202 residue as the likely site of enzyme modification by BITZ compounds. Consequently, it was predicted that mutation of Cys-202 to an Ala residue would decrease the sensitivity of PfIspD to the BITZ compound class since formation of the covalent disulfide bond would no longer be able to occur.
Indeed, introducing this PfspD mutation resulted in a six-fold decrease in sensitivity of the PfspD enzyme to inhibition by BITZ compounds (Table 4.10 and Figure 4.23). This was highlighted following treatment of both wildtype (PfspD-wt) and mutant (PfspD-C202A) PfspD enzymes over prolonged 50 minute incubations with 1.

<table>
<thead>
<tr>
<th>P. falciparum IspD enzyme variant</th>
<th>Enzyme inhibition IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfspD-wt</td>
<td>81 ± 13</td>
</tr>
<tr>
<td>PfspD-C202A</td>
<td>470 ± 39</td>
</tr>
</tbody>
</table>

Table 4.10 Inhibitory activity of 1 against wildtype PfspD and the mutant PfspD-C202A enzyme. Mean IC₅₀ and SEM displayed; representative data shown: n ≥ 3.¹²⁷

This clear change in enzyme sensitivity (Figure 4.23) reiterates the importance of Cys-202 for BITZ inhibitor activity since we find that mutation of active site Cys-202 to an alanine residue significantly reduces the sensitivity of recombinant PfspD to the effect of BITZ derived inhibitors. However, it should be acknowledged that this sensitivity change may reflect both a loss of covalent bond formation as well as a change in compound fit and complementarity within the active site, following introduction of the C202A mutation to the PfspD inhibitor binding region.¹²⁷ Furthermore, introduction of the PfspD-C202A mutation is only observed as causing reduced sensitivity of the PfspD enzyme to the BITZ chemotype rather than complete resistance against it (Figure 4.23).

Figure 4.23 Dose-dependent inhibition of recombinant PfspD and PfspD-C202A following treatment with 1; representative data shown; n ≥ 3.

The observation of weakened BITZ inhibition against the PfspD-C202A mutant enzyme reinforces the indication of an initial non-covalent inhibitory effect of BITZ compounds which 1 is still able to produce against the mutant PfspD-C202A, likely due to complementary shape-fit, despite reduced protein-ligand recognition and the absence of covalent mechanistic interactions. Alternatively, the
observation of weak, but detectable, BITZ compound inhibition of *Pf*ispD-C202A may suggest an independent mode of weak inhibitory activity at the mutant *Pf*ispD enzyme.

### 4.4.2 *Pf*ispD and *Pf*ispD-C202A Enzymatic Assays

To further assess and compare the behaviour of BITZ compounds at both the wildtype and mutant *Pf*ispD enzymes, a series of *Pf*ispD and *Pf*ispD-C202A enzymatic assays were conducted with inhibitor 1. These assays involved pre-incubating the respective enzymes with 1 and varying the time points at which IC\textsubscript{50} measurements were taken.

During reversible inhibition, an equilibrium is typically established between component enzymes, inhibitors, and enzyme-inhibitor complexes.\textsuperscript{[10]} In contrast, covalent inhibition is often marked by an increase in the apparent potency of an inhibitor over time, as irreversible associations are gradually made between the inhibitor and the enzyme.\textsuperscript{[28]} To evaluate this premise with respect to the proposed mechanism of enzyme inhibition, *Pf*ispD enzyme variants were evaluated in time-dependent IspD enzyme assays using BITZ inhibitor 1. Various concentrations of 1 were pre-incubated in the presence of *Pf*ispD prior to measurement of enzyme activity. As expected for covalent inhibition, the potency of 1 was seen to increase over time when pre-incubated with the wildtype *Pf*ispD enzyme (*p* = 0.001, corresponding to *Pf*ispD-wt data Figure 4.24 and Table 4.11).

![Figure 4.24](image)

**Figure 4.24** Time-dependent changes in inhibition of recombinant *Pf*ispD-wt enzyme activity following treatment with 1. Mean IC\textsubscript{50} and SEM displayed; n ≥ 4 for each data point.

In contrast, no such substantial or significant change in *Pf*ispD-C202A inhibition was observed over the same time period with exposure to and pre-incubation with 1 (*p* = 0.32, corresponding to *Pf*ispD-C202A data Table 4.11) which suggests that covalent enzyme inhibition was no longer occurring. These results highlight the importance and validity of the proposed covalent interactions with Cys-202 during inhibition of *Pf*ispD by BITZ compounds.
Table 4.11 Inhibitory activity of 1 against wildtype PfISP and mutant PfISP-C202A following pre-incubation of the enzyme and inhibitor prior to measurement of enzyme activity.

Considering the mechanism of covalent enzyme inhibition, once an inhibitor-enzyme adduct has formed, increasing concentrations of the natural enzyme substrate should not relieve covalent inhibition of the protein.\textsuperscript{38} Consistent with this model, the susceptibility of PfISP-wt to inhibition by 1, following prolonged incubation, was not substantially affected by varying concentrations of the natural enzyme substrate, CTP (Figure 4.25, b). In contrast, inhibition of PfISP-wt by 1 was somewhat relieved under higher CTP concentrations over shorter incubation periods (Figure 4.25, a), as is typically observed for reversible competitive inhibitors.\textsuperscript{38}

<table>
<thead>
<tr>
<th>PfISP enzyme variant</th>
<th>Pre-incubation time (minutes)</th>
<th>Enzyme inhibition IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfISP-wt</td>
<td>5</td>
<td>330 ± 57</td>
</tr>
<tr>
<td>PfISP-wt</td>
<td>50</td>
<td>81 ± 13</td>
</tr>
<tr>
<td>PfISP-C202A</td>
<td>5</td>
<td>860 ± 220</td>
</tr>
<tr>
<td>PfISP-C202A</td>
<td>50</td>
<td>470 ± 39</td>
</tr>
</tbody>
</table>

Figure 4.25 Inhibition of recombinant PfISP enzymes by BITZ inhibitor 1.\textsuperscript{27}
Together, these observations are consistent with relatively slow inhibitor-enzyme adduct formation within the CTP binding site, as predicted by our molecular docking model. In contrast, inhibition of PfIspD-C202A activity was not significantly affected by CTP concentrations over short or long incubation periods with 1 (Figure 4.25, c and d). This demonstrates that the CTP-site binding does not substantially contribute to inhibition of the mutant PfIspD-C202A enzyme; and therefore suggests an independent mode of inhibition.

4.4.3 Kinetic Evaluation of the PfIspD and PfIspD-C202A Enzymes

In order to evaluate the function of the PfIspD-C202A mutant enzyme, kinetic parameters ($K_{cat}$, and the Michaelis constant, $K_m$)\textsuperscript{[38]} were characterised for the enzyme. Kinetic analysis demonstrated that introduction of the C202A mutation, replacing the active site Cys-202 residue with Ala, results in decreased $K_{cat}$ and increased $K_m[CTP]$ values (Figure 4.26, A and B). This result is consistent with changes to the CTP binding site, as expected in the PfIspD-C202A mutant.

![Figure 4.26 Michaelis-Menten kinetics of the PfIspD-wt and PfIspD-C202A enzymes; A: direct activity plot of enzyme activity vs. [CTP]; B: Lineweaver-Burke plot: $1/ V_{max}$ vs. $-1/ K_m$, where changes in the y-intercept indicate changes in $V_{max}$ and changes in the x-axis indicate changes in $K_m$.](image)

$K_{cat}$ is a measure of maximum enzyme velocity ($V_{max}$) expressed in activity per amount of enzyme (i.e. μmol of substrate converted per μmol of enzyme per second) and therefore provides a measure of enzyme turnover. $K_m$ provides a measurement of substrate concentration at half-maximal enzyme velocity and is therefore a measure of the affinity of a particular enzyme for its substrate; as a result, $K_m$ is often used as an indicator of the change in the enzyme’s ability to use (or process) a particular substrate.\textsuperscript{[38]}
Kinetic evaluation of the PfIspD-C202A enzyme highlights that introduction of the C202A mutation confers changes to both the $K_{cat}$ (or $V_{max}$) and the $K_m$ [CTP] (Figure 4.2, A and B and preliminary data, Table 4.12). The notable increase in $K_m$ [CTP] values for PfIspD-C202A indicates that the mutant enzyme has a reduced ability to use CTP as a substrate. This therefore supports our prediction, based on the PfIspD homology model, that the Cys-202 residue is located within the CTP binding site.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{cat}$ [CTP] (s$^{-1}$)</th>
<th>$K_m$ [CTP] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfIspD-wt</td>
<td>0.42</td>
<td>64</td>
</tr>
<tr>
<td>PfIspD-C202A</td>
<td>0.31</td>
<td>160</td>
</tr>
</tbody>
</table>

Table 4.12 Preliminary $K_{cat}$ and $K_m$ kinetic data with respect to [CTP] for the PfIspD-wt and PfIspD-C202A enzymes.

The finding that a change to the PfIspD inhibitor binding site, by introduction of the C202A mutation, has an effect on the utilisation of CTP in the PfIspD-C202A enzyme is also consistent with our hypothesis that BITZ inhibitor binding and CTP binding occur within the same site.


### 4.4.4 Crystallography

SDM rendered the EcIspD-wt enzyme sensitive to the effect of BITZ inhibitors following introduction of the A14C mutation at the EcIspD active site. This single point mutation replaced the Ala residue of the *E. coli* IspD homologue with a Cys residue, facilitating BITZ compound activity at the mutant enzyme. To support these studies, EcIspD-A14C was co-crystallized in the presence of 5 mM of 1 ([Table 4.13](#table413)) which revealed the formation of a new intramolecular disulfide bond between Cys-14 and Cys-25, providing supportive structural evidence for our mutant enzyme activity data.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>EcIspD-A14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>C2</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
</tr>
<tr>
<td>$a, b, c$ (Å)</td>
<td>94.68, 47.67, 53.95</td>
</tr>
<tr>
<td>$a, \beta, \gamma$ (°)</td>
<td>90.00, 99.515, 90.00</td>
</tr>
<tr>
<td>Resolution (Å)*</td>
<td>20-1.60 (1.70-1.60)*</td>
</tr>
<tr>
<td>$R_{meas}$</td>
<td>7.3% (46.4%)</td>
</tr>
<tr>
<td>$I/\sigma I$</td>
<td>11.29 (2.56)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.3% (98.2%)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.52 (3.43)</td>
</tr>
</tbody>
</table>

**Table 4.13** Data collection and refinement statistics for co-crystallization of EcIspD-A14C with inhibitor 1 at 5 mM.[27]

Previous crystal structures of the enzyme show that in the absence of CTP, loop spanning residues 14-25 are disordered and flexible in the EcIspD protein (PDB ID: 1VGT,[39] 1H3M,[40] 3N9W,[41]). Whereas, in the presence of CTP (PDB ID: 1I52[12]), this loop region becomes ordered and makes several important contacts with CTP. In the mutant EcIspD-A14C structure, while electron density corresponding to 1 was not observed, the resulting crystal structure revealed formation of a now-accessible disulfide bridge in a closed conformation of the loop which caused occlusion of the EcIspD-A14C CTP binding site ([Figure 4.27](#figure427)).
Introduction of the cysteine residue into EcIspD-A14C therefore enables formation of a disulfide bridge which can occlude and inhibit the mutant E. coli IspD enzyme in an analogous manner to the proposed inhibition of the PfIspD enzyme. This provides a direct contrast to EcIspD-wt which is insensitive to inhibition by BITZ compounds since disulfide bond formation, required to occlude the active site and inhibit the enzyme, is not viable in the absence of the active site Cys-202 residue.

This crystallographic data therefore provides modest additional evidence for a temporary covalent interaction between BITZ inhibitor 1 and the Cys-14 residue in the mutant EcIspD-A14C enzyme. Together with SDM work and molecular modelling evaluations, these studies provide evidence that the PfIspD enzyme is inhibited by BITZ compounds via covalent bond formation between the active site Cys-202 residue and the BITZ core and that this same effect can be induced and observed in IspD enzyme homologues which do not naturally possess the required active site protein sequence to facilitate this mode of enzyme inhibition.

4.5 Considering BITZ Reactivity at Cysteine Residues

The demonstrated cysteine reactivity of BITZ compounds suggests that development of a BITZ inhibitor series may be hampered or compromised by possible polypharmacology. However, several recent literature reviews have noted that covalent mechanisms of action are prevalent amongst successful drugs and that the covalent modification of proteins and drug targets is becoming increasingly popular, representing an expanding approach to drug discovery and development. Indeed, aspirin which is also a covalent modifier, is one of the most widely used drug in the world.
The polypharmacology of bioactive molecules\textsuperscript{[43,44]} and approved drugs\textsuperscript{[45]} has similarly been studied and is consistent with the concept that safe and effective drugs are invariably active at many targets. One such study has calculated that, on average, approved therapeutic drug entities are active against approximately seven different molecular targets and still produce the desired pharmacology and phenotypic effect.\textsuperscript{[46]} It is clear that continued vigilance will be required as BITZ compounds and their derivatives are progressed further into antimalarial drug development with continued monitoring for potential inhibitor promiscuity and off-target reactivity, which could lead to toxicity in the human host.

However, it should be also noted that other compounds based around the BITZ chemotype have demonstrated successful medicinal chemistry optimisation within a number of drug development programs.\textsuperscript{[47–53]} Recently, a molecule containing a BITZ construct began Phase II clinical trials as an antiviral agent, thus providing further supportive evidence that the potential reactivity of the BITZ moiety alone is not incompatible with a safe therapeutic profile.\textsuperscript{[54]}

Separate research by Dahl \textit{et al}.\textsuperscript{[55]} provides further support for use of this chemical chemotype within drug design and medicinal chemistry optimisation. As part of their studies into benzoisothiazolone phosphomannose isomerase inhibitors, Dahl and co-workers conducted a glutathione (GSH) binding assay with BITZ-derived compound 7 (Figure 4.28) which is highly analogous to compound 8 (Figure 4.28), a simplified BITZ derivative from our own studies.

\textbf{Figure 4.28} Chemical structures of BITZ derived compounds 7\textsuperscript{[55]} and 8.

The S9-based GSH transferase assay provides a means of identifying compounds which have a tendency to react with GSH \textit{in vivo} and therefore may present a risk of generating GSH-trapped reactive metabolites.\textsuperscript{[55]} This is therefore an efficient means of determining the extent of promiscuous binding and non-specific binding events associated with a given chemical chemotype. When tested in the S9-based GSH transferase assay, Dahl \textit{et al}. identified no GSH adducts of 7 using liquid chromatography–mass spectrometry (LCMS).\textsuperscript{[55]} This result gives further support for the inclusion of the BITZ warhead and chemical chemotype within drug design since these studies show that indiscriminate chemical or enzymatic turnover is limited with GSH and goes some way to suggest that the BITZ chemotype does not behave promiscuously at non-specific GSH residues \textit{in vivo}. 
4.6 Conclusions and Future Work

A docking protocol suitable for the molecular modelling of small molecules binding to PfIspD was determined using the EcIspD enzyme homologue as a surrogate protein. A lack of availability of the PfIspD crystal structure required that the EcIspD enzyme to be selected as a substitute homologue, enabled by the active site similarity between the two enzymes.

EcIspD was consequently used to identify a suitable modelling protocol with which to dock small molecules at the IspD active site; the aim of which was to apply the identified protocol to a homology model of the PfIspD protein to examine the binding of small molecules at the active site. Extracting and re-docking the natural IspD enzyme product, CDP-ME, co-crystallised in the known 1INI E. coli crystal structure,\textsuperscript{[12]} enabled the docking protocol parameters to be identified which were capable of accurately reproducing the known CDP-ME binding pose, thus verifying the protocol both accurate and reproducible.

Following generation of the PfIspD homology model, further molecular modelling studies were conducted to investigate the alignment and orientation of BITZ derived molecules at the PfIspD active site; where examination of the resulting docking poses revealed a consistent molecular alignment of BITZ compound at PfIspD. As a result, a mechanism of enzyme inhibition has been proposed which is initiated by non-covalent active site recognition, promoted by complementary shape-fit and non-covalent protein-inhibitor interactions, but ultimately relies on the formation of a covalent enzyme-inhibitor adduct which renders the enzyme inactive.

A combination of further molecular modelling studies, SDM, enzyme activity assays and crystallographic evaluation has enabled us to substantiate this proposed mechanism of enzyme inhibition as the mechanism by which the BITZ chemotype and its analogues are able to inhibit the PfIspD enzyme. More specifically, a mechanism of enzyme inhibition has been deciphered whereby a disulfide protein-inhibitor adduct is formed between the PfIspD active site Cys-202 residue and the electrophilic sulfur atom of the BITZ inhibitor core, which is facilitated by the proximal alignment of these entities in the PfIspD binding site. The nucleophilic thiol group of Cys-202 is responsible for attacking the sulfur atom of the N-S heteroatom bond within the BITZ core, this causes N-S bond cleavage and subsequent formation of a sulfur-Cys-202 disulfide bond which results in inhibition of the PfIspD enzyme. In line with this proposed mechanism, the demonstrated species-selectivity of BITZ compounds for PfIspD over other enzyme homologues relies on the presence of the active site Cys-202 residue.
Several biological methods have been effectively used to confirm the proposed inhibitory mechanism of the BITZ chemotype at \( PfIspD \). DTT enzyme rescue experiments have shown that the addition of this reducing agent to the \( PfIspD \) enzymatic assay, following treatment with BITZ compounds, produces rapid enzyme rescue and relieves the effects of BITZ inhibition, restoring \( PfIspD \) enzyme activity. This result therefore indicates that DTT is able to reduce the inhibitory sulfur-Cys-202 bond, proposed as the key interaction within the enzyme-inhibitor complex, releasing the \( PfIspD \) active site Cys-202 residue and re-establishing \( PfIspD \) enzyme activity. DTT rescue experiments therefore provide evidence that the BITZ chemotype specifically targets the \( PfIspD \) enzyme through formation of a disulfide bond, strongly supporting our proposed model of enzyme inhibition.

Since the IspD active site Cys-202 residue is not conserved in bacterial homologues of the enzyme, site directed mutagenesis (SDM) has been used to confirm the specific requirement for this key cysteine residue in facilitating \( PfIspD \) enzyme inhibition by our proposed model of covalent inhibition with the BITZ chemotype. BITZ compounds have been found to be ineffective against wildtype \( EcIspD \). However, generation of the \( EcIspD\)-A14C enzyme (possessing the specified active site Cys residue) and subsequent treatment with BITZ compounds, has shown this mutant enzyme is sensitive to inhibition by BITZ compounds. This result therefore demonstrates that \( EcIspD \) can be rendered sensitive to BITZ-derived compounds through specific mutation and introduction of the active site Cys-202 residue, providing further evidence of this protein residue in \( PfIspD \) active site inhibition. In addition, crystallography work has provided supportive structural evidence for our demonstrated mutant A14C-\( EcIspD \) enzyme activity data.

SDM also generated the \( PfIspD\)-C202A mutant enzyme, replacing the active site Cys-202 residue with an alanine residue in the \( PfIspD \) homologue. Subsequent treatment of the resulting mutant enzyme with BITZ compounds showed this mutation caused around a six-fold decrease in enzyme sensitivity, compared to inhibition of wildtype \( PfIspD \) by BITZ compounds. This observation indicates that covalent enzyme inhibition was no long able to occur in the \( PfIspD\)-C202A enzyme, further highlighting the essential involvement of the Cys-202 residue in achieving covalent inhibition of \( PfIspD \). However, the weakened inhibitory activity shown against \( PfIspD\)-C202A is likely to signify that the non-covalent recognition element of BITZ compound inhibition at the enzyme is still able to produce a diminished level of activity against \( PfIspD\)-C202A. This weak, non-covalent inhibitory contribution is most likely a consequence of some complementary shape-fit between the mutant protein and BITZ inhibitor, despite reduced recognition and the absence of covalent mechanistic interactions. In addition, the lack of effect varying CTP concentration during inhibition of \( PfIspD\)-C202A with BITZ compound 1,
suggests that blocking the CTP binding site does not substantially contribute to inhibition of the mutant enzyme, therefore indicating an alternative mode of PfIspD-C202A inhibition.

Altogether, these studies provide a robust explanation for the marked species-selectivity of BITZ compounds and their inhibitory activity amongst IspD homologues. In addition, kinetic evaluation of wildtype PfIspD and PfIspD-C202A has demonstrated that the PfIspD-C202A mutant enzyme has a reduced ability to utilise CTP as an enzyme substrate, supporting our prediction, based on the PfIspD homology model, that the Cys-202 residue is located within the CTP binding site.

In addition to the described molecular docking studies, computational techniques could be used to further rationalise the behaviour and alignment of BITZ derived inhibitors within the PfIspD active site. As a result, more extensive docking studies and molecular evaluation may facilitate a predictive approach to compound design, providing informed indications of ‘drug-like’ compounds which should be prioritised for lead optimisation. Such an approach would enhance computational contributions to the molecular design loop. Indeed, refined evaluation of identified docking poses, prediction of ligand-protein binding energies and calculating molecular properties could be used in a data mining approach that is able to accurately determine if a given compound or chemical structure will show activity against the PfIspD enzyme, more significantly informing compound design.

It has been acknowledged that the cysteine reactivity of BITZ compounds at PfIspD must be carefully monitored to ensure that there is no significant off-target reactivity or risk of toxicity towards the human host. Indeed, the potential promiscuity of the BITZ warhead means that non-specific binding events at additional protein contacts may be possible and must continue to be evaluated and monitored throughout continuing further compound development. However, the inclusion of the BITZ chemical motif in a number of successful medicinal chemistry optimisation programs, has demonstrated that the promiscuity and potential reactivity of the BITZ chemotype itself, is not incompatible with a safe therapeutic profile and the development of effective chemotherapeutic agents. Therefore, as interest in the covalent modification of drug targets increases, similar techniques and experiments to those outlined here may be used to probe other identified cysteine modifiers which may be capable of mirroring this mode of covalent modification.
4.7 Experimental

For general chemical and biological methodologies see Chapter 2, Section 2.7.1.

4.7.1 Computational Methods

Molecular Modelling

A homology model of PfIspD was constructed using the PHYRE online homology modelling program.[24] PfIspD primary sequence Q8I273 was obtained from UNIPROT (http://www.uniprot.org/, accessed 10/12/14). A number of protein alignments and homology models were constructed by PHYRE, and the model with 98.82% confidence was selected, which was based on an E. coli IspD structure (PDB accession code 1I52).[12] 1I52 is a 1.50-Å resolution crystal structure of E. coli IspD, complexed with CDP-ME in the active site. The structure of the model was validated using the WHATIF web interface.[25] BITZ inhibitors 1, 5 and 6 were modelled in silico using the homology model described above in order to visualize the interactions between each analogue and the active site. Using GOLD, protons were added and docking was performed with default parameters, except that GoldScore was used and 50 docking poses were obtained for comparison and analysis. Covalent docking was performed using GOLD with the C-alpha atom of Cys-202 as the link atom to BITZ inhibitors 1, 5 and 6.[16]

4.7.2 Biological Methods

Cloning of IspD Orthologues

P. falciparum: The construct used for expression of the codon-optimized PfIspD protein has been described previously.[56] To generate the PfIspD variants containing the C202A mutation, N- and C-terminal fragments of the optimized PfISP D gene were amplified from the above vector using the following primers. C202A N-terminal fragment: T7 Fwd primer, 5'-TAATACGACTCACTATAGGG-3', with C202A Rev primer, 5'-GTTTGCCAATACCGCCGGCTAGCAGGATACTATGAATG-3'; C202 C-terminal fragment, C202A Fwd primer, 5'-CATTCATAGTATCTGCTAGGCCGGGTATTGGCAAAC-3', with T7 Rev primer, 5'-GCTAGTTATTGCTCAGCGG-3'. Amplicons containing the entire PfISP D gene were generated using PfIspD Fwd and Rev primers, which introduce a 6-His tag, with N- and C-terminal fragments as template (PfIspD Fwd primer: 5'-CTCACCACCACCACCACCATATGATGCACATCTCAGATATATGTAATA-3'; PfIspD Rev: 5'-ATCCTATCTTTACTCTTATTATTGAGGAGTAGAGTAAT-3'). Following amplification, PfISP D variants were cloned into pBG1861 by ligation-independent cloning as previously described,[56,57] and verified by Sanger sequencing.
P. vivax: The construct used for expression of codon-optimized PvIspD (P. vivax Sal-1; PVX_081425) has been previously described.\textsuperscript{[56]}

E. coli: The construct used for expression of EcIspD-wt has been previously described.\textsuperscript{[58]} The A14C mutation was introduced by first generating N- and C-terminal gene fragments containing the mutation. These fragments were generated using the following primers: A14C N-terminal fragment: T7 Fwd primer, 5'-TAATACGACTCACTATAGGG-3', with A14C Rev primer, 5'-CATTGACGGCCAATCCGGCACCACGGCACAACATC-3'; A14C C-terminal fragment, A14C Fwd primer, 5'-GATGTGTCGCCCTTGGCCTCGGATTCGCGCCCGGAATAGTG-3', with T7 Rev primer, 5'-GCTAGTTATTGCTACGCG-3'. Amplicons containing the entire E. coli IspD gene were generated using EcIspD Fwd and Rev primers, which introduce a 6-His tag, with N- and C-terminal fragments as template (EcIspD Fwd primer: 5' - CTCACCACCACCCATATGATGGCAACCTCGTTG-3'; EcIspD Rev primer: 5' - ATTCATTATCCTACCTATGTATTTGCTCTGAG-3'). Following amplification, E. coli A14C-IspD was cloned into pBG1861 by ligation-independent cloning as previously described,\textsuperscript{[57]} and verified by Sanger sequencing.

**Crystallisation**

Following nickel affinity purification, EcIspD-A14C was further purified by gel chromatography using a HiLoad 16/60 Superdex 200pg column (GE Healthcare) equilibrated with 10 mM Tris (pH 8.0) and 150 mM NaCl. The fractions containing EcIspD-A14C were pooled and concentrated to 15 mg/mL using an Amicon centrifugal filter. The protein sample was pre-incubated on ice with 5 mM final concentration of compound 1. The protein crystallized in 0.2 M calcium acetate, 0.1 M HEPES (pH 7.5), 10% PEG 8000. Crystals were flash-frozen under liquid nitrogen for data collection. X-ray data were collected from a single crystal using a wavelength of 1 Å at synchrotron beamline 4.2.2 of the Advanced Light Source in Berkeley, CA. Data were processed with XDS and the coordinates from PDB ID: 1VGT\textsuperscript{[39]} were used as a search model for phase determination by molecular replacement using PHENIX. Automatic model rebuilding was performed in PHENIX. Subsequent iterated manual building/rebuilding and refinement of models were performed using Coot and PHENIX, respectively. The structure validation server MolProbity was used to monitor refinement. A complete summary of final crystallographic refinement statistics is given in Table 4.13. Figures were generated and rendered in PyMOL.\textsuperscript{[13]}
4.7.3 Organic Synthesis

Preparation of 2-(ethylisulfanyl)-N-phenylbenzamide 3

![Chemical Structure](image)

To a solution of 2-phenylbenzo[d]isothiazol-3(2H)-one 2 (0.05 g, 2.19 mmol, 1.0 eq) in anhydrous THF (5 ml), sodium ethanethiolate (0.02 g, 0.264 mmol, 1.2 eq) was added and the N₂ atmosphere restored. The resulting solution was stirred at room temperature for 30 minutes before heating to 60°C under reflux conditions for a further 40 minutes. THF was removed under reduced pressure and the crude material was quenched with water (25 ml). The organic material was extracted into DCM (3 x 25 ml) and the combined organic layers dried with Na₂SO₄, filtered and concentrated under reduced pressure to give the pure product as a white solid in a 63.1% yield. \( R_f = 0.51 \) (50:50 EtOAc:hexane). **1H NMR** (400 MHz, DMSO) \( \delta \) 10.57 (s, 1H, NH), 7.80 – 7.71 (m, 4H), 7.52 (dt, \( J = 7.5, 1.4 \) Hz, 1H), 7.43 – 7.34 (m, 3H), 7.14 (tt, \( J = 7.4, 1.1 \) Hz, 1H); **ES+HRMS** m/z 479.0868 [M+Na]^+ \( C_{15}H_{15}NOS_2 \) requires 289.06; \( C_{26}H_{20}N_2O_2S_2 \) (corresponding to 4 from the self-dimerization of 3) requires 456.10.
4.8 References


Chapter 5

An Alternative Inhibitor of the

PfIspD Enzyme
5.1 MMV-008138: A Stereospecific Chemotype and PfISPΔ Inhibitor

It has been previously acknowledged that covalent modification and the use of covalent modifiers can be a risky strategy within drug design programmes and the development of chemotherapeutic agents.[1,2] The cysteine reactivity demonstrated by 1,2-benzo[d]isothiazol-3(2H)-one (BITZ) compounds (Chapter 4) poses a possible issue that corresponding BITZ inhibitors may be compromised by polypharmacology. Indeed, vigilance will be required should BITZ compounds progress further in antimalarial drug development. The noted potential for BITZ compounds to interact with off-target proteins in promiscuous binding events highlights a risk of unpredictable reactivity and presents a need for monitoring the extent of inhibitor promiscuity to minimise toxicity risks in the human host.[3–5] It was therefore decided reasonable, and within the scope of this research, to consider alternative means of achieving PfISPΔ inhibition.

Research efforts remain focused on achieving inhibition of the PfISPΔ enzyme target as a method of disrupting MEP pathway biosynthesis in Plasmodium spp. parasites, consequently preventing essential isoprenoid precursor synthesis as a means of inhibiting Plasmodium parasite growth. However, synthetic, biological, computational and mechanistic evaluations are now focused on a complementary strategy towards an alternative inhibitor scaffold that is constructed around a discrete chemical chemotype. Since research thus far has focused on optimising inhibition of the PfISPΔ enzyme through covalent means, with the identification and development of the BITZ chemotype, attentions are now directed towards a non-covalent method of achieving PfISPΔ enzyme inhibition.

This chapter will discuss and evaluate the potential of a tetrahydro-β-carboline chemotype (Figure 5.1) as a potent inhibitor of the PfISPΔ enzyme operating via an alternative non-covalent mechanism of action. We will consider the similarities and differences of β-carboline-derived compounds with respect to the BITZ chemotype, since both are inhibitors of PfISPΔ enzyme. In addition, development of the structure-activity-relationship (SAR) around the tetrahydro-β-carboline chemotype will also be explored.

5.1.1 Identification of MMV-008138

Recently the Malaria Box compound, MMV-008138 [(2,4-dichloro-phenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-2-ium-3-carboxylate], was identified through a phenotypic screen of the large GlaxoSmithKline (GSK) screening library.[6] MMV-008138, based around the tetrahydro-β-carboline chemical construct (Figure 5.1), was identified in an isopentenyl pyrophosphate (IPP) chemical rescue screen. MMV-008138 was found to be a potent antimalarial compound, inhibiting P. falciparum growth as a result of activity against the Plasmodium parasite apicoplast.[7,8] Since MMV-008138 was
identified in a phenotypic screen (Chapter 2, Section 2.1 and 2.1.1), the specific antimalarial target of the compound was initially unknown and as such, the mechanism by which MMV-008138 was capable of apicoplast dysfunction was unclear.\footnote{9}

An interesting structural feature of MMV-008138 is that the compound contains two stereogenic centres, meaning that four distinct stereoisomers of the compound are possible (Figure 5.2).\footnote{9} The presence of chirality within a potential drug molecule represents a point of consideration for forward drug design and SAR development since chemical chirality can dictate precise 3D structural requirements that must be adhered to or preserved within chiral analogues in order to maintain activity. Chirality within chemical analogues and synthetic species also presents hurdles of additional synthetic complexity, stereo-specificity and the performance of required reactions, as well as the ease by which particular diastereoisomers may be isolated.

\begin{figure}[h]
  \centering
  \includegraphics[width=0.5\textwidth]{mmv_008138.png}
  \caption{Chemical structure of MMV-008138 with the tetrahydro-β-carboline chemotype and chemical core highlighted in blue.}
  \label{fig:mmv_008138}
\end{figure}
Chapter 5

Figure 5.2 Chemical structures of the four diastereoisomers of MMV-008138 where the stereochemistry of each is depicted.

There are also some benefits to molecular chirality and the presence of stereo-centres in potential chemotherapeutic agents, in terms of the longevity and success of compounds moving through the drug design process. A number of diverse research programs have shown that the survival rate of compounds in the drug design process can be improved by increasing the number of chiral centres within a molecule. Indeed, it has been demonstrated that both the complexity and presence of chiral centres within drug molecules correlates, in some cases, with the successful transition of compounds from drug discovery to clinical candidates.\[^{10}\]

It is argued that chemical chirality constructively increases molecular complexity and can therefore enhance compound potency through more appropriate complementarity of a particular isomer to a specified target which is achieved without increasing the molecular weight (MW) of a compound.\[^{11}\] The appropriate use of chiral motifs can therefore be used to enhance compound specificity, and potentially potency, at an enzyme or protein without needing to alter other structural aspects of a compound, which may otherwise compromise other physicochemical properties such as MW, lipophilicity and therefore compound solubility, permeability and distribution.

In relation to our research, use of molecular chirality may prove to be a useful tool in enriching compound specificity towards the PfIspD enzyme target. This may help to circumvent current concerns
regarding the promiscuity and potential off-target cysteine reactivity of BITZ inhibitors by considering the development of alternative \( Pf\text{IspD} \) inhibitors that show enhanced selectivity for \( Pf\text{IspD} \). Indeed, the increased molecular complexity associated with a chiral motif, may provide an additional degree of molecular recognition and therefore specificity in resulting compounds which may in turn enhance potency at the \( Pf\text{IspD} \) molecular target.\(^{[12]}\)

Initial evaluation of MMV-008138, including its identification as an IPP rescue phenotype in the Malaria Box screen, was carried out using racemic mixtures of the compound which contained unspecified ratios of diastereoisomers.\(^{[9]}\) The initial screen identifying MMV-008138 as a compound that acts against the \( Plasmodium \) apicoplast, demonstrated that MMV-008138 could produce > 95% growth inhibition at 5 \( \mu \)M of the compound alone with an IC\( _{50} \) value of 350 ± 50 nM against the \( P. falciparum \) Dd2 strain, in the absence of IPP.\(^{[8]}\)

Further investigations by Wu et al. confirmed the IPP rescue phenotype of MMV-008138, with an EC\( _{50} \) of 772 nM (664 to 741 nM) against the \( P. falciparum \) W2 strain in the absence of IPP.\(^{[9]}\) Whilst such evaluations clearly highlighted the activity of MMV-008138 against \( Plasmodium \) spp. growth, these initial studies only indicated that MMV-008138 targets an apicoplast pathway, but provided no further insight into how stereochemistry affects activity or the specific molecular target at which the compound acts.

In a study to determine the activity of the four MMV-008138 stereoisomers, Wu et al. identified that the \( 1R,3S \) isomer is the most active stereoisomer of the MMV-008138 compound; demonstrating an EC\( _{50} \) of 110 nM and IPP rescue up to 25 \( \mu \)M (Table 5.1).\(^{[9]}\)

<table>
<thead>
<tr>
<th>MMV-008138 diastereoisomer</th>
<th>Parasite growth EC( _{50} ) (( \mu )M)</th>
<th>Parasite growth EC( _{50} ) (( \mu )M) + IPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1R,3S )</td>
<td>0.11 (0.10 – 0.12)</td>
<td>&gt; 25</td>
</tr>
<tr>
<td>( 1R,3R )</td>
<td>3.8 (2.7 – 5.3)</td>
<td>&gt; 25</td>
</tr>
<tr>
<td>( 1S,3R )</td>
<td>18.6 (12.4 – 28.0)</td>
<td>&gt; 25</td>
</tr>
<tr>
<td>( 1S,3S )</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
</tbody>
</table>

Table 5.1 Inhibitory activities of the four MMV-008138 stereoisomers against \( Plasmodium \) parasite growth, both with and without the presence of IPP; data represents means values with 95% confidence intervals.\(^{[9]}\)

Both the \( 1R,3R \) and \( 1S,3R \) isomers showed notably reduced activity against parasite growth compared to the \( 1R,3S \) isomer; where measured activity of these two isomers showed more than a 30-fold and 160-fold decrease in activity compared to the \( 1R,3S \) isomer. However, all three of the aforementioned MMV-008138 isomers demonstrated IPP-rescueable activity of up to 25 \( \mu \)M.\(^{[9]}\) In contrast, the \( 1S,3S \)
isomer was found to be completely inactive against parasite growth, both with and without the presence of IPP. Taken all together, the stereospecificity of parasite growth inhibition by MMV-008138 stereoisomers is clearly evident and strongly indicates that a specific MMV-008138 stereoisomer is likely to bind to and inhibit a specific cellular target.\[9\]

### 5.1.2 Biological Target of MMV-008138

Following its identification, there is now substantial evidence that MMV-008138 is capable of inhibiting *P. falciparum* growth through potent and selective activity against the *Pf*IspD enzyme, which is already established as a key enzyme of the MEP pathway, involved in mediating isoprenoid precursor biosynthesis within the apicoplast of *Plasmodium* parasites.\[9,13-17\]

To determine the biological target of MMV-008138, and thus consider a possible mechanism of action, *Plasmodium* parasites resistant to MMV-008138 were generated in work conducted by others.\[9\] Whole-genome sequencing of three drug-resistant populations revealed three non-synonymous mutations that conferred resistance to MMV-008138. Within all three resistant populations, the mutated gene was of common identity and encoded the 4-diphosphocytidyl-2C-methyl-o-erythritol cytidylyltransferase (IspD) enzyme of the MEP isoprenoid precursor pathway, highlighting this enzyme as the likely molecular target of MMV-008138.\[9\]

In order to determine if MMV-008138 was indeed capable of directly inhibiting *Pf*IspD, and therefore if this was the molecular target of the compound, Wu *et al.* expressed and purified the *Pf*IspD enzyme to enable measurement of its enzymatic activity following treatment with MMV-008138 and the use of a pyrophosphate release assay.\[9\] Comparable to the pattern of inhibitory activity against *Plasmodium* parasite growth observed with the four MMV-008138 stereoisomers, inhibition of the *Pf*IspD enzyme by the different MMV-008138 stereoisomers was also stereospecific and analogous to that demonstrated in parasite growth inhibition (Table 5.2).\[9\]

<table>
<thead>
<tr>
<th>MMV-008138 diastereoisomer</th>
<th>Parasite growth EC(_{50}) (μM)</th>
<th><em>Pf</em>IspD activity IC(_{50}) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R,3S</td>
<td>0.11 (0.10-0.12)</td>
<td>7.1 (6.1-8.3)</td>
</tr>
<tr>
<td>1R,3R</td>
<td>3.8 (2.7-5.3)</td>
<td>170 (140-200)</td>
</tr>
<tr>
<td>1S,3R</td>
<td>18.6 (12.4-28.0)</td>
<td>2500 (1900-3100)</td>
</tr>
<tr>
<td>1S,3S</td>
<td>&gt; 50</td>
<td>&gt; 2500</td>
</tr>
</tbody>
</table>

**Table 5.2** Inhibitory activities of the four MMV-008138 stereoisomers against purified *Pf*IspD; mean data values given with 95% confidence intervals.\[9\]
The most active diastereoisomer of MMV-008138 was again the 1R,3S isomer which displayed potent inhibition of the PfIspD enzyme with activity in the low nanomolar region: PfIspD IC₅₀ value = 7.1 nM. All other diastereoisomers of MMV-008138 were considerably less potent and the 1S,3S isomer completely inactive (Table 5.2). A combination of genetic and biochemical data has therefore identified the PfIspD enzyme of the MEP apicoplast pathway as the specific cellular target of the MMV-008138 compound (Scheme 5.1). MMV-008138 inhibition of blood-stage *P. falciparum* growth is both potent and stereospecific, with the most active 1R,3S stereoisomer demonstrating an EC₅₀ value of 110 nM against parasite growth and directly inhibiting PfIspD *in vitro* at an IC₅₀ value of 7.1 nM (Table 5.2). These findings are strongly supported by the work of others who also suggest that *in vivo* 1R,3S-MMV-008138 inhibition of PfIspD is likely to result in decreased cellular levels of MEP pathway metabolites that are enzymatically downstream of the IspD enzyme.

![Scheme 5.1 Proposed inhibition of IspD enzymatic activity by 1R,3S-MMV-008138](image)

Further studies conducted by Imlay *et al.* have addressed the question of species-selectivity and the potential antimicrobial activity spectrum of the 1R,3S-MMV-008138 isomer. Consistent with previous reports, 1R,3S-MMV-008138 was found to be inactive against bacterial orthologues of the IspD enzyme.
enzyme. *E.coli* IspD (*Ec*IspD) is not inhibited with treatment of 1R,3S-MMV-008138 up to 30 μM and *Mycobacterium tuberculosis* IspD (*Mt*IspD) is insensitive to 1R,3S-MMV-008138 (Figure 5.3). These results are well aligned with previous studies which also found MMV-008138 to lack activity against the *A. thaliana* IspD (*At*IspD) enzyme homologue.\(^\text{[9]}\)

![Figure 5.3](image.png)

**Figure 5.3** Species selectivity of 1R,3S-MMV-008138 against various IspD homologues.\(^\text{[15]}\)

In contrast to the lack of activity against bacterial IspD orthologues, 1R,3S-MMV-008138 was shown to potently inhibit *P. vivax* IspD (*Pv*IspD) with an IC\(_{50}\) value of 310 nM (95% CI 240-400 nM); however, the *Pf*IspD enzyme was shown to remain the most sensitive IspD orthologue evaluated with a measured IC\(_{50}\) value of 47 nM (95% CI 37-60 nM).\(^\text{[15]}\) The 1R,3S MMV-008138 isomer therefore shows promise as broad spectrum antimalarial agent with the potential to treat *P. vivax* malaria infections.

This result is highly comparable to the activity spectrum and species selectivity that we have observed with the BITZ inhibitor chemotype which successfully inhibits *P. falciparum* and *P. vivax* IspD enzymes but shows no activity against bacterial IspD enzyme orthologues.\(^\text{[17]}\) We have shown that BITZ compounds are species-selective IspD inhibitors and do not possess activity against the *Ec*IspD enzyme (Chapter 4, Section 4.4.1.1, Figure 4.21 and Table 4.9),\(^\text{[17]}\) but in contrast show activity against the *Pv*IspD enzyme, which is comparable to that demonstrated against *Pf*IspD. Enzymatic assays using both purified *P. vivax* and *P. falciparum* IspD enzymes have shown that a range of BITZ analogues (Chapter 3, Section 3.11, BITZ compounds 4-6 and 13-15, Table 3.9) demonstrate low micromolar to nanomolar activity against these two *Plasmodium* IspD enzyme species. Considered together, these recent studies suggest that, like the BITZ chemotype, further modifications to the MMV-008138 scaffold may widen the therapeutic scope of this chemotype, facilitating broad-spectrum activity against multiple malaria species.
5.1.3 MMV-008138: Mechanism of Action

Targeted metabolic profiling of MEP pathway metabolites in *P. falciparum* cells has been performed by Imlay *et al.* in order to establish the anti-parasitic mechanism of action of the 1R,3S-MMV008138 isomer. Already knowing *Pf*IspD to be the cellular target of 1R,3S-MMV008138, Imlay *et al.* suggested that *in vivo* inhibition of *Pf*IspD is therefore likely to result in decreased cellular levels of MEP pathway metabolites that are enzymatically downstream of IspD. Targeted metabolic profiling studies therefore aimed to confirm that 1R,3S-MMV008138 is capable of arresting the MEP pathway though specific and targeted inhibition of the *Pf*IspD enzyme.

It was found that treatment of *P. falciparum* with 1R,3S-MMV-008138 dramatically reduced cellular levels of the most distal MEP metabolite, methylenolyltetritol cyclic diphosphate (MEcPP) (*Figure 5.4*). 1R,3S-MMV008138 was shown to reduce levels of MEcPP to 12 ± 4% of control levels which is consistent with cellular inhibition of MEP pathway metabolism upstream from MEcPP. Of note, this observed effect was not replicated with the 1S,3R-MMV008138 isomer, previously shown to be inactive against *Pf*IspD.

![The MEP Pathway](image)

*Figure 5.4 The MEP Pathway.*

This result is consistent with work previously described determining the downstream effects of fosmidomycin (FSM), the established phosphonic acid IspC inhibitor, on distal metabolite levels within the MEP pathway. Comparably, treatment of *P. falciparum* with FSM causes a 95 ± 2% decrease in cellular levels of MEcPP compared to control levels. A further consistency, observed through targeted metabolic profiling, is the comparable effect of the BITZ chemotype on MEP pathway metabolite levels which had also been shown to produce a significant decrease in MEcPP levels (*Chapter 3, Section 3.12, Figure 3.19*).
Treatment of *P. falciparum* with selected BITZ compounds and 1R,3S-MMV-008138 does not affect the levels of the DOXP metabolite which is metabolically upstream from the IspC and IspD MEP pathway enzymes. Together, these studies confirm that both the BITZ chemotype and 1R,3S-MMV-008138 are capable of inhibiting the *Plasmodium* MEP pathway at levels which affect asexual parasite growth. In addition, 1R,3S-MMV-008138-treated parasites which are then supplemented with 200 μM IPP, one of the key isoprenoid precursors (Figure 5.4), are found to be viable. This further establishes that the metabolic effects and inhibition of parasite growth by 1R,3S-MMV-008138 are specific to MEP pathway inhibition since it has been demonstrated that the antimalarial potency of this compound is relieved by media supplementation with IPP. [8,15]

5.1.4 MMV-008138: Competitive Behaviour at PfIspD

It is well established, [13,16,19,20] and has been previously discussed (Chapter 1, Section 1.4.2 and 1.5.2) that the third enzyme of the MEP pathway (IspD) catalyses the transfer of a cytidyl group from CTP to MEP, therefore utilising the two substrates of the enzyme and generating the enzyme product CDP-ME. In order to determine the molecular mechanism of action by which 1R,3S-MMV-008138 is capable of inhibiting the PfIspD enzyme, kinetic characterisation of PfIspD was carried out by Imlay *et al.* [15]

Kinetic analysis supports a model of inhibition whereby 1R,3S-MMV-008138 behaves in a competitive manner with respect to the CTP substrate at the PfIspD enzyme (Figure 5.5; a and b). In contrast, parallel studies demonstrate a non-competitive model of inhibition of 1R,3S-MMV-008138 with respect to the MEP substrate at PfIspD (Figure 5.5; c and d). Figure 5.5 depicts the Michaelis-Menten kinetics of PfIspD following treatment with various concentrations of 1R,3S-MMV-008138, showing that inhibition of PfIspD with 1R,3S-MMV-008138 is competitive with respect to CTP but not MEP. [15]
Since 1R,3S-MMV-008138 has been shown to be capable of inhibiting the PfISPD enzyme homolog (Figure 5.3), subsequent kinetic enzyme studies were also conducted to determine the mode of 1R,3S-MMV-008138 inhibition at PfISPD. Kinetic evaluations indicate that, as seen for PfISPD, inhibition of PfISPD also occurs via competition with the CTP substrate. Therefore, this work not only establishes that PfISPD represents the enzymatic target of 1R,3S-MMV-008138, but also demonstrates that 1R,3S-MMV-008138 inhibits PfISPD competitively with the CTP enzyme substrate.

5.2 Molecular Modelling of 1R,3S-MMV-008138 at PfISPD

In addition to enzymatic and cellular analysis of 1R,3S-MMV-008138, molecular modelling studies were carried out to provide insight into the spatial alignment and binding mode of 1R,3S-MMV-008138 at the PfISPD enzyme. This body of work denotes the beginning of our involvement in the evaluation and development of the MMV-008138 compound (and more broadly the tetrahydro-β-carboline chemical chemotype) as a PfISPD inhibitor and potential antimalarial chemotherapeutic agent.

Equivalent to our previous molecular modelling studies with the BITZ chemotype and derived compounds, the PfISP homology model (Chapter 4, Section 4.3) was used to dock 1R,3S-MMV-008138 into the CTP binding site of the enzyme using the previously defined docking protocol (Protocol 4; Chapter 4, Section 4.2.1, Table 4.7). The docking program GOLD 5.0.1 was used to assess the strength of interactions between 1R,3S-MMV-008138 and the PfISP homology model as well as visualise non-covalent interactions between the inhibitor and the enzyme binding site.
Previous studies have demonstrated that IspD enzyme homologues in other organisms are amenable to inhibition by small molecules (Chapter 1, Section 1.5.2); most notable perhaps is inhibition of the herbaceous \textit{Arabidopsis thaliana} IspD (AtIspD) orthologue which occurs by a number of structurally diverse small molecules.\textsuperscript{[24,25]} However, it is significant that these previously described molecules all demonstrate allosteric inhibition of the AtIspD orthologue. In contrast, initial docking of 1R,3S-MMV-008138 highlighted that the tetrahydro-β-carboline inhibitor appears to target the CTP binding site of \textit{Pf}IspD (Figure 5.6). Indeed, visualisation of 1R,3S-MMV-008138 in the \textit{Pf}IspD CTP binding site shows the identified pose of 1R,3S-MMV-008138 to be comparable to the alignment of the natural enzyme product, CDP-ME, closely mimicking the binding mode of the cytidine phosphate moiety, as defined by the \textit{E. coli} crystal structure (PDB: 1I52).\textsuperscript{[26]}

\textbf{Figure 5.6} 1R,3S-MMV-008138 and CDP-ME modelled in the CTP binding site of the \textit{Pf}IspD homology model. The active site is rendered as a white surface; selected residues are depicted as sticks: carbon atoms are orange; oxygen atoms are red; nitrogen atoms are blue. 1R,3S-MMV-008138 is displayed as sticks: carbon atoms are dark grey. CDP-ME is displayed as sticks: carbon atoms are light-grey. Image created using PyMOL.\textsuperscript{[27]}

This observation draws strong parallels to the assessment of the BITZ chemotype at \textit{Pf}IspD since our previous modelling studies have also shown BITZ derived inhibitors to target and occupy the CTP binding site of the \textit{Pf}IspD enzyme (Chapter 4, Section 4.3.2). Indeed, overlaying identified binding poses of the two inhibitor chemotypes, using representative BITZ compound 1 (Table 5.3) and 1R,3S-MMV-008138, clearly highlights the similar alignment of the two compounds which both broadly mirror the known pose and orientation of the CDP-ME enzyme product\textsuperscript{[26]} (Figure 5.7).
Figure 5.7 $1R,3S$-MMV-008138, 1 and CDP-ME modelled in the CTP binding site of the PfIspD homology model. The active site is rendered as a white surface; selected residues are depicted as sticks: carbon atoms are orange; oxygen atoms are red; nitrogen atoms are blue. $1R,3S$-MMV-008138 is displayed as sticks: carbon atoms are pink; 1 is displayed as sticks: carbon atoms are dark grey; CDP-ME is displayed as sticks: carbon atoms are light-grey. Image created using PyMOL.\cite{27}

Using the previously specified Protocol 4 (Chapter 4, Section 4.2.1, Table 4.7), 50 GA docking poses were calculated for $1R,3S$-MMV-008138 at PfIspD to further assess the predicted alignment and orientation of the compound. $1R,3S$-MMV-008138 demonstrated excellent spatial fit in the CTP binding pocket and was predicted to bind to the PfIspD active site with an average GoldScore\cite{23,28} of 49.6 ± 3; where the CTP enzyme substrate itself has a GoldScore of 72.0 ± 4. The higher GoldScore of CTP, compared to that predicted for $1R,3S$-MMV-008138, is likely due to the negative charge on the triphosphate group of CTP, forming enhanced hydrogen bonds to Arg208, compared to that of the neutral $1R,3S$-MMV-008138 compound.\cite{15}

$1R,3S$-MMV008138 is predicted to form an array of four hydrogen bonds between the carboxylic acid functionality of the compound and the three active site protein residues: Thr-664, Arg-208, and Lys-215 (Figure 5.8). These predicted enzyme-inhibitor contacts mirror those formed by the phosphate group of CTP in the 1I52 crystal structure\cite{26} and therefore support the competitive nature of $1R,3S$-MMV-008138 with CTP, as previously observed in enzymology studies.\cite{15} It can therefore be deduced that $1R,3S$-MMV-008138 has a non-covalent mechanism of action at PfIspD, targeting the CTP binding site of the enzyme, as a competitive inhibitor of CTP.
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**Figure 5.8** Docking pose of 1R,3S-MMV-008138 in the CTP binding site of the PfIspD homology model. The active site is rendered as a white surface and residues forming hydrogen bonds with 1R,3S-MMV-008138 are depicted as sticks: carbon atoms are orange; oxygen atoms are red; nitrogen atoms are blue. 1R,3S-MMV-008138 is displayed as sticks: carbon atoms are dark grey. Hydrogen bonds are shown as dotted black lines with distance given in angstroms. Image created using PyMOL.[15,27]

The requirement for the 1R,3S diastereoisomer of MMV-008138 to achieve potent PfIspD enzyme inhibition (Table 5.2) can now be rationalised through the spatial demands of the PfIspD CTP binding site, as shown by the enzyme surface depicted in Figures 5.6 and 5.8. It is evident from docking predictions (Figure 5.8) that 1R,3S-MMV-008138 adopts a binding pose that spatially, is very tight-fitting within the CTP binding pocket and therefore may be strongly influenced by PfIspD active site protein residues with which the compound closely aligns and associates. Consequently, it is plausible that there may be little room for significant structural variation around certain regions of the 1R,3S-MMV-008138 compound which must be taken into consideration for future inhibitor design; most notably, restrictions seem likely around the carboxylic acid functionality.

As already established, both the 1R,3S-MMV-008138 compound and BITZ inhibitors (represented here by 1) bind to the CTP binding pocket of PfIspD where some pose overlap is observed between the two chemotypes, as well as close to alignment of both compounds to the natural product CDP-ME (Figure 5.7). However, the tetrahydro-β-carboline and BITZ chemotypes both occupy slightly different spatial alignments with respect to each other in the CTP binding pocket and as such, display subtle differences in proximity to various protein residues in the PfIspD active site (Figure 5.9).
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Figure 5.9 1R,3S-MMV-008138 and 1 modelled in the CTP binding site of the PfIspD homology model. The active site is rendered as a white surface; selected residues are depicted as sticks: carbon atoms are orange; oxygen atoms are red; nitrogen atoms are blue. 1R,3S-MMV-008138 is displayed as sticks: carbon atoms are pink; 1 is displayed as sticks: carbon atoms are dark grey. Image created using PyMOL.[27]

The depiction of the two PfIspD inhibitors in the CTP binding pocket (Figure 5.9) highlights comparable proximities of the two compounds to the Thr-664 and Arg-208 active site residues. Noticeably, BITZ inhibitor 1 appears to extend further into the binding site, facilitating alignment with and therefore covalent attachment to the Cys-202 residue. In contrast, the identified protein-ligand interactions between 1R,3S-MMV-008138 and PfIspD consist mainly of hydrogen bonding interactions between the carboxylic acid functionality and the active site protein residues: Thr-664, Arg-208, and Lys-215 (Figure 5.8). This strongly suggests a non-covalent mechanism of enzyme inhibition regarding 1R,3S-MMV-008138 which outcompetes CTP for occupancy of PfIspD the binding site.

Two distinct mechanisms of action can therefore be rationalised for the BITZ and tetrahydro-β-carboline chemotypes, despite the extent of similarities displayed by these chemical motifs in targeting and inhibiting the PfIspD enzyme. As already established and providing further evidence that these two chemical motifs are capable of producing the same cellular effects against Plasmodium parasites, metabolic profiling of MEP pathway intermediates when treated with these two chemotypes also yields highly comparable results (Section 5.1.3).

The comparable features identified between the tetrahydro-β-carboline and BITZ chemotypes led us to ask the question: how would these two chemical motifs behave if administered in combination and
what would be the resulting effects on inhibition of parasite growth and overall antimalarial efficacy? Put simply, are these two chemotypes capable of behaving with synergy at the PfIspD enzyme target, producing an enhanced level of potency against the enzyme and subsequently parasite growth as a result of co-administration? In order to answer these questions, a series of competition experiments were undertaken to determine the antimalarial effects of the combination of the two compounds, selecting a derivative of both the BITZ and tetrahydro-β-carboline chemotypes to be representative.

5.3 Can a Combined Administration of the BITZ and Tetrahydro-β-carboline Chemotypes Produce Enhanced Antimalarial Activity?

5.3.1 Rationale and Background

It is widely accepted that there is a dual benefit to the use of combination therapies within drug design. The combination of chemical agents, known to produce the desired biological effect, can not only optimise the resulting therapeutic outcome, but may also protect the components of the combined therapy, minimising the risk of resistance development against either chemical entity.\[29\]

Therefore, a desirable advantage in the use of combination chemotherapies within antimalarial drug design is the potential that the resulting therapy may be capable of delaying the onset of \textit{Plasmodium} resistance to new chemical agents, as well as reduce the effects of resistance to therapies that may already have diminished antimalarial efficacy when administered alone.\[30,31\] However, for a combination therapy to be successful, the component drugs must be capable of working synergistically, producing a heightened therapeutic effect than could be achieved by administering either component drug alone. Consequently, a combined therapy approach can enable effective and efficacious antimalarial therapies to be administered that contain smaller doses of the component drugs; therefore producing effective, lower-dose therapies that may be beneficial for the development of alternative dosing regimens in poor and disadvantaged regions which are often hard to access and are where malaria is endemic.\[32\]

The use of combination therapies is by no means a new phenomenon; over the last century, advances in genetics and cell biology have facilitated the increasing use of drug combinations to tackle therapeutic challenges in modern medicine.\[33\] It is now well understood that biological disease states, often interconnected by multiple molecular pathways, are likely to be more susceptible to the simultaneous action of multiple drugs due to the chances of improved efficacy, decreased toxicity and a reduced risk of resistance development.\[34,35\] For exactly these reasons, combination therapies have become treatment standards in several areas of disease therapy including cancer,\[36\] hypertension\[37\] and AIDS,\[38\] as well as antimalarial therapy.\[34\] Indeed, artemisinin-based combination therapies
(ACTs) were introduced in the mid-1990’s and became recommended at the first-line treatment for all cases of *P. falciparum* malaria infection by the WHO in 2005.\[^{31}\]

Whilst ACTs have remained the gold standard of care for over a decade for the treatment of non-complicated malaria infection, some highly effective non-artemisinin based combination therapies have also been developed and successfully administered.\[^{29,39}\] The combination of atovaquone-proguanil, brand name: Malarone (Chapter 1, Section 1.3.2.3, refer to Figure 1.8), is a good example of a successful combination therapy that has been used primarily for the treatment and prevention of malaria infection in non-immune travellers; wider administration of this combination has been limited due to the high cost of the treatment regimen and the requirement for daily dosage.\[^{29,39}\]

Until recently, a combination of sulfadoxine-pyrimethamine (Chapter 1, Section 1.3.2.3, Figure 1.8) was also used as a chemoprotectant against malaria. This combination was used to prevent infection in some of the most vulnerable groups, predominantly expectant mothers and young children, in areas with an absence of intense malaria transmission which often means that partial immunity against the disease doesn’t develop within a population.\[^{39}\] It is therefore evident that combination therapies have already played a vital role in malarial treatment and prevention strategies and should continue to be used and developed as the fight against *Plasmodium* parasite resistance intensifies.

Interactions between known chemotherapeutics or potential chemical agents, investigated using isobologram competition experiments, can provide essential insight and knowledge into the combined effects of two chemical agents on a disease phenotype.\[^{30}\] When two or more drugs are given in combination, there are three potential outcomes of the resulting drug-drug interactions: these are the effect of synergy, antagonism or simply an additive effect.\[^{30,40}\]

If two or more drugs behave with synergy when given in combination, they may demonstrate activity or produce a phenotypic effect that is greater than expected from the individual potencies and efficacies of the component drugs when administered alone. In contrast, some drug combinations show simple additivity where the combined effect of the drugs is simply consistent with the individual drug potencies. The term additivity does not mean an addition of magnitudes but rather provides a basis for assessing the extent of synergy or antagonism observed and is derived from a more basic concept of dose equivalence.\[^{41}\]

At the other end of the spectrum, an effect of antagonism represents a lesser or reduced effect produced by drugs given in combination than the simple additive effect expected from the knowledge of each component drug, and its associated potency when administered alone.\[^{34}\] An ideal scenario in this method of drug design and administration is one where a drug combination synergies the desired
therapeutic effect but exhibits a simple additive effect for any associated and undesirable side effects either of the combined treatments; this can be rationalised since all chemotherapies are likely to be associated with some adverse effects.\textsuperscript{[41]}

The most common method for the quantitative assessment of interactions between drugs is the method of isoboles.\textsuperscript{[41]} The isobologram provides a graphical method of assessing the extent of synergism or antagonism between drug combinations. The isobole approach is therefore applicable to pairs of drugs that produce overtly similar and measurable effects, i.e. two drugs of the same classification, such as two antimalarials, which are each known to induce the desired biological effect or phenotype.\textsuperscript{[40]}

An isobole is the set of doses (or concentrations) of the two distinct compounds from within the same drug classification that, when administered together produce a specific and measurable effect in the system under study. Importantly the two component drugs should be capable of producing the required biological effect when administered alone. When this is the case, the resulting isobole is displayed as a curve in a Cartesian coordinate system (Figure 5.10) in which the doses (or concentrations) of the two component drugs are represented on the x- and y-axes.\textsuperscript{[42]} The straight-line joining these intercepts (between the top left of the y-axis to the bottom right of the x-axis) represents the dose pairs of component drugs and is called an isobole. The isobole represents where there is no interactions between the co-administered drugs and conveys the reduction required in the dose of one drug that accompanies the presence of a specified dose of the second drug. In other words, the negative slope of the line shows that the increase in quantity of Drug A means a reduced quantity of Drug B is needed to achieved to a specified level of therapeutic effect.\textsuperscript{[41]}
Figure 5.10 Schematic isobologram showing the Cartesian plane for a generic combination of Drug A and Drug B at a specified levels; figure reproduced from [43].

When the doses of Drug A and Drug B required to achieve the desired biological or phenotypic effect are less than expected, the combined effect of Drug A and Drug B is synergistic and the coordinate location within the isobologram plane is below the line of additivity, as represented by the green region of the isobologram graph (Figure 5.10). Conversely, when greater than expected doses of Drug A and Drug B are required to achieve the desired therapeutic effect, the combined effect of the two drugs is antagonistic. Such a result demonstrates that the administration of this particular drug combination has the effect of reducing and impeding the efficacy of the component drugs within the combination. The graphical representation of an antagonistic effect is a coordinate location above the additivity line of the isobologram plane, as represented by the red region of the graph (Figure 5.10).[43]

5.3.2 1R,3S-MMV-008138-BITZ Inhibitor Combinations and Possible Outcomes

We have explored the possibility of antagonism or synergy between 1R,3S-MMV-008138 and three of the most active BITZ derived compounds (1-3), using a modified fixed-ratio isobole method.[38] Structures and activities of each selected component inhibitor, when administered as a single agent are given in Table 5.3.
Two arguments were made for the possible outcomes of these studies as we sought to evaluate whether the combined use of these inhibitors, from covalent and non-covalent inhibitor classes, would be capable of producing a more enhanced antimalarial effect. Considering the two selected chemotypes and an argument for likely antagonism, it could be rationalised that one motif is likely to outcompete the other, since both the tetrahydro-β-carboline and BITZ chemotype target the same MEP pathway enzyme and are predicted to occupy the CTP binding site of PfISP\(D\). It is therefore plausible that the presence of both components may simply prevent full and effective binding with respect to each other at the PfISP\(D\) active site and consequently render the presence of one compound ineffective.

Equally, an argument for synergy was also considered. Research to date\(^{15,17}\) has outlined that BITZ derived compounds and 1R,3S-MMV-008138 behave via different mechanisms of action to achieve inhibition of the PfISP\(D\) enzyme. We have established that the BITZ chemotype operates via a distinctive mechanism of action that relies on formation of a covalent enzyme-inhibitor adduct between the sulfur atom of the BITZ core and the PfISP\(D\) Cys-202 residue. Formation of this disulfide bridge subsequently occludes the active site and prevents substrate binding rendering PfISP\(D\) inactive.\(^{17}\)
In contrast, 1R,3S-MMV-008138 (and structural analogues) work via a mechanism of non-covalent inhibition, behaving competitively with the CTP enzyme substrate at PfISPD. Since both chemical motifs induce inhibition of the PfISPD enzyme by distinct mechanisms, it is reasonable that these two chemotypes may work simultaneously, and synergistically, via these contrasting mechanisms of action to produce enhanced inhibition of PfISPD. Furthermore, it is known that the PfISPD enzyme exists as a c_2-symmetric homodimer in the whole cell; it is therefore plausible that the binding of one inhibitor chemotype may facilitate enhanced binding of the second to its own unique binding region as a result of induced conformational changes in the PfISPD enzyme, a consequence of protein-ligand interactions with one component inhibitor motif.

Such an effect would be consistent with a model of allosteric modulation where one chemotype is able to affect the potency and binding of the other component drug. However, such an effect may be considered unlikely within this research scenario since neither the tetrahydro-β-carboline or BITZ chemotypes are allosteric modulators of PfISPD; with both known to bind at the CTP binding site of the PfISPD enzyme.

5.3.3 1R,3S-MMV-008138 and BITZ Inhibitors have Antagonistic Anti-Parasitic Activity

Parasite growth was assayed after co-treatment with a variety of 1R,3S-MMV008138 and BITZ inhibitor concentrations. Pairs of concentrations (e.g. [1R,3S-MMV008138] vs. [1] Figure 5.11) at which 50% growth inhibition (EC_{50}) was observed were plotted to indicate the resulting antiparasitic activity of the specified chemotype combinations. The isoboles generated from these competition experiments, using a modified fixed-ratio isobole method as demonstrated previously by others, indicate that the anti-parasitic action of 1R,3S-MMV-008138 in combination with various BITZ inhibitors (1-3) is antagonistic.
Chapter 5

Figure 5.11 Combined antiparasitic effects of 1R,3S-MMV008138 and 1. The linear dotted line is representative of additive antiparasitic activity; the black data points are representative of measured antiparasitic effects between 1R,3S-MMV008138 and 1; data averaged from three independent experiments and indicates antagonistic antiparasitic activity.

A result of antagonism is quickly rationalised from the three isobologram experiments (Figures 5.11-5.13), since the Cartesian plane for the combination of the tetrahydro-β-carboline and BITZ chemotypes lies above the line of additivity (Figure 5.10) and is consistent across all three 1R,3S-MMV008138-BITZ inhibitor combinations (Table 5.3). The data obtained from these studies was further quantified through the calculation of sum fractional inhibitory concentrations (sum FICs) where a sum FIC value of < 0.5 reflects a measurement of synergy and a sum FIC value of > 2.0 signifies a measurement of antagonism.[44]

Figure 5.12 Combined antiparasitic effects of 1R,3S-MMV008138 and 2. The linear dotted line is representative of additive antiparasitic activity; the black data points are representative of measured antiparasitic effects between 1R,3S-MMV008138 and 2; data averaged from three independent experiments and indicates antagonistic antiparasitic activity.
We found that the sum FIC value of 1R,3S-MMV-008138 and 1 (Figure 5.11) was 1.15 ± 0.14; the sum FIC value of 1R,3S-MMV-008138 and 2 (Figure 5.12) was 1.32 ± 0.15; and the sum FIC value of 1R,3S-MMV-008138 and 3 (Figure 5.13) was 1.40 ± 0.26. Where the data corresponding to each isobologram (Figures 5.11-5.13) is representative of n = 3 replicates.

Figure 5.13 Combined antiparasitic effects of 1R,3S-MMV008138 and 3. The linear dotted line is representative of additive antiparasitic activity; the black data points are representative of the measured antiparasitic effects between 1R,3S-MMV008138 and 3; data averaged from three independent experiments and indicates antagonistic antiparasitic activity.

The isobolograms generated illustrate a consistent pattern of antagonism across all combinations of 1R,3S-MMV008138 with 1, 2, and 3 respectively (Figures 5.11-5.13); however, the calculated sum FIC values across the three combinations do not meet the specified value of > 2.0 to be categorised as significantly antagonistic.\[44\] Considering the sum FIC values alone, we can therefore conclude that the interactions between the tetrahydro-β-carboline and BITZ chemotypes are at least mildly antagonistic.

Yet, it is clear graphically that the measured data points lie consistently above the line of additivity (Figures 5.11-5.13) and in no way indicate any synergy within the combinations explored. It is therefore evident that drug combinations of these chemical chemotypes is not feasible and would not produce the desired biological effect of a potent anti-parasitic activity and inhibition of P. falciparum growth. A drug combination with tetrahydro-β-carboline and BITZ derivatives as component drugs can be concluded as incompatible and need not be pursued further.

However, as previously discussed, a drug combination such as this may never have been feasible since neither the tetrahydro-β-carboline nor BITZ chemotype are allosteric modulators of PfISP\(_D\), and both are known to act at the same binding site. Indeed, few drug combination strategies have been employed where the component drugs are known to act at a single molecular target. A rare example of such a combination was between Ritonavir and Lopinavir, generating a fixed-dose combination for
the treatment of HIV, where both component drugs are of the protease inhibitor class. Administered alone, lopinavir shows insufficient bioavailability however, a strategy of co-administration with sub-therapeutic doses of ritonavir (an inhibitor of cytochrome P450 3A4) was enough to boost the pharmacokinetic parameters of the resulting combination providing an effective therapy. However, this unique example aside, the isobologram experiments conducted and the resulting data obtained, provides further evidence that both the tetrahydro-β-carboline and BITZ chemical chemotypes target the PfISP-D MEP pathway enzyme.

5.3.4 1R,3S-MMV-008138 and FSM have Additive Anti-Parasitic Activity

In contrast to the effects of antagonism demonstrated between 1R,3S-MMV-008138 and BITZ inhibitors 1-3 (Table 5.3), similar studies evaluating the combination of 1R,3S-MMV-008138 and FSM demonstrate a different result. In work carried out by Imlay et al., a modified fixed-ratio isobole was used to measure the extent of synergy or antagonism between 1R,3S-MMV-008138 and the potent IspC inhibitor, FSM. The isobole generated from these studies (Figure 5.14) indicates that the anti-parasitic action of 1R,3S-MMV-008138 and FSM is additive, not synergistic, since the resulting isobole is linear. In other words, no substantial interaction occurs between 1R,3S-MMV-008138 and FSM and as such, no enhancement of anti-parasitic activity is observed when these chemical entities are combined, compared to the potency of 1R,3S-MMV-008138 and FSM when administered alone.

![Figure 5.14](image)

**Figure 5.14** A linear isobole indicating that the anti-parasitic effects of 1R,3S-MMV-008138 and FSM are additive; data points represent averages from at least three independent experiments.

Comparing the effects of combining 1R,3S-MMV-008138 with both BITZ derived compounds and FSM, a noteworthy observation is that an antagonistic effect is seen when two components are combined which act against the same molecular target (here the PfISP-D enzyme). In contrast, combining two components which act against different molecular targets within the same biological pathway (i.e.
*Pf*ispC and *Pf*ispD with the combination of FSM and 1R,3S-MMV-008138 respectively) produces an additive anti-parasitic effect. The possible inference is therefore that combining two chemical components which display an antimalarial phenotype but which act against distinct targets in discrete biological pathway, may produce a synergistic antiparasitic effect.

### 5.4 Development of SAR Around the Tetrahydro-β-carboline Chemotype

#### 5.4.1 Preliminary SAR Around the Tetrahydro-β-carboline Chemotype

Having concluded that the BITZ and tetrahydro-β-carboline chemotypes need to be considered as distinct chemical entities for their continued development as inhibitors of the *Pf*ispD enzyme, SAR development around the tetrahydro-β-carboline chemotype was next considered. Organic synthesis was used to facilitate structural modifications to the tetrahydro-β-carboline chemotype in order to develop SAR around the chemical motif and determine whether enhanced phenotypic activity could be achieved through structural refinement and modification of the chemotype, aiming to promote improved binding and potency at the *Pf*ispD enzyme. Key objectives were to enhance the enzymatic and whole cell efficacy of the 1R,3S MMV-008138 isomer as well as improve the metabolic profile of this current lead inhibitor.

It was established from our molecular modelling studies that 1R,3S-MMV-008138 isomer binds tightly within the *Pf*ispD enzyme active site (Section 5.2; Figure 5.8 is representative) therefore highlighting that only small structural refinements to the chemotype may be tolerated. Indeed, significant alteration to chemical features and misplaced steric bulk may prevent resulting MMV-008138 derivatives efficiently accessing and inhibiting the *Pf*ispD active site. The preliminary SAR map (Figure 5.15) depicts the SAR requirements that were known at this time, and the subsequent questions and regions of molecular development that were to be explored through chemical modifications to 1R,3S-MMV-008138 and the tetrahydro-β-carboline structural framework.
As previously discussed (Section 5.1.2, Table 5.2), the requirement for 1R,3S stereochemistry across the C ring of the tetrahydro-β-carboline chemotype (Figure 5.15) has been shown by others to be essential for inhibitory activity against the PfISP-D target enzyme and parasite growth.\textsuperscript{9}

\textbf{5.4.1.1 Modifications to the R Group at C-3}

Work by Yao \textit{et al.} evaluating the phenotypic activity of MMV-008138 compound analogues against parasite growth, using the \textit{P. falciparum} Dd2 parasite strain, has highlighted some key structural requirements and constraints relating to the R functionality of the carboxylate group at the C-3 position of the C ring. Of note, the \textit{P. falciparum} Dd2 parasite strain is both pyrimethamine-resistant and mefloquine-resistant, and shows intermediate resistance to chloroquine.\textsuperscript{46} To determine the precise influence of modifications in the C-3 substituent, Yao \textit{et al.} made a number of R group variations, keeping all other structural features of the 1R,3S MMV-008138 isomer constant, including 2',4'-dichloro substitution in the D ring (Table 5.4).\textsuperscript{46}

The presence of a carboxylic acid group is often considered a liability in drug development; therefore, this functionality at C-3 was selected for modification. The carboxylic acid functionality can be an important contributor to a pharmacophore and is indeed present in a large number of drugs that have been successfully marketed world-wide, including nonsteroidal anti-inflammatory drugs (NSAIDs) and antibiotics as key examples. However, the presence of this functionality commonly leads to compound liabilities and idiosyncrasies with respect to drug bioactivation.\textsuperscript{5,47,48} Acidic compounds and groups tend to be associated with poor permeability as they cannot readily cross negatively charged lipid membranes, due to their frequent existence in a deprotonated state at physiological pH.\textsuperscript{47,49} In addition, acid-containing compounds often to display high levels of protein binding in a cell\textsuperscript{50} and are particularly susceptible to glucuronide-adduct formation, mediated by glucuronidation in Phase II
metabolism;\textsuperscript{\[51,52\]} glucoronidation is a second-pass metabolic pathway that can generate undesirable side effects.\textsuperscript{\[47\]}

Since carboxylic acid groups can compromise the metabolism profile of drugs, Yao \textit{et al.} replaced this functionality to promote metabolic stability and increase compound half-life (t\textsubscript{1/2}), whilst aiming to retain activity against \textit{P. falciparum} growth. Replacing the C-3 carboxylic acid of 1\texttextit{R},3\texttextit{S}-MMV-008138 with a methyl ester, producing 5, was found only to achieve weak inhibition of parasite growth (Table 5.4). A methyl ester functionality therefore does not mirror the binding capacity of the carboxylic acid, although Yao \textit{et al.} concluded that 5 might serve well as prodrug. This result was supported by further work examining a number of other ester functionalities at the C-3 position; all of which were found to be inactive.\textsuperscript{\[46\]}

![Diagram](image)

\textit{4 - 6}

<table>
<thead>
<tr>
<th>Compound number</th>
<th>R\textsuperscript{1}</th>
<th>X</th>
<th>\textit{P. falciparum} Dd2 strain IC\textsubscript{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>OH</td>
<td>2',4'-Cl\textsubscript{2}</td>
<td>250 ± 70</td>
</tr>
<tr>
<td>5</td>
<td>OCH\textsubscript{3}</td>
<td>2',4'-Cl\textsubscript{2}</td>
<td>6800 ± 1400</td>
</tr>
<tr>
<td>6</td>
<td>NHCH\textsubscript{3}</td>
<td>2',4'-Cl\textsubscript{2}</td>
<td>190 ± 30</td>
</tr>
</tbody>
</table>

\textbf{Table 5.4} Phenotypic whole cell activity of compounds 4-6 against \textit{P. falciparum} strain Dd2, demonstrating the effect of 3-C substitution on \textit{P. falciparum} growth inhibition; data collected and reported by Yao \textit{et al.}.\textsuperscript{\[46\]}

In contrast, the methylamide derivative 6 was shown to be slightly more potent against \textit{P. falciparum} Dd2 growth than 4, consequently highlighting that small functional group modifications at the C-3 (R) position are tolerated.\textsuperscript{\[46\]} As a result, compound 6 was re-synthesised in our lab and tested using the \textit{Pf}spD enzymatic assay (\textbf{Chapter 2, Section 2.2.3}) to determine its activity against the \textit{Pf}spD target enzyme and provide a direct comparison to 4 (1\texttextit{R},3\texttextit{S}-MMV-008138), which has also been re-synthesised in our lab (Table 5.5).
Table 5.5 Inhibitory activity of 4 and 6 against the PfIspD enzyme and P. falciparum growth in culture: 3D7 parasite strain. Mean data and SEM given, representative of ≥ n = 3.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>R¹</th>
<th>X</th>
<th>PfIspD IC₅₀ (nM)</th>
<th>Whole cell 3D7 EC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>OCH₃</td>
<td>2',4'-Cl₂</td>
<td>25.3 ± 5.6</td>
<td>349 ± 30</td>
</tr>
<tr>
<td>6</td>
<td>NHCH₃</td>
<td>2',4'-Cl₂</td>
<td>21.6 ± 2.7</td>
<td>373 ± 85.5</td>
</tr>
</tbody>
</table>

Encouragingly, our results are highly comparable to those outlined by Yao et al. as 6 was shown to be a potent inhibitor of the PfIspD enzyme, demonstrating slightly increased potency against the target, compared with 4. This was also mirrored by highly comparable activity of 4 and 6 against P. falciparum growth with EC₅₀ values in the mid-nanomolar region for both analogues (Table 5.5). Whilst a slight decrease in phenotypic activity was observed in our study, compared to the work by Yao et al., this can perhaps be rationalised given that two different strains of P. falciparum parasite culture were used in the phenotypic whole cell assays conducted by the two groups.

The outcome of this follow-up study gave us confidence to adopt this R group modification, exchanging the carboxylic acid functionality at C-3 position (represented by 4) for the methyl amide (represented by 6): this adaptation was implemented and applied to the synthesis of all 1R,3S-MMV-008138 derivatives from this point. An additional benefit to the inclusion of the methyl amide functionality was a notable improvement in the synthetic methodology required to generate the resulting target compounds. Since carboxylic acid-containing derivatives of 4 required ester hydrolysis to generate target compounds, which was found to be problematic, the synthesis, purification and crystallisation of alternative methyl amide derivatives of 6 was notably more straightforward, since no hydrolysis step was required.

The ability to more easily crystallise target analogues is also a significant advantage as it facilitates the use of crystallography. Such studies not only informs knowledge of inhibitor-protein interactions, supporting mechanism of action evaluations, but also, in the case of MMV-008138 derivatives, enables the absolute stereochemistry of synthetic analogues to be determined. Since accurate isolation and characterisation of the required 1R,3S isomers is essential with this chemotype, the ability to crystallise target compounds for this purpose is a notable advantage.

Considering further evaluation of the R group, Yao et al. explored alternative amide functionalities to a methyl group at the R position of the MMV-008138 motif (Figure 5.15). These additional studies, featuring amides with larger alkyl groups such as iPr and Bu, found the resulting compounds to be less potent than the methyl amide derivative 6. It can therefore be rationalised that a narrow SAR around the R¹ group and C-3 substituent (Figure 5.15) reflects the nature of a very tight COOH binding
pocket in the PfISP-D active site, as previously highlighted by molecular modelling studies (Figure 5.8). Consequently, this may explain why groups larger than a methyl amide (6) are not tolerated. As a result, further modifications to R₁ were not explored further in our own SAR studies.

5.4.1.2 Modifications to the D Ring

To establish more structural determinants of the \textit{P. falciparum} inhibitory growth activity of 4, Yao \textit{et al.} generated a number of compounds to explore the effect of varying D ring substituents.\[46\] The majority of compounds generated involving D ring substitutions which were found to be inactive phenotypically. The only example of 3′,4′-disubstitution explored in the D ring did not inhibit parasite growth with a resulting IC₅₀ value > 20 μM; cases of mono-chloro substitution, at the 2′- and 4′-positions, demonstrated poor activity against the whole cell with inhibitory activity in the single digit micromolar region. An entirely un-substituted D ring was also shown to produce an inactive derivative with an IC₅₀ value of > 10 μM against parasite growth.\[46\]

These results demonstrate that the steric requirements for D ring substituents are also very restricted since the majority of modifications resulted in compounds that were incapable of inhibiting \textit{P. falciparum} parasite growth.\[46\] This mirrors our expectations, established as a result of docking studies of 1R,3S-MMV-008138 in the PfISP-D homology model. Molecular modelling highlighted that 1R,3S-MMV-008138 occupies a tight binding pocket within the PfISP-D active site (Figure 5.8), indicating that only small structural refinements are likely to be possible around particular regions of the tetrahydro-β-carboline chemotype. Indeed, the structural explorations made by Yao \textit{et al.} seem to confirm that substitution at the 2′- and 4′-positions of the D ring complement the shape of the rather tight PfISP-D binding pocket which 1R,3S-MMV-008138 is shown to occupy (Figure 5.8).\[15,46\] In addition, Yao \textit{et al.} have suggested that the presence of a 2′-substituent may induce constraints on the D ring, causing it to lie perpendicular to the C ring which may promote compound binding and shape fit within the PfISP-D active site.\[46\]

5.4.1.3 Modifications to the 2° Amine in the C Ring

Early synthetic explorations within our group have revealed that the 2° amine functionality in the C ring of the tetrahydro-β-carboline core cannot be converted into a 3° amine if PfISP-D and parasite growth inhibition are to be maintained. Structural modifications to the 2° amine, replacing the NH functionality with larger R² groups, 7 and 8 respectively, highlight that additional steric bulk at this position is not tolerated since both 7 and 8 demonstrate a loss of activity against the PfISP-D enzyme and 3D7 parasite growth. Indeed, this small study indicates that increasing steric bulk at the C ring 2°
amine correlates with an increasing loss of inhibitory activity against both the PfispD enzyme and Plasmodium parasite growth.

\[
\begin{array}{cccccc}
\text{Compound} & \text{R}^1 & \text{R}^2 & \text{X} & \text{PfispD IC}_{50} (\mu\text{M}) & \text{Whole cell 3D7 EC}_{50} (\mu\text{M}) \\
7 & \text{NHCH}_3 & \text{CH}_3 & 2',4'-\text{Cl}_2 & > 2 & 8.07 \pm 0.115 \\
8 & \text{OH} & \text{Bn} & 2'-\text{Cl},4'-\text{CF}_3 & > 25 & 8.36 \pm 0.439 \\
\end{array}
\]

Table 5.6 Enzymatic and phenotypic activity of 7 and 8 against the PfispD enzyme and 3D7 P. falciparum growth in culture.

5.5 Further Modifications to the D Ring

5.5.1 Design Rationale and Synthesis

Since D ring modifications explored by Yao et al. focused mainly on variations around a di-substituted D ring motif, examination of tri-substituted functionalisation within the D ring was now considered. Molecular modelling suggests that the D ring and its alignment within the PfispD binding site (Figure 5.8) is less spatially restricted in the protein pocket that it occupies compared to the very tight binding pocket occupied by the C-3 group. Consequently, there may be potential for further exploitation and structural development within the D ring as small additional substituents may be tolerated. Yao et al. highlighted that steric requirements at this site are very specific and that therefore, any modifications must be subtle and not introduce significant steric bulk which may hinder compound binding.

Consequently, it was decided to introduce an additional substituent at the 6-position of the D ring, generating a 2’,4’,6’-trisubstitution pattern (Figure 5.16), which was also mirrored by a parallel study in our group considering 1’,2’,5’-trisubstitution at the D ring using the same chemical functionalisation. In the two parallel studies, di-chloro substitution at the 2’- and 4’-D ring positions was maintained, along with the methyl amide functionality at C-3, simply varying the substituent at the 6’- (and comparably 5’-) position of the D ring. Work conducted by others, as well as in our group,\cite{21,46,54} indicates that the presence of polar functionalities in the D ring tend to assist and promote the inhibitory activity of resulting analogues against the PfispD enzyme and Plasmodium
parasite growth. As a result, a small range of polar substituents were selected to functionalise the 6’-position of the D ring (Figure 5.16).

![Chemical structure and substitution pattern of 1R,3S-MMV-008138 derivatives 10-14.](image)

Figure 5.16 Chemical structure and substitution pattern of 1R,3S-MMV-008138 derivatives 10-14.

Considering the reasonably tight binding site occupied by the D ring (Figure 5.8), it clear that only the introduction of relatively small groups was likely to be tolerated. However, this subtle functionalisation had the potential to facilitate and capture additional hydrogen bonding interactions around the aromatic phenyl ring that could push compound activity into the single digit nanomolar region.

With this in mind 6’-fluoro substitution (10) was first considered since fluorine is a good isostere of hydrogen, most notably due its small size, making it more likely to be tolerated within the tight PfIspD binding pocket. The presence of fluorine may facilitate additional hydrophobic and hydrogen bond acceptor contacts between compound 10 and the PfIspD protein, potentially increasing the strength of compound binding, as was the rationale with this inhibitor series. In addition, the deployment of fluorine can also be used to improve compound stability through its ability to modulate metabolic processes. The high strength of the C-F bond and the powerful electron withdrawing properties of fluorine make the C-F bond almost inert under most biological conditions and can therefore slow down metabolism at the site at which it is introduced.

Introduction of chlorine at the 6’-position was also considered due to its analogous hydrogen bonding capacity as a halogen. Compound 11 therefore also has the potential to promote the strength of compound binding at PfIspD through additional hydrogen bonding interactions with active site protein residues. However, the increased atomic size of chlorine, compared to fluorine, could hinder efficient binding of the resulting analogue due to the limited space of the PfIspD binding pocket. This substitution therefore enables the tolerance to steric bulk at the 6’-position to be determined. In direct contrast to the hydrogen bond accepting capabilities of halogens, synthesis of the 6’-hydroxy derivative 12 allowed the effects of a hydrogen bond donating group at the same position to be assessed.
Compound 13 involved introduction of a methoxy group at the 6’-position of the D ring allowing further assessment of the tolerance to steric bulk at this position. In addition, 13 also evaluates the ability of the methoxy group to lock the conformation of the D ring with respect to the tetrahydro-β-carboline motif. It is plausible that hydrogen bonding between a methoxy group at the 6’ position and the indole nitrogen of the tetrahydro-β-carboline motif could lock the conformation of the molecule, preventing rotation or ring flipping of the D ring within the PfIspD active site. Such an effect may exert influence on the binding affinity of 13 and the resulting inhibitory activity of the compound. In addition, evaluation of 1R,3S-MMV-008138 docking poses at PfIspD (Figure 5.8) suggests that the presence of a 6’-methoxy group could facilitate additional hydrogen bond contacts to the Arg-208 PfIspD protein residue, increasing the number of non-covalent protein-inhibitor interactions in the binding pocket and promoting the strength of compound binding.

In contrast to the previously described analogues (10-13), the final compound of this series, 14, differs slightly in that the carbon atom at the 6’-position of the D ring is replaced with a nitrogen atom, generating a pyridinyl analogue of the 1R,3S-MMV-008138 compound. Nitrogen atoms are spatially smaller than fluorine, therefore occupying a smaller area of space and contributing a reduced amount of steric bulk. Consequently, compound 14 has the potential to demonstrate an improved spatial and steric fit at the PfIspD binding site compared to analogues 10-13. Moreover, the introduction of a nitrogen atom into the aromatic D ring removes outward projections from the ring at the 6’-position, compared to the functionalization of 10-13, further reducing steric clashes with the active site protein residues. An additional benefit to a pyridinyl D ring is the hydrogen bonding capacity of nitrogen, due to its available lone pair, providing a hydrogen bond acceptor site in the compound. Finally, the polarity of nitrogen also promotes compound solubility and reduces lipophilicity, as highlighted by the order of magnitude drop in calculated LogD compared to other series analogues (Table 5.7), therefore enhancing the pharmacokinetic profile of compound 14.

Generation of analogues 10-14 was achieved using a two-step synthesis for each compound (Scheme 5.2). The first step of the synthesis involved a Pictet-Spengler reaction\[^{56,57}\] between various tri-substituted benzaldehydes, the component providing D-ring functionalisation, and the L-isomer of tryptophan methyl ester (L-Trp-OMe) which was purchased as the corresponding hydrochloride salt and neutralised before use in the synthesis. The second and final synthetic step involved conversion of the C-3 methyl ester into the corresponding methyl amide, previously discussed in Section 5.4.1.1, via a substitution reaction with methyl amine (Scheme 5.2).
Scheme 5.2 Synthetic strategy for the generation of 1R,3S-MMV-008138 derivatives 10-14.

The Pictet-Spengler reaction is an acid-catalysed transformation used to generate the tetrahydro-β-carboline chemotype from carbonyl compounds, here aromatic benzaldehydes, and tryptamine derivatives. When the carbonyl components used within this procedure are aldehydes, but not formaldehyde, the reaction is asymmetric. Reviews of the asymmetric methodology have suggested that tryptophan esters are more reactive in the Pictet-Spengler reaction than tryptophan derivatives, providing sufficient justification for the use of L-Trp-OMe (Scheme 5.2) as the tryptophan regent in our syntheses.

The Pictet-Spengler reaction is characterised by formation of an iminium ion, mediated by an acid-catalysed reaction between the selected tryptamine and carbonyl components. Intramolecular attack of the resulting iminium ion A (Scheme 5.3) by electrons from within the pyrrole ring causes ring closure and generation of the specified tetrahydro-β-carboline derivative. There has been much debate regarding the mechanism by which ring closure occurs in the Pictet-Spengler reaction since ring closure is facilitated by attack of electrons from the pyrrole ring onto the sp² carbon atom of iminium ion A. It is therefore plausible that attack could occur from either the 2- or 3- position of pyrrole, assisted by the lone pair of the 2° amine ring nitrogen, facilitating generation of intermediates: B or C (Scheme 5.3).
Scheme 5.3 Possible ring closure mechanisms of the Pictet-Spengler reaction generating the tetrahydro-β-carboline chemotype; representative example provided using 1R,3S-MMV-008138.\textsuperscript{[60]}

Generation of \textit{spiro}-indolenine intermediate B is suggested to occur following attack of pyrrole from the 3'-position onto the sp\textsuperscript{2} carbon of intermediate A and has been proven to exist by isotopic labelling experiments conducted by Bailey \textit{et al.}\textsuperscript{[61]} Intermediate B is able to rearrange via C-C bond migration to intermediate C, the tetrahydro-β-carboline structure, which can also form directly as a result of attack of pyrrole from the 2'-position onto the sp\textsuperscript{2} carbon of intermediate A (Scheme 5.3). However, research by others has indicated that rearrangement of B to C is energetically unfavourable and that direct attack of pyrrole from the 2'-position (Scheme 5.3) is more likely to represent the mechanistic rearrangement, generating intermediate C directly in the Pictet-Spengler condensation.\textsuperscript{[60,62]}

Since it is sometimes the case that only one enantiomer of a chiral compound has the ability to produce a desired biological effect, when applied as a therapeutic agent, the development of an enantioselective Pictet-Spengler synthesis has long been of interest within synthetic and medicinal chemistry.\textsuperscript{[60]} A number of approaches aiming to develop enantioselective methodologies have used enantiopure tryptophan esters\textsuperscript{[63]} which we mirrored in our synthetic approach by selecting the enantiomerically pure L-Trp-OMe as our tryptophan starting reagent (Scheme 5.2). However, this does not make the Pictet-Spengler synthesis enantioselective as a mixture of the \textit{cis-} (1S,3S) and \textit{trans-} (1R,3S) diastereoisomers are still produced. In other words, the stereoselectivity of the method comes

\[1R,3S-MMV-008138\]
from selecting the major (or required) diastereoisomer in the starting material (L-Trp-OMe). Formation of the cis-isomer tends to predominate in the asymmetric Pictet-Spengler reaction, which is slightly problematic for our work since it has already been established that the 1R,3S (trans) isomer of tetrahydro-β-carboline derivatives is exclusively required to achieve inhibitory activity against PfISPd and Plasmodium parasite growth.\[9,15\]

Nonetheless, we found that isolation of the cis- and trans- isomers generated within the Pictet-Spengler reaction can be achieved with relative ease using flash column chromatography. The ease of separation and indeed the stereoselectivity of each synthesis seems to be dependent on the functionalisation and substitution pattern of the specific reagents used. Both synthetic yield and stereoselectivity were seen to vary considerably using different benzaldehydes to generate analogues 10-14. However, we have found it possible to separate the resulting mixture of diastereoisomers following either step of the reaction scheme (Scheme 5.2). It was therefore possible to select the optimal point of isolation for the required trans isomer, as determined by the extent of polar separation between the cis and trans isomers when present as either the methyl ester intermediates 9a-e or methyl amide target compounds 10-14 (Scheme 5.2).

Adding further complication to the isolation and characterisation of these chiral target compounds, varying the benzaldehyde component (Scheme 5.2) not only altered synthetic yields and stereoselectivity, but also affected the polarity of the resulting cis and trans isomers with respect to each other. It was therefore impossible to tell by polarity (monitored by tlc) whether an isolated isomer corresponded to either the cis or the trans product in each synthesis. As a result, both isomers of each analogue require isolation and characterisation to determine the identity and stereochemistry of each material.

NOE spectroscopy (NOESY) NMR experiments were employed to determine the stereochemistry of the various materials isolated. NOESY is a homonuclear (\(^1\)H) 2D technique that detects “through-space” coupling between atoms of the same nuclei, here \(^1\)H atoms, and can therefore be used as a method of structural elucidation. In this instance, NOSEY was used to detect interactions between the hydrogen atoms at the C-1 and C-3 positions of the tetrahydro-β-carboline motif (Figure 5.15) in order to determine the stereochemistry of the isolated isomer.\[64\] The rationale was such that, in the case of the cis (1S,3S) isomer, the hydrogen atoms at the C-1 and C-3 positions would occupy the same face of the tetrahydro-β-carboline molecule (Figure 5.17) and therefore be able to “see each other” and interact “through-space”, producing a correlating signal in the 2D NOE spectrum.
Conversely, in the case of the *trans* (1R,3S) isomer, the hydrogen atoms at the C-1 and C-3 positions occupy opposite faces of the tetrahydro-β-carboline motif ([Figure 5.17](#)) and so would not be able interact via “through-space” coupling due to increased obstruction and separation between the two atoms preventing their interaction. Therefore, the absence of an interaction between the C-1 and C-3 hydrogens in the 2D NOE spectra represents a positive result, indicating isolation of the *trans* (1R,3S) isomer.

In this study, 2D NOE spectroscopy represents an acceptable form of analysis for the initial characterisation of compound stereochemistry since work with *L*-Trp-OMe ([Scheme 5.2](#)) within our group, as well as studies conducted by others,[65] suggests that this material is not prone to racemisation. We were therefore confident that the undesirable scenario of generating the four MMV-008138 diastereoisomers ([Figure 5.2](#)) from the Pictet-Spengler reaction was a low risk outcome. Rather, we were only likely to generate the 1S,3S and 1R,3S isomers ([Figure 5.17](#)) if *L*-Trp-OMe remained stable to racemisation, as it is believed to. So far, we have found this to be the case. However, as research with this chemotype progresses, further structural and stereochemical elucidation, by means of both crystallography and chiral HPLC, should be undertaken to more definitively confirm the absolute stereochemistry of 1R,3S-MMV-008138 derived compounds.

### 5.5.2 Enzymatic PfIspD and Whole Cell Activity Data

Following the described synthesis of MMV-008138 derivatives 10-14 ([Scheme 5.2](#)) and the successful isolation and characterisation of each required *trans* (1R,3S) isomer; all target compounds were tested in the PfIspD enzymatic assay ([Chapter 2, Section 2.2.3](#)) and *P. falciparum* whole cell growth assay ([Chapter 2, Section 2.3.5](#)) to determine the extent of their inhibitory activity against the PfIspD enzyme and *P. falciparum* parasite growth. The resulting data is given below in [Table 5.7](#).
Chapter 5

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Y</th>
<th>R²</th>
<th>PfispD IC₅₀ (μM)</th>
<th>Whole cell 3D7 EC₅₀ (μM)</th>
<th>MW (gmol⁻¹)</th>
<th>ClogD</th>
<th>CaqSol</th>
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<tr>
<td>10</td>
<td>C</td>
<td>F</td>
<td>2.22</td>
<td>1.00</td>
<td>391.07</td>
<td>3.86</td>
<td>13.64</td>
</tr>
<tr>
<td>11</td>
<td>C</td>
<td>Cl</td>
<td>&gt; 5</td>
<td>-</td>
<td>407.04</td>
<td>3.96</td>
<td>5.98</td>
</tr>
<tr>
<td>12</td>
<td>C</td>
<td>OH</td>
<td>&gt; 5</td>
<td>-</td>
<td>389.07</td>
<td>3.64</td>
<td>17.90</td>
</tr>
<tr>
<td>13</td>
<td>C</td>
<td>OCH₃</td>
<td>&gt; 5</td>
<td>-</td>
<td>403.09</td>
<td>3.76</td>
<td>13.99</td>
</tr>
<tr>
<td>14</td>
<td>N</td>
<td>-</td>
<td>&gt; 2</td>
<td>2.68</td>
<td>374.07</td>
<td>2.82</td>
<td>77.11</td>
</tr>
</tbody>
</table>

Table 5.7 Inhibitory activity of compounds 10-14 against the PfispD enzyme and *P. falciparum* growth in culture (strain 3D7); MW of compounds and calculated ClogD and CaqSol values given.

Unfortunately, evaluation of compounds 10-14 in the PfispD enzyme assay highlights that 2',4',6'-trisubstitution in the D ring of the MMV-008138 chemotype is not tolerated (Table 5.7). In comparison to the initial 1R,3S-MMV-008138 hit 4 (Table 5.5), consistent loss of activity (greater than 150 fold) against the PfispD enzyme is observed across the of MMV-008138 derivatives 10-14.

These results further reflect the tight PfispD binding pocket that is occupied by 1R,3S-MMV-008138 derived compounds, again demonstrating that there is very little tolerance to structural variation and steric bulk around the tetrahydro-β-carboline motif, therefore supporting the results demonstrated by Yao *et al.*[46] For compounds 11-13, whole cell data, determining inhibitory activity against *P. falciparum* parasite growth, was not obtained since the measured activities of these compounds against the PfispD enzyme fell outside the specified activity threshold of < 5 μM (Chapter 2, Section 2.3.5) and therefore are not classified as active against the target enzyme. Interesting, these results show that 10 is the most tolerated analogue in the series, highlighting a clear advantage to reduced (or minimal) steric bulk at the 6'-position and corresponding pocket in the PfispD active site.

5.5.3 Further SAR Explorations

Additional work in the group, exploring further modifications to the D ring, continues to confirm that steric requirements around this group are very precise and only subtle variations are tolerated without causing a loss of inhibitory activity against PfispD or *Plasmodium* parasite growth. Indeed, the majority of compounds generated around the 1R,3S-MMV-008138 chemotype display suboptimal activity against the PfispD enzyme compared to the initial 1R,3S-MMV-008138 hit 4 (Table 5.5).[53] This is seen in an analogous study within our group considering 1',2',5'-trisubstitution around the D ring, using the same chemical functionalities at the 5'-position as were introduced initially at the 6'-position (Figure 5.16). Disappointingly, this compound series was also inactive against PfispD.
Overall, the majority of modifications made on the previously unsubstituted face of the D ring (specifically the 5’- and 6’-positions) have been unsuccessful, generating compounds where inhibitory activity is completely abolished or only moderate against the PfIspD enzyme and parasite growth. It can therefore be concluded that binding pocket of PfIspD that is occupied by the D ring (Figure 5.8) is too tight to tolerate substitution at the 5’- and 6’-positions of the ring. However, in slight contrast to this assessment, compound 15 (Figure 5.18), the 6’-bromo variant of inhibitor 6 (Table 5.5), displays potent activity against the PfIspD enzyme with an IC_{50} value of 160 ± 24.2 nM (n = 3 replicates). This is an interesting result as, whilst the 5’- and 6’-positions of the D ring remain un-substituted, bromine has a much larger atomic radius than chlorine [atomic radius of Br is 1.12 Å, compared to 0.97 Å and 0.57 Å for chlorine and fluorine respectively][66] therefore introducing increased steric bulk at the 4’-position compared to 4’-chloro substitution, but that is shown to be tolerated and active against PfIspD.

![Chemical structure of compound 15](image)

**Figure 5.18** Chemical structure of compound 15; currently the most potent tetrahydro-β-carboline-derived inhibitor against the PfIspD enzyme.

In addition, not only is compound 15 more than twice as potent against PfIspD than the corresponding 2’,4’-dichloro analogue 6 (Table 5.5), but current biological analysis also suggests that 15 is capable of inhibiting *Plasmodium* parasite growth with a measured parasite EC_{50} value of 0.248 μM. This therefore seems to suggest that MMV-008138 analogue activity is not solely an issue of sterics, at least at the para-position of the D ring, but that SAR around this ring could actually be determined to some extent by electronics.
5.6 Conclusions and Future Work

Having identified potential off-target reactivity with the BITZ chemotype (see Chapter 3) where additional off-target contributions to those against the PfIspD enzyme may contribute to inhibition of *P. falciparum* parasite growth, an alternative chemical chemotype, active against PfIspD, has also been explored. Robust biological data from a range of experiments confirms a proposed mechanism of PfIspD enzyme inhibition by BITZ compounds as occurring *via* a covalent modification strategy (see Chapter 4). However, we are aware that the covalent modification of enzymes and proteins can be a risky strategy within drug design which has encouraged our consideration of an alternative chemical chemotype that inhibits the PfIspD enzyme by an alternative, non-covalent mechanism of action.

As a result, our group is now involved in research focused around MMV-008138 derived compounds, based on the tetrahydro-β-carboline chemotype *(Figure 5.1)* as a potent and selective inhibitor of the PfIspD enzyme. This work was initially pioneered by our collaborators (the Odom Molecular and Cellular Biology lab at Washington University in St Louis)[15] and others.[9,46] The MMV-008138 compound was first recognised in phenotypic screen carried out by GSK, showing it to be a potent antimalarial compound that was capable of inhibiting of *P. falciparum* growth in culture. Since its identification, extensive biological investigations have shown the MEP pathway PfIspD enzyme to be the protein target of MMV-008138. Enzymatic data has also revealed that only the 1R,3S-MMV-008138 diasteroisomer of MMV-008138 is active against PfIspD, giving a high degree of specificity and selectivity to this compound.

Assessment of species-selectivity and the potential antimicrobial activity spectrum of the 1R,3S-MMV-008138 isomer produced highly comparable results to those observed with the BITZ inhibitor chemotype. Indeed, the 1R,3S-MMV-008138 isomer potently inhibits *P. falciparum* and *P. vivax* homologues of the IspD enzyme but shows no inhibition against *E. coli* and *Mycobacterium tuberculosis* IspD, highlighting the species-selectivity of the MMV-008138 scaffold and its therapeutic scope as a broad-spectrum antimalarial agent. Metabolic profiling of MEP pathway metabolites in *P. falciparum* cells has demonstrated that 1R,3S-MMV-008138 is capable of inhibiting the *Plasmodium* MEP pathway at levels which affect asexual parasite growth. In addition, IPP rescue experiments have further established that the inhibition of parasite growth, shown by 1R,3S-MMV-008138, is specific to MEP pathway inhibition since the antimalarial effects of this compound are rescued by supplementation with IPP.

Since we are interested in alternative methods of PfIspD inhibition to that demonstrated by the BITZ chemotype, molecular modelling studies and kinetic analysis of 1R,3S-MMV-008138 have rationalised
that the compound inhibits PfIspD via a non-covalent mechanism of action, competing with the IspD enzyme substrate, CTP. Molecular modelling studies have also revealed that 1R,3S-MMV-008138 assumes a comparable binding alignment to that of the natural enzyme product, CDP-ME, which draws a direct comparison to the previously determined binding alignment of the BITZ chemotype (see Chapter 4). Therefore, it is now known that both the 1R,3S-MMV-008138 compound and BITZ derived compounds bind to the CTP binding pocket of PfIspD with a degree of pose overlap. However, it is also clear that both the tetrahydro-β-carboline and BITZ chemotype occupy some distinct space within the CTP binding pocket, showing varying proximity to different protein residues in the PfIspD active site.

The increasing number of comparable features between the tetrahydro-β-carboline and BITZ chemotypes prompted us to ask the question: how would these two chemical motifs behave if co-administered, and what would be the resulting effects on phenotypic activity? However, extensive competition experiments, generating isobolograms which combined 1R,3S-MMV-008138 with several distinct BITZ inhibitors, showed that these two chemical motifs behave antagonistically when co-administered and do not promote phenotypic antimalarial activity.

A number of exploratory SAR investigations have been conducted around the 1R,3S-MMV-008138 compound and tetrahydro-β-carboline chemotype. Whilst these investigations have been extensive, the measured activities of resulting compounds consistently indicate that the SAR around this compound is relatively limited, with little room for significant structural variation or additional functionality. Indeed, this notion is supported by molecular modelling studies and can be rationalised through the spatial demands of the PfIspD CTP binding site in which 1R,3S-MMV-008138 adopts a binding pose that spatially, is very tight-fitting.

It was already known that the 1R,3S stereochemical relationship across the C ring is essential to observe activity against PfIspD and Plasmodium parasite growth. It has also been shown that structural modifications to the 2° amine in the C ring are not tolerated. In addition, it is now evident that inclusion of a methyl-amide functionality, replacing the carboxylic acid group at the C-3 position of the C ring, promotes enzymatic and phenotypic activity. Most recently, SAR development have focused on modifications to the A and D rings of the 1R,3S-MMV-008138 motif, to determine if small structural variations in these regions are able to promote protein-ligand contacts within the PfIspD active site and therefore promote inhibitor activity.

Considering the D ring, recent 1R,3S-MMV-008138 analogues have demonstrated that 2°,4°,6°-trisubstitution, varying functionalities at the 6°-position but maintaining 2°,4°-dichloro substitution, is not tolerated since a loss of activity is seen against both PfIspD and parasite growth. The lack of
enzymatic activity across these analogues, subsequently translating into loss of phenotypic activity, is likely due to limited free space within the PfIspD binding pocket which the D ring occupies. Introducing functionalisation at the 6’-position of the D ring therefore prevents complementary binding within the PfIspD active site, compromising the inhibitory capabilities of resulting analogues. A comparable effect is also observed introducing 2’,4’,5’-trisubstitution to the D ring of 1R,3S-MMV-008138.

From the broad SAR investigations conducted in the group, a close structural analogue of 1R,3S-MMV-008138 has been identified as one of the most potent tetrahydro-β-carboline analogues against PfIspD to date. Compound 15 (Figure 5.18) has a 2’-chloro-4’-bromo substitution pattern in the D ring and a methyl-amide functionality at the C-3 position of the C ring. Compound 15 displays potent activity against the PfIspD enzyme with an IC₅₀ value of 160 nM and initial whole cell data that suggests enhanced inhibitory activity against Plasmodium parasite growth, compared to the corresponding 2’,4’-dichloro analogue 6 and the original 1R,3S-MMV-008138 hit compound (Table 5.5).

SAR development around 1R,3S-MMV-008138 and the tetrahydro-β-carboline chemotype is ongoing, with the aim of progressing towards an optimised lead structure that is suitable for clinical development as an antimalarial agent, targeting the PfIspD enzyme. To assist SAR development, molecular modelling using the PfIspD homology model to dock proposed 1R,3S-MMV-008138 analogues into the PfIspD active site will be used to inform compound design. Further molecular modelling will therefore guide SAR development by predicting structural analogues likely to be tolerated at the PfIspD active site, based on docking observations and identified binding poses. More extensive use of molecular modelling may better inform structural and chemical modifications by use of the molecular feedback loop. Indeed, docking inactive 1R,3S-MMV-008138 analogues 10-14 could be highly informative and may reveal specific structural clashes that prevent complementary binding of these compounds at the PfIspD active site.

Continuing research will also focus on improving the metabolism and pharmacokinetic profiles of 1R,3S-MMV-008138 analogues to enable movement towards a fully optimised lead compound. In particular, structural modifications to the A ring of the tetrahydro-β-carboline motif, such as fluorine substitution at the 6’- and 7’-positions or alternatively, conversion of the phenyl A ring to a pyridinyl ring, will be undertaken.
5.7 Experimental

5.7.1 Computational Methods

Molecular Modelling

A homology model of PfIspD was constructed using the PHYRE online homology modelling program.\textsuperscript{[67]} PfIspD primary sequence Q8I273 was obtained from UNIPROT (http://www.uniprot.org/, accessed 10/12/2014). A number of protein alignments and homology models were constructed by PHYRE, and the model with 98.82% confidence was selected, which was based on an \textit{E. coli} IspD structure (PDB accession code 1I52).\textsuperscript{[68]} 1I52 is a 1.50 Å resolution crystal structure of \textit{E. coli} IspD, complexed with CDP-ME in the active site. The structure of the model was validated using the WHATIF web interface.\textsuperscript{[69]} 1R,3S-MMV008138 and selected BITZ inhibitor 1 were modelled \textit{in silico} using the homology model described above in order to visualize the interactions between each analogue and the active site. Using GOLD, protons were added and docking was performed with default parameters, except that GoldScore was used and 50 docking poses were obtained for comparison and analysis.\textsuperscript{[23]}

5.7.2 Biological methods

\textit{1R,3S-MMV008138} and BITZ Isobolograms

Asynchronous cultures of \textit{P. falciparum} strain 3D7 were diluted to 0.5% parasitemia and cultured over a range of concentrations of \textit{1R,3S-MMV008138} and/or \textit{1, 2 and 3} in 100 μL culture volumes. Stock solutions of \textit{1, 2, 3} and \textit{1R,3S-MMV008138} were prepared at concentrations such that the EC\textsubscript{50} values for these compounds alone would fall around the midpoint of a 10-point 2-fold dilution series. As previously described,\textsuperscript{[30]} drug response curves were then performed at \textit{1, 2, 3/1R,3S-MMV008138} ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5. After 72 h, parasite growth was quantified using PicoGreen dye (Invitrogen) to measure the DNA content as previously described.\textsuperscript{[18]} EC\textsubscript{50} values were calculated, treating each \textit{1, 2, 3/1R,3S-MMV008138} combination as if it were a single unique drug, by nonlinear regression analysis using GraphPad Prism software. Following these calculations, EC\textsubscript{50} values were prorated between compounds according to \textit{1, 2, 3/1R,3S-MMV008138} ratios and plotted as an isobologram. Each data point represents the average of three independent experimental replicates.
5.7.3 Organic Synthesis

Preparation of \textit{L-tryptophan methyl ester}

\[ \begin{array}{c}
\text{L-tryptophan methyl ester hydrochloride (3.0 g, 11.8 mmol) was stirred in 1:1 EtOAc:NaHCO}_3 (150 ml),
\text{extracted into EtOAc (3 x 50 ml), washed with brine (100 ml), dried over MgSO}_4 \text{ and concentrated}
\text{under reduced pressure to give \textit{L-tryptophan methyl ester} as an off-white crystalline solid in 90.6%}
\text{yield.} \\
\text{\textsuperscript{1}H NMR (500 MHz, CDCl}_3 \delta 7.77 (d, } J = 7.9 \text{ Hz, 1H), 7.57 (d, } J = 8.1 \text{ Hz, 1H), 7.54 (s, 1H), 7.37 (t,}
\text{ } J = 7.2 \text{ Hz, 1H), 7.29 (t, } J = 7.5 \text{ Hz, 1H), 4.02 (t, } J = 5.1 \text{ Hz, 1H), 3.92 (s, 3H), 3.48 (dd,}
\text{ } J = 14.5, 4.7 \text{ Hz, 1H), 3.28 (dd, } J = 14.5, 7.6 \text{ Hz, 1H).} \\
\text{\textsuperscript{13}C NMR (101 MHz, CDCl}_3 \delta 175.4, 136.4, 127.2, 123.4, 121.8, 119.2, 118.3, 111.4, 109.6, 54.4, 52.1, 30.3; CI+HRMS m/z 219.1133 [M+H\text{]}}^+ \text{ requires 218.11.}
\end{array} \]

Preparation of \textit{methyl (S)-1-phenyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylates 9a-e}

\textbf{General Procedure (1)}

\[ \begin{array}{c}
\text{\textit{L-tryptophan methyl ester} (2.29 mmol), as the free base, was combined with the required 2',4',6'-}
\text{trisubstituted benzaldehyde (2.52 mmol, 1.1 eq) in anhydrous DCM (15 ml) under a N}_2 \text{ atmosphere.}
\text{The resulting solution was cooled to 0°C before the dropwise addition of TFA (1.0 eq) and gradually}
\text{warming to room temperature. The solution was stirred at room temperature over 18 hours to 3 days.}
\text{The solution was quenched with 10% NH}_4\text{OH (30 ml), extracted into DCM (3 x 30 ml), washed with}
\text{brine (50 ml), dried over MgSO}_4 \text{ and concentrated under reduced pressure. The crude products were}
\text{purified by flash column chromatography.}
\end{array} \]
Preparation of methyl (S)-1-(2,4-dichloro-6-fluorophenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate 9a

The general procedure (1) was followed using 2,4-dichloro-6-fluorobenzaldehyde to give 9a, purified by flash column chromatography (1% EtOAc:DCM) to give a 1:4 (trans:cis) diastereoisomer mixture as a white-yellow fine, crystalline solid in 83.9% yield. **Rf = 0.41 (2:98 EtOAc:DCM).** \(^1^H\) NMR (500 MHz, DMSO) \(\delta\) 10.64 (s, 1H, cis NH), 10.60 (s, 0.4H trans NH), 7.65 (s, 1H, cis), 7.63 (s, 0.25H, trans), 7.47 – 7.37 (m, 2.5H, cis/trans), 7.20 (dt, \(J = 7.9, 0.8\) Hz, 1H, cis), 7.19 (dt, \(J = 7.9, 0.9\) Hz, 0.25H, trans), 7.05 – 6.94 (m, 2.5H, cis/trans), 6.02 (s, 0.25H, trans), 5.79 (d, \(J = 5.7\) Hz, 1H, cis), 3.98 (m, 1.25H, cis/trans), 3.73 (s, 3H, cis CH\(_3\)), 3.62 (s, 0.75H, trans CH\(_3\)), 3.11 – 3.02 (m, 1.25H, cis/trans), 2.78 (ddd, \(J = 21.6, 12.0, 6.5\) Hz, 2H); \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 173.2, 162.3 (d, \(J_{CF} = 239.8\) Hz), 136.5, 136.4, 135.9 (d), \(J_{CF} = 6.4\) Hz), 127.0 (d, \(J_{CF} = 6.5\) Hz), 125.8 (d, \(J_{CF} = 3.5\) Hz), 121.2, 120.8 (d, \(J_{CF} = 29.5\) Hz), 118.9 (d), \(J_{CF} = 23.3\) Hz), 118.1 (2C), 111.5, 111.4, 106.5, 56.8, 52.3, 52.1, 25.7; **Elemental analysis (%) found:** C, 57.92; H, 3.87; N, 7.01%. \(\text{C}_{19}\text{H}_{15}\text{ClF}_2\text{N}_2\text{O}_2\) requires C, 58.03; H, 3.84; N, 7.12%; **CI+HRMS m/z** 393.0579 [M+H]**\(^+\) C\(_{19}\)H\(_{15}\)ClF\(_2\)N\(_2\)O\(_2\) requires 392.05.

Preparation of methyl (S)-1-(2,4,6-trichlorophenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate 9b

The general procedure (1) was followed using 2,4,6-trichlorobenzaldehyde to give 9b, purified by flash column chromatography to give a mixture of diastereoisomers as an off-white/cream solid in 42.7% yield. **Rf = 0.32 (2:98 EtOAc:DCM).** \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.51 (dd, \(J = 6.9, 1.6\) Hz, 2H, cis/trans), 7.46 (d, \(J = 2.1\) Hz, 2H cis/trans), 7.43 (br s, NH indole), 7.30 (d, \(J = 2.1\) Hz, 2H cis/trans), 7.24 (dd, \(J = 6.8, 1.8\) Hz, 2H cis/trans), 7.18 – 7.09 (m, 4H cis/trans) 6.44 (s, 1H, trans), 6.13 (s, 1H, cis), 4.22 (dd, \(J = 5.9, 2.7\) Hz, 1H, trans), 4.02 (dd, \(J = 11.2, 4.3\) Hz, 1H, cis), 3.82 (s, 3H, CH\(_3\) cis), 3.71 (s, 3H, CH\(_3\) trans), 3.38 (dt, \(J = 15.4, 1.9\) Hz, 1H, trans), 3.25 (ddd, \(J = 15.5, 6.0, 2.3\) Hz, 2H, cis/trans), 2.94 (ddd, \(J = 15.1, 11.2, 2.7\) Hz, 1H, cis), 2.28 (br s, NH); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 173.2, 137.4, 136.8, 135.9, 135.2, 133.1, 132.2, 130.4, 128.6, 127.0, 122.0, 119.9, 118.1, 111.0, 109.1, 57.2, 54.0, 52.4, 25.8; **ES+HRMS m/z** 409.0269 [M+H]**\(^+\) C\(_{19}\)H\(_{15}\)Cl\(_3\)N\(_2\)O\(_2\) requires 408.02.

Preparation of methyl (S)-1-(2,4-dichloro-6-hydroxyphenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate 9c

The general procedure (1) was followed using 2,4-dichloro-6-hydroxybenzaldehyde to give 9c, purified, and with diastereoisomers separated, by flash column chromatography in a 1:3:1 ratio of cis: trans isomers, as a white solid and yellow viscous oil respectively, with a combined 84.2% yield. **Rf of cis isomer = 0.54 (2:98 EtOAc:DCM); Rf of trans isomer = 0.25 (2:98 EtOAc:DCM).** \(15\)\(_{3}\)S (cis isomer) \(^1^H\) NMR (400 MHz, CDCl\(_3\)) \(\delta\) 11.72 (br s, 1H, NH), 7.61 (br s, 1H, OH), 7.50 (d, \(J = 7.5\) Hz, 1H), 7.25 (dd, \(J = 126.3\) Hz), 7.18 (m, 2.5H, cis/trans), 7.05 – 6.94 (m, 2.5H, cis/trans), 6.02 (s, 0.25H, trans), 5.79 (d, \(J = 5.7\) Hz, 1H, cis), 3.98 (m, 1.25H, cis/trans), 3.73 (s, 3H, cis CH\(_3\)), 3.62 (s, 0.75H, trans CH\(_3\)), 3.11 – 3.02 (m, 1.25H, cis/trans), 2.78 (ddd, \(J = 21.6, 12.0, 6.5\) Hz, 2H); \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 173.2, 162.3 (d, \(J_{CF} = 239.8\) Hz), 136.5, 136.4, 135.9 (d), \(J_{CF} = 6.4\) Hz), 127.0 (d, \(J_{CF} = 6.5\) Hz), 125.8 (d, \(J_{CF} = 3.5\) Hz), 121.2, 120.8 (d, \(J_{CF} = 29.5\) Hz), 118.9 (d), \(J_{CF} = 23.3\) Hz), 118.1 (2C), 111.5, 111.4, 106.5, 56.8, 52.3, 52.1, 25.7; **Elemental analysis (%) found:** C, 57.92; H, 3.87; N, 7.01%. \(\text{C}_{19}\text{H}_{15}\text{ClF}_2\text{N}_2\text{O}_2\) requires C, 58.03; H, 3.84; N, 7.12%; **CI+HRMS m/z** 393.0579 [M+H]**\(^+\) C\(_{19}\)H\(_{15}\)ClF\(_2\)N\(_2\)O\(_2\) requires 392.05.
7.4 Hz, 1H), 7.18 - 7.08 (m, 2H), 7.00 (d, J = 2.1 Hz, 1H), 6.73 (d, J = 2.1 Hz, 1H), 6.04 (s, 1H), 3.94 (dd, J = 11.3, 4.1 Hz, 1H), 3.88 (s, 3H), 3.33 (dd, J = 15.5, 4.1, 1.7 Hz, 1H), 3.06 (dd, J = 15.4, 11.3, 2.6 Hz, 1H); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 171.6, 159.9, 136.3, 135.4, 132.8, 130.3, 126.5, 122.6, 120.3, 120.0, 119.9, 118.3, 117.3, 111.2, 108.3, 56.2, 54.3, 52.8, 24.6. 1R,3S (trans isomer) $^1$H NMR (400 MHz, CDCl$_3$) δ 14.57 (br s, 1H, NH, R2), 8.23 (s, 1H, NH), 8.08 (br s, 1H, OH), 7.60 (d, J = 7.7 Hz, 1H), 7.35 (d, J = 8.1 Hz, 1H), 7.20 (dt, J = 7.2, 1.0 Hz, 1H), 7.12 (dt, J = 0.9, 1.0 Hz, 1H), 6.94 (d, J = 2.3 Hz, 1H), 6.87 (d, J = 1.9 Hz, 1H), 6.78 (d, J = 2.0 Hz, 1H), 4.33 (dd, J = 9.1, 4.4 Hz, 1H), 3.79 (s, 3H), 3.58 (dd, J = 14.5, 4.4 Hz, 1H), 3.24 (dd, J = 14.5, 9.1 Hz, 1H); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 171.3, 164.3, 163.4, 138.7, 136.3, 136.2, 126.9, 123.6, 122.4, 119.8, 119.5, 118.5, 117.1, 113.8, 111.4, 110.2, 70.7, 52.7, 30.1; ES+HRMS m/z 391.0614 [M+H]$^+$ C$_{19}$H$_{18}$Cl$_2$N$_2$O$_3$ requires 390.05.

Preparation of methyl (S)-1-(2,4-dichloro-6-methoxyphenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate 9d

The general procedure (1) was followed using 2,4-dichloro-6-methoxybenzaldehyde to give 9d purified by flash column chromatography (2% EtOAc:DCM) as a white solid, a mixture of diastereoisomers in 92.0% yield. RF = 0.26 (2:98 EtOAc:DCM). cis rotamers $^1$H NMR (400 MHz, DMSO) δ 10.47 (s, 1H, NH, R1), 10.42 (s, 1H, NH, R2), 7.40 (d, J = 7.6 Hz, 1H), 7.35 (d, J = 2.0 Hz, 1H, R2), 7.27 (d, J = 2.0 Hz, 1H, R1), 7.20 (d, J = 7.5 Hz, 1H), 7.16 (d, J = 1.9 Hz, 1H, R2), 7.12 (d, J = 2.0 Hz, 1H, R1), 7.02 – 6.92 (m, 2H), 5.96 (d, J = 8.8 Hz, 1H, R1), 5.75 (d, J = 10.7 Hz, 1H, R2), 3.93 (m, 1H), 3.72 (s, 3H), 3.06 (m, 1H), 2.73 (m, 1H); $^1$H NMR (400 MHz, CDCl$_3$) δ 174.0, 173.5, 160.4, 160.0, 136.4, 136.2, 135.7, 135.6, 135.0, 134.5, 134.4, 134.3, 127.3, 127.1, 125.7, 125.3, 122.7, 121.8, 120.9, 118.8, 118.7, 117.8, 112.7, 111.6, 111.5, 111.5, 107.1, 106.0, 57.4, 57.3, 57.0, 56.9, 53.2, 52.3, 52.2, 48.8, 26.2, 25.9. trans isomer (lacking single CH$_3$ peak) $^1$H NMR (500 MHz, CDCl$_3$) δ 7.52 (m, 2H), 7.21 (d, J = 5.0 Hz, 1H, NH), 7.11 (m, 2H), 7.07 (s, 1H), 6.80 (s, 1H), 6.22 (s, 1H), 4.23 (t, J = 4.2 Hz, 1H), 3.72 (s, 3H), 3.60 (br s, 3H), 3.25 (m, 2H), 1.27 (s, 1H, NH); $^{13}$C NMR (101 MHz, CDCl$_3$) δ 174.1, 159.9, 136.3, 135.7, 135.0, 127.3, 125.6, 121.4, 119.3, 118.0, 110.8, 56.5, 54.6, 52.2, 48.0, 24.3; Elemental analysis (%) found: C, 59.11; H, 4.50; N, 6.79%. C$_{20}$H$_{18}$Cl$_2$N$_2$O$_2$ requires C, 59.27; H, 4.48; N, 6.91%; ES+HRMS m/z 405.0764 [M+H]$^+$ C$_{20}$H$_{18}$Cl$_2$N$_2$O$_2$ requires 404.07.

Preparation of methyl (S)-1-(3,5-dichloropyridin-2-yl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate 9e

The general procedure (1) was followed using 3,5-dichloropicolinaldehyde to give 9e purified by flash column chromatography, giving a 1:2 ratio of mixed diastereoisomers as a cream-orange clumpy solid in 83.0% yield. RF = 0.28 (5:95 EtOAc:DCM). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.45 (d, J = 2.1 Hz, 1H, cis), 8.38 (d, J = 2.1 Hz, 1H, trans), 7.84 (d, J = 2.2Hz, 1H, cis), 7.80 (br s, trans NH indole), 7.78 (d, J = 2.1 Hz,
1H, trans), 7.65 (br s, cis NH indole), 7.53 (d, J = 8.0 Hz, 1H, trans), 7.50 (d, J = 7.6 Hz, 1H, cis), 7.24 (d, J = 7.6 Hz, 2H), 7.13 (m, 4H, cis/trans), 5.99 (s, 1H, trans), 5.92 (s, 1H, cis), 4.49 (dd, J = 7.6, 5.2 Hz, 1H, trans), 4.03 (d, J = 6.0 Hz, 1H, cis), 3.84 (s, 3H, CH₃), 3.77 (s, 3H, CH₃, trans), 3.32 – 3.18 (m, 2H, cis/trans), 3.10 (br s, cis/trans NH), 3.09 – 2.93 (m, 2H, cis/trans); ¹³C NMR (101 MHz, CDCl₃) δ 173.4, 154.0, 147.1, 136.1, 130.4, 126.9, 122.4, 119.9, 118.3, 111.0, 109.5, 56.3, 54.3, 52.3, 26.0 (peaks more intense: likely cis isomer); ¹³C NMR (101 MHz, CDCl₃) δ 174.0, 156.1, 146.8, 137.0, 136.2, 131.5, 131.2, 130.2, 126.9, 122.3, 119.6, 118.4, 110.9, 109.2, 53.0, 52.2, 51.6, 25.1 (peak intensity reduced: likely trans isomer); ES+HRMS m/z 376.0614 [M+H]⁺ C₁₈H₁₅Cl₂N₃O₂ requires 375.05.

Preparation of (1R,3S)-1-(2,4,6-trisubstituted-phenyl)-N-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamides 10-14

**General Procedure (2)**

The appropriate compound 9a-e was transferred to a sealed tube and dissolved in methylamine solution, 33 wt. % in absolute EtOH (130.0 eq). The resulting solution was allowed to stir for 16hrs-3 days at room temperature under an atmospheric atmosphere. Excess methylamine was removed under reduced pressure and the crude products were purified and diastereoisomers separated by flash column chromatography.

Preparation of (1R,3S)-1-(2,4-dichloro-6-fluorophenyl)-N-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide 10

The general procedure (2) was followed using 9a overnight to give 10 as an off-white/yellow solid in 98.1% total yield, isolating 0.0504 g (15.5% yield) of the trans diastereoisomer. Rf of trans isomer = 0.30 (40:60 EtOAc:DCM); Rf of cis isomer = 0.49 (40:60 EtOAc:DCM). ¹H NMR (500 MHz, DMSO) δ 10.64 (s, 1H, NH), 7.87 (q, J = 4.7 Hz, 1H), 7.58 (s, 1H, NH), 7.42 (m, 2H), 7.21 (dt, J = 7.1, 0.8 Hz, 1H), 7.02 (td, J = 7.01, 1.2 Hz, 1H), 6.96 (td, J = 7.9, 1.1 Hz, 1H), 5.78 (m, 2H), 5.21 (t, J = 5.6 Hz, 1H), 3.67 (d, J = 5.3 Hz, 1H), 3.21 (t, J = 7.4 Hz, 1H, NH), 2.95 (dd, J = 15.2, 5.1 Hz, 1H), 2.84 (dd, J = 15.2, 7.5 Hz, 1H), 2.62 (d, J = 4.6 Hz, 3H), ¹³C NMR (126 MHz, DMSO) δ 173.5, 161.9 (d, J CF = 253.6 Hz), 136.4, 136.4 (d, J CF = 253.6 Hz).
Preparation of (1R,3S)-N-methyl-1-(2,4,6-trichlorophenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide 11

The general procedure (2) was followed using 9b overnight to give 11 as an off-white solid in 91.0% total yield, isolating 0.0360 g (13.6% yield) of the trans diastereoisomer. 

\[ \text{RF of cis isomer = 0.28 (10:80 EtOAc:DCM).} \]

\[ ^{1}H \text{ NMR (500 MHz, DMSO)} \delta 10.51 (s, 1H, indole NH), 7.86 (q, J = 4.8 Hz, 1H, amide NH), 7.79 (d, J\text{H-H} = 1.9 Hz, 1H), 7.54 (d, J\text{H-H} = 1.8 Hz, 1H), 7.40 (d, J = 7.7 Hz, 1H), 7.18 (dd, J = 7.8, 0.9 Hz, 1H), 6.99 (dt, J = 7.1, 1.2 Hz, 1H, NH), 6.94 (dt, J = 7.1, 1.2 Hz, 1H), 6.05 (d, J\text{H-H} = 6.0 Hz, 1H), 3.89 (dd, J = 4.9, 5.6 Hz, 1H), 3.17 (t, J = 6.1 Hz, 1H), 3.08 (ddd, J = 15.1, 4.3, 1.7 Hz, 1H), 2.92 (ddd, J = 15.1, 5.6, 1.7 Hz, 1H), 2.62 (d, J = 4.6 Hz, 3H); ^{13}C \text{ NMR (101 MHz, CDCl}_3) \delta 172.6, 135.9, 135.0, 133.6, 131.1, 130.3 (2C), 128.7 (2C), 127.2, 121.7, 119.4, 118.3, 110.8, 108.2, 54.9, 50.2, 26.1, 21.8; \]

\[ \text{Elemental analysis (%) found: C, 55.42; H, 4.04; N, 9.90%.} \]

\[ C_{39}H_{46}Cl_{3}N_{2}O \text{ requires C, 58.18; H, 4.11; N, 10.71%; ES+HRMS m/z 408.0431 [M+H]^+ C_{39}H_{46}Cl_{3}N_{2}O \text{ requires 407.04.} \]

Preparation of (1R,3S)-1-(2,4-dichloro-6-hydroxyphenyl)-N-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide 12

The general procedure (2) was followed using 9c (pure trans diastereoisomer) and 140.0 eq methylamine solution, 33 wt. % in absolute EtOH overnight to give 12 as a yellow solid in 54.2% yield. 

\[ \text{RF = 0.33 (10:80 EtOAc:DCM).} \]

\[ ^{1}H \text{ NMR (500 MHz, CDCl}_3) \delta 12.14 (br s, 1H, indole NH), 8.17 (br s, 1H, OH), 7.59 (d, J = 8.0 Hz, 1H), 7.37 (dt, J = 8.1, 0.9 Hz, 1H), 7.21 (td, J = 8.1, 1.0 Hz, 1H, 7.12 (td, J = 8.0, 1.0 Hz, 1H), 7.09 (d, J = 2.2 Hz, 1H, amide NH), 6.89 (d, J = 2.1 Hz, 1H), 6.76 (d, J = 2.1 Hz, 1H), 5.96 (d, J = 1.9 Hz, 1H), 3.98 (dt, J = 7.7, 2.3 Hz 1H), 3.60 (ddd, J = 14.8, 3.3, 0.9 Hz, 1H), 2.96 (dd, J = 15.0, 4.9 Hz, 1H), 2.71 (s, 3H), 1.26 (s, 1H, 2° amine); ^{13}C \text{ NMR (101 MHz, CDCl}_3) \delta 170.7, 161.0, 136.8, 136.4, 135.3, 126.9, 123.1, 122.4, 120.4, 119.8, 118.5, 117.5, 114.3, 111.4, 111.1, 71.1, 58.7, 28.1, 26.6; \]

\[ \text{Elemental analysis (%) found: C, 58.55; H, 4.784; N, 9.70%.} \]

\[ C_{39}H_{27}Cl_{2}N_{3}O_2 \text{ requires C, 58.48; H, 4.39; N, 10.77%; ES+HRMS m/z 390.0767 [M+H]^+ C_{39}H_{27}Cl_{2}N_{3}O_2 \text{ requires 389.07.} \]

Preparation of (1R,3S)-1-(2,4-dichloro-6-methoxyphenyl)-N-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide 13

The general procedure (2) was followed using 9d and 160eq methylamine solution, 33 wt. % in absolute EtOH over 3 days to give 13 as an off-white solid in 61.7% yield. 

\[ \text{RF = 0.23 (2:98 EtOAc:DCM).} \]

\[ ^{1}H \text{ NMR (500 MHz, DMSO)} \delta 10.37 (s, 1H, NH), 7.84 (q, J = 4.8 Hz, 1H), 7.40 (d, J = 7.5 Hz, 1H), 7.17 (d,
$J = 7.6 \text{ Hz, 1H}$, $7.15 (d, J = 4.6 \text{ Hz, 1H}), 6.95 (dtd, J = 15.9, 7.1, 1.2 \text{ Hz, 2H}), 5.81 (s, 1H), 3.89 (unresolved dd, $J = 5.3 \text{ Hz, 1H}), 3.04 (dd, J = 15.0, 5.0, 1.6 \text{ Hz, 1H}), 2.91 (dd, J = 15.0, 5.6, 1.5 \text{ Hz, 1H}), 2.64 (d, J = 4.7 \text{ Hz, 3H}), 1.25 (s, 1H, NH); $^{13}C \text{ NMR} (101 \text{ MHz, CDCl}_3) \delta 172.6, 159.8, 136.1, 135.7, 135.3, 132.8, 127.6, 124.6, 122.5, 121.4, 119.3, 118.3, 111.5, 110.6, 65.9, 56.5, 55.1, 29.7, 26.2; \text{ Elemental analysis} (%) \text{ found: C, 60.10; H, 5.18; N, 9.82\%. $C_{20}H_{19}Cl_2N_2O_2$ requires C, 59.42; H, 4.74; N, 10.39\%; \text{ ES+HRMS m/z} 404.0922 [M+H$^+$]. $C_{20}H_{19}Cl_2N_2O_2$ requires 403.09.

Preparation of (15,3S)-1-(3,5-dichloropyridin-2-yl)-N-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxamide 14

The general procedure (2) was followed using 9e and 200.0 eq methylamine solution, 33 wt. % in absolute EtOH over 3 days to give 14 as an off-white solid in 55.9% total yield, isolating 0.0733 g (14.4% yield) of the trans diastereoisomer. $R_f = 0.32 (2:98 \text{ EtOAc:DCM}). \text{ $^1H NMR} (500 \text{ MHz, DMSO}) \delta 10.53 (s, 1H, indole NH), 8.43 (d, J = 2.2 \text{ Hz, 1H}), 8.29 (d, J = 2.2 \text{ Hz, 1H}), 7.74 (q, J = 4.5 \text{ Hz, 1H, amide NH}), 7.43 (d, J = 7.7 \text{ Hz, 1H}), 7.21 (d, J = 8.0 \text{ Hz, 1H}), 7.02 (dt, J = 7.9, 1.0 \text{ Hz, 1H}), 6.96 (dt, J = 7.8, 0.8 \text{ Hz, 1H}), 5.71 (d, J = 5.8 \text{ Hz, 1H}), 3.66 (td, J = 10.0, 4.4 \text{ Hz, 1H}), 2.95 (dd, J = 15.2, 4.5 \text{ Hz, 1H}), 2.67 (dd, J = 14.2, 10.0 \text{ Hz, 10H}), 2.59 (d, J = 4.6 \text{ Hz, 3H}); \text{ $^{13}C NMR} (126 \text{ MHz, DMSO}) \delta 173.0, 156.9, 145.3, 136.9, 136.1, 133.0, 130.9, 130.0, 126.7, 120.7, 118.2, 117.5, 110.9, 108.6, 52.0, 51.3, 25.4, 25.2; \text{ Elemental analysis} (%) \text{ found: C, 58.01; H, 4.46; N, 14.56\%. $C_{18}H_{16}Cl_2N_4O$ requires C, 57.61; H, 4.30; N, 14.93\%; \text{ ES+HRMS m/z} 375.0767 [M+H$^+$]. $C_{18}H_{16}Cl_2N_4O$ requires 374.07.

Overlay of $^1H NMR Spectra

Overlaid $^1H NMR spectra of the cis and trans isomers of compound 11 can be found in Appendix 3. The overlaid spectra highlight the observed change in chemical shift of the H-1 proton for corresponding cis and trans isomers of tetrahydro-β-carboline derivatives.

2D Nuclear Overhauser effect spectroscopy (NOESY) NMR

2D NOESY NMR experiments were recorded on Bruker Avance III HD 500 MHz spectrometer at ambient temperature. Standard parameters were used for each 2D NMR experiment; altering only the mixing time and number of scans (NS) specified. A mixing time of 1.0 second was used in order to verify an NOE build-up and identify through-space correlations; NS = 16 for each experiment. Gradient-enhanced 2D NOESY sequence (noesygpph) was used, and standard 2D processing was applied. Some non-through space couplings were also see but these were discounted as they were not NOESY based. Representative NOE spectra for compound 11 can be found in Appendix 4. Positive enhancement observed between chemical shift values of H-1 and H-3 confirms presence of a tetrahydro-β-carboline cis isomer. Conversely, presence of the trans isomer is confirmed by lack of
positive enhancement between assigned chemical shift values for H-1 and H-3 protons within derived tetrahydro-β-carboline structures.
5.8 References


Chapter 6

Considering Off-Target Interactions of the BITZ Chemotype:

A Chemical Biology Approach
Chapter 6

6.1 Potential Off-Target BITZ Chemotype Reactivity

As previously alluded to (see Chapter 3), it is possible that the 1,2-benzo[d]isothiazol-3(2H)-one (BITZ) chemotype and derived inhibitors may be capable of exerting inhibitory effects at off-target proteins, in addition to the PfIspD enzyme, which may contribute to observed inhibition of *Plasmodium* parasite growth. In particular, an indication of off-target reactivity was highlighted by the alkyl-amino biphenyl BITZ compound series 1-3 and the biphenyl-pyridinyl BITZ compound series 4-10 (Table 6.1 and 6.2 respectively) since both inhibitor series displayed a marked increase in potency against *P. falciparum* parasite growth, compared to that observed against the PfIspD enzyme (see Chapter 3). The inhibitory activity of compounds in the alkyl-amino biphenyl BITZ compound series 1-3 is ~ four to five times more potent against *Plasmodium* parasite growth than against the PfIspD enzyme (Table 6.1).

![BITZ Compound 1-3](image)

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<th>Compound number</th>
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<th>PfIspD IC₅₀ (µM)</th>
<th>Whole cell 3D7 EC₅₀ (µM)</th>
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<td>Morpholine</td>
<td>0.501</td>
<td>0.736</td>
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<td>2</td>
<td>Diethylamine</td>
<td>2.33</td>
<td>0.438</td>
</tr>
<tr>
<td>3</td>
<td>Pyrrolidine</td>
<td>0.507</td>
<td>0.133 ± 0.016</td>
</tr>
</tbody>
</table>

Table 6.1 Inhibitory activity of alkyl-amino biphenyl BITZ analogues 1-3 against the PfIspD enzyme and *P. falciparum* growth in culture (strain 3D7).

This observation is strongly echoed by the biphenyl pyridinyl BITZ analogue series. Indeed, BITZ compounds 4-10 demonstrate a complete loss of inhibitory activity against PfIspD but show activity in the low micromolar region against *Plasmodium* parasite growth (Table 6.2). This clear distinction between enzymatic and phenotypic antimalarial activity therefore indicates that BITZ derived compounds may be capable of exerting off-target inhibitory effects which are not specific to the PfIspD enzyme but that contribute to *P. falciparum* parasite growth inhibition.
Table 6.2 Inhibitory activity of biphenyl pyridinyl BITZ analogues 4-7 against the PfISPd enzyme and *P. falciparum* growth in culture (strain 3D7).

If this assumption is correct, further knowledge regarding an additional molecular target would be highly beneficial for ongoing and informed antimalarial design. It was therefore appropriate to ask the question: are there any additional protein targets within *P. falciparum* parasites that the BITZ chemotype and derived compounds are capable of targeting and inhibiting?

Consequently, a chemical-biology approach was employed aiming to identify the localisation and identity of any additional molecular target within malarial-causing *P. falciparum* parasites that the BITZ chemotype may be able to interact with. In order to facilitate this study, a small range of tagged-chemical probes were designed and synthesised which were used to monitor the behaviour of BITZ derived compounds in the cell.

### 6.2 Biological Probes

It is not uncommon that potential clinical compounds are unsuccessful due to a lack of specificity (prominent off-target effects) and a limited understanding of their role or behaviour at a suggested protein target. It is therefore important to have knowledge of the overall behaviour of a compound, looking beyond target-directed design and *in vitro* optimisation. A broad range of technologies are available to assess and enhance the understanding of drug and chemotype selectivity within cells and animal models including chemical probes, proteomics and screening tools. In particular, activity-based probes (ABPs) are widely used to more fully understand the biological roles of chemical agents, measuring both off-target effects and on-target inhibition within *in vitro* and *in vivo* studies.
Chemical probes can be described as small, often synthetic molecules that are used to establish the relationship between a molecular target and a drug, or to explore the role of a protein or a chemical agent in the broader biological context of cells and organisms. A chemical probe is therefore a selective small-molecule that is able to modulate a protein’s function and can itself be monitored within the cell. Such a tool enables specific biological questions to be addressed regarding the behaviour of drugs and the cellular function of proteins in biochemical, cell-based and animal studies.

### 6.2.1 Using Biological Probes to Address Research Questions

Chemical probes are used widely in modern research programs since they facilitate the study of specific binding events between chemical agents and specified cellular components in organisms and cells. Recent work by Wang et al. highlights the use of a fluorescent mD1 probe to study the specific binding of the mD1 protein to an inhibitor and its target HIV protein for the development of HIV detection methods. The designed fluorescent mD1 probe (or protein sensor) contained a Föster resonance energy transfer (FRET pair) that can directly detect the gp120 protein of the HIV virus, potentially enabling earlier detection of HIV infection.

The use of ABPs have also been successfully applied within a number of drug discovery projects focused on the development of protease inhibitors. Proteases are well-established drug targets within pharmaceutical development due to their regulatory roles in many human pathologies, including degenerative, inflammatory and infectious diseases. However, the development of protease inhibitors as drugs remains a challenge since achieving target specificity represents a significant hurdle, despite major advances in many biochemical technologies. The use of ABPs has enabled the enhanced assessment of drug selectivity within this area, as well as improved understandings of the biological role of proteases. Indeed, Xiao et al. developed ABPs to label and inhibit engineered caspases, which are cysteine proteases with essential roles in apoptosis and inflammation. The engineered labelling of these proteins enabled the study of caspase function within a timeframe suitable for monitoring the events of apoptosis.

In a contrasting area of research, development of BET bromodomain inhibitors provides an appropriate demonstration of the power of chemical probes in opening up new areas of biology and developing biochemical and molecular understanding. BET proteins govern the assembly of histone complexes which regulate inflammatory gene expression and therefore represent a point of potential intervention in inflammatory gene expression. The use of chemical probes in this field was initiated
by the development of small molecule BET inhibitor probes that were structurally related to previously identified small molecule inhibitors of the BET family of bromodomains (Figure 6.1).[^7,^8]

![Chemical structures of (+)-JQ1 and GSK525762A](image)

**Figure 6.1** Chemical structures of (+)-JQ1[^8] and GSK525762A[^7]; structurally related small molecule BET inhibitor probes which highlighted key protein-protein interactions between BET bromodomains and histone N-acetyl lysine residues, highlighting the therapeutic potential of BET inhibition in tumour cell proliferation therapy.

These structurally similar probes were shown to inhibit protein-protein interactions (PPIs) between BET bromodomains and N-acetyl lysine residues on histones; subsequently regulating gene expression and suppressing transcription of pro-inflammatory genes involved in the propagation of inflammation and tumour cell proliferation. Facilitated and assisted by the use of chemical probes, these discoveries broadened the therapeutic potential of BET inhibition and ultimately led to the initiation of more BET inhibitor drug discovery programs.[^6]

It is therefore clear that biological probes can be highly diverse and designed with specific purpose to tackle individual research questions within a wide range of clinical and biochemical settings.

In order to address our own research question, a small series of chemical probes were designed and synthesised to monitor the localisation of BITZ derived inhibitors within *Plasmodium* parasites, with the aim of determining any additional proteins to which the BITZ chemical chemotype may be capable to bind. Two distinct chemical probes were used to evaluate whether the BITZ chemotype is indeed capable of binding to additional molecular proteins within the *Plasmodium* parasite and where these protein targets may localise. Consequently, these studies aimed to determine whether enhanced inhibitory activity of BITZ compounds against *Plasmodium* parasite growth, compared to the isolated PfIsPD enzyme, is the result of interaction with additional cellular targets within the parasite in addition to the known and confirmed activity against the PfIsPD enzyme.

A fluorescent-labelled BITZ inhibitor was designed as an initial tool to evaluate localisation of the BITZ chemotype with *Plasmodium* parasites. In addition, a biotin-tagged probe was generated to facilitate
pull-down assays, using streptavidin-agarose beads, for the purpose of determining the parasite proteins to which the BITZ chemotype can bind.

6.3 Identification of Suitable Chemical Motifs to Generate BITZ and Control Probes

It was proposed that the use of a simple BITZ derivative as the core recognition structure for our fluorescent-labelled and biotin-tagged chemical probes would be appropriate for these studies. Chemical- or activity-probes are generally small-molecule reporters that are designed to be selective for a specified biological target, providing a representative assessment of a particular chemical motif and its behaviour within living cells and organisms.\[3] Generally, chemical probes consist of three distinct parts: a chemical warhead (the drug motif), a spacer region and a specified tag (i.e. a fluorophore or biotin tag).\[3]

With a chemical probe construct such as this, designed probes can also behave as inhibitors of a target enzyme, allowing them to function as suitable mimetics of the chemical chemotype in question, enabling activity, localisation and distribution studies to be conducted within organisms and cells. It was decided that compound 8 (Figure 6.2) would be used as the chemical warhead of BITZ probe molecule 10 (Figure 6.2) with added functionalities to incorporate the required spacer region and appropriate tag molecule, as required for the described probe construct.\[1]

![Figure 6.2 Chemical structures of simple BITZ derivative 8 and related isostere 2-phenylisoindoline-1,3-dione, 9.](image)

As described in Chapter 2, 8 is a simple BITZ core derivative with an un-substituted N-phenyl ring that functions as a basic side chain and is therefore a very simplified derivative of the original DT2009-0168020 HTS hit (Chapter 2, Figure 2.8). Following PfIspD enzymatic and 3D7 whole cell growth assays, we have shown 8 to be an active inhibitor against the PfIspD enzyme and Plasmodium parasite growth (Table 6.3). 8 has consequently been used as a robust reference point for SAR development around the BITZ chemotype (Chapter 2, Section 2.4) and therefore represents a suitable starting point for the design of inhibitory chemical probes that function as an accurate representative of the BITZ chemotype and derived inhibitors.
<table>
<thead>
<tr>
<th>Compound number</th>
<th>PfISPd Inhibition IC\textsubscript{50} (μM)</th>
<th>3D7 Whole Cell Growth Inhibition IC\textsubscript{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.599</td>
<td>5.49</td>
</tr>
<tr>
<td>9</td>
<td>&gt; 100</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 6.2** Inhibitory activity of 8 and 9 against PfISPd and *P. falciparum* 3D7 growth in culture.

It was also necessary to generate a control probe which could be used in inhibitor localisation studies and pull-down assays that would be inactive against both the PfISPd enzyme and *Plasmodium* parasite growth. A suitable control motif must be deficient in the ability to inhibit PfISPd and parasite growth and therefore provide a distinction between reactive and non-reactive probes. An appropriate chemical motif for the control probe must therefore lack the ‘active’ electrophilic sulfur atom and N-S heteroatom bond of the BITZ thiazolone ring. As a result, the related BITZ isostere, 2-phenylisoindoline-1,3-dione (9, Figure 6.2) was selected as the warhead for the control probe 11 (Figure 6.3) as this motif has been shown to be inactive against the PfISPd enzyme (Table 6.3) in previous chemotype validation studies (see Chapter 2, Section 2.4). Having identified compounds 4 and 5 (Figure 6.1) as the warheads for the reactive and non-reactive chemical probe species, these motifs were used for the development of novel BITZ probes and isoindoline-1,3-dione derived control probes respectively.

### 6.4 Localisation Studies

#### 6.4.1 Fluorescent Molecular Probes

A fluorescent 7-nitrobenza-2-oxa-1,3-diazole (NDB) tagged BITZ probe, and corresponding NBD-tagged control probe were designed and synthesised to facilitate the use of laser scanning confocal microscopy in order to observe the localisation of the BITZ motif and derived inhibitors with in the *Plasmodium* parasite.

Fluorescence is highly exploited in the employment of chemical probes and biosensors. To date, a huge variety of fluorescent probes have been designed to enable the recognition and evaluation of a diverse range of molecules and their biochemical effects within biological systems. Many analytical methods are available for gathering information about the interaction of chemical agents with living cells including absorption and fluorescence spectroscopy, electrochemical methods, fluorescent microscopy, and isotope-based methods. Fluorescent microscopy is a particularly valuable fluorescence imaging technique since it is both straightforward and easily initiated, and now supported by advanced optical and microscopy equipment, with better access to specialised confocal
laser scanning microscopy instruments which can provide enhanced cellular images.\textsuperscript{[9]} The operational simplicity of fluorescent sensors and probes, alongside their sensitivity and suitability for bio-imaging within living systems, has led to widespread use and development of this detection technique.\textsuperscript{[10]}

Various types of fluorescent probe are widely used by analytical scientists, organic chemists, and medical scientists across a vast number of research programs to provide insight into the behaviours of chemical agents within cells and organisms. Fluorescent probes can determine the function of specific proteins and the structural and biological responses of a cell to molecular modifiers. Successful applications of fluorescent probes have been demonstrated by many researchers and are well reviewed in literature.\textsuperscript{[4,5,9,11–13,13–24]} Whatever the function or application of a fluorescent probe, several requirements need to be accounted for when designing such a molecular probe. A fluorescent molecule should contain: a fluorophore capable of displaying an efficient level of emission which can be observed by the detection instrument, show minimal interference with auto fluorescence from the biological sample, and contain specific functional groups that allows the recognition and interaction of the probe with the appropriate molecular features of the cell or organism under study.\textsuperscript{[9]}

Fluorophores emit electromagnetic radiation when luminescing at an appropriate excitation wavelength ($\lambda_{\text{ex}}$). The absorption of a photon of light at $\lambda_{\text{ex}}$ excites the fluorophore from its ground state to an accessible excited electronic state; subsequent relaxation (or radiative deactivation) of the excited fluorophore back to the original ground state results in fluorescence.\textsuperscript{[17,25]} The fluorescence quantum yield ($\phi_F$) of any given fluorophore gives the ratio of photons absorbed to photons emitted via fluorescence.\textsuperscript{[26]}

Considering the requirements outlined above, the NBD fluorophore (highlighted in green, \textbf{Figure 6.3}) was selected and incorporated into the NBD-tagged BITZ probe \textbf{10} and corresponding NBD-tagged control probe \textbf{11} (\textbf{Figure 6.3}). \textbf{10} and \textbf{11} were specifically designed as a fluorescent-labelled inhibitor and a fluorescent-labelled non-inhibitor of the PfIspD enzyme respectively, to facilitate imaging of the BITZ motif at the cellular level. The chemical warhead of probes \textbf{10} and \textbf{11} are highlighted in red with the spacer region of each probe highlighted in blue (\textbf{Figure 6.3}).
Figure 6.3 Chemical structures of the NBD-tagged BITZ probe 10 and NBD-tagged control probe 11; chemical warheads are highlighted in red, the spacer regions in blue and NBD fluorophore in green.

4-chloro-7-nitrobenza-2-oxa-1,3-diazole (NDB-Cl) (Figure 6.4) is a commercially available, amine-reactive chemical compound where NBD-amine adducts display a fluorescent excitation/emission spectrum of $\lambda_{\text{ex}}/\lambda_{\text{em}} = 464/512$ nm respectively, in aqueous solutions. NDB-Cl is widely used in the labelling of peptides, proteins, drugs and other biomolecules for a broad range of applications.\[17,27]\ NDB-Cl itself is non-fluorescent but forms highly fluorescent compounds on reaction with amine groups in synthetic amines, amino acids, peptides and proteins; NDB-Cl also reacts with thiol groups to form fluorescent adducts.\[28\]

Figure 6.4 Chemical structure of 4-chloro-7-nitrobenza-2-oxa-1,3-diazole (NDB-Cl).

Literature data has illustrated that the nature of substituents attached to the NBD platform have significant influence on the fluorescence quantum yield of the NBD fluorophore. NBD-derivatives with electron withdrawing groups at both the 4- and 7-poitsons are generally not emissive,\[17\] as is the case with the non-fluorescent reagent NBD-Cl (Figure 6.4). The transformation of one of these two functionalities into an electron donating group, as is achieved through substitution with an amine, subsequently enabling radiative deactivation of the excited fluorophore and consequently fluorescence emission from the NBD-derivative.\[17\] The strong electron withdrawing nitro-group
makes the para C-Cl bond highly reactive towards nucleophilic substitution and therefore able to react readily with a wide range of amines, forming fluorescent NBD-derivatives.\cite{10}

Altogether, the established chemistry of the 2-nitrobenzyl group, alongside the synthetic accessibility of NBD derivatives, the ability to excite the NBD fluorophore with visible photons and the high fluorescence quantum yield of NBD derivatives, all contributed to the section of the NBD fluorophoric group for the generation of our fluorescent-labelled probes.\cite{17,27}

6.4.2 Synthesis of the NBD-Tagged BITZ Probe and NBD-Tagged Control Probe

The generation of NBD-tagged BITZ probe 10 required a linear five-step synthesis involving several coupling and de-protection reactions (Scheme 6.1). The synthetic strategy was initiated by a simultaneous amide coupling and ring closure between N-Boc-m-phenylenediamine and the stable and isolatable acid chloride, 2-(chlorothio)-benzoyl chloride X (Figure 6.5, previously described in Chapter 2, Section 2.3.3).\cite{29,30}

Following coupling of X with the mono-Boc protected diamine, the Boc-group of 10a was removed mediated under acidic conditions by TFA,\cite{31} to provide the free amine 10b. 10b was subsequently coupled with a second mono-Boc protected reagent, Boc-S-Ava-OH, using a standard amide-coupling reaction\cite{32,33} to give 10c. The Boc-group of 6c was also removed under acidic conditions using TFA to generate 10d.\cite{34} Boc-S-Ava-OH was incorporated into the outlined syntheses (Scheme 6.1 and 6.2) to facilitate the introduction of the required spacer region (highlighted in blue, Figure 6.3) within both the NBD-tagged BITZ probe 10 and NBD-tagged control probe 11.

![Chemical structure of the stable and isolatable acid chloride 2-(chlorothio)-benzoyl chloride X.](image)

**Figure 6.5** Chemical structure of the stable and isolatable acid chloride 2-(chlorothio)-benzoyl chloride X.

The final step of the illustrated synthesis (Scheme 6.1) involved a nucleophilic substitution reaction between 10d and NBD-Cl to incorporate the NBD fluorophore and generate the fluorescent NBD-amine probe 10. A similar approach was previously demonstrated by Ellis et al. where a comparable synthetic approach and research strategy was employed to determine the parasitic accumulation sites of tetraoxane antimalarial agents using fluorescent microscopy to visualise NBD-tagged tetraoxane derivatives.\cite{21}
Scheme 6.1 Synthetic strategy for generation of NBD-tagged BITZ probe 10.

All reactions within the described synthetic route (Scheme 6.1) preceded in moderate to high yields, with the exception of the final coupling between 10d and NBD-Cl to generate 10. For the generation of the control probe 11, a shorter three-step synthesis (Scheme 6.2) was feasible due to the commercial availability of 2-(3-aminophenyl)-2,3-dihydro-1H-isoindole-1,3-dione, which is the meta-amine derivative of 2-phenylisoindoline-1,3-dione, 12 (Figure 6.2), the structural warhead of 11.

The first of the three-step synthesis involved amide-coupling of 2-(3-aminophenyl)-2,3-dihydro-1H-isoindole-1,3-dione and the mono-Boc protected species, Boc-5-Ava-OH, generating 11a in an analogous amide-coupling reaction to that describe in the synthesis of 10. Using HATU as the coupling reagent, this amide-coupling reaction again performed well and proceeded in high yield. TFA-mediated Boc-deprotection of 11a subsequently generated the free amine 11b which was able to undergo nucleophilic substitution with NBD-Cl to incorporate the specified fluorescent NBD functionality and afford the designed control probe 11.
Scheme 6.2 Synthetic strategy for generation of NBD-tagged control compound 11.

Across both outlined synthetic strategies (Scheme 6.1 and 6.2), the incorporated Boc-deprotection reactions, mediated by TFA,[31] showed varying efficiency where poor reaction yields appear to correlate with increasing reagent complexity, potentially impeding reaction efficiency. HATU-mediated amide-coupling reactions were efficient and high yielding for both syntheses. Nucleophilic substitution of NBD-Cl with 10d and 11b, as outlined by Niu et al.[10] was successful and generated the target compounds in both instances. However, the aromatic nucleophilic substitution was consistently low yielding in the synthesis of both 10 and 11 which was a contrast to the results demonstrated by Niu et al.[10]

6.4.3 Inhibitory Activity of the NBD-Tagged BITZ Probe and NBD-Tagged Control Probe Against PfIspD

Having generated both the NBD-tagged BITZ probe 10 and the NBD-tagged control probe 11, it was necessary to determine the extent of their inhibitory activity against the PfIspD enzyme. If selection of the 2-phenylisoindoline-1,3-dione motif (9, Figure 6.2) was appropriate for the chemical warhead of the control probe 11 (Figure 6.3), a lack of inhibitory activity against PfIspD on exposure to 11 should be seen. In direct contrast, there was a strong requirement for the NBD-tagged BITZ probe 10 to exhibit inhibitory activity against the PfIspD enzyme. Ideally, a comparable or enhanced IC50 value to that displayed by (8, Table 6.2) would be seen therefore enabling 10 to function as an accurate BITZ inhibitor mimic, providing a representative picture of BITZ chemotype behaviour within the parasite.
Evaluation of 10 and 11 in the PfIspD enzymatic assay\(^{[35]}\) (previously discussed in Chapter 2, Section 2.2.3) highlighted that we were correct in our assessment and selection of the required chemical warheads for the BITZ probe (10) and control probe (11). The NBD-tagged BITZ probe 10 was found to have an IC\(_{50}\) value of 0.335 μM against the PfIspD enzyme and is therefore almost twice as potent against the enzyme as the simple BITZ derivative 8. Whereas 11 was shown to be inactive against PfIspD, with an IC\(_{50}\) value of > 40 μM (Table 6.4).

<table>
<thead>
<tr>
<th>Compound number</th>
<th>PfIspD Inhibition IC(_{50}) (μM)</th>
<th>SEM value (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.335</td>
<td>0.073</td>
</tr>
<tr>
<td>11</td>
<td>&gt; 40</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.4 Inhibitory activity of NBD-BITZ probe 10 and control probe 11 against the PfIspD enzyme; mean IC\(_{50}\) and SEM values given which are representative of n = 3 replicates.

The lack of activity displayed by 11 confirms that the 2-phenylisoindoline-1,3-dione derived headgroup is a suitable chemical control, again demonstrating that loss or replacement of the sulfur heteroatom in the thiazolone ring (B ring) of BITZ abolishes PfIspD inhibitory activity. It is also evident that the extent of PfIspD activity loss, comparing 10 and 11, is of a similar magnitude to that observed between 8 and 9; where greater than a 100-fold potency loss was observed in each case on alteration of the BITZ headgroup. Overall, the measured PfIspD enzymatic activity data illustrates that 10 and 11 can be used effectively as fluorescent probes to assess the behaviour and localization of BITZ derived inhibitors in Plasmodium parasites, monitoring the cellular accumulation of these compounds by fluorescent microscopy.

6.4.4 Inhibitory Activity of the NBD-Tagged BITZ Probe and NBD-Tagged Control Probe Against 3D7 P. falciparum Parasite Growth

Since use of the NBD-tagged BITZ probe in fluorescent microscopy aims to assess localization of the BITZ chemical motif within Plasmodium parasites by observation of the whole cell, it was also appropriate to determine the inhibitory activity of 10 and 11 against P. falciparum growth in culture. As previously described (see Chapter 2, Section 2.3.5), a DNA-based microfluorometric assay measuring the extent of P. falciparum growth in culture, after treatment with various chemical agents, is used to determine the ability of a compound to inhibit parasite growth. This assay is facilitated by the detection of parasite DNA intercalation with PicoGreen®, an ultrasensitive fluorescent nucleic acid dye.\(^{[36]}\)
Having generated the fluorescent BITZ probe and control probe, 10 and 11, incorporating the NBD fluorophore to achieve compound fluorescence, the described whole cell *P. falciparum* growth assay using PicoGreen® as the nucleic acid detection dye, was no longer appropriate to assess levels of parasite growth inhibition. This is a result of overlap between the excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) wavelengths of the NBD-amine fluorophore present in 10 and 11 and PicoGreen® (Table 6.5).

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation/Emission Wavelengths (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBD-amine</td>
<td>464/512</td>
</tr>
<tr>
<td>PicoGreen®</td>
<td>485/520</td>
</tr>
<tr>
<td>Hoechst 33258</td>
<td>352/455</td>
</tr>
<tr>
<td>Acridine Orange</td>
<td>460/530</td>
</tr>
</tbody>
</table>

*Table 6.5* Excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) wavelengths (in nm) of various fluorophores.

The overlapping excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) spectra of the NBD-amine and PicoGreen® fluorophores prevents the use of the *P. falciparum* growth assay as previously employed.\[37,38\] Interference between the two fluorophores would distort measurement of the PicoGreen® nucleic acid detection dye, therefore misrepresenting the magnitude of *P. falciparum* growth inhibition. Consequently, an alternative nucleic acid dye was required, displaying a $\lambda_{ex}/\lambda_{em}$ spectrum distinct from the NBD-amine fluorophore to enable the activity of 10 and 11 against *P. falciparum* parasite growth in culture to be determined.

Hoechst 33258 was identified as a suitable nucleic acid dye to replace PicoGreen® in our *P. falciparum* growth assay since the $\lambda_{ex}/\lambda_{em}$ spectra of NBD-amine and Hoechst 33258 are appropriately distinct (Table 6.5). In order to assess the efficiency of Hoechst 33258 as a nucleic acid detection dye within the *P. falciparum* growth assay, a test assay was conducted using untreated *P. falciparum* culture and a two-fold parasitaemia (Pt) dilution series. The parasite DNA content of untreated cultures in two parallel assays was measured independently with both Hoechst 33258 and PicoGreen® to provide a measure of assay efficiency and read-ability using each dye (Figure 6.6).

The linear relationship observed between the detected of levels Hoechst 33258 fluorescence and the parasitaemia of *P. falciparum* culture, illustrates that Hoechst 33258 is a suitable replacement for PicoGreen® as a nucleic acid dye within the *P. falciparum* growth assay. This result therefore enabled measurement of the inhibitory *P. falciparum* parasite growth activity of 10 and 11 using the standard *P. falciparum* parasite growth assay.
**Figure 6.5** Comparison of the detected fluorescence from *P. falciparum* infected cultures determined using microfluorimetrics, measured separately using Hoechst 33258 and PicoGreen® as nucleic acid dyes. Asynchronous *P. falciparum* infected culture was used in the assay which was set up using a two-fold series dilution of parasitaemia.

Further analysis of the collected data highlighted that both high and comparable R\(^2\) values were determined with the use of each nuclear dye (PicoGreen® R\(^2\) = 0.9385; Hoechst 33258 R\(^2\) = 0.9773). R-squared (R\(^2\)), the coefficient of determination (ranging from 0 and 1), defines the proportion of variance in a dependent variable that is predictable from an independent variable. The resulting value of R\(^2\) therefore indicates the extent to which the dependent variable can be predicted by the independent variable, consequently providing a measure of accuracy.\(^{[39,40]}\) R\(^2\) values close to a value of one indicate that a model can explain the majority of variability seen in measured data and therefore gives confidence in the parameters or protocol being measured.\(^{[39]}\) Hence R\(^2\) analysis of the DNA-based microfluorometric assay (**Figure 6.6**) gives confidence in the use of Hoechst 33258 as a nucleic acid dye to provide an accurate measurement of the inhibitory activity of 10 and 11 against *P. falciparum* parasite growth.

Despite this encouraging assessment, application of the adapted whole cell assay using Hoechst 33258 did not produce interpretable results. It is possible that reduced sensitivity of Hoechst 33258, as a DNA intercalator, compared to PicoGreen® is responsible for the lack of interpretable results in the adapted assay protocol.\(^{[36]}\) As such, further work is needed to identify a suitable fluorescent nucleic acid dye, or an alternative assay procedure, to enable the inhibitory activity of fluorescent probes 10 and 11 to be determined against *P. falciparum* parasite growth.
6.4.5 Localisation Studies using Fluorescent Microscopy

To identify the cellular localisation of the inhibitory PfispD BITZ motif, a series of incubation and localization experiments were performed to enable the fluorescent imaging of BITZ probe 10 in 3D7 culture. These studies were also mirrored by analogous control experiments using the non-BITZ control probe 11, shown to be inactive against the PfispD enzyme (Table 6.4).

To initiate this body of work, protocol modifications were conducted to determine optimal conditions for incubation of 3D7 P. falciparum cultures with fluorescent probes 10 and 11, prior to visualisation of the treated cultures by fluorescent microscopy. It was found that an overnight incubation period of ~ 20 - 22 hours, maintaining 500 µL of parasite culture at 37 °C in an atmosphere of 5% O₂; 5% CO₂ and 90% N₂, with a 1:1000 dilution factor of 10 and 11, to be most optimal. Asynchronous parasite cultures were used to enable visualisation of Plasmodium parasites at all stages of the parasite life cycle. A high level of parasitaemia was maintained within cultures to ease the identification of parasite infected cells and therefore enable cellular images to be easily captured during fluorescent, live-imaging microscopy.

Fluorescent microscopy was carried out using Olympus BH8 and Olympus Fluoview FV1000 confocal microscopes to visualise the fluorescent NBD-tagged BITZ probe 10 and the fluorescent NBD-tagged control probe 11 within P. falciparum infected cultures. Hoechst 33258 nucleic acid dye was also added to the treated cultures to enable the identification of parasite nuclei by blue ultraviolet (UV) light and therefore inform the assessment of BITZ compound localisation with respect to parasite nuclei.

Imaging of treated parasite cultures (Figures 6.6 - 6.9) immediately revealed that both the fluorescent BITZ probe 10 and control probe 11 are able to penetrate the cell and continue to emit green fluorescence at an easily detectable level. An extensive range of images were obtained using live microscopy imaging, facilitated with the Olympus Fluoview FV1000 confocal microscope; a selection of representative images are presented in Figures 6.6 - 6.9. Figures 6.6 and 6.7 depict 3D7 P. falciparum parasite cultures that were treated with fluorescent BITZ probe 10; whereas Figures 6.8 and 6.9 exemplify selected parasite cultures that were treated with the fluorescent control probe 11.
Figure 6.6 Representative images of BITZ probe 10 after incubation with 3D7 P. falciparum cultures; images of infected cells were taken using white light, blue UV light and green UV light providing a phase image, visualisation of parasite nuclei and the fluorescent BITZ probe respectively.

Figure 6.7 Representative images of BITZ probe 10 after incubation with 3D7 P. falciparum cultures; images of infected cells were taken using white light, blue UV light and green UV light providing a phase image, visualisation of parasite nuclei and the fluorescent BITZ probe respectively.

The microscopy images taken using natural (or white) light provide phase contrast and were used to initially identify and focus on a parasite infected cells of interest for further fluorescent imaging. A short exposure time of 1/200 of a second was required to capture phase images. Blue UV light was used to view the nuclear Hoechst 33258 dye which enables cells to be seen under UV light and consequently parasite nuclei to be identified; a slightly longer exposure time of 1/10 of a second enabled these images to be obtained. The GFP (green fluorescent protein) images were obtained using green UV light and depict the NBD-tagged probe compounds following incubation with the parasite cultures. An exposure time of 2 seconds was required for these images.

These described localisation studies, facilitated by fluorescent microscopy, are only in preliminary stages but some initial conclusions have been drawn. As illustrated in Figures 6.6 and 6.7, a clear distinction in cellular localisation is observed between the Hoechst nuclear dye and our fluorescent BITZ probe 10, highlighting that the BITZ chemotype and derived compounds are excluded from the parasite nucleus. It can also be seen that green fluorescence distribution is cytoplasmic, with reasonably uniform distribution across the cell. In addition, green fluorescence appears also to be
excluded from the parasite food vacuole, although further microscopy studies should be conducted to validate this.

Whilst it may not be apparent from the representative images provided here, more extensive and ongoing microscopy work has highlighted that the accumulation of fluorescent BITZ probe 10 appears to be parasite selective. In other words, penetration of the fluorescent NBD-tagged probes 10 and 11 was not observed in red blood cells (RBCs) that were not infected with Plasmodium parasites.

Figures 6.8 and 6.9 provide representative images of the microscopic observations following the treatment of 3D7 P. falciparum cultures with the fluorescent control probe 11. Interestingly, no distinct contrast in fluorescence patterns are observed by comparison of these images (Figures 6.8 and 6.9) to those obtained after treatment of 3D7 P. falciparum cultures with fluorescent BITZ probe 10 (Figures 6.6 and 6.7). Figures 6.8 and 6.9 highlight that green fluorescence distribution remains cytoplasmic with probe compound 11 and is excluded from both the parasite nucleus and food vacuole. This observation is perhaps somewhat unexpected since it has already been shown that control probe 11 is inactive against the PfIspD enzyme (Table 6.4). As a result of this, we may have
expected to see a difference in cellular distribution compared to BITZ probe 10, which is known to actively inhibit PfispD (Table 6.4).

It is therefore clear that additional fluorescent microscopy is required to further evaluate BITZ treated Plasmodium parasite cultures and determine a more precise localisation pattern of the BITZ chemotype. In turn, it may then be possible to highlight any contrasting patterns of distribution that may be observed with chemical motifs that do not inhibit the PfispD enzyme. Additional microscopy studies should also be considered to shed further light on the behaviour of these chemical probes within Plasmodium parasite and RBCs. Indeed, the use of an apicoplast marker could be introduced to more explicitly highlight the organelle within the parasite, providing a clearer indication of BITZ localisation with respect to this cellular component. The apicoplast is a plastid organelle that is a unique feature of Apicomplexan species and houses the MEP biosynthetic pathway and constituent enzymes. Therefore, analysis of BITZ probe localisation in Plasmodium infected RBCs, with an added awareness of this crucial and highly relevant parasite organelle, may enable more informed fluorescent microscopy studies from here-on in.

Furthermore, an assessment of cellular morphology, facilitated by GIEMSA staining of cultures in a non-fluorescent microscopy setting, could highlight and physiological differences that may be apparent between BITZ-treated and untreated cells. Consequently, such a study has the potential to highlight any effects on cell that may be induced by treatment with BITZ compounds in terms of cellular health and viability.

6.5 Protein Binding Assays

6.5.1 Streptavidin Affinity Pull-down Assays

An alternative biochemical method, suitable for determining the parasite proteins to which the BITZ chemotype can bind, is a peptide pull-down assay. Pull-down assays are used to identify the natural binding partners of captured proteins or chemical agents and can also confirm the presence of a protein species or an enriched protein within a biological or cellular system. Therefore, pull-down experiments can be used to determine the interactions that occur between a biochemical probe and un-identified proteins, as well as confirm suspected interactions between a probe protein and a known protein.

Pull-down assays represent an in vitro method of affinity purification where a ‘bait molecule’ is used to tag and capture proteins, quickly isolating the proteins which are able to bind to the ‘bait molecule’ (or tag) and allowing their identification. The ‘bait molecule’ must therefore contain a functional or chemical recognition element (or ‘warhead’: represented by a red triangle, Figure 6.10) that is able to
capture certain proteins within cell lysate. The ‘bait molecule’ must also contain a separate reactive ‘tag’ (represented by a green star, Figure 6.10) that is able to bind to a specific affinity ligand, enabling capture of the probe and consequently identification of the proteins which are bound to the chemical recognition element of the probe.

Figure 6.10 Illustration of the general components and construct of a chemical probe for use in protein binding, pull-down assays: image reproduced from [44].

A number of reactive ‘tags’ are commonly used within these labelling experiments where each ‘tag’ has a specific affinity ligand to which it is able to bind; a number of tag-affinity ligand pairs are summarised in Table 6.6 below.\(^{[43]}\)

<table>
<thead>
<tr>
<th>Tag Molecule</th>
<th>Affinity Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione S-transferase (GST)</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Poly-histidine</td>
<td>Nickle or Cobalt chelate complexes</td>
</tr>
<tr>
<td>Biotin</td>
<td>Streptavidin</td>
</tr>
</tbody>
</table>

Table 6.6 Commonly used protein-reactive ‘tags’ and their associated affinity binding ligands.\(^{[43]}\)

The binding of a reactive ‘tag’ to its immobilised affinity ligand generates a secondary affinity support, enabling proteins that have become attached to the chemical recognition element (‘warhead’) of the probe following exposure to a protein source (i.e. cell lysate), to be identified. Following incubation with nuclear extract, the secondary affinity support is extensively washed, allowing the separation of bound and un-bound proteins; bound proteins are then released and eluted from the support to enable their purification and identification. The method of protein release and elution is generally specific to the affinity ligand used, and can be modified as appropriate.\(^{[43,45]}\) In addition to its simplicity, another advantage to a peptide pull-down assay is the unbiased nature of the approach, which allows the identification of binding partners, even if they had not been considered likely binding
candidates.\textsuperscript{[45–47]} As a result, this methodology is appropriate for addressing research questions considering off-target effects of molecular and chemical agents.

A commonly used peptide pull-down experiment is the streptavidin-agarose pull-down assay as it is easy to perform and does not require the use of radiolabelled probes.\textsuperscript{[48]} Employment of the biotin-streptavidin tag-affinity ligand pair (Table 6.6) facilitates this assay, which involves the incubation of nuclear extract from a specific cell with biotinylated molecular probes and streptavidin-agarose beads. These beads capture and immobilise any proteins within the nuclear extract that are able to bind to the chemical recognition element (biotin) of the molecular probes. The cellular proteins bound to the resulting biotin-streptavidin complex can then be dissociated from the support and identified using a number of methods, such as Western blotting and LC-MS/MS.\textsuperscript{[48]} A streptavidin-agarose pull-down assay can therefore quantitatively analyse an array of proteins to which a probe molecule is found to bind.\textsuperscript{[48]} Biotinylated probes are compounds or proteins that have been labelled with biotin (Figure 6.11) and also contain a specific chemical recognition element (or ‘warhead’, see Figure 6.10), the cellular reactivity of which is likely under investigation.

![Chemical structure of biotin](image)

**Figure 6.11** Chemical structure of biotin.

The model pull-down technique is an invaluable tool for enabling the enhanced study of cellular pathways, the PPIs (protein-protein interactions) that occur within them and how small molecular or chemical agents may interact with components in a defined biological pathway. It is perhaps most common for pull-down assays to be used in the confirmation or detection of PPIs within a cell or biological system, where a ‘bait protein’ is tagged and captured using a specific affinity ligand, enabling the identification of cellular proteins which interact with the bait protein.\textsuperscript{[43]} Within the context of our research, the use of a streptavidin pull-down assay will involve tagging a chemical probe as a ‘bait molecule’ to both confirm and detect the proteins to which the BITZ chemical motif is able to bind within *Plasmodium* parasites.

Consequently, the aim of our own streptavidin pull-down assay is to incubate *Plasmodium* parasite nuclear extract with a biotinylated BITZ probe in the presence of streptavidin-conjugated agarose beads. Proteins in the parasite nuclear extract, with which the BITZ chemotype can interact, will bind to the BITZ motif that is incorporated as the ‘warhead’ of our biotinylated chemical probes (Figure
6.12). Following BITZ-protein binding events, the entire complex can bind to the streptavidin-agarose beads via interaction of the biotin tag. Having immobilised the biotinylated probe on streptavidin-agarose beads, the proteins bound to the BITZ motif can then be identified and analysed.\cite{48} The streptavidin-agarose pull-down has been shown to be a suitable tool for the analysis of PPIs and protein-chemical interactions in a number of diverse research projects \cite{1, 6, 44, 45, 48–56} and therefore represents an appropriate assay for our own research, enabling the question of promiscuous binding of the BITZ motif to be addressed.

The biochemical streptavidin pull-down method was therefore employed to facilitate the unbiased identification of proteins to which the BITZ chemotype and derived inhibitors can bind in *Plasmodium* parasites.

6.5.2 Design of Biotinylated BITZ and Control Probes

It was necessary to generate a biotinylated BITZ probe compound, accompanied by an appropriate control probe, to enable application of the described streptavidin pull-down technique. The ‘bait molecules’, as discussed, are represented by the designed biotinylated chemical probes 12 and 13 (Figure 6.12). In order to generate a biotinylated BITZ probe and control probe, a similar design strategy, mirroring the rationale employed for generation of the fluorescent NBD-tagged BITZ probe and control probe, 10 and 11 (Section 6.3 and 6.4) was used. The simple BITZ derivative 8 and related 2-phenylisoindoline-1,3-dione isostere, 9 (Figure 6.2), were again selected as warheads for the active and non-active biotin-tagged BITZ probe and biotin control probe respectively.

![Figure 6.12 Biotin probe motif and resulting structures of the biotinylated BITZ probe 12 and biotinylated control probe 13; image reproduced from 44.](image-url)
Work by others has found that carbamate-biotin and amide-biotin connections are amongst the most stable linkages that can be used in the biotinylation of chemical compounds.\textsuperscript{[44,56]} Since an amide-biotin linkage is easily generated between our selected chemical warheads and the carboxylic acid side chain of biotin (Figure 6.12), this linkage was incorporated into both the biotinylated BITZ probe 12 and biotinylated control probe 13, reflecting a design strategy that has been successfully demonstrated in previous work by others.\textsuperscript{[44]}

\textbf{6.5.3 Synthesis of the Biotinylated BITZ Probe and Control Probe}

The generation of biotinylated BITZ probe 12 was achieved using a three-step synthesis, in which all reactions proceeded in moderate to high yields (Scheme 6.3). Analogous to the synthesis of NBD-tagged BITZ probe 10 (Figure 6.3), the first two synthetic steps involved an amide coupling and ring closure between the mono-Boc protected diamine, \(N\)-Boc-\(m\)-phenylenediamine, and the stable and isolatable acid \(X\) (Figure 6.5) to give 10a.\textsuperscript{[29,30]} This was followed by removal of the Boc-protecting group, mediated by TFA,\textsuperscript{[31]} to generate the free amine 10b.

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {\includegraphics[width=\textwidth]{synthetic_strategy}};
\end{tikzpicture}
\end{center}

\textit{Scheme 6.3} Synthetic strategy for generation of biotinylated BITZ probe 12.

The final step of the synthesis required the use of an amide-coupling reaction\textsuperscript{[32,33]} in which 10b was coupled to biotin to generate the target biotinylated BITZ probe 12. Due to the commercial availability of 2-(3-aminophenyl)-2,3-dihydro-1H-isodindole-1,3-dione, which comprises the structural warhead of 13, this biotinylated control probe was generated in a single synthetic step using the same synthetic procedure (Scheme 6.4).
Scheme 6.4 Synthetic strategy for generation of biotinylated control probe 13.

The depicted single-step amide-coupling\textsuperscript{[32,33]} between 2-(3-aminophenyl)-2,3-dihydro-1H-isindole-1,3-dione and biotin proceeded in moderate yield. It was noted that the amide-coupling reactions carried out with biotin (see Schemes 6.3 and 6.4) generally performed less well than amide-coupling reactions with the Boc-5-Ava-OH reagent as the carboxylic acid coupling partner, in similar reactions for the synthesis of 10 and 11 (Schemes 6.1 and 6.2).

6.5.4 Inhibitory Activity of the Biotin-Tagged BITZ Probe and Biotin-Tagged Control Probe Against PfIspD

Having generated the biotinylated BITZ probe 12 and corresponding control probe 13, the inhibitory activity of these two compounds was determined against the PfIspD enzyme using the discussed PfIspD enzymatic assay\textsuperscript{[35]} (see Chapter 2, Section 2.2.3). The biotinylated BITZ probe 12 was found to have an IC\textsubscript{50} value of 0.632 μM against the PfIspD enzyme (Table 6.7) and therefore demonstrates comparable activity to that of the simple BITZ derivative 8 (Table 6.3). In contrast, the biotinylated control probe 13 was found to be inactive against PfIspD, with an IC\textsubscript{50} value of > 40 μM (Table 6.7).

<table>
<thead>
<tr>
<th>Compound number</th>
<th>PfIspD Inhibition IC\textsubscript{50} (μM)</th>
<th>SEM value (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.632</td>
<td>0.028</td>
</tr>
<tr>
<td>13</td>
<td>&gt; 40</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.6 Inhibitory activity of biotinylated BITZ probe 12 and control probe 13 against the PfIspD enzyme; mean IC\textsubscript{50} and SEM values given which are representative of n = 3 replicates.

The determined activities of 12 and 13 (Table 6.7) are highly analogous to the PfIspD inhibitory activities of the fluorescent NBD-tagged probes 10 and 11 (Table 6.4). The BITZ-derived chemical probes 10 and 12 demonstrate potent and comparable activity, in the low micromolar region, against the PfIspD enzyme. Whereas, the non-BITZ control probes 11 and 13, using the 2-phenylisoindoline-1,3-dione motif 9 (Figure 6.2) as the chemical ‘warhead’ were both found to be inactive against the
**Chapter 6**

\(*Pf*IspD enzyme, with measured IC\textsubscript{50} values consistently > 40 \textmu M. This therefore validates the use of the 2-phenylisoidine-1,3-dione motif, 9 (Figure 6.2), as an inactive chemical motif against the \(*Pf*IspD enzyme and an appropriate headgroup for the biotinylated control probe 13. Consequently, these results show that the biotinylated probes 12 and 13 are suitable for use in the previously described streptavidin affinity pull-down assays (Section 6.5.1) to determine to the *Plasmodium* parasite proteins to which the BITZ chemotype, and therefore derived inhibitors, are able to bind and potentially inhibit.

**6.5.5 Inhibitory Activity of the Biotin-Tagged BITZ Probe and Biotin-tagged Control Probe Against 3D7 *P. falciparum* Parasite Growth**

The antimalarial activity of the biotinylated BITZ probe 12 and the biotinylated 2-phenylisoidine-1,3-dione control probe 13 must also to be evaluated against 3D7 *P. falciparum* parasite growth. Demonstration of significant phenotypic antimalarial activity by 12 and a contrasting lack of antimalarial activity by 13 would confirm these two compounds ideal antimalarial probes with which to conduct the described streptavidin affinity pull-down experiments. Unfortunately, very poor solubility of 12 and 13 has meant that, as yet, it has not been possible to determine the inhibitory activity of either of these biotinylated probes against 3D7 *P. falciparum* parasite growth. As a result, the discussed streptavidin affinity pull-down experiments are currently suspended until this data can be obtained.
6.6 Conclusions and Future Work

A small series of chemical probes have been designed and synthesised to address the question of BITZ chemotype localisation with *Plasmodium* parasite infected cells and potential off-target reactivity of the BITZ chemotype and derived *PfIspD* inhibitors.

In addition to fluorescent NBD-tagged and biotin-tagged probes generated directly around the BITZ chemotype, the related but distinct 2-phenylisoindoline-1,3-dione chemotype and BITZ isostere was employed as a suitable motif around which control compounds were generated for each probe. Use of the *P. falciparum* IspD enzymatic assay has highlighted a strong activity distinction between the BITZ probes and control probes, consistent in both the fluorescent and biotinylated probe series. The control probes (11 and 13) display ~100-fold loss of inhibitory activity against the *PfIspD* enzyme compared to the corresponding BITZ derived probes (10 and 12).

The fluorescent NBD-tagged BITZ probe was generated to facilitate localisation studies of the BITZ chemotype by means of fluorescent microscopy. Incubation of the BITZ probe with 3D7 *P. falciparum* cultures and visualisation of the resulting cultures using the Olympus Fluoview FV1000 confocal microscope has shown that the designed BITZ probe is able to penetrate host RBCs and emit detectable fluorescence from within the cell. The resulting fluorescence pattern shows that distribution of the BITZ probe is be broadly cytoplasmic, where the compound is excluded from both the parasite nucleus and food vacuole. Since comparable patterns of fluorescence are also observed with the corresponding non-BITZ (and *PfIspD* inactive) control compound, further investigations must be conducted to further evaluate BITZ treated *Plasmodium* cultures and determine more precise localisation patterns of the BITZ chemotype and how they may be distinct from the distribution of non-*PfIspD* inhibitors within the cell. Addition of an apicoplast marker as a supplementary visualisation tool represents an initial approach that could be taken. Additional studies will also be conducted to assess cellular morphology and the physiological effects of BITZ compound treatment on both parasite-infected and non-infected RBCs.

A biotinylated probe of the BITZ chemotype, and a non-BITZ biotinylated control probe, have been prepared to facilitate a controlled streptavidin pull-down strategy to provide a direct assessment of the proteins to which BITZ is able to bind within a solution of *P. falciparum* parasite lysate. Elution of proteins bound to the BITZ probe and subsequent analysis by LC-MS/MS would identify the parasite proteins bound to the BITZ chemotype in an unbiased BITZ-protein binding assessment. Disappointingly, a lack of compound solubility in the resulting biotinylated probes has prevented use
of the *P. falciparum* parasite growth assay and, as a result, a measure of inhibitory activity against *Plasmodium* parasite growth has not yet been obtained for these compounds.

Since this phenotypic activity data is required before protein pull-down assays can be initiated, attentions must first focus on improving the solubility of biotinylated BITZ probes, enabling the *P. falciparum* parasite growth assay to be conducted. An initial approach should consider modifying chemical components of the probe structures to promote the solubility of resulting compounds. Since the chemical recognition element (the BITZ chemotype) and ‘tag’ component (biotin) cannot be modified, variation of the linker group within the probe motif, should be addressed. Previously, amide-biotin linkages were incorporated into designed biotinylated probes since this linkage was easily generated between the BITZ warhead and the carboxylic acid side chain of biotin. Since work by others has identified that a carbamate-biotin group also represents a stable linkage,\[44,56\] this functionality should be incorporated as an alternative group to the amide-biotin linker with the purpose of improving the solubility of the resulting probe structure. Following this, more structurally divergent biotinylated BITZ probes should also be explored in order to generate the required chemical probes with appropriate pharmacokinetic properties. This will enable the inhibitory activity of the required BITZ probes to be assessed within the *P. falciparum* parasite growth assay, ultimately allowing the streptavidin pull-down assays to be conducted to determine the extent of BITZ chemotype off-target binding with *Plasmodium* parasite proteins.
6.7 Experimental

For general chemical and biological methodologies see Chapter 2, Section 2.7.1.

6.7.1 Biological Methods

Fluorescent microscopy

For fluorescent microscopy, cells at ~ 10% parasitaemia were fixed in 4% paraformaldehyde in PBS and stored in fixative at 4 °C as required. 200 µL aliquots of cells were incubated with the following dilutions: 1:1000 of 10 and 1:1000 of 11 for the detection of GFP. Hoechst 33258 (Life Technologies) was used as a nuclear counterstain. Images were obtained using the Olympus Fluoview FV1000 confocal microscope. For imaging of live cells, cells were stained with 10 mg mL⁻¹ Hoechst 33258 for 10 minutes and mounted under coverslips on polysine adhesion slides using ProLong Gold mounting media. Cells were visualised on an Olympus BH8 microscope at room temperature for no longer than 15 minutes. For all microscopy, minimal adjustments in brightness and contrast were applied equally to images.

6.7.2 Organic Synthesis

Preparation of 2-(chlorothio)benzoyl chloride X

Thionyl chloride (22.8 mmol, 7 eq) and N,N-dimethylformamide (3 drops) were added to a solution of 2,2'-dithiobenzoic acid (3.26 mmol) in anhydrous dichloroethane (25 ml) under a N₂ atmosphere at room temperature. The stirring solution was heated to reflux at 80°C for 1.5 hours. Sulfuryl chloride (4.24 mmol, 1.3 eq) was added to the cooled solution at 25°C before heating the solution to 50°C for 45 minutes. Solvent, excess thionyl chloride and excess sulfuryl chloride were removed under reduced pressure to generate X, a yellow-green solid as pure product in quantitative yield. Rₓ = 0.79 (50:50 EtOAc:hexane). ¹H NMR (400 MHz, CDCl₃) δ 8.34 (dd, J = 8.0, 1.2 Hz, 1H), 7.93 (dd, J = 8.2, 0.4 Hz, 1H), 7.77 (dt, J = 8.5, 1.3 Hz, 1H), 7.42 (dt, J = 8.0, 0.8 Hz, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 169.5, 146.1, 135.8, 134.7, 127.4, 125.8, 124.4.
Preparation of tert-butyl (3-(3-oxobenzo[d]isothiazol-2(3H)-yl)phenyl)carbamate 10a

To a solution of N-Boc-m-phenylenediamine (1.16 g, 5.57 mmol) in DCM (20 ml), 2-(chlorothio)benzoyl chloride (X) (1.50 g, 7.24 mmol, 1.2 eq) was added at 0°C under a N₂ atmosphere. Triethylamine (2.56 ml, 18.4 mmol, 3.3 eq) was added to the solution which was allowed to warm to room temperature and stirred overnight. The solution was quenched with NaHCO₃ (30 ml), the organic layer was extracted into DCM (3 x 40 ml), washed with brine (40 ml) and dried over MgSO₄ before concentrating under reduced pressure. The crude product was purified by flash column chromatography and by trituration with Et₂O to give 10a as an off white-cream solid in 63.0% yield. Rf = 0.37 (3:7 EtOAc:hexane and 1% triethylamine). ¹H NMR (400 MHz, CDCl₃) δ 8.10 (d, J = 7.9 Hz, 1H), 7.87 (s, 1H), 7.66 (dt, J = 8.2, 1.1 Hz, 1H), 7.57 (d, J = 8.1 Hz, 1H), 7.44 (dt, J = 7.9, 0.8 Hz, 1H), 7.42 – 7.28 (m, 3H), 6.67 (br s, 1H, NH) 1.52 (s, 9H); ¹³C NMR (101 MHz, CDCl₃) δ 164.1, 152.5, 139.9, 139.5, 137.8, 132.4, 129.8, 127.2, 125.8, 124.9, 120.1, 118.71, 116.8, 114.41, 80.9, 28.3; ES+HRMS m/z 365.0929 [M+Na]⁺ C₁₈H₁₈N₂O₅S requires 342.10.

Preparation of 2-(3-aminophenyl)benzo[d]isothiazol-3(2H)-one 10b

To a solution of 10a (3.49 mmol) in DCM (20 ml) under a N₂ atmosphere, trifluoroacetic acid (TFA) (4.0 ml, 52.4 mmol, 15.0 eq) was added and allowed to stir at room temperature overnight. After this time, DCM and excess TFA were removed under reduced pressure. The crude material was re-dissolved in DCM, quenched with NaHCO₃ and the organic layer extracted into DCM (3 x 40 ml), washed with brine (40 ml) and dried over MgSO₄ and concentrated in vacuo to give the pure product 10b as a yellow-orange solid in a 84.4% yield. Rf = 0.31 (65:35 EtOAc:hexane and 1% triethylamine). ¹H NMR (400 MHz, CDCl₃) δ 8.09 (d, J = 7.9 Hz, 1H), 7.65 (t, J = 7.5 Hz, 1H), 7.57 (d, J = 8.0 Hz, 1H), 7.43 (t, J = 7.5 Hz, 1H), 7.22 (t, J = 8.0 Hz, 1H), 7.16 (t, J = 1.9 Hz, 1H), 7.01 (dd, J = 8.0, 1.2 Hz, 1H), 6.63 (dd, J = 8.0, 1.9 Hz, 1H), 3.52 (br s, 2H, NH₂); ¹³C NMR (101 MHz, CDCl₃) δ 164.1, 147.4, 139.9, 138.2, 132.3, 130.2, 127.4, 125.2, 125.1, 120.0, 114.2, 113.8, 111.1; CI+HRMS m/z 243.0597 [M+H]⁺ C₁₃H₁₀N₂O₃S requires 242.05.
Preparation of tert-butyl (5-oxo-5-((3-(3-oxobenzo[d]isothiazol-2(3H)-yl)phenyl)amino) pentyl)carbamate 10c

Boc-5-Ava-OH (0.404 g, 1.86 mmol) and HATU (1.059 g, 2.79 mmol, 1.5 eq) were dissolved in DMF (10 ml) and allowed to stir at room temperature for 20 minutes. 10b (0.45 g, 1.86 mmol, 1.0 eq), dissolved in DMF (5 ml), was added after this time, followed by DIPEA (0.96 ml, 5.57 mmol, 3.0 eq) and the solution was stirred at room temperature for a further 20 hours, after which time the reaction was complete. Ninhydrin solution required to visualise Boc-5-Ava-OH. The solution was quenched with NaHCO$_3$ (40 ml) and the organic layer extracted into EtOAc (3 x 40 ml), then washed with H$_2$O (2 x 40 ml) and brine (40 ml), dried over MgSO$_4$, filtered through a pad of celite and concentrated in vacuo. The crude product was purified by trituration with Et$_2$O and EtOAc to give 10c as an off-white solid in a 92.4% yield. $R_f = 0.23$ (50:50 EtOAc:hexane and 1% triethylamine). $^1$H NMR (400 MHz, DMSO) $\delta$ 10.13 (s, 1H, NH), 8.09 (s, 1H), 8.06 (d, $J = 8.1$ Hz, 1H), 7.95 (d, $J = 7.8$ Hz, 1H), 7.76 (dd, $J = 7.2$, 0.9 Hz, 1H), 7.55 – 7.47 (m, 2H), 7.43 (d, $J = 5.2$ Hz, 2H), 6.83 (t, $J = 5.4$ Hz, 1H, NH), 2.93 (unresolved dt, $J = 7.8$, 6.2 Hz, 2H), 2.33 (t, $J = 7.3$ Hz, 2H), 1.63 – 1.51 (m, 2H), 1.46 – 1.37 (m, 2H), 1.36 (s, 9H); $^{13}$C NMR (101 MHz, DMSO) $\delta$ 172.0, 163.7, 156.1, 140.7, 140.5, 137.9, 133.1, 131.0, 126.6, 126.5, 124.9, 122.5, 119.1, 117.6, 115.0, 77.8, 36.5, 29.6, 28.7 (3C), 28.7, 22.9; ES+HRMS m/z 464.1622 [M+Na]$^+$ C$_{23}$H$_{27}$N$_3$O$_4$S requires 441.17.

Preparation of 5-amino-N-(3-(3-oxobenzo[d]isothiazol-2(3H)-yl)phenyl)pentanamide 10d

To a solution of 6c (0.523 g, 1.19 mmol) in DCM (20 ml) under a N$_2$ atmosphere, trifluoroacetic acid (TFA) (2.74 ml, 35.5 mmol, 15.0 eq) was added and allowed to stir at room temperature overnight. After this time, DCM and excess TFA were removed under reduced pressure. The crude material was re-dissolved in DCM, quenched with NaHCO$_3$ (40 ml) and the organic layer extracted into DCM (3 x 40 ml), washed with brine (40 ml), dried with MgSO$_4$ and concentrated in vacuo to give 10d as an orange-brown viscous oil in a 66.1% yield. $R_f = 0.00$ (5:95 MeOH:EtOAc). $^1$H NMR (400 MHz, DMSO) $\delta$ 10.15 (s, 1H, amide NH), 8.10 (s, 1H), 8.06 (d, $J = 8.1$ Hz, 1H), 7.95 (d, $J = 7.8$ Hz, 1H), 7.76 (t, $J = 7.5$ Hz, 1H), 7.68 – 7.61 (m, 2H), 7.57 – 7.41 (m, 2H), 7.43 (d, $J = 5.2$ Hz, 2H), 7.25 (t, $J = 7.3$ Hz, 2H), 6.82 (t, $J = 5.4$ Hz, 1H, NH), 2.90 (unresolved dt, $J = 7.8$, 6.2 Hz, 2H), 2.33 (t, $J = 7.3$ Hz, 2H), 1.63 – 1.51 (m, 2H), 1.46 – 1.37 (m, 2H), 1.36 (s, 9H); ES+HRMS m/z 462.1580 [M+Na]$^+$ C$_{23}$H$_{25}$N$_3$O$_4$S requires 441.17.
7.51 (t, J = 7.6 Hz, 2H), 7.42 (d, J = 4.5 Hz, 2H), 2.95 (s, NH₂), 2.55 (t, J = 6.7 Hz, 2H), 2.32 (t, J = 7.2 Hz, 2H), 1.61 (tt, J = 7.3, 7.3 Hz, 2H), 1.38 (tt, J = 7.2, 6.8 Hz, 2H); ¹³C NMR (101 MHz, DMSO) δ 172.2, 163.7, 140.8, 140.5, 137.9, 133.1, 130.1, 126.6, 126.5, 124.9, 122.5, 119.1, 117.7, 115.0, 41.8, 36.8, 33.2, 23.0; ES+HRMS m/z 342.1270 [M+H]⁺ C₁₈H₁₉N₃O₂S requires 341.12.

Preparation of 5-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)-N-(3-(3-oxobenzo[d]isothiazol-2(3H)-yl)phenyl)pentanamide 10

To a solution of 6d (0.120 g, 0.351 mmol) in anhydrous ethanol (12 ml) under a N₂ atmosphere, 4-chloro-7-nitrobenzofurazan (NBD-chloride) (0.077 g, 0.387 mmol, 1.1 eq) was added and allowed to stir at room temperature overnight. Additional anhydrous ethanol (5 ml) was added and the solution heated to 50°C for 4 hours under a restored N₂ atmosphere. The solution was then cooled to room temperature and stirred overnight. Ethanol was removed under reduced pressure and the crude product purified by column chromatography to give 10 a red-brown solid in a 30.6% yield. Rf = 0.38 (7:3 EtOAc:hexane). ¹'H NMR (400 MHz, DMSO) δ 10.16 (s, 1H, amide NH), 9.57 (s, 1H, amide NH), 8.50 (d, J = 8.6 Hz, 1H), 8.10 (s, 1H), 8.06 (d, J = 8.1 Hz, 1H), 7.95 (d, J = 7.8 Hz, 1H), 7.77 (t, J = 7.8 Hz, 1H), 7.51 (t, J = 7.2 Hz, 2H), 7.46 – 7.39 (m, 2H), 6.43 (d, J = 8.9 Hz, 1H), 3.52 (s, 2H), 2.41 (s, 2H), 1.74 (s, 4H); ¹³C NMR (101 MHz, DMSO) δ 171.7, 163.7, 145.7, 140.6, 140.5, 138.4, 137.9, 133.1, 130.1, 126.6 (2C), 126.5 (2C), 124.9, 122.4 (2C), 119.1, 117.7, 115.0, 99.6, 43.6, 36.4, 27.7, 23.0; Elemental analysis (%) found: C, 57.46; H, 4.29; N, 15.73; S, 6.01%. C₂₄H₁₉N₆O₂S requires C, 57.14; H, 4.00; N, 16.66; S, 6.36%; ES+HRMS m/z 527.1115 [M+Na]⁺ C₂₄H₂₀N₆O₂S requires 504.12.

Preparation of tert-butyl (5-((3-(1,3-dioxoisindolin-2-yl)phenyl)amino)-5-oxopentyl) carbamate 11a

Boc-5-Ava-OH (0.40 g, 1.84 mmol) and HATU (1.1 g, 2.76 mmol, 1.5 eq) were dissolved in DMF (10 ml) and allowed to stir at room temperature for 20 minutes. 2-(3-aminophenyl)-2,3-dihydro-1H-isoisodole-
1,3-dione (0.48 g, 2.03 mmol, 1.1 eq), dissolved in DMF (5 ml), was added after this time, followed by DIPEA (0.95 ml, 5.52 mmol, 3.0 eq) and the solution was stirred at room temperature for a further 20 hours, after which time the reaction was complete. Ninhydrin solution required to visualise Boc-5-Ava-OH. The solution was quenched with NaHCO₃ (40 ml) and the organic layer extracted into EtOAc (3 x 40 ml), then washed with H₂O (2 x 40 ml) and brine (40 ml), dried over MgSO₄ and concentrated in vacuo. The crude product was purified by trituration with Et₂O and EtOAc to give 11a as a light brown solid in a 91.7% yield. \( R_f = 0.28 \) (6:4 EtOAc:hexane and 1% triethylamine). \(^1\)H NMR (400 MHz, MeOD) \( \delta \) 7.99 – 7.83 (m, 4H), 7.72 (t, \( J = 1.9 \) Hz, 1H), 7.63 (t, \( J = 8.2, 1.1 \) Hz, 1H), 7.44 (t, \( J = 8.1 \) Hz, 1H), 7.17 (dd, \( J = 7.9, 0.9 \) Hz, 1H), 3.07 (t, \( J = 6.9 \) Hz, 2H), 2.41 (t, \( J = 7.3 \) Hz, 2H), 1.77 – 1.66 (m, 2H), 1.60 – 1.49 (m, 2H), 1.43 (s, 9H); \(^{13}\)C NMR (101 MHz, MeOD) \( \delta \) 173.1, 167.3 (2C), 157.2, 139.3, 134.4 (2C), 131.7, 129.7, 127.4, 123.2 (2C), 122.1, 119.1, 118.2, 116.01, 78.5, 39.55 36.1, 29.1, 27.4 (3C), 22.6; ES+HRMS m/z 460.1843 \[ M+Na \]^⁺ 

Preparation of 5-amino-N-(3-(1,3-dioxoisindolin-2-yl)phenyl)pentanamide 11b

To a solution of 11a (0.732 g, 1.67 mmol) in DCM (20 ml) under a N₂ atmosphere, trifluoroacetic acid (TFA) (1.9 ml, 25.1 mmol, 15.0 eq) was added and allowed to stir at room temperature overnight. After this time, DCM and excess TFA were removed under reduced pressure. The crude material was redissolved in EtOAc, quenched with NaHCO₃ and the organic layer extracted into EtOAc (3 x 40 ml), washed with brine (40 ml), dried with MgSO₄ and concentrated in vacuo. The crude product was purified by washing with MeOH to give 11b as a grey-brown solid in a 44.2% yield. \( R_f = 0.00 \) (EtOAc and 1% triethylamine). \(^1\)H NMR (400 MHz, DMSO) \( \delta \) 10.31 (s, 1H, NH), 8.01 – 7.90 (m, 4H), 7.88 (br s, 2H, NH₂), 7.76 (s, 1H), 7.66 (d, \( J = 8.2 \) Hz, 1H), 7.45 (t, \( J = 8.0 \) Hz, 1H), 7.12 (d, \( J = 7.9 \) Hz, 1H), 2.80 (t, \( J = 7.2 \) Hz, 2H), 2.39 (t, \( J = 6.8 \) Hz, 2H), 1.63 (m 4H); \(^{13}\)C NMR (101 MHz, DMSO) \( \delta \) 171.6, 167.4, 140.3, 135.2 (2C), 132.6, 132.0 (2C), 129.5, 123.9 (2C), 122.5, 119.1, 118.4, 39.0, 36.1, 27.0, 22.5; ES+HRMS m/z 338.1508 \[ M+H \]^⁺ 

C₁₉H₁₉N₃O₅ requires 337.14.
Chapter 6

Preparation of \(N\)-(3-(1,3-dioxoisindolin-2-yl)phenyl)-5-((7-nitrobenzo[c][1,2,5]-oxadiazol-4-yl) amino)pentanamide 11

![Chemical Structure]

To a solution of 11b (0.235 g, 0.698 mmol) in anhydrous DCM (12 ml) under a N\(_2\) atmosphere, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (0.116 g, 0.581 mmol, 1.0 eq) was added. The solution was evacuated and purged with N\(_2\) before the addition of triethylamine (0.08 ml, 0.581 mmol, 1.0 eq) and was allowed to stir at room temperature overnight. The crude solution was diluted with DCM, washed with brine (3 x 30 ml) and the organic layer dried with MgSO\(_4\) and concentrated in vacuo. The crude product was purified by column chromatography to give 11 a red-brown solid in a 35.4% yield. **Rf** = 0.63 (5:95 MeOH:EtOAc and 1% triethylamine). \(^1\)H NMR (400 MHz, DMSO) \(\delta\) 10.13 (s, 1H, amide NH), 9.60 (s, 1H, NH), 8.49 (d, \(J = 8.9\) Hz, 1H), 8.03 – 7.86 (m, 4H), 7.72 (s, 1H), 7.61 (d, \(J = 8.1\) Hz, 1H), 7.43 (t, \(J = 8.0\) Hz, 1H), 7.11 (d, \(J = 7.8\) Hz, 1H), 6.43 (d, \(J = 9.0\) Hz, 1H), 3.51 (unresolved t, 2H), 2.40 (t, \(J = 6.2\) Hz, 2H), 1.72 (m, 4H); \(^{13}\)C NMR (126 MHz, DMSO) \(\delta\) 171.7, 167.4, 145.7, 145.0, 144.7, 140.2, 138.4 (2C), 135.2, 132.6, 132.0 (2C), 129.5, 123.9 (2C), 122.5, 121.0, 119.1, 118.4, 99.6, 58.4, 43.6, 36.4, 27.7, 23.0; **Elemental analysis** (%) found: C, 60.03; H, 4.32; N, 15.85%; \(C_{25}H_{20}N_6O_6\) requires C, 60.00; H, 4.03; N, 16.79%; **ES+HRMS** m/z 523.1348 [M+Na]\(^+\); \(C_{25}H_{20}N_6O_6\) requires 500.14; **HPLC** (purity analysis %) found: 94.67%; **RT**: 14.12 min/ 40 min (66.5/33.5, 0.3 ml/min IPA:HEXANE, Signal = 254 nm, SYNCHROSILICA).

Preparation of \(N\)-(3-(3-oxobenzo[d]isothiazol-2(3H)-yl)phenyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide 12

![Chemical Structure]

Biotin (0.231 g, 0.944 mmol) and HATU (0.538 g, 1.42 mmol, 1.5 eq) were dissolved in DMF (10 ml) and allowed to stir at room temperature for 20 minutes. 10b (0.252 g, 1.04 mmol, 1.1 eq), dissolved in DMF (5 ml), was added after this time, followed by DIPEA (0.49 ml, 2.83 mmol, 3.0 eq) and the solution was stirred at room temperature for a further 20 hours, after which time the reaction was
complete. The solution was quenched with NaHCO$_3$ (40 ml) and the organic layer extracted into DCM (3 x 40 ml), then washed with H$_2$O (2 x 40 ml) and brine (40 ml), dried over MgSO$_4$ and concentrated in vacuo. The crude product was purified by trituration with Et$_2$O and EtOAc to give 12 as an off-white solid in a 76.3% yield. $R_f$ = 0.08 (5:95 MeOH:EtOAc and 1% triethylamine). $^1$H NMR (400 MHz, DMSO) $\delta$ 10.14 (s, 1H, amide NH), 8.09 (s, 1H), 8.06 (d, $J$ = 8.2 Hz, 1H), 7.95 (d, $J$ = 7.8 Hz, 1H), 7.76 (t, $J$ = 7.5 Hz, 1H), 7.51 (m, 2H), 7.44 (d, $J$ = 4.8 Hz, 2H), 6.45 (s, 6H, NH), 6.37 (s, 6H, NH), 4.30 (t, $J$ = 6.8 Hz, 1H), 4.14 (t, $J$ = 6.4 Hz, 1H), 3.13 (dd, $J$ = 11.1, 7.5 Hz, 1H), 2.82 (dd, $J$ = 12.4, 5.0 Hz, 1H), 2.58 (d, $J$ = 12.4 Hz, 1H), 2.34 (t, $J$ = 7.2 Hz, 2H), 1.72 – 1.29 (m, 6H); $^{13}$C NMR (101 MHz, DMSO) $\delta$ 172.0, 163.7, 163.2, 140.7, 140.5, 137.9, 133.1, 130.1, 126.6, 126.5, 124.9, 122.4, 119.1, 117.7, 115.0, 61.5, 59.7, 55.9, 40.3, 36.7, 28.7, 28.6, 25.5; Elemental analysis (%) found: C, 57.30; H, 5.16; N, 11.71; S, 13.34%. C$_{23}$H$_{24}$N$_4$O$_3$S$_2$ requires C, 58.95; H, 5.16; N, 11.96; S, 13.96%; ES+HRMS m/z 491.1190 [M+Na]$^+$ C$_{23}$H$_{24}$N$_4$O$_3$S$_2$ requires 468.13.

Preparation of N-(3-(1,3-dioxoisodolin-2-yl)phenyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide 13

Biotin (0.202 g, 0.819 mmol) and HATU (0.467 g, 1.23 mmol, 1.5 eq) were dissolved in DMF (7 ml) and allowed to stir at room temperature for 20 minutes. 2-(3-aminophenyl)-2,3-dihydro-1H-isooindole-1,3-dione (0.215 g, 0.901 mmol, 1.1 eq), dissolved in DMF (5 ml), was added after this time, followed by DIPEA (0.42 ml, 2.46 mmol, 3.0 eq) and the solution was stirred at room temperature for a further 20 hours, after which time the reaction was complete. The solution was quenched with NaHCO$_3$ (40 ml) and the organic layer extracted into EtOAc (3 x 40 ml), then washed with H$_2$O (2 x 40 ml) and brine (40 ml), dried over MgSO$_4$ and concentrated in vacuo. The crude product was purified by trituration with EtOAc and MeOH to give 13 as a yellow-cream solid in a 58.7% yield. $R_f$ = 0.13 (5:95 MeOH:EtOAc and 1% triethylamine). $^1$H NMR (400 MHz, DMSO) $\delta$ 10.11 (s, 1H), 8.04 – 7.86 (m, 4H), 7.74 (s, 1H), 7.63 (d, $J$ = 8.2 Hz, 1H), 7.44 (t, $J$ = 8.0 Hz, 1H), 7.12 (d, $J$ = 7.9 Hz, 1H), 6.46 (s, 1H, NH), 6.38 (s, 1H, NH), 4.31 (t, $J$ = 7.3 Hz, 1H), 4.14 (t, $J$ = 5.9 Hz, 1H), 3.17 – 3.08 (m, 1H), 2.83 (dd, $J$ = 12.4, 5.0 Hz, 1H), 2.58 (d, $J$ = 12.4 Hz, 1H), 2.34 (t, $J$ = 7.3 Hz, 2H), 1.72 – 1.31 (m, 6H); $^{13}$C NMR (101 MHz, DMSO) $\delta$ 171.9, 167.4 (2C), 163.2, 140.3, 135.2 (2C), 132.6, 132.00 (2C), 129.5, 123.9 (2C), 122.4, 119.1, 118.4, 61.5, 59.7,
55.9, 49.1, 36.7, 28.7, 28.6, 25.6; **Elemental analysis** (%) found: C, 60.66; H, 5.31; N, 11.77; S, 6.73%.

C_{24}H_{24}N_{4}O_{4}S requires C, 62.05; H, 5.21; N, 12.06; S, 6.90%; **ES+MS** m/z 487.1417 [M+Na]^+ C_{24}H_{24}N_{4}O_{4}S requires 464.15.
6.8 References


Chapter 6


Chapter 7
Final Conclusions and Perspectives
7.1 Final Conclusions and Perspectives

Malaria is a life-threatening, vector-borne disease which causes over 200 million cases per year and is responsible for nearly half a million deaths. Despite significant scientific progress over the last two decades, achieving a 30% decrease in the global incidence of malaria between 2000 and 2013, malaria remains a major global health problem, putting almost half the world’s population at risk of contracting the disease. Consequently, the development of new and effective antimalarial chemotherapeutics is a fundamental priority but represents a significant challenge within drug discovery. It is the ability of the Plasmodium parasite to develop drug resistance which significantly hinders the fight against malaria, where the present acceleration of Plasmodium resistance is seriously threatening advances made in malaria treatment and prevention. With 95 countries registering malaria transmission in 2015, the development of new antimalarial drugs and battle to overcome Plasmodium parasite resistance has never been more crucial.

Amidst significant research efforts pursuing the identification of novel drugs and novel drug targets to expand the antimalarial drug pipeline, considerable interest has developed around the chemical manipulation of isoprenoid biosynthesis. Isoprenoids are a diverse class of natural products which play vital roles in many cellular functions and are essential for Plasmodium parasite survival. Plasmodium parasites use the non-mevalonate (MEP) pathway to generate isoprenoid precursors, delivering a key biological selectivity to humans and other mammals which use the mevalonate (MVA) pathway for this purpose. Research by others into fosmidomycin, a potent and well-characterised MEP pathway inhibitor, have validated the pathway as a viable target for antimalarial drug discovery. These results provoked our interest in the third enzyme of the MEP pathway, IspD, a novel and underexplored biological target with which to advance antimalarial drug development.

Virtual screening and chemoinformatics studies facilitated the generation of a focused ligand library of 5000 compounds that was assessed in a HTS to identify novel inhibitors of the PfIspD target enzyme. The HTS identified 54 novel compound structures that demonstrate inhibitory activity against PfIspD. From the structures identified, the 1,2-benzol[d]isothiazol-3(2H)-one (BITZ) chemotype was selected as the hit chemotype for PfIspD inhibitor design and SAR development. A number of synthetic strategies have been identified which facilitate the efficient generation of the BITZ chemotype and derived inhibitors. These strategies have been employed throughout the generation of all BITZ compounds discussed and were used to initiate preliminary SAR work which confirmed the specific chemical construct of the BITZ headgroup essential to achieve inhibition of the PfIspD enzyme.
Following the transition into hit-to-lead optimisation, extensive SAR investigations around the BITZ chemotype have generated a broad and diverse range of BITZ compounds which include three of the most potent BITZ derived PfIspD inhibitors to date. Indeed, the most potent BITZ inhibitor demonstrates an impressive IC$_{50}$ value of 73 nM against PfIspD, which is six-times more potent than the HTS hit. In addition, potent PfIspD inhibition translates into efficacious phenotypic activity, with several BITZ compounds displaying EC$_{50}$ values as low as 206 nM against *P. falciparum* parasite growth. Subsequent BITZ compound evaluation demonstrates good correlation between PfIspD enzyme inhibition and inhibition of *Plasmodium* parasite growth, indicating that inhibition of the MEP pathway, via the PfIspD target, is able to cause parasite death. This is also supported by metabolic profiling of MEP pathway intermediates which highlights that anti-parasitic activity is the result of MEP pathway inhibition which is sufficient to disrupt *de novo* production of isoprenoid precursors. These results, further backed up by the work of others, enables one of our key research questions to be answered, as we have demonstrated that arrest of the MEP pathway via PfIspD inhibition is able to cause *Plasmodium* parasite death through the disruption of isoprenoid precursor generation.

In addition, biological studies have shown BITZ compounds to offer potential as a broad spectrum antimalarial agents with several BITZ analogues demonstrating low micromolar to nanomolar activity against PvIspD, therefore meeting a key developmental requirement of antimalarial drug design. Biological evaluations have also established that BITZ derived compounds express inhibitory activity against *P. falciparum* parasite strains which show drug resistance to existing antimalarial therapies. This is of significant importance given the current wide spread and accelerating *P. falciparum* species drug resistance which means existing drug-resistant parasites must be sensitive to novel antimalarial therapies if new drugs and research efforts are to succeed.

In order to determine the mechanism of action by which the BITZ chemotype inhibits PfIspD, a combination of molecular modelling studies and biological assays have been conducted. Since a PfIspD crystal structure is unavailable, EcIspD was used to identify a suitable modelling protocol with which to dock small molecules at the IspD active site, that was then applied to a PfIspD homology model. Molecular modelling studies investigated the orientation of BITZ compounds at the PfIspD active site; where the examination of docking poses revealed a consistent molecular alignment. As a result, a mechanism of enzyme inhibition has been proposed which is initiated by non-covalent active site recognition but ultimately relies on the formation of a covalent PfIspD-BITZ adduct, rendering the enzyme inactive. More specifically, the proposed mechanism involves formation of a disulfide protein-inhibitor adduct between the PfIspD active site Cys-202 residue and the electrophilic sulfur atom of the BITZ inhibitor core, facilitated by their proximal alignment in the PfIspD binding site.
A range of biological methodologies, including site directed mutagenesis (SDM), enzyme activity assays and crystallographic evaluation, confirm and support the proposed mechanism of enzyme inhibition. SDM has been used to verify the specific requirement for the Cys-202 active site residue in facilitating PfIspD enzyme inhibition. The wildtype EcIspD enzyme, against which BITZ compounds are ineffective, has been rendered sensitive to the effects of BITZ inhibitors through introduction of the active site Cys-202 residue and generation of the EcIspD-A14C mutant enzyme. This result therefore supports the role of the Cys-202 protein residue in PfIspD active site inhibition. In addition, generation of the PfIspD-C202A mutant enzyme, replacing Cys-202 with Ala, causes a six-fold decrease in enzyme sensitivity when treated with BITZ compounds compared to wildtype PfIspD. This observation highlights that covalent enzyme inhibition is no long able to occur at PfIspD-C202A, but that weak inhibitory effects are retained and likely signify non-covalent recognition contributions of BITZ compounds at the enzyme.

Several lines of evidence now suggest that some BITZ derived compounds may be capable of eliciting off-target inhibitory effects that may contribute to observed inhibition of Plasmodium parasite growth. A loss of PfIspD enzymatic activity is observed within several BITZ compound series but where a notable level of anti-parasitic activity is maintained and in some instances markedly enhanced. Such an activity trend is indicative of off-target interactions that are capable of inhibiting Plasmodium parasite growth, despite a lack of activity against the PfIspD enzyme. This observation is supported by the negative result of IPP rescue experiments, indicating that parasite growth inhibition by BITZ compounds does not solely result from activity against the IspD enzyme. Recent research has therefore focused on the question of potential off-target reactivity of the BITZ chemotype, as well as an alternative chemical chemotype which has been shown to be a potent inhibitor of the PfIspD enzyme.

A small series of chemical probes have been designed to address the question of BITZ chemotype localisation with Plasmodium infected cells as well as the potential off-target reactivity of BITZ. NBD-tagged and biotin-tagged BITZ probes were generated, along with suitable non-PfIspD active control probes, for the purpose of fluorescent microscopy and streptavidin pull-down assays. Fluorescent microscopy showed distribution of the NBD-tagged BITZ probe to be broadly cytoplasmic and excluded from both the parasite nucleus and food vacuole. Further microscopy is needed to determine more precise BITZ localisation patterns and how these may be distinct from the distribution of non-PfIspD inhibitors within Plasmodium culture. A biotinylated BITZ probe was designed to facilitate a streptavidin pull-down experiment to determine the proteins to which the BITZ chemotype is able to bind in a solution of P. falciparum parasite lysate. As yet, assessment of off-target BITZ reactivity by
this means has not been feasible; research must first focus on the design of an alternative biotinylated BITZ probe to enable inhibitory activity against *Plasmodium* parasite growth to be confirmed.

Robust biological data has confirmed the proposed mechanism of *PfIspD* inhibition by BITZ compounds as occurring via covalent modification. However, the covalent modification of enzymes and proteins can be a risky strategy within drug design. This has therefore driven our consideration of a distinct chemical chemotype shown to inhibit the *PfIspD* enzyme by an alternative, non-covalent mechanism of action. MMV-008138, a tetrahydro-β-carboline derivative, was recognised in phenotypic screen showing it to be a potent inhibitor of *P. falciparum* growth in culture. Further work has highlighted that only the 1R,3S-MMV-008138 diastereoisomer is active against *PfIspD*, providing a high degree of specificity and selectivity to this compound.

Metabolic profiling of MMV-008138 and assessments of species-selectivity have revealed comparable results to those of the BITZ chemotype, highlighting therapeutic scope of MMV-008138 as a broad-spectrum antimalarial agent as well as its ability to inhibit the MEP pathway at levels which affect asexual parasite growth. In addition, positive IPP rescue experiments show that the inhibition of *Plasmodium* parasite growth is specific to MEP pathway inhibition. The comparable features identified between the tetrahydro-β-carboline and BITZ chemotypes inspired a series of competition experiments, generating isobolograms which combined 1R,3S-MMV-008138 with several BITZ inhibitors. However, the resulting data highlighted that the two chemical motifs behave antagonistically when co-administered, producing a lessened phenotypic effect.

SAR development around 1R,3S-MMV-008138 is currently quite limited, with little room for significant structural variation or additional functionality. This can be rationalised through the spatial demands of the *PfIspD* binding site, in which 1R,3S-MMV-008138 adopts a spatially tight-fitting binding pose, as demonstrated by molecular modelling. More recent SAR development have focused on modifications to the A and D rings of 1R,3S-MMV-008138 and again highlight that structural modifications must be both precise and discrete to maintain complementary binding and activity against *PfIspD*. In analogues where this is achieved, the resulting activity against both *PfIspD* and *Plasmodium* parasite growth is of nanomolar potency. Further work must address the metabolism and stability profiles of 1R,3S-MMV-008138 derivatives to enable movement towards an optimised lead structure. As such, development of 1R,3S-MMV-008138 analogues remains ongoing, with the aim of progressing towards a lead compound that is suitable for clinical development as an antimalarial agent, targeting the *PfIspD* enzyme.
Appendix
Appendix 1. HTS Output: 54 Hit Compounds Identified as Active Inhibitors of the \textit{Pfi}spD enzyme.

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Appendix 2. Summary of preliminary SAR studies around close structural analogues of the DT2009-0168020 hit.\(^{[1]}\)

![Chemical structure](attachment:structure.png)

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Overlay of $^1$H NMR for separated cis and trans isomers of II. Red spectra represents cis isomer and green spectra represents trans isomer.
Appendix 3. Pg 341

Compound 11

trans isomer

1H NMR
Positive enhancement of chemical shifts between H-1 and H-3 confirms the presence of the cis isomer.

Appendix 4. Pg 342
No observation of positive enhancement between the chemical shifts of H-1 and H-3 confirms presence of the trans isomer.