Investigating the Potential of Primary Sulfonamides as Antimalarial Drug Leads

Gillian Marie Fisher

Bachelor of Science (Hons)

School of Natural Sciences
Griffith Sciences
Griffith University

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Abstract

Malaria remains one of the world’s most important infectious diseases causing over 600,000 deaths annually, mainly in African children under the age of five [1]. In the absence of a licenced vaccine, malaria prevention and treatment relies on drugs and vector control [1]. Unfortunately malaria parasite resistance has emerged to currently used antimalarial drugs, including the current gold standard artemisinin combination therapies (ACTs) [1-3]. Added to this, the majority of agents in the current antimalarial drug discovery and development portfolio are based on known antimalarial pharmacophores [4], which may compromise their widespread use due to potential issues of cross resistance. With only one new antimalarial chemical class (chemotype) presently under development, the spiroindolones [5], there is an urgent need to ensure that the antimalarial drug discovery pipeline is primed with new chemotypes, ideally with novel modes of action in order to combat resistance. In this thesis project this problem was addressed by investigating the antimalarial potential of primary sulfonamides (PS), a chemotype not currently used for malaria, but with a proven track record for treatment of other diseases, including glaucoma, renal disorders and epilepsy [6].

In the first part of this work, the \textit{in vitro} antimalarial activity of 65 PS containing compounds was investigated. Compounds were selected based on various strategies, including examining compounds already used clinically for other human diseases in a repurposing or repositioning strategy (16 compounds), investigation of experimental
compounds originally tested against cancer cells in a “piggyback” strategy (14 compounds), investigation of PS compounds arising from a large antimalarial high throughput (HTS) screen of a GlaxoSmithKline (GSK) pharmaceutical library (26 compounds), and the investigation of novel “hybrid” compounds that combine a PS moiety with the 7-chloro-4-aminoquinoline antimalarial pharmacophore (9 compounds).

All of the clinically used sulfonamides tested in this study were found to have poor in vitro activity against *Plasmodium falciparum* malaria parasites (IC$_{50}$ ≥6 µM). While there are potential advantages in identifying a potent antimalarial PS compound that is already used as a drug for other diseases, such as a known safety profile and reduced development costs, given that these drugs were designed to target human cells and not malaria parasites, these findings were not completely unexpected. In contrast, “piggybacking” onto previous work on experimental PS glycoside compounds with reported anti-tumour cell activity yielded more promising results. Of 14 experimental PS glycosides examined, less than ten had *P. falciparum* in vitro IC$_{50}$’s of 1-3 µM, with some having >40 fold better activity for malaria parasites versus human cells. A structure activity relationship analysis using derivatives lacking a PS group indicated that the PS moiety in these compounds contributes to their antimalarial activity. Importantly, the compound with the best antimalarial activity, **PS-3**, also showed no evidence of cross resistance with the antimalarial drugs chloroquine and mefloquine, as determined using drug-sensitive (3D7) and resistant (Dd2) *P. falciparum* parasite lines (IC$_{50}$~1 µM). **PS-3** also has good selectivity for *P. falciparum* versus human cells (Selectivity Index (SI) >50). The next PS compounds to be investigated were 26 compounds identified as antimalarial “hits” (≥50% inhibition of *P. falciparum* growth at
2 µM) in the publically available GSK TCAMS library [7]. Of these, 13 potential lead-like compounds were identified (Pf3D7 IC50 <1 µM; SI>10). In particular one compound, **GSK-15** (Pf3D7 IC50 0.23µM; SI>40), meets the *in vitro* criteria required for a hit antimalarial (*P. falciparum* IC50 <1µM SI > 10; [8]), and if proven to possess appropriate pharmacokinetic properties, will be progressed to *in vivo* studies in the future. Finally, the antimalarial activity of a group of novel 7-chloro-4-aminoquinoline PS hybrids was assessed in an attempt to investigate whether such “hybrid” molecules might have a suitable antimalarial lead-like profile. The 7-chloro-4-aminoquinoline moiety is present in chloroquine and other clinically approved antimalarial drugs. While none of the PS-containing hybrid compounds had better antimalarial activity than chloroquine itself, surprisingly three control hybrid compounds, lacking the PS moiety, were shown to overcome the resistance profile of chloroquine. The most promising of these non-PS compounds, **H9** (*P. falciparum* IC50 0.23 µM; SI >833), exhibits properties of an early lead-like compound and may be an interesting starting point for future studies.

In the second part of this thesis the targets of several of the antimalarial PS compounds identified above were investigated using “target-biased” and “target-unbiased” approaches. These approaches included investigating recombinant *P. falciparum* carbonic anhydrase (PfCA) as a putative target of antimalarial PS compounds, testing photoffinity labelling (PAL) probes as potential tools to identify PS compound targets in protein lysates, and generation of *P. falciparum* lines resistant to the most potent PS glycoside, **PS-3**, in order to examine the resulting phenotype. As PS-containing compounds are known to target carbonic anhydrases (CAs) in human cells and other
organisms, the first approach utilized was to investigate \textit{Pf}CA as a possible target of the compounds examined in this study. As a result of collaboration with colleagues in Italy, an active recombinant form of \textit{Pf}CA was successfully generated and used to assess the inhibitory activity of some of the antimalarial PS glycosides and clinically used sulfonamides identified in this project. While inhibition of \textit{Pf}CA activity was observed for several of the compounds tested (Ki 85-189 nM), none of the compounds demonstrated selectivity for the parasite enzyme versus human CA I and II isoforms, with one exception. The secondary sulfonamide saccharin, was found to have more potent activity against \textit{Pf}CA versus human CA I (SI =102) and II enzymes (SI = 33). This is an intriguing result and has resulted in a panel of saccharin analogues being investigated in ongoing work outside this project.

The first unbiased target identification approach to be investigated was the use of photoaffinity labelling (PAL) which involves the use of specifically designed small molecule PAL probes that can be cross-linked to their respective target proteins using UV irradiation, then detected using click chemistry and in-gel fluorescence [9]. In this study four probes were examined, including three benzenesulfonamide based probes (one with a pre-installed fluorophore), and one probe based on the clinically used PS compound, acetazolamide. All probes were successfully validated in PAL experiments with recombinant human CA II protein, however their use in cell lysates was hampered by non-specific labelling issues. Future studies will involve confirming the binding of these probes to recombinant \textit{Pf}CA (now available) and generation of new PAL probes designed to block non-specific binding.
The final unbiased target identification strategy to be employed focused on generation of *P. falciparum* parasites resistant to the PS glycoside PS-3 combined with phenotypic analyses. A PS resistant line (3D7-C3^{PS3}) with a >6 fold higher IC_{50} for PS-3 (IC_{50} 10.4 \mu M) compared to wild type parasites (3D7-C3 IC_{50} 1.6 \mu M) was generated. These resistant parasites were shown to have no cross-resistance with the antimalarial drugs chloroquine, artesunate, or pyrimethamine. Importantly, there was no evidence of increased copy number of the *pfmdr1* gene, which encodes a multi-drug resistance transporter associated with resistance by several currently used antimalarials [10, 11]. Unexpectedly, it was found that the PS moiety of the PS-3 compound did not appear to be linked to the resistance phenotype of 3D7-C3^{PS3} parasites, as control compounds lacking a PS group also displayed >5 fold reduced activity to 3D7-C3^{PS3} versus 3D7-C3. Further screening of various PS glycoside analogues against 3D7-C3^{PS3} parasites suggested an association with the glucose component of these compounds in conferring the resistance phenotype. These data led to the working hypothesis that the selection compound PS-3 may target a parasite hexose transporter. While additional studies were not able to be carried out to test this hypothesis within the scope of this project, genomic DNA from PS-3 resistant clones is currently being subjected to whole genome sequencing in order to identify mutations that may confer the PS-3-resistance phenotype.

In summary, work in this thesis has provided new insights into the antimalarial potential of PS compounds. *In vitro* studies have led to the identification of two PS-containing compounds and three non-PS compounds that have early-phase lead-like antimalarial profiles (IC_{50} <1\mu M; SI >40) and that will be pursued in future work. Additionally, this
Work led to the generation of recombinant form of PfCA, identified as a target of some of the compounds in this study (although perhaps not the only target). This recombinant PfCA enzyme will be used in future protein X-ray crystallography studies that may in turn aid in the design of next generation PS inhibitors with improved potency and selectivity for malaria parasites. Finally, the generation of a PS resistant P. falciparum line may aid in determining the mechanism of action of PS-3, possibly identifying a novel “druggable” target in P. falciparum parasites. The PS-3 resistant line also provides a tool for future screening of next generation compounds that may act on the same target or pathway within the parasite.
Statement of Originality

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

_____________________________
Gillian Marie Fisher
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<table>
<thead>
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<th>Description</th>
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<tr>
<td>AAZ</td>
<td>acetazolamide</td>
</tr>
<tr>
<td>ACT</td>
<td>artemisinin-based Combination Therapies</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
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<td>celecoxib</td>
</tr>
<tr>
<td>Cpd</td>
<td>compound</td>
</tr>
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</tr>
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<td>furosemide</td>
</tr>
<tr>
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<td>gram</td>
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<tr>
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<td>GlaxoSmithKline</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrogen chloride</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HTS</td>
<td>high throughput screening</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>concentration of component where response is reduced by half</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est (that is)</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kₐ</td>
<td>affinity constant</td>
</tr>
<tr>
<td>Ki</td>
<td>inhibitory constant</td>
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mL  millilitre
min  minutes
NaAc sodium acetate
NFF Neonatal Foreskin Fibroblast
nm  nanometres
nM  nanomolar
OGTR Office of the Gene Technology Regulator
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
QIMR Queensland Institute of Medical Research
RBCs Red Blood Cells
RI  Resistance Index
RT  Room Temperature
RPMI Roswell Park Memorial Institute
SAR Structure-activity Relationship
SD  Standard deviation
SDS Sodium Dodecyl Sulphate
sec seconds
SFA sulfanilamide
SI  Selectivity Index
SPR Structure property relationship
TEMED Tetramethylethylenediamine
WHO World Health Organisation
Ethics and agreements

All research was conducted in accordance with approved protocols and OGTR guidelines from Griffith University Biosafety Committee (NLRD/002/13 and DNR535) and QIMR Berghofer Medical Research Institute (NLRD P655 and DNR P655).

Human blood and sera were provided by the Brisbane Red Cross service under approved research agreement with the QIMR Berghofer Medical Research Institute (11-06QLD-12) and Griffith University (13-02QLD-15).

Human blood and sera were utilized under approved human research ethics committee protocol from the QIMR Berghofer Medical Research Institute (HREC approval number P655) and Griffith University (ESK/01/12/HREC).
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1 Introduction

Publications arising from this chapter


**Contribution:** I wrote the sections on sulfonamide drugs and contributed to editing sections of the publication and addressing reviewer’s comments.
1.1 Malaria

Malaria is a potentially life threatening disease caused by parasites of the genus *Plasmodium* that are transmitted to humans via the bite of infected female *Anopheles* mosquitoes. Malaria is responsible for approximately 200-300 million clinical cases and >600,000 deaths annually [1]. The majority of malaria morbidity and mortality occurs in sub-Saharan Africa, however, Asia, Latin America and, to a lesser extent, the Middle East and parts of Europe are also affected (Figure 1.1). It is estimated that in sub-Saharan Africa, where most malaria mortality occurs, a child dies every 60 seconds due to this disease [12].

![Map of Malaria transmission risk](image)

**Figure 1.1: Malaria, countries or areas at risk of transmission**
Map generated with the Global Malaria Mapper 2014, MMV

There are six *Plasmodium* species capable of infecting humans; *P. falciparum, P. vivax, P. malariae, P. ovale* (two species) and *P. knowlesi* [13-15]. *P. falciparum* and *P. vivax* are the most significant human-infecting malaria parasites, with *P. falciparum* being the most lethal [1]. Recently, a large number of human cases of malaria have also occurred
with *P. knowlesi* – a zoonotic species that normally causes malaria in macaque monkeys [16-22].

The initial symptoms of malaria include fever, headache, chills and vomiting. If left untreated, this can sometimes lead to severe complications such as anaemia, metabolic acidosis and cerebral malaria symptoms [23]. At present there is no licensed vaccine for malaria and the most promising candidate, the RTS,S vaccine, has only shown 30-40% efficacy in African children in phase III clinical trials [24-26]. Vector control such as insecticide treated mosquito nets and indoor residual spraying are used widely to prevent transmission of malaria [1]. However these methods rely on a single class of insecticide, the pyrethroids, and continued use is likely to lead to resistance in the mosquito [1]. Current antimalarial drugs are becoming less effective due to widespread parasite drug resistance brought about by the continued use of monotherapies, inappropriate/incomplete therapeutic regimens and counterfeit drugs [2, 27]. These findings highlight the necessity for research into the identification of new prevention and treatment strategies for malaria, including the development of a malaria vaccine and new antimalarial drugs.

1.2 Lifecycle of the Malaria parasite

The lifecycle of the malaria parasite (Figure 1.2) is complex and requires both invertebrate and vertebrate hosts. In humans, the lifecycle consists of several stages beginning with sporozoite entry into the bloodstream via the salivary glands of an infected female Anopheles mosquito when she takes a blood meal [28] . Once in the
bloodstream, sporozoites enter hepatocytes where they remain for 9-16 days and undergo asexual replication. *P. ovale* and *P. vivax* have dormant liver stages called hypnozoites that can remain in the liver for weeks up to many years [29]. This can result in relapse of malaria symptoms [30, 31]. Each sporozoite gives rise to tens of thousands of merozoites inside an infected hepatocyte and, once released, merozoites travel to the hosts blood stream and are able to invade erythrocytes [28]. The invasion of erythrocytes by *Plasmodium* parasites involves highly specific molecular interactions, therefore the key molecules involved in this process are considered prime candidates for the development of vaccine and drug therapies [32].
Once inside the erythrocyte the parasite continues to develop and progresses through the asexual intraerythrocytic developmental cycle. This begins with the ring form, followed by maturation to a trophozoite-stage parasite that is highly metabolically active (Figure 1.2). During this stage, the parasite ingests host cell components for survival, including haemoglobin which it degrades, ultimately releasing free amino acids that are used for parasite growth [23]. The waste product of haemoglobin degradation, heme, is polymerised into hemozoin, an inert crystalline substance (also called malaria pigment) that is stored within the digestive vacuole [23]. The trophozoite stage concludes with multiple rounds of asynchronous nuclear division resulting in the formation of the schizont (Figure 1.2). Each schizont contains up to 32 merozoites which are released after red blood cell lysis and go on to invade new red blood cells, completing the intraerythrocytic asexual part of the lifecycle [33]. This stage coincides with the manifestations of the clinical symptoms of malaria and many proteins involved in merozoite invasion are potential vaccine targets [23, 32]. This recurring intraerythrocytic cycle continues approximately every 44-48h for P. falciparum, P. ovale and P. vivax infection, every 72h for P. malariae infection and every 24h for P. knowlesi [34, 35]. Utilizing mechanisms that are not yet well defined, a small percentage of parasites in the red blood cells are able to differentiate and form male and female gametocytes (Figure 1.2), which are sexual forms of the parasite [36-38]. Gametocytes are essential for transmission of malaria parasites to the mosquito vector and are ingested during a blood meal then converted to gametes in the mid-gut of the mosquito. The gametes then undergo fertilisation and form a zygote which traverses the gut wall and develops into an oocyst. Sporogony within the oocyst produces numerous sporozoites and when the oocyst ruptures the sporozoites travel to the salivary glands
for onward transmission into another host [33]. Infective sporozoites can remain in the salivary glands of the mosquito for one to two months. The Plasmodium lifecycle begins again when an infected mosquito bites a susceptible host and injects sporozoites into the blood stream [33].

1.3 Antimalarial drugs and drug resistance

In the absence of a clinically approved vaccine, malaria prevention is highly dependent on chemoprophylaxis and chemotherapy. The first synthetic antimalarials appeared in the 1930s and currently there are a limited number of effective compounds for treating malaria parasite infection, particularly due to the ability of malaria parasites to develop drug resistance [39-42]. Even the current first choice drug combination for treatment of uncomplicated P. falciparum malaria, the artemisinin combination based therapies (ACTs), are under threat [1-3]. The following is an overview of the some of the main antimalarial drug classes currently in use clinically, including available information on their mode of action and disease resistance mechanisms. A more detailed review can be found in [40, 43].

1.3.1 Quinolines

The 4-aminoquinoline chloroquine, a synthetic relative of the plant derived drug quinine, was introduced for malaria treatment in the 1930s [44] and was the antimalarial drug of choice for many decades. This was due to chloroquine being safe, cheap and highly effective. However, resistance to this drug was first observed in Thailand in 1957 [45] and by 1988 resistance had spread to all of sub-Saharan Africa [46]. Today P.
*Plasmodium falciparum* parasite resistance to chloroquine is present in most areas of the world, although chloroquine is still used to treat *P. vivax* malaria in endemic countries with resource constraints and no evidence of resistance [1, 47]. Chloroquine accumulates in the food vacuole of the parasite [48] and acts by interfering with the polymerisation of toxic heme moieties, the by-products of haemoglobin digestion in the intraerythrocytic cycle of the parasite [49-51]. Resistance has been linked to mutations in the *pfcr* gene which encodes the chloroquine resistant transporter (CRT) protein and to a lesser extent the *pfmdr* gene which encodes the P-glycoprotein homologue 1 [52-54]. The aryl amino alcohols, analogues of chloroquine, namely mefloquine, halofantrine and lumefantrine, were introduced in the 1980s as alternatives to chloroquine [55-57], however these drugs are currently not recommended for use as monotherapies due to resistance and/or dangerous side effects [58-62]. Another chloroquine analogue, primaquine, an 8-aminoquinoline, has been used since the 1950s to eradicate the liver stage parasites in *P. vivax* infections and is the only drug available that is used to eliminate late stage gametocytes [1, 63]. Unfortunately, a lengthy 14 day treatment regime with primaquine is needed which often leads to problems with non-compliance [63]. The mode of action of primaquine remains unclear and it can lead to haemolytic anaemia in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals, compromising its widespread use [64]. A new 8-aminoquinoline Tafenoquine is currently under development however this compound still has the haemolytic liabilities of primaquine [65, 66].
1.3.2 Antifolates

The first antifolates for malaria use, pyrimethamine and proguanil (metabolised \textit{in vivo} to the active form cycloguanil), were introduced in the 1940s/50s \cite{67, 68}. However, resistance emerged rapidly which lead to the introduction of the antifolate combination therapy sulfadoxine-pyrimethamine (SP) \cite{69}. Despite the rapid development of SP resistance in South East Asia in the 1960s, this affordable combination therapy has been in use in Africa since 1990, albeit with reducing efficacy \cite{70-72}. More recently sulfadoxine pyrimethamine and amodiaquine (SPAQ) are being rolled out as treatments for seasonal malaria chemoprevention (SMC) for children under the age of five \cite{73} and this has reduced malaria incidence by 75\% in the Sahel region of Africa \cite{74}. Antifolate drugs inhibit essential enzyme production via interactions with the folate pathway. Pyrimethamine and cycloguanil act on the dihydrofolate reductase enzyme (DHFR), while the sulfonamides inhibit dihydropterate synthetase (DHPS). DHPS is an enzyme exclusive to the parasite and is responsible for the biosynthesis of key folate coenzymes. DHFR is present in both the host and parasite and is involved in the provision of nucleotides for DNA synthesis and the metabolism of certain amino acids \cite{75}. Point mutations of the target genes \textit{dhfr} and \textit{dhps} have contributed to the development of parasite resistance to antifolates \cite{76-78}.

1.3.3 Naphthoquinones

The antimalarial atovaquone is a structural analogue of co-enzyme Q and acts on the parasites mitochondria \cite{79}. Atovaquone targets the cytochrome bc complex (complex
III) without affecting the hosts mitochondria. This is due to differences in the parasite (CoQ₈) and human (CoQ₁₀) complex [80]. Resistance occurs rapidly when atovaquone is used alone, with point mutations occurring in the cytochrome b gene [81, 82]. However, atovaquone in combination with proguanil (marketed as Malarone®) has been used to counteract resistance development [83]. Despite this, cases of Malarone® treatment failure have been reported, not all of which were associated with cytochrome b mutations, which indicates that there may also be alternative resistance mechanisms to this drug [84-86].

1.3.4 Peroxides

Artemisinin, a sesquiterpene lactone, derived from the wormwood plant *Artemisia annua*, emerged in the 1960s as an antimalarial [44, 87]. Initially, artemisinin was used successfully to treat malaria in China, including chloroquine resistant cases [87]. Several artemisinin derivatives, namely, artemether, arteether, and artesunate emerged in the 1980s as antimalarial therapies [88]. Artemisinins and their derivatives have a short biological half-life (t₁/₂=2-5h) and require lengthy treatment regimens when used as monotherapies to avoid parasite recrudescence [89]. To minimise the potential for parasite resistance developing, artemisinin-based combination therapies (ACTs) were developed [90]. However continued use of artemisinin monotherapies in places such as Western Cambodia [91] may have contributed to the spread of artemisinin resistance. Currently resistance to ACTs has been reported in four south east Asian countries [1-3]. In addition there is already evidence that resistance to one of the major ACTs, artemether-lumefantrine, is developing in parts of East Africa [92, 93].
The exact mode of action of artemisinin derivatives against *Plasmodium* parasites remains unclear and historically there have been four main models proposed that have been discussed extensively in various reviews [94-99]. These models suggest that artemisinins, once activated through the cleavage of the intrinsic peroxide bond, may: i) inhibit heme detoxification; ii) induce the alkylation of the transcriptionally controlled tumour protein (PfTCTP) and other proteins; iii) inhibit the sarco/endoplasmic reticulum membrane calcium ATPase 6 (PfATPase 6); and/or iv) disrupt *Plasmodium* mitochondrial functions [100]. Mutations in the PfATPase6 gene have been implicated in resistance to artemisinin [94, 101], thus supporting the ATPase 6 inhibition theory.

More recently a kelch protein (K13) was found to be linked to resistance in an *in vitro* artemisinin selected *P. falciparum* line [102, 103] and this association was also confirmed in clinical measures of artemisinin resistant and sensitive parasites in Cambodia [102, 103]. It was reported that levels of the PfP13K enzyme (an enzyme that promotes cell signalling and survival) are regulated by PfKelch13 which mediates the ubiquitination and degradation of PfP13K [104]. Mutations in PfKelch13 that prevent it from binding to PfP13K were shown to increase levels of PfP13K and thus overcome the effects of artemisinin [104]. Furthermore, a cell cycle regulator (PfE415w) was linked to artemisinin dormant parasites (a proposed mechanism of artemisinin resistance) using the same *in vitro* artemisinin resistant line mentioned above [105].

Despite World Health Organisation (WHO) recommendations to cease the use of artemisinin monotherapies, as of November 2013, nine countries were still marketing artemisinin monotherapies and thirty pharmaceutical companies continue to
manufacture them [1]. If continued, use of artemisinin monotherapy is likely to further increase the spread of resistance, potentially further compromising the use of ACTs which is currently the WHO's drug of choice for treating malaria.

1.3.5 Antimalarials currently under development

Currently there are several drugs in various stages of preclinical or clinical development, however most of these are reformulations or new combinations of existing antimalarial drugs [106]. Only one new chemical class (chemotype) of antimalarial, the spiroindolones [5], has progressed to the point of clinical trials in the past decade. Figure 1.3 is a summary of the global portfolio of current antimalarials in development and their chemotypes, a more detailed account of the current status of these compounds are reviewed in [106].

Widespread and increasing drug resistance to current antimalarials, along with manufacturing and supply problems [107], substantiate the need for new antimalarial therapies. The next generation of antimalarials will ideally require different modes of action to their predecessors, as well as complementary pharmacokinetics, in order to prevent cross-resistance to existing drugs.
### Figure 1.3: 2015 global portfolio of antimalarial medicines under development [73]

<table>
<thead>
<tr>
<th>Translational</th>
<th>Human volunteers</th>
<th>Development</th>
<th>Patient exploratory</th>
<th>Patient confirmatory</th>
<th>Under review*</th>
<th>Access Post Approval</th>
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<tr>
<td>P218 DHFR BIOTEC (Monsanto/LSHTM)</td>
<td>MMV/048 UCT/TTA</td>
<td>OZ439a/PQP Sanofi</td>
<td>Tafenoquine GSK</td>
<td>Rectal Artesunate CPLA/Strokes/TDR</td>
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<td>Artemether- Luminofantrine Novartis</td>
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<tr>
<td>SJ733 St Jude Buzau</td>
<td>ACT840 Actelion</td>
<td>OZ439/FQ Sanofi</td>
<td>DHA-Piperazine Paediatric Sigma-Tau</td>
<td></td>
<td><strong>2</strong></td>
<td>Artemether- Luminofantrine Dosage Forms Novartis</td>
</tr>
<tr>
<td>DDD498 Mercer Sonera (Dundee)</td>
<td>CDR1 97-76 Ipca</td>
<td>KAU009 Novartis</td>
<td>Pyronaridine- Artesunate Paediatric Shionogi</td>
<td></td>
<td><strong>4</strong></td>
<td>Artesunate for Injection Gailin</td>
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<tr>
<td>FAS92 (Drexel/IVRG/NIH)</td>
<td>DF12 Dosier</td>
<td>KAF156 Novartis</td>
<td>Co-Immune Bacillus Cont. of Top. Med.</td>
<td></td>
<td><strong>5</strong></td>
<td>DHA- Piperazine Sigma-Tau</td>
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<td>MMV253 (Astrazeneca)</td>
<td>Tierra et al. (Imperial College London/LSHTM)</td>
<td>DSM205 NihTakeda</td>
<td>Artemisinin Naphthoquine KHC</td>
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<td><strong>6</strong></td>
<td>Pyronaridine- Artesunate Shionogi</td>
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<td>Antiamoether sublingual spray ProPharma Ltd</td>
<td>Formononcycin Piperazine James-Paine Group</td>
<td>Anthelminthics ProPharma Ltd</td>
<td></td>
<td><strong>9</strong></td>
<td>Artemether Amodiaquine Sanofi MSD</td>
</tr>
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<td>NL4815 Merck</td>
<td>ACTs Endoperoxides</td>
<td>Severe Malaria</td>
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<td>Artemether Mefloquine CPLACNS</td>
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<tr>
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<td>Severe Malaria</td>
<td>Antibiotics</td>
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<tr>
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<td>P. vivax</td>
<td>Molecular Mechanisms</td>
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<td>Artemether Mefloquine CPLACNS</td>
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*First review or approval by WHO Prequalification, or by regulatory bodies who are CH members or observers

**Approval in India but not under review by WHO**
1.4 Antimalarial drug discovery and development – available resources and key bottlenecks

The discovery and development of any antimalarial drug is a long term project and can take up to fifteen years or more. Ideally new drugs for uncomplicated *P. falciparum* malaria should be efficacious against drug resistant strains, fast acting to ensure compliancy, be safe and be suitable for small children and pregnant women [41, 108]. According to the Medicines for Malaria Venture, the worlds’ leading not-for-profit malaria drug discovery and development organization, a multi-pronged approach is needed to eliminate malaria [8]. This includes the development of fast acting compounds that can reduce the initial parasite burden, a long duration partner drug to prevent relapses, a transmission blocking agent and chemoprotection drugs [109]. The current target profiles for new antimalarial drugs are discussed in greater detail in Chapter 3, with key criteria for an early drug lead being *in vitro* potency (<0.1µM), parasite specific selectivity (>100), compound novelty and lack of chemical liabilities and oral efficacy or protection in a malaria mouse model (Appendix 2). To deliver and develop new antimalarial drug leads requires effective interaction across a number of scientific disciplines. As discussed below, advances in genomics, proteomics, transfection technologies, *in vivo* and *in vitro* models, and high throughput screening have provided effective tools for identifying and developing new drug targets.

The sequencing of the first complete malaria parasite genome in 2002, with several since then, has enabled the rapid identification of putative *P. falciparum* targets that are
homologous to validated drug targets from other biological systems [110, 111]. However approximately 50% of the genes in the *P. falciparum* genome encode proteins of unknown function. These so called “hypothetical proteins” are untapped resources for drug development, however further research into characterisation of these genes is required and is ongoing [112]. Currently PlasmoDB, a functional genomic sequence data base for *Plasmodium spp*, contains fully sequenced and annotated genomes for nine *Plasmodium* species [113]. Transcripts for approximately ninety five percent of *P. falciparum* genes are available and offer vital information pertaining to the when and where known and putative drug targets are expressed in the parasite [114-117]. New proteomic approaches such as analysis of post translational modifications may also provide new avenues for drug discovery research [118-121]. In addition to advances in genomics and proteomics, progress in transfection technologies has allowed the production of *P. falciparum* transgenic cell lines (i.e. gene knockouts, gene disruption mutants and gene over-expression mutants), some of which have been fundamental in improving our understanding of parasite biology, and in validating potential drug targets [122-125].

Typically antimalarial drug discovery begins with compound or natural product screening *in vitro* against cultured *P. falciparum* infected erythrocytes in cell based assays or using *in vitro* target-based assays [126]. Medium or high throughput whole-cell based screening allows the screening of millions of compounds to identify lead drug targets for antimalarial drug development. Whilst this still leaves the question of the target unresolved, this approach has recently led to information sharing by large pharmaceutical companies to further the development of antimalarial therapies. For
example a recent high throughput screening of a GlaxoSmithKline (GSK) library of approximately two million compounds identified approximately eight thousand compounds that inhibited the in vitro growth of drug-sensitive (3D7) and resistant (Dd2) *P. falciparum* parasites (≥80% and ≥50% at 2 µM, respectively) [7]. The structures of GSKs hit compounds were made publicly available through deposition in the open access European Bioinformatics Institute ChEMBL Neglected Tropical Disease archive. As discussed later in this thesis, this is a valuable resource for malaria drug discovery and has provided a source of compounds for work described in this study (see Chapter 3).

Another useful resource that is sparking current interest is the repurposing or repositioning of drugs previously developed as treatments or drug leads, for other diseases [127, 128]. As repurposed drugs are typically approved for clinical use, the discovery of a new application, such as antimalarial efficacy, has the potential to result in significant time and cost savings. A good example of this in the context of malaria is the use of certain broad spectrum antibiotics, such as synthetically derived tetracyclines (e.g. doxycycline) which have been used successfully for malaria chemoprophylaxis and treatment [129, 130]. Likewise clindamycin, a lincosamide antibiotic originally used for acne treatment in the 1940-1950s [131], with numerous applications since, has been used clinically for malaria since the 1970’s [132]. Additional information can be found in recent reviews, including one published as part of this thesis project [133, 134].

Other approaches to antimalarial drug development include the use of existing drugs and/or drug leads to create combination therapies. Combination therapies are widely
used for antimalarial treatment as they have the potential to delay the onset of resistance compared to monotherapies [135]. More recently efforts have focused on synthesising single drugs with dual functionality and/or targets, commonly known as ‘hybrid drugs’ [136-138]. Recent examples that employ pharmacophore hybridization as an approach to antimalarial drug discovery include chloroquine-triazine hybrids [139-141], chloroquine-pyrimidine hybrids [142], trioxaquines [4, 136, 137], the mefloquine-artesunate hybrid MEFAS [143], the ferrocene-chloroquine hybrid, ferroquine [144-146] and others [147-151]. Part of the work in this PhD uses this approach to investigate the antimalarial activity of a panel of novel hybrid compounds based on the clinically used drug chloroquine and is discussed in more detail in Chapter 3.

1.5 Antimalarial target-identification approaches

While it is not essential to know the target of an antimalarial agent prior to using it clinically, as was the case for artemisinin and other drugs, knowledge of how a drug acts and what target or pathway it affects can provide important information. This can include guiding generation of more potent and/or selective compounds, selection of suitable partner drugs, or a means to monitor emerging resistance once clinically employed. A range of approaches can be employed to investigate the target of antimalarial compounds, some of which were utilized in this thesis project (Chapters 4-6). While additional information on the specific strategies used in this thesis are presented within relevant chapters, a discussion of some of the main antimalarial target identification approaches currently available are presented below. These include the use
of *in silico* methods, phenotypic approaches, genomics, metabolomics, proteomics and *in vitro* resistance selection strategies [152].

### 1.5.1 *In silico* target identification/prediction

Potential information on putative targets can be obtained via *in silico* approaches that involve comparing chemical structures to the structure of compounds that may have a known target in another organism. This approach can be facilitated by resources such as the TDR Targets Database (http://tdrtargets.org), which is an online tool for the identification and prioritization of molecular targets for drug development for neglected human diseases, including malaria [153]. However, it is important to note that there are limitations to this predictive strategy. For example cycloguanil and methotrexate both contain a diaminopyrimidine group that is essential for binding to DHFR, but differ structurally otherwise and therefore would not be recognised by this computational method [152]. In another example, an in silico predictive approach was used to assess 8457 “hit” antimalarial compounds from a Novartis screening library, however only 5% of compounds were able to be assigned to a probable mode of action [154]. This low success rate combined with the fact that ~50% of the *Plasmodium* genome does not have homologues in other organisms [110], highlights the importance of experimental methods to identify antimalarial compound mode of action.
1.5.2 Phenotypic approaches

Investigating phenotypic changes in parasites can help identify putative mechanisms of action or pathways associated with drug activity. For example assessing the stage and/or speed of action of a compound in combination with molecular approaches may provide clues to potential biological targets. For example inhibition of \textit{P. falciparum} calcium–dependent protein kinase 1 (PfCDPK1) and dipeptidyl aminopeptidase 3 (PfDPAP3) were shown to block merozoite egress in the mature shizont [155, 156]. Stage specific activity has also identified the type II fatty-acid biosynthesis pathway to be essential in liver stages but not in intraerythrocytic stages [157]. Likewise, the antimalarial effects of the broad spectrum antimicrobial agent fosmidomycin, which targets the \textit{Plasmodium} non-mevalonate pathway of isoprenoid biosynthesis (DOXP), can be rescued by supplementation by isoprenoid metabolites [158]. Chemical rescue has also been used to validate the \textit{P. falciparum} apicoplast as an essential, and therefore druggable, organelle. Isoprenoid synthesis has been established as the only essential apicoplast metabolic function and cultures supplemented with isopentenyl pyrophosphate (IPP) are rescued from antibiotic treatment [159]. Parasites continue to grow and replicate in the presence of IPP even in the absence of the apicoplast organelle [159].

1.5.3 Metabolomic approaches

Metabolomic-based strategies have been around for several years and are proving to be beneficial for antimalarial target identification, and understanding mechanisms of antimalarial drug resistance [158, 160, 161]. This approach was used to reveal methylerthritol phosphate cytidyltransferase (IspD) as a secondary target of
fosmidimycin, an inhibitor of DOXP in the apicoplast [158]. In another approach, analysis of metabolites from chloroquine sensitive and resistant parasites revealed a significant difference in levels of peptides relating to haemoglobin digestion with peptide accumulation being linked to the *P. falciparum* chloroquine resistance transporter gene (*pfert*) [160].

### 1.5.4 Proteomic approaches

Relatively few proteomic approaches have been used to understand the mechanism of action of antimalarial drugs or drug resistance in *P. falciparum*. The differential expression of proteins treated with artemisinin and chloroquine were investigated in a large scale study using mass spectrometry with the expression of 41 and 38 proteins, respectively, found to be up regulated [120]. Additionally a redox proteomics approach was used to identify proteins that were oxidatively modified in different intraerythrocytic stages of chloroquine resistant parasites [162]. More recently 2D fluorescence difference gel electrophoresis (2D-DIGE) and isobaric tagging reagents for relative and absolute quantitation (iTRAQ) were employed to identify proteins that are deregulated by doxycycline. Although mitochondrial and apicoplast proteins were identified no specific target or pathway was found [163].

Other proteomic approaches include the use of chemical probes designed to label target proteins in cells or cell lysates [9]. These probes include a reactive group to bind with the target, a linker group capable of covalent binding and a biotin or fluorophore group to allow visualisation and/or separation of the probe-labelled protein [9]. A biotin based
probe approach has been used in *P. falciparum* to identify the serine protease (PfSUB1), metallo-aminopeptidases and DPAP1/DAP3 as antimalarial drug targets [164, 165]. However these probes are not effective for labelling in live cells due to their bulky nature [166]. To overcome this problem a dual step ‘clickable’ probe has evolved which enables the addition of the reporter group after the incorporation of the reactive group to the target protein in live cells or cell extracts [167-169]. This click chemistry based protein profiling method is yet to be exploited for antimalarial drug target identification and will be introduced as part of the work in this thesis in Chapter 5.

### 1.5.5 *In vitro* resistance selection

The identification of new antimalarial targets has recently been facilitated by the generation of parasites that are resistant to an inhibitory compound of interest followed by analysis of resistant clones versus parental clones [5, 170, 171]. Generation of resistant lines via *in vitro* compound selection essentially relies on the parasites natural acquisition of resistance and is achieved by culturing parasites in the presence of compound until a resistance phenotype is acquired. Once a stable phenotype has been achieved, a number of approaches can be used to investigate the resistance profile including cross-resistance studies with other compounds, candidate gene sequencing, and whole genome sequencing [172-175]. Genomic changes can then be verified in genetic complementation studies using various techniques including allelic exchange and *piggyBac* strategies [176]. Several novel and previously known antimalarial targets have been identified using *in vitro* resistance selection (discussed in detail in Chapter 6) and the use of this method has led to a better understanding of the mechanism of action.
of artemisinin, a component of the WHO’s recommended first line treatment for \( P. falciparum \) infections \([5, 170, 171, 177, 178]\).

As discussed in Chapter 6 this approach has been used to begin to understand how the primary sulfonamide compound class targets \( P. falciparum \) parasites.

### 1.6 Primary sulfonamides - a potential new antimalarial

Despite the critical need for antimalarial drugs with novel modes of action, no new chemical class of antimalarial drug has been approved since 1996 \([179]\), and only one, a spiroindolone, has progressed to clinical trials \([5, 180]\). To address the need for discovery of new drug starting points, work in this thesis focused on investigating compounds containing a primary sulfonamide (PS) moiety as a potential new antimalarial chemotype.

#### 1.6.1 Primary Sulfonamides

The sulfonamide moiety has an established history of efficacy and safety in clinically used drugs \([181]\). While historically the term “sulfa allergy” has been associated with sulfonamide containing drugs, this effect is now known to be associated with the aniline structure of some of the bacterial sulfonamides and not the sulfonamide functional group as previously thought \([181]\). For example, the antimalarial drug sulfadoxine (an antifolate drug) contains a secondary sulfonamide group with an aniline toxicophore \([181]\). Secondary sulfonamides are substituted on the sulfonamide nitrogen in contrast
to primary sulfonamides (Figure 1.4), making them structurally distinct in terms of possible interactions with potential biological targets, to the extent that primary sulfonamides are a separate class of compound [182]. Therefore antimalarial primary sulfonamides are not predicted to act as antifolates in malaria parasites, although as with any new drug lead each compound needs to be examined for cross-resistance to existing antimalarial drugs, a criterion for classification as an early phase hit or lead (Appendix 2). Primary sulfonamides are known mainly to target carbonic anhydrase (CA) enzyme activity [183-185]. CA enzymes are important for many biochemical processes as discussed in detail below and in Chapter 4 of this thesis.

1.6.2 Primary sulfonamides and Carbonic anhydrases

Classical CA inhibitors (CAIs) are PS compounds and have been in clinical use for over fifty years as diuretics and systemically acting anti-glaucoma drugs, and more recently as anti-epileptics and anti-cancer agents [6, 183, 186]. There are over 100 FDA approved sulfonamide containing drugs and many of these compounds show significant CA inhibitory activity and CA inhibition may be responsible for their therapeutic effect. [186]. Carbonic anhydrases (CA; EC 4.2.1.1) are zinc metalloenzymes that catalyse the reversible reaction of carbon dioxide and water to bicarbonate and a proton. In 1933 the first CA was purified from bovine red cells [187]. CAs are involved in important
Figure 1.4: Primary sulfonamides are a distinct chemical class of compound

Acetazolamide is an example of a primary sulfonamide (R-SO₂NH₂; panel A) a distinct chemical class of compound to the secondary sulfonamides (R-SO₂NH-R'; panel B). The antimalarial drug sulfadoxine (panel B) is an antibacterial secondary sulfonamide and comprises an aniline toxicophore (red) and a secondary sulfonamide group (blue).

physiological processes connected with respiration and transport of CO₂/bicarbonate, pH and CO₂ homeostasis, electrolyte secretion, biosynthetic reactions, bone resorption, calcification, tumorogenicity, and many more physiologic and pathologic processes [186, 188-192]. Several of the mammalian CAs are therapeutic targets and human α-CA inhibitors, including those containing PS moieties, are established diuretics and antiglaucoma drugs [186]. Furthermore α-CA inhibitors, many of which contain a PS, have shown potential for the development of anti-obesity, anti-cancer and anti-infective therapies [186].
However, the lack of isozyme selectivity of the presently available inhibitors, along with the high number of CA isoforms in humans [186] and their diffuse localisation in many tissue types [186], can pose problems in the progression of CAIs as therapeutic agents. It is therefore promising that progress is taking place in the design of CA selective and isozyme specific CAIs. For example, the ubiquitous CA II isoform was found to have an Ala65 amino-acid residue which is not present in any other isoforms, a difference that may be exploited in the design of compounds with lower affinity for CA II versus other human CAs [193]. Furthermore, several approaches have recently been reported allowing the identification of compounds that specifically target the tumour associated isoforms CA IX and CA XII. These include the use of fluorescent sulfonamides, membrane impermeant, hypoxia-activatable and sugar containing compounds and nanoparticles coated with CAIs [194-205].

Advances in identification of specific inhibitors that exploit key target enzyme differences are promising for the potential development of PS compounds for other applications, including use against infectious diseases. In addition to the aforementioned clinical applications of CAIs, their potential as anti-infective agents has recently emerged as a new research direction. The CAs have been cloned and characterised for such pathogens as Helicobacter pylori, Mycobacterium tuberculosis, Candida albicans and Cryptococcus neoformans, and have been shown to be essential for microbial growth and virulence [206-211]. Of particular interest to this study is the presence of a CA in the P. falciparum genome (PfCA), a protein historically classified as belonging to the α-CA class of enzymes [206]. Given that sulfonamide containing compounds can inhibit Plasmodium growth in vivo and in vitro [206], a hypothesis addressed in this
thesis project (Chapter 4) was that the antimalarial PS compounds identified in Chapter 3 of this study may target the PfCA.

1.6.3  *P. falciparum* carbonic anhydrase

Over a decade ago the first evidence of in situ CA activity in *P. falciparum* was shown using electron microscopy and the CA-specific Hanssen’s stain [212]. Several years later a more comprehensive study demonstrated the existence of CA in *P. falciparum* and the protein was reported to differ in both physical and kinetic properties from the human CA II enzyme (Table 1.1). This study also reported that *P. falciparum* infected erythrocytes contained approximately double the amount of CA than uninfected erythrocytes. In addition the activity of PfCA increased with parasite maturation, with trophozoite and schizont stage CA activity being 7.5 and 18 fold with respect to ring stage CA activity. In 2004, the PfCA gene was identified from the malarial parasite genome database and a fragment of this gene was cloned and expressed in *E. coli*. The recombinant PfCA enzyme (amino acid sequence: 211-445; PlasmoDB gene ID PF3D7_1140000) was shown to be catalytically active and sensitive to the primary sulfonamides, acetazolamide ($K_i = 315$ (±26) nM) and sulfanilamide ($K_i = 84$ (±10) µM).

<table>
<thead>
<tr>
<th>Source</th>
<th>$K_m$ (mM)</th>
<th>$K_{cat}$ (min$^{-1}$)</th>
<th>$K_i$ Acetazolamide (nM)</th>
<th>$K_i$ Sulfanilamide (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CA II</td>
<td>10.1 ± 0.8</td>
<td>74.1 ± 5.7</td>
<td>99 ± 6</td>
<td>145 ± 2</td>
</tr>
<tr>
<td>PfCA</td>
<td>3.7 ± 0.2</td>
<td>10.4 ± 1.2</td>
<td>247 ± 14</td>
<td>56 ± 4</td>
</tr>
</tbody>
</table>

Data taken from [213]
Importantly, in terms of design of potential *P. falciparum* specific inhibitors, the deduced amino acid sequence of the PfCA gene has low identity (~3-10%) with human CA amino acid sequences, several of which are shown in Table 1.2. While the *Plasmodium* CA sequences share greater amino acid identity, the identity between the human infecting parasite and the rodent malaria parasites is low (<50%; Table 1.2).

<table>
<thead>
<tr>
<th>Organism</th>
<th>PfCA</th>
<th>PcCA</th>
<th>PyCA</th>
<th>PbCA</th>
<th>hCA I</th>
<th>hCA II</th>
<th>hCA III</th>
<th>hCA VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfCA</td>
<td>100</td>
<td>42</td>
<td>40</td>
<td>47</td>
<td>8</td>
<td>3</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>PcCA</td>
<td>42</td>
<td>100</td>
<td>65</td>
<td>84</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>PyCA</td>
<td>40</td>
<td>65</td>
<td>100</td>
<td>85</td>
<td>19</td>
<td>17</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>PbCA</td>
<td>47</td>
<td>84</td>
<td>85</td>
<td>100</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>hCA I</td>
<td>8</td>
<td>10</td>
<td>19</td>
<td>7</td>
<td>100</td>
<td>60</td>
<td>53</td>
<td>31</td>
</tr>
<tr>
<td>hCA II</td>
<td>3</td>
<td>9</td>
<td>17</td>
<td>5</td>
<td>60</td>
<td>100</td>
<td>58</td>
<td>33</td>
</tr>
<tr>
<td>hCA III</td>
<td>8</td>
<td>6</td>
<td>16</td>
<td>6</td>
<td>53</td>
<td>58</td>
<td>100</td>
<td>32</td>
</tr>
<tr>
<td>hCA VI</td>
<td>10</td>
<td>7</td>
<td>13</td>
<td>12</td>
<td>31</td>
<td>33</td>
<td>32</td>
<td>100</td>
</tr>
</tbody>
</table>

CA = carbonic anhydrase; Pf = *P. falciparum*; Pc = *P. chabaudi*; Py = *P. yoelii*; Pb = *P. berghei* and h = human. Data taken from [214]
Table 1.3: Antimalarial activity of Compound 10

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Structure</th>
<th>rPfCA1 Structure</th>
<th>Pf IC50 (μM)a</th>
<th>Pb ID50 (mg/kg)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpd 10</td>
<td><img src="image" alt="Structure Image" /></td>
<td>0.18</td>
<td>1.00</td>
<td>10.00</td>
</tr>
<tr>
<td>AAZ</td>
<td><img src="image" alt="Structure Image" /></td>
<td>0.32</td>
<td>20.00</td>
<td>No effect</td>
</tr>
<tr>
<td>CQ</td>
<td><img src="image" alt="Structure Image" /></td>
<td>No inhibition</td>
<td>-</td>
<td>5.00</td>
</tr>
</tbody>
</table>

*aIn vitro inhibition data of recombinant purified PfCA; bIn vitro inhibition of growth of P. falciparum; cIn vivo antimalarial activity in P. berghei infected mice. Chloroquine (CQ) used as a control for in vivo antimalarial activity and acetazolamide (AAZ) as a control for PfCA inhibition. Compound (Cpd) 10, 4-(3, 4-dichlorophenylureido) thioureido-benzenesulfonamide. Data taken from [215].

Despite this, the amino acids responsible for putative binding and catalysis are identical among the P. falciparum, P. yoelii, human CA II and the Drosophila melanogaster sequences [216]. A PfCA homologue has not yet been identified in the genomes of other human infecting Plasmodium species [113] including P. vivax, although given that ~50% of the Plasmodium genome is not yet annotated orthologues of this protein may exist.

While relatively little work has been conducted on inhibition of PfCA, one compound has been identified from a series of aromatic sulfonamides that had previously been under investigation as antiglaucoma and anticancer agents [206]. The lipophilic 4-(3, 4-dichlorophenylureido-ethyl)-benzenesulfonamide was found to be a moderately effective inhibitor of P. falciparum in vitro growth (IC50 2μM). However this compound was also shown to inhibit human CA II enzyme activity with similar profiles to PfCA.
More recently a thioureido derivative of this compound, namely 4-(3, 4-dichlorophenylureido) thioureido-benzenesulfonamide (compound 10, Table 1.3), showed increased activity against *P. falciparum in vitro* growth (IC$_{50}$ 1μM) and was also effective *in vivo* against *P. berghei* (ID$_{50}$ of 10mg/kg) compared to chloroquine as a control (ID$_{50}$ of 5mg/kg) (Table 1.3) [215]. Beyond these limited studies, target validation of these two sulfonamides has not been reported.

### 1.6.4 *PfCA may be essential for *P. falciparum* pyrimidine synthesis*

The malaria parasite requires purines and pyrimidines for nucleic acid synthesis for growth and replication during the intraerythrocytic part of the life cycle [217-223]. The parasites salvage pre-formed purines from the human host, but must synthesise pyrimidines *de novo* from bicarbonate, adenosine 5’-triphosphate, glutamine and aspartate [217-223]. *PfCA* is proposed to be involved in the first step of this process, being the synthesis of carbamoylphosphate from glutamine in the presence of carbomoylphosphate synthase, with the substrate for this reaction being bicarbonate (Figure 1.5) [213]. Many of the clinically available antimalarials have been shown to directly or indirectly affect parasite pyrimidine metabolism (eg. pyrimethamine, cycloguanil, atovaquone, sulfonamides and sulfones) [224]. If antimalarial PS compounds target *PfCA* they may block pyrimidine synthesis at the beginning of this pathway which would be a viable therapeutic strategy. Despite this hypothesis, it should be noted that *PfCA* has not yet been experimentally shown to be essential to *P. falciparum* parasites and other possible sources of bicarbonate and/or the involvement of the host CA has not been investigated thus far.
Figure 1.5: Malaria parasite pyrimidine metabolic pathway.  
OMP, orotidine 5'-monophosphate; UMP, uridine 5'-monophosphate; UTP, uridine 5'-triphosphate; CTP, cytidine 5'-triphosphate; PfCPSII, *P. falciparum* carbamoyl phosphate synthetase II; PfATCase, *P. falciparum* aspartate carbamoyltransferase; PfDHOase, *P. falciparum* dihydroorotase; PfDHODH, *P. falciparum* dihydroorotate dehydrogenase; PfOPRT, *P. falciparum* orotate phosphoribosyltransferase; PfODC, *P. falciparum* orotidine 5'-monophosphate decarboxylase; PfCTps, *P. falciparum* cytidine 5'-triphosphate synthase.
As mentioned previously the PS group is in the structure of several anti-infective drugs where the mode of action is attributed to CA inhibition [186, 225]. In addition to this a recombinantly expressed *P. falciparum* CA (*Pf*CA) has been shown to be inhibited by several aromatic PS compounds [206, 215], one of which displayed promising *in vitro* and *in vivo* antimalarial activity (*Table 1.3*). This together with the current dogma that *Pf*CA is possibly important in the essential *P. falciparum* pyrimidine synthesis pathway led to the hypothesis that *Pf*CA may also be a target to the antimalarial PS compounds identified in this study. However it should be noted that other potential targets were also investigated using unbiased approaches.
1.7 Project Aims

Malaria is a significant global infectious disease, resulting in 200-300 million clinical cases and >0.6 million deaths annually. In the absence of a clinically approved vaccine, malaria prevention and treatment relies on vector control and drug therapy [1]. Unfortunately, all current antimalarial drugs are failing due to the widespread emergence of parasite resistance [1-3]. Therefore next generation antimalarial drugs, particularly new chemotypes that have novel drug targets in the parasite, are urgently needed. This project addresses this problem by investigating the antimalarial potential of primary sulfonamides, a clinically validated class of compounds that has not yet been exploited for malaria therapy. The hypotheses and specific aims of the project are listed below.

Hypothesis

*That novel primary sulfonamide compounds can be identified as antimalarial drug leads that act in a different way to existing antimalarial drugs*

This hypothesis was addressed by two broad aims:

**Aim 1:** To identify antimalarial primary sulfonamide (PS) compounds that may represent new antimalarial drug starting points.

This Aim is addressed in Chapter 3, where the *in vitro* activity of 65 compounds against *P. falciparum* and mammalian cell lines was assessed. Compounds examined included
15 clinically used drugs containing a primary sulphonamide (PS) moiety, 14 new experimental PS glycoside compounds originally designed for cancer, a panel of 26 PS containing compounds derived from recent antimalarial high throughput screening of a GlaxoSmithKline (GSK) library of approximately two million compounds, and a small panel of 9 hybrid molecules containing PS and 4-aminoquinoline moieties. Key compounds arising from this Chapter were further investigated in under Aim 2 for their mode of action against *P. falciparum* parasites.

**Aim 2: To investigate the antimalarial target/targets of primary sulphonamide containing compounds using both biased and unbiased target identification approaches.**

Under this aim a target-biased approach, based on the hypothesis that the *P. falciparum* CA may be a target of antimalarial PS compounds, was employed to assess the inhibitory activity of antimalarial PS compounds against recombinant *PfCA* (Chapter 4). In Chapter 5 and 6, primary sulfonamide bioaffinity probes and *in vitro* resistance selection, respectively, were used to investigate the mode of action of antimalarial primary sulphonamide compounds in an unbiased (target-independent) approach.
2 General methods
2.1 *P. falciparum in vitro cultures*

*P. falciparum* infected erythrocytes were cultured in O positive blood in RPMI 1640 (Gibco, USA) supplemented with 10% heat-inactivated pooled human sera and 5µg/mL gentamicin (Sigma, USA). Cultures were maintained at 37°C in gas mixture composed of 5% O₂, 5% CO₂, and 90% N₂, as described previously [126]. Cultures were maintained between 0.1 – 10% parasitemia and 5% hematocrit, and monitored daily via microscopic examination of Giemsa-stained thin blood smears. Parasite cultures were fed by replacing the old culture media with freshly prepared complete media (as described above) every 1 – 2 days. Alternatively, the parasite cultures were split by diluting a fraction of the blood pellet in new O positive blood and complete culture media to 5% hematocrit.

2.2 Sorbitol synchronisation

Ring-stage *P. falciparum* infected erythrocytes were enriched by sorbitol treatment as described previously [226]. Briefly, *P. falciparum* infected erythrocytes cultures were pelleted by centrifugation at 726 x g for 2 min in a 10 – 50mL conical tube and medium was removed. The packed red blood cell pellet was resuspended in 10 volumes of sterile 5% sorbitol solution then incubated at room temperature for 5 min. The suspension was pelleted by centrifugation as above and the sorbitol solution removed. The ring-stage enriched red blood cells pellet (>90% rings) was resuspended in complete culture media and returned to culture (Section 2.1).
2.3 Cryopreservation of *P. falciparum* infected erythrocytes

Predominant ring stage *P. falciparum* parasitized RBCs were pelleted using an Allegra® X-15R Beckman COULTER centrifuge at 726 x g for 2 min and resuspended in an equal volume of sterile freezing solution (Appendix 1). The solution was then aliquoted into cryovials (Greiner Bio-one, Belgium) and frozen in an ethanol dry ice bath. Cryovials were transferred to -80°C for at least 24 hr and then stored in liquid nitrogen for long term storage.

2.4 Thawing *P. falciparum* infected erythrocytes

Cryovials containing frozen RBCs infected with *P. falciparum* parasites were removed from liquid nitrogen storage and thawed quickly at 37 °C in a water bath. Thawed cells were immediately transferred to a 50 ml centrifuge tube (Greiner Bio-One, Germany) and 0.2 pellets volume of sterile 12% NaCl solution added drop wise with continuous mixing followed by 5 min incubation at room temperature. Following the addition of 10 pellet volumes of 1.6% NaCl drop by drop, the solution was centrifuged at 726 x g for 2 min in an Allegra® X-15R Beckman COULTER centrifuge. The resulting pellet was resuspended in complete parasite culture media (Appendix 1) at 5% hematocrit and cultured as in Section 2.1.
2.5 *In vitro* P. *falciparum* growth inhibition assays

The antimalarial activity of compounds was tested against *P. falciparum* using a 72 hr isotopic microtest, as previously described [227]. Briefly, synchronous ring-stage *P. falciparum* infected RBCs (0.5% parasitemia and 2.5% final hematocrit) were seeded into 96-well tissue culture plates (3596 Costar®, Corning, USA) containing serial dilutions of control or test compounds. After incubating for 48 hr under standard *P. falciparum* culture conditions in incomplete parasite culture media (Appendix 1), 0.5 µCi [³H]-hypoxanthine (PerkinElmer®, USA) was added to each well followed by culturing for a further 24 hr. Cells were harvested onto 1450 MicroBeta filter mats (Wallac, USA) and [³H] incorporation was determined using a 1450 MicroBeta liquid scintillation counter (PerkinElmer®, USA). The percentage inhibition of growth compared to that of matched DMSO (0.5%; Sigma-Aldrich, USA) controls was determined for at least three independent experiments, each carried out in triplicate wells. IC₅₀ values were calculated using linear interpolation of inhibition curves [228]. Each compound was assayed in triplicate wells, in at least three independent experiments and chloroquine (Sigma-Aldrich, USA) was used as a positive control in all assays.

2.6 *In vitro* cytotoxicity assays

Neonatal foreskin fibroblast (NFF) cells were cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (CSL Biosciences, Parkville, Victoria, Australia), 1% streptomycin (Life Technologies, Inc., Rockville,
MD; complete medium) at 37°C and 5% CO₂. Cells were maintained in LogPhase growth and then seeded (3,000/well) into 96-well tissue culture plates (Corning, USA) and were grown for 24h before treatment. Compounds were dissolved in 100% DMSO and diluted in complete medium; the DMSO concentration in the medium did not exceed 1%. Control cells were treated with the equivalent dose of DMSO. Three days after treatment initiation, the cells were washed with PBS and fixed in methylated spirits and total protein was determined using sulforhodamine B as described previously [229]. Percentage inhibition of growth was compared to matched 1% DMSO controls. IC₅₀ values were calculated using linear interpolation of inhibition curves. The mean IC₅₀ (+/- SD) is shown for three independent experiments, each carried out in triplicate.

2.7 Saponin lysis

*P. falciparum* infected erythrocyte cultures were pelleted by centrifugation at 726 x g for 2 min and the culture medium removed. Parasites were extracted by resuspending the culture pellet in 10 volumes of 0.15% saponin (Sigma, USA; diluted in PBS) and incubating on ice for 5 min. The lysed red blood cell mixture was transferred into microfuge tubes (Eppendorf, Germany) and centrifuged at 17,000 x g at 4°C for 5 min. The resulting pellets were washed three times in 500 µL of cold PBS or until the supernatant was clear. The parasite pellet was stored at -80°C until needed.
2.8 Genomic DNA extraction

*P. falciparum* parasite pellet (Section 2.7) was washed once in 1 mL of TKM1 buffer (10 mM Tris, pH 7.6, 10 mM KCl, 10 mM MgCl₂, and 2 mM EDTA) for every 50 µL packed cell pellet. The pellet was resuspended in 100 µL of TKM1 buffer and then 400 µL of TKM2 buffer (10 mM Tris, pH 7.6, 10 mM KCl, 10 mM MgCl₂, 2 mM EDTA, and 0.4 M NaCl) was added. The sample was mixed by vortexing, then 25 µL of 20% SDS added, followed by incubation at 56°C for 15 min. Next, 150 µL of 6 M NaCl was added, the sample centrifuged for 5 min at 17,000 x g and the soluble fraction containing the genomic DNA transferred to a microfuge tube. To precipitate DNA, 2 volumes of 100% ethanol was added and the sample centrifuged at 4°C for 15 min. The supernatant was then decanted and the DNA pellet washed once in 500 µL of 70% ethanol, air dried, and then resuspended in 50 µL of sterilised deionised water or TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). DNA was stored at -20°C.

2.9 Polymerase chain reaction (PCR)

All PCRs were carried out in 25 – 50 µL reaction containing 1× PCR reaction buffer, 1 – 2 mM MgCl₂, 200 µM dNTPs (Peqlab, Germany), 200 nM primers (forward and reverse), 2.5 units *Taq* polymerase (AmpliTaq GOLD, Applied Biosystem, USA) or *Pfu Taq* polymerase (Strategene, USA) and 20 – 50 ng DNA template. For PCR cycling, samples were first denatured at 95°C for 5 min, then 30 cycles of denaturation (95°C for 30 sec), annealing (55°C – 65°C for 30 sec), and DNA extension (70°C) for 3 – 4 min,
a final extension step was carried out at 70°C for 10 min then the samples were stored at 4°C.

2.10 DNA sequencing

Sequencing was carried out using ABI Bigdye version 3.1 (Applied Precision, Australia) in quarter reaction volume. A reaction mix consisted of 1.2 µL of dye terminator ready reaction mix, 3.2 pmol of primer, 20 -150 ng of template, and milliQ water to a final volume of 12µL per reaction. The sample was subjected to 25 cycles of denaturation (96°C for 30 sec), annealing (50°C for 15 sec), and extension at 60°C for 4 min. Isopropanol precipitation was carried out following the sequencing reaction to remove dye terminator. The sequencing reaction was extracted with 120 µL of 70% isopropanol, incubated at room temperature for 10 min, then centrifuged at 17,000 x g for 30 min in a bench top centrifuge (Eppendorf, USA). The supernatant was discarded by aspiration, and the pellet washed with 500 µL 70% isopropanol followed by centrifugation at 17,000 x g for 30 min. The supernatant was removed by gentle aspiration and the pellet dried in a SpeedyVac vacuum for 5 min at RT. DNA sequence analysis was carried out at the QIMR /Griffith University DNA sequencing facilities.

2.11 Plasmid extraction

*E. coli* were grown overnight at 37°C in Luria Broth (LB) containing the appropriate antibiotic (up to 5 mL culture volume) and pellet by centrifugation at 17,000 x g for 10 min in a bench top centrifuge (Eppendorf, USA). Mini-prep plasmid extractions (5 – 10
µg) were carried out using the QIAprep Spin Miniprep Kit (Qiagen, US) as per manufacturer’s instructions. Plasmid DNA was eluted in 30 – 50 µL EB buffer (provided with the kit) or sterilised deionised water and stored at -20°C.

2.12 Restriction digest

Restriction digest of DNA was carried out in a 50 µL reaction volume containing 0.5 – 1 µg DNA, 10 – 20 units of restriction enzymes and 1× reaction buffer (provided with enzymes, New England Biolabs, UK). In some cases, depending on the enzyme, BSA was included in the reaction mix to stabilize the enzyme. Restriction digest reaction was carried out at 37°C for 2 h or overnight.

2.13 DNA gel electrophoresis

DNA (0.5 – 1 µg) was separated on 0.8 – 1% agarose gel at 80 – 100V in 1× TAE buffer containing 0.5µg/mL ethidium bromide. Agarose gel was analysed using a KODAK Gel Logic 100 System (Kodak™, US)

2.14 Gel purification

Extraction of DNA bands from agarose gel were carried in a microfuge tube using the Qiagen Gel Purification kit as per manufacturer’s instructions (Qiagen, USA). DNA was eluted out in 30 – 50µL EB buffer (provided with the kit) and stored at -20°C or until use.
2.15 *E. coli* transformation

Plasmid DNA was transformed into TOP10, Dh5 alpha or BL21 *E. coli* cells (Invitrogen, USA), by chemical transformation. Briefly, 0.1 – 0.5 µg of plasmid DNA was added to 1 – 2 × 10^9 competent cells in a 50-100 µL volume and the samples mixed gently by stirring with a pipette tip. The mixture was immediately incubated on ice for 30 min, then heat-shocked at 42°C for 30 sec for uptake of the plasmid. The tube was then immediately transferred to ice for 2 min, then 250 µL of SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 2% glucose) added and the transformation mixture incubated at 37°C for 1 h, with shaking (200 rpm using a Bioline Shaker, Edwards Instruments Company Australia, Australia). A sample of the transformation mixture (100 – 200 µL) was then plated onto LB agar plates containing an appropriate antibiotic and the plates incubated at 37°C overnight to obtain single colonies.

2.16 SDS PAGE

Protein samples (Section 4.2.3) or parasite extracts (Section 2.7) were resuspended in 6 x SDS loading dye (Appendix 1), heated at 93°C for 3 min in a heating block (Select BioProducts, Australia), then immediately passed 3–4 times through a blunt end Terumo 27x1/2 gauge needle to shear DNA and membranes that might interfere with loading. Samples were chilled on ice for at least 5 min, and then centrifuged for 1 min at 17,000 x g in a microcentrifuge (Eppendorf, USA). The supernatants of the resulting
samples (usually 5–20% of the total sample volume) were separated using 12–15% SDS-PAGE in 1x SDS running buffer (Appendix 1).

2.17 Protein quantification

Protein concentrations were determined using the Bradford protein estimation method [230]. Samples were diluted in a total volume of 50 µL in PBS. After addition of 200 µL Bradford solution (BioRad, USA) to each well in 96-well plate, the samples were incubated for 15 min at room temperature. Absorbance of samples was measured at 595 nm in a Synergy™ 2 plate reader (Biotek® Instruments, Inc, USA) in 96 well microtitre plates. Protein concentrations were calculated in comparison with a bovine serum albumin standard curve (0.5 – 10 µg BSA, in triplicate measurements).

2.18 Western Blot

Protein samples prepared as per Section 2.16 were separated via 10 – 15% SDS-PAGE and then protein transferred to polyvinylidene fluoride (PVDF) membrane (Roche, Germany). The membrane was pre-blocked in BLOTTO (5% skim milk powder-PBS solution) for at least 1 h at room temperature and then incubated overnight at 4°C in BLOTTO containing Anti-His G-HRP antibody (Invitrogen, USA) diluted to 1:1000. The membrane was then washed with 1×PBS-Tween 20 (0.1%) and then incubated in enhanced chemiluminescence solution (ECL; Amersham, UK) for 5 min and exposed to X-ray film (FujiFilm, Japan) and processed in a Kodak 3000RA XOMAT developer.
3 Evaluation of antimalarial properties of primary sulfonamides

Publications arising from this chapter


Contribution: I generated Pf3D7 and Dd2 IC$_{50}$ data in Table 3.2 and contributed to writing and editing sections of the publication. Where relevant, work carried out by others is acknowledged in this chapter.


Contribution: I generated all of the biological data included in this manuscript. I wrote the first draft of this manuscript, edited subsequent drafts and contributed to addressing reviewers comments. Where relevant, work carried out by others is acknowledged in this chapter.
3.1 Introduction

Despite the critical need for antimalarial drugs with novel modes of action, no new chemical class of antimalarial drug has been approved since 1996 [179], and only one, a spiroindolone, has progressed to clinical trials [5, 180]. To address the need for discovery of new drug starting points, work in this Chapter focused on investigating compounds containing a primary benzenesulfonamide moiety as a potential new antimalarial chemotype. The sulfonamide moiety has an established history of efficacy and safety in clinically used drugs, and apart from their common sulfonamide moiety, these compounds exhibit variable drug-like properties [181].

Prior evidence supports the investigation of primary sulfonamides as antimalarials. As discussed in Chapter 1, published work from another group on a panel of primary benzenesulfonamides led to the identification of a thiouredo benzenesulfonamide; [215], which displayed moderate growth inhibitory activity against \( P. falciparum \) parasites \textit{in vitro} (IC\(_{50} \sim 1 \mu M)\) and \textit{in vivo} activity (ID\(_{50} 10\text{mg/kg})\) against \( Plasmodium berghei \) in a mouse malaria model (Table 1.3). In addition, information sharing by large pharmaceutical companies has led to further evidence that primary sulfonamides may be promising new antimalarial starting points. As mentioned previously (Section 1.4), GlaxoSmithKline (GSK) made publicly available the structures of eight thousand compounds that inhibited the \textit{in vitro} growth of drug-sensitive (3D7) and resistant (Dd2) \( P. falciparum \) parasites (\( \geq 80\% \) and \( \geq 50\% \) at 2 \( \mu M \), respectively) [7]. By searching this database, 31 compounds comprising a primary sulfonamide (PS) group were identified, the majority of which displayed good antimalarial activity and
selectivity and good drug like properties (Appendix 3; Rule of 5 violations ≤ 3; Table 3.3). The 'rule of five' guidelines are intended to help improve the probability of success in drug discovery. The guidelines are a set of physicochemical properties for predicting the likelihood that drug candidates would be orally bioavailable and have been extremely successful in reducing the drug discovery pipeline attrition rate that has been attributed to poor compound properties. For antimalarial drugs the ‘rule of five’ guidelines are of some value in triaging compounds as oral bioavailability is a must, however satisfying these guidelines is not sufficient. For example the guidelines do not extend to predicting a compounds potential off target toxicity, or the ability of a compound to subsequently enter a malaria parasite within a red blood cell or to be selective for parasite over host or to not be toxic. It should be noted that there are clinically used drugs that do not obey the 'rule of five', for example may antibiotics, as well as drugs that are dosed as pro-drugs [231]. As with any guidelines, there are limitations, and an awareness of these limitations is vital.

An important part of the early phase of drug development is to investigate new chemical classes of compounds with novel modes of action to help limit potential cross resistance with existing drugs. Likewise combinations of antimalarial compounds with different modes of action are important to further delay the onset of drug-resistance in a clinical setting. This approach can either involve a drug combination formulation/regimen or exploit synthetic chemistry approaches to design hybrid compounds. In the latter approach, two antimalarial pharmacophores, with different modes of action, are chemically combined to produce ‘hybrid drugs’. Advantages of hybrid drugs include potential improvements in potency, stability, and/or solubility. Recent examples that employ pharmacophore hybridization as an approach to antimalarial drug discovery
include chloroquine-triazine hybrids [139-141], chloroquine-pyrimidine hybrids [142], trioxaquines [4, 136, 137], the mefloquine-artesunate hybrid MEFAS [143], the ferrocene-chloroquine hybrid, ferroquine [144-146] and others [147-151]. **Figure 3.1.** The majority of these compounds have shown improved antimalarial activity over the parent compound when tested *in vitro* against drug resistant parasites [4, 136, 137, 143-145, 147, 148, 150, 151, 232]. Furthermore several of these hybrid compounds have also shown comparable or better *in vivo* efficacy when tested in mouse malaria models [4, 136, 137, 143-145, 148]. Three hybrid compounds have advanced further along the malaria drug discovery pipeline with the mefloquine-artesunate hybrid MEFAS and the trioxaquine PA11103/SAR116242 currently undergoing pre-clinical development, while the organometallic drug candidate ferroquine is in phase II clinical trials [4, 143, 144]. Many hybrid compounds have focused on the antimalarial drug chloroquine, comprising a chlorinated 4-aminoquinoline scaffold [137, 144, 145, 149-151, 232-234]. Although *P. falciparum*, the most lethal human malaria parasite species, has developed resistance to chloroquine in most areas of the world there is evidence of re-emergence of chloroquine sensitive parasites in Africa [235]. Furthermore this drug remains effective for the treatment of other malaria parasite species such as *P. vivax* [236, 237].These favourable biopharmaceutical properties make strategies aimed at overcoming issues of parasite resistance to this drug attractive, although naturally any hybrid compound would need to undergo full *in vivo* safety and pharmacokinetic profiling.
In this Chapter, different strategies were employed to investigate PS compounds as antimalarial agents. First a drug repurposing approach was employed to investigate the antimalarial activity of a group of clinically used PS drugs. Next, the antimalarial activity of a panel of novel PS glycoside compounds and non-PS controls was evaluated [238]. The antimalarial activity of 26 PS containing compounds identified from the published in vitro screening of the GSK TCAMs library [7] were also profiled. Finally, the antimalarial activity of novel 7-chloro-4-aminoquinoline:PS hybrid compounds was investigated. The overall aim of this work was to ascertain if these PS compounds represent starting points for development of new antimalarial drug leads and to investigate structure-activity relationship (SAR) and structure-property relationship (SPR) profiles that may aid in further development of this chemotype to target malaria parasites.

Figure 3.1: The antimalarial drug chloroquine and representative hybrid antimalarial compounds.
3.2 Materials and methods

3.2.1 Compounds

Clinically approved primary sulfonamides: Celecoxib, zonisamide and 7-chloro-1,2,3,4-tetrahydro-2-methyl-3-(2-methylphenyl)-4-oxo-6-quinazolinesulfonamide were purchased from IS Chemical Technology®, China. Valdecoxib and sulthiam were a gift from Dr Claudiu Supuran, University of Florence, Italy. Hydrochlorothiazide, metolazone, bendroflumethiazide, furosemide, saccharin, methazolamide, indapamide, trichlormethiazide, bumetanide, acetazolamide, 4-Aminomethylbenzenesulfonamide HCl and chloroquine were purchased from Sigma-Aldrich®, USA. Stock solutions of 10 mM were prepared in 100% DMSO (Sigma-Aldrich®, USA), stored at -20°C and diluted when required.

Novel PS glycosides: PS glycosides (PS-1-14) and matched control compounds (PS-1'-10') were synthesised in Assoc. Prof Sally-Ann Poulsen’s laboratory at the Eskitis Institute for Drug Discovery, Griffith University, Australia. Compounds were supplied as 100 mM and 10 mM stocks diluted in 100% DMSO (Sigma-Aldrich®, USA), stored at -20°C and diluted as required.

GSK PS compounds: 26 PS compounds identified in the European Bioinformatics Institute ChEMBL Neglected Tropical Disease TCAMS database (www.ebi.ac.uk/chemblIntd/download/#tcams) were supplied by GSK [7] under a
Material Transfer Agreement (MTA). All compounds were supplied as 10 mM stocks diluted in 100% DMSO, stored at -20°C and diluted as required.

PS hybrid compounds: Six PS hybrid compounds (H4, H5, H7, H8, H10 and H11) comprising the 7-chloro-4-aminoquinoline scaffold of chloroquine and a primary benzenesulfonamide, and three control compounds (H6, H9 and H12) lacking the PS group were synthesised in Assoc. Prof Sally-Ann Poulsen’s laboratory at the Eskitis Institute for Drug Discovery, Griffith University, Australia. Compounds were supplied as solids and diluted in 100% DMSO (Sigma-Aldrich®, USA) as required and stored at -20°C.

3.2.2 Physicochemical profiling of compounds

[Note: these data were generated by Assoc. Prof Sally-Ann Poulsen, Griffith University]

Software tools for multi-parameter profiling are inexpensive and provide rapid feedback, and when used with an awareness of the limitations around accuracy of prediction may provide insight into drug-like properties of a compound class to guide the direction of follow-on studies [239]. QikProp (Schrödinger Suite 2009) is a Pharma industry gold standard software package that allows the calculation of compound properties and prediction of absorption, distribution, metabolism and elimination (ADME) properties [240]. For the PS glycoside compounds of this study a selection of key property values were generated using QikProp (Table 3.3). The selected parameters include LogP (octanol/water partition coefficient); Log S (aqueous solubility); Pcaco (predicted apparent caco-2 cell permeability); % human oral absorption and Ro5 (rule of 5 violations). The recommended values for parameters (i.e. representing 95% of known
drugs) are LogP (-2.0 to +6.5), Log S (-6.5 to +0.5), Pcaco (>500 good, <25 poor), % human oral absorption (>80% high, <20% poor) and Ro5 violations (maximum 4) [240].

3.2.3 *Plasmodium falciparum* lines

The drug sensitive line *P. falciparum* 3D7 [241] and the drug resistant line *P. falciparum* Dd2 [242] were cultured as per Section 2.1 and assessed in $^3$H-Hx growth inhibition assays (Section 2.5). Line Dd2 is resistant to chloroquine, quinine, pyrimethamine and sulfadoxine, while line 3D7 is sensitive to these compounds.

3.2.4 Statistical analysis

For comparing *in vitro* antimalarial activities a two-tailed *t* test was used using GraphPad Prism® data analysis software.
3.3 *In vitro* activity of PS compounds against *P. falciparum* parasites

3.3.1 Clinically approved human primary sulfonamide-containing drugs have poor *in vitro* antimalarial activity

To ascertain whether clinically approved sulfonamide containing drugs have antimalarial activity and therefore a potential drug starting point, a panel of sixteen compounds, 15 PS compounds and one secondary sulfonamide (saccharin) ([Figure 3.2](#)) was screened *in vitro* against *P. falciparum* 3D7 parasites. All compounds are known to inhibit human carbonic anhydrase (CA) enzymes, although for some their mode of action (MOA) is attributed to non-CA targets. Compounds were tested for growth inhibitory activity in a primary screen at concentrations of 12.5 µM, 6.25 µM and 1.25 µM, in three independent assays, using a standard ³H-Hx uptake growth inhibition assay ([Section 2.5](#)). With the exception of celecoxib, all compounds exhibited <50% inhibition of growth of *P. falciparum* parasites at the highest concentration tested ([Table 3.1](#); IC₅₀ > 12.5 µM). Although celecoxib inhibited *P. falciparum* growth by >90% at 12.5 µM, with a calculated IC₅₀ of 6.88 ± 2.97 µM ([Table 3.1](#)), this level of activity is poor – well above that required for even an early stage hit antimalarial compound ([Appendix 2](#)). In addition, a comparison of reported toxicity against HepG2 cells (IC₅₀ 23.8 µM [243]) indicates that this compound also has low selectivity for *P. falciparum* versus mammalian cells (SI < 4). Overall these data indicate that these inhibitors are largely ineffective against *P. falciparum* malaria parasites and not worth pursuing as antimalarials.
Figure 3.2: Structures of clinically approved sulfonamide compounds tested for in vitro antimalarial activity
All compounds contain a PS motif denoted in red \((R-SO_2NH_2)\), with the exception of saccharin, which contains a secondary sulfonamide motif denoted in blue \((R-SO_2NH-R')\).
Table 3.1: Antimalarial activity of clinically used sulfonamide compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>LogP</th>
<th>Pf3D7 % Inhibition @ 12.5 µM</th>
<th>Pf3D7 IC50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrochlorothiazide</td>
<td>-0.1</td>
<td>1.0 (±1.7)</td>
<td>ND</td>
</tr>
<tr>
<td>Metolazone</td>
<td>2.5</td>
<td>0.5 (±0.6)</td>
<td>ND</td>
</tr>
<tr>
<td>Bendroflumethiazide</td>
<td>1.2</td>
<td>5.7 (±9.1)</td>
<td>ND</td>
</tr>
<tr>
<td>Furosemide</td>
<td>2.0</td>
<td>2.2 (±11.1)</td>
<td>ND</td>
</tr>
<tr>
<td>Saccharin</td>
<td>0.9</td>
<td>7.4 (±15.2)</td>
<td>ND</td>
</tr>
<tr>
<td>Methazolamide</td>
<td>0.1</td>
<td>2.8 (±2.8)</td>
<td>ND</td>
</tr>
<tr>
<td>Indapamide</td>
<td>2.2</td>
<td>9.2 (±9.7)</td>
<td>ND</td>
</tr>
<tr>
<td>Trichlormethiazide</td>
<td>1.0</td>
<td>12.8 (±16.8)</td>
<td>ND</td>
</tr>
<tr>
<td>Bumatane</td>
<td>2.6</td>
<td>0.0</td>
<td>ND</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>-0.3</td>
<td>0.9 (±1.5)</td>
<td>ND</td>
</tr>
<tr>
<td>4-Aminomethylbenzenesulfonamide HCl</td>
<td>NA</td>
<td>0.7 (±7.6)</td>
<td>ND</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>3.5</td>
<td>94.6 (±3.5)</td>
<td>6.9 (±3.0)</td>
</tr>
<tr>
<td>Zonisamide</td>
<td>0.4</td>
<td>0.0</td>
<td>ND</td>
</tr>
<tr>
<td>7-chloro-1,2,3,4-tetrahydro-2-methyl-3-(2-methylphenyl)-4-oxo-6-quinazolinesulfonamide</td>
<td>NA</td>
<td>5.0 (±7.4)</td>
<td>ND</td>
</tr>
<tr>
<td>Valdecoxib</td>
<td>2.7</td>
<td>3.9 (±19.0)</td>
<td>ND</td>
</tr>
<tr>
<td>Sulthiam</td>
<td>-0.3</td>
<td>4.6 (±11.2)</td>
<td>ND</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>4.6</td>
<td>-</td>
<td>0.02(±0.002)</td>
</tr>
</tbody>
</table>

Data from three independent experiments performed in triplicate wells. ND: not determined; NA: not available.

3.3.2 Antimalarial activity of a panel of primary sulfonamide glycosides

Next, the in vitro antimalarial activity of a panel of novel synthetic PS glycosides (PS-1-14) was examined. To determine if the PS component conferred antimalarial activity, matched control compounds (1'-10') with the PS group substituted with a hydrogen...
group were also examined (Figure 3.3). PS-1-14 include a range of variable structural features, including a sugar fragment (either glucose, galactose, glucuronic acid or maltose; where R = Ac or H), a glycosidic linker (X = S, O, or SO₂) and a triazole substituent (Y = I or H). Carbohydrate-based drugs have been shown to have improved solubility, stability and/or targeting when compared to non-carbohydrate drugs [244-248] and thus the rationale for the inclusion of a sugar group in these compounds.

Figure 3.3: Glycoside-based primary benzenesulfonamide compounds.
Structure of PS Compounds 1-14 and control compounds, where the sulfonamide moiety is replaced by a hydrogen atom, 1'-10'

When tested against *P. falciparum* parasites *in vitro* using a standard ³H-Hx growth inhibition assay (Section 2.5) similar IC₅₀s were obtained for both drug sensitive (3D7) and drug resistant (Dd2) lines for PS compounds 3, 4, 7, 9, 10, 13 and 14 (Table 3.2 P<0.05). PS Compounds 2, 5 and 6 displayed no activity at the highest concentration tested (50 µM), while a further five compounds displayed moderate activity with IC₅₀ values between ~5 and 50 µM (PS-7, 9, 13, 8 and 1; Table 3.2). Six compounds had
IC$_{50}$ values less than 6 µM (PS-3, 4, 11, 12, 14 and 10), with PS-3 and PS-4 having the best activity (IC$_{50}$s $\sim$1-2 µM; Table 3.2). A comparison of these data with that of the matched control compounds lacking the PS moiety (1'-'10') revealed that four compounds (PS-3', 4', 9' and 10') had significantly higher IC$_{50}$ values (P<0.05) than their corresponding sulfonamide containing partners (PS-3, 4, 9, and 10; Table 3.2). PS-7 showed similar activity to its related control compound PS-7'. PS- 8' was the only compound to show better activity (~7 fold lower IC$_{50}$) than its sulfonamide containing equivalent PS-8. PS-2, 5 and 6, like their respective non PS derivative, had very poor biological activity (IC$_{50}$s $>$50 µM).

3.3.2.1 Antimalarial structure-activity relationship (SAR) analysis of PS glycosides

Observed trends in antimalarial SAR of PS-1-14 include (i) the triazole substituted compounds (where $Y = I$) generally have greater potency than compounds where $Y = H$; (ii) the different sugars have minimal affect (compare PS-3, 11, 12, 14); and (iii) the acetylated sugars ($R = Ac$) either have similar or improved potency over the corresponding free sugars ($R = H$). A combination of the three substituents ($R$, $Y$ and $X$) appears to impact on the overall antimalarial activity, however the triazole substituent ($Y = I$ or $H$) dominates SAR, followed by the glycosidic linker $X$ and the sugar $R$ group, both with lesser effects. Compounds where $Y = I$ have the best antimalarial activity and have greater activity than the corresponding compounds where $Y = H$, notably PS-3 ($Y = I$, IC$_{50}$ 0.9 µM) and PS-4 ($Y = I$, IC$_{50}$ 1.0 µM) compared to analogues PS-1 ($Y = H$, IC$_{50}$ 31.0 µM) and 2 ($Y = H$, IC$_{50}$ $>$50 µM).
Table 3.2: *In vitro* antimalarial activity of primary benzenesulfonamide compounds

<table>
<thead>
<tr>
<th>Cpd&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Structural features</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sugar</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS-3 (3')</td>
<td>Glc</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-4 (4')</td>
<td>Glc</td>
<td>H</td>
</tr>
<tr>
<td>PS-11</td>
<td>Gal</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-12</td>
<td>GlcOMe</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-14</td>
<td>Mal</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-10 (10')</td>
<td>Glc</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-7 (7')</td>
<td>Glc</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-9 (9')</td>
<td>Glc</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-13</td>
<td>Mal</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-8 (8')</td>
<td>Glc</td>
<td>H</td>
</tr>
<tr>
<td>PS-1 (1')</td>
<td>Glc</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-2 (2')</td>
<td>Glc</td>
<td>H</td>
</tr>
<tr>
<td>PS-5 (5')</td>
<td>Glc</td>
<td>Ac</td>
</tr>
<tr>
<td>PS-6 (6')</td>
<td>Glc</td>
<td>H</td>
</tr>
<tr>
<td>CQ</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Analogues lacking the sulfonamide group are shown in brackets.<br><sup>b</sup> Mean IC<sub>50</sub> (±SD) for primary benzenesulfonamide compounds (Z = SO₂NH₂) and corresponding analogues lacking the sulfonamide group (Z = H) against *P. falciparum* line 3D7 for 3-6 independent experiments, each carried out in triplicate wells; Mean IC<sub>50</sub> against normal NFF cells for two independent experiments, each carried out in triplicate wells; nd – not determined, ns – not synthesised; Glc – glucose; Gal – galactose; Mal – maltose; GlcOMe – Glucuronic acid; CQ, chloroquine
3.3.2.2 Physicochemical property parameters of PS glycosides

[Note: These data were generated by Assoc. Prof. Sally-Ann Poulsen, Griffith University]

Physicochemical property parameter values for the compounds of this study (Table 3.3) provide a guide to correlate and rationalise SPR with the SAR identified. LogP represents intrinsic lipophilicity and compounds with LogP < 0 typically have good solubility but poor lipid bilayer permeability [239]. This relationship correlates well with the poorer activity of the compounds where X = SO\(_2\) as all have a calculated LogP (cLogP) value less than 0, and LogP values lower (by ~1) than the corresponding compound where X = S. Compounds where Y = I have the best antimalarial activity and have greater activity than the corresponding compounds where Y = H, notably PS-3 (Y = I, IC\(_{50}\) 0.9 µM, cLogP = +1.4) and PS-4 (Y = I, IC\(_{50}\) 1.0 µM, cLogP = -1.5) compared to analogues PS-1 (Y = H, IC\(_{50}\) 31.0 µM, cLogP +0.9) and PS-2 (Y = H, IC\(_{50}\) >50 µM, cLogP -2.0). Interestingly, the effect of the triazole substituent Y = I on activity appears independent of cLogP as compounds PS-3 and PS-4 have opposing LogP values and likely opposing membrane permeability, yet almost identical IC\(_{50}\)s at 1 µM or less. However, it is important to note that while the human serum used for parasite culture is heat treated, any residual esterase activity [249, 250] may hydrolyze the acetate groups (R = Ac) of sugars converting compounds PS-3, 7 and 1 to the less permeable free sugars (R = H), compounds PS-4, 8 and 2, respectively. Sulfonamide compounds PS-3, 4, 10 and 9 are predicted to have better cell membrane permeability when the primary sulfonamide is removed to give PS-3', 4', 10' and 9' respectively, so the ~5-fold reduction in antimalarial activity is indicative of a target based effect.
3.3.3 Mammalian cell cytotoxicity of PS glycosides

To determine whether the in vitro antimalarial activity of the PS compounds examined in this study was selective for the malaria parasite, mammalian cell-based assays using a normal cell line (neonatal foreskin fibroblast (NFF) cells; Section 2.6) were carried out on all PS compounds exhibiting a P. falciparum IC$_{50}$ ≤5 µM (Table 3.2). Six of the seven compounds PS-4, 11, 12, 14, 10 and 7 had IC$_{50}$ values >100 µM (the highest concentration tested), while PS-3 gave an IC$_{50}$ 51.1 (±2.8) µM. The calculated selectivity indices (SI; NFF IC$_{50}$/P. falciparum IC$_{50}$) were ≥40 for the two most potent antimalarial compounds (PS-3 and 4). While this is lower than the SI of the antimalarial drug chloroquine (SI >1,000), the lack of general cytotoxicity is encouraging for antimalarial compound starting points.
Table 3.3: Predicted properties for PS glycosides and non-PS controls

<table>
<thead>
<tr>
<th>Cpd</th>
<th>LogP</th>
<th>Log S</th>
<th>Pcaco (nm/s)</th>
<th>Human Oral Absorption (%)</th>
<th>Ro5 violations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-3 (3')</td>
<td>1.4 (3.2)</td>
<td>-6.2 (-5.9)</td>
<td>6.3 (135.6)</td>
<td>23.4 (58.2)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>PS-4 (4')</td>
<td>-1.5 (0.6)</td>
<td>-3.3 (-3.5)</td>
<td>4.2 (88.7)</td>
<td>0 (65.5)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>PS-11</td>
<td>1.4</td>
<td>-6.1</td>
<td>5.4</td>
<td>22.1</td>
<td>2</td>
</tr>
<tr>
<td>PS-12</td>
<td>1.2</td>
<td>-5.9</td>
<td>9.7</td>
<td>25.58</td>
<td>2</td>
</tr>
<tr>
<td>PS-14</td>
<td>-1.2</td>
<td>-1.5</td>
<td>2.0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>PS-10 (10')</td>
<td>0.7 (2.5)</td>
<td>-5.5 (-5.1)</td>
<td>7.1 (152.8)</td>
<td>20.6 (54.9)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>PS-7 (7')</td>
<td>-0.7 (0.8)</td>
<td>-3.2 (-1.7)</td>
<td>3.7 (138.9)</td>
<td>6.9 (44.3)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>PS-9 (9')</td>
<td>0.2 (1.8)</td>
<td>-5.1 (-3.9)</td>
<td>7.2 (153.2)</td>
<td>17.6 (50.5)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>PS-8 (8')</td>
<td>-2.4 (-0.4)</td>
<td>-2.0 (-2.0)</td>
<td>3.3 (69.1)</td>
<td>0 (44.6)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>PS-13</td>
<td>-1.5</td>
<td>-1.9</td>
<td>1.7</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>PS-1 (1')</td>
<td>0.9 (2.7)</td>
<td>-5.8 (-5.1)</td>
<td>6.6 (155.0)</td>
<td>21.1 (56.3)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>PS-2 (2')</td>
<td>-2.0 (0.1)</td>
<td>-2.8 (-3.0)</td>
<td>3.2 (68.8)</td>
<td>0 (60.4)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>PS-5 (5')</td>
<td>-1.1 (0.3)</td>
<td>-2.6 (-1.7)</td>
<td>4.4 (106.1)</td>
<td>6.3 (39.3)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>PS-6 (6')</td>
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<td>-1.6 (-1.6)</td>
<td>2.1 (45.4)</td>
<td>0 (50.8)</td>
<td>2 (0)</td>
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</table>

*Data for analogues lacking the sulfonamide group are shown in brackets. LogP – octanol/water partition coefficient; Log S – aqueous solubility; Pcaco – Predicted apparent Caco-2 cell permeability; % human oral absorption; Ro5 – rule of 5 violations; Glc – glucose; Gal – galactose; Mal – maltose. Data calculated using QikProp 3.5 from Shrödinger [240].

3.3.4 Antimalarial activity of GlaxoSmithKline (GSK) PS compounds

Additional evidence that PS compounds have antimalarial potential comes from recent high throughput screening of a GlaxoSmithKline (GSK) library of ~2,000,000 compounds. From this library GSK identified ~8,000 compounds that inhibited the in vitro growth of drug-sensitive (3D7) and resistant (Dd2) *P. falciparum* parasites (≥80% and ≥50% at 2 μM, respectively) [7]. The structures of GSKs’ hit compounds were
made publically available through deposition in the open access European Bioinformatics Institute ChEMBL Neglected Tropical Disease archive. Following a sub-structure search (carried out by Assoc. Prof. Sally-Ann Poulsen, Griffith University) using the PS chemotype (-SO₂NH₂) as the search fragment, 26 PS compounds were identified in the European Bioinformatics Institute ChEMBL Neglected Tropical Disease TCAMS database (Figure 3.4 and Figure 3.5). Compounds were sourced from GSK (Section 3.2.1) and evaluated in $³$H-Hx uptake growth inhibition assays (Section 2.5) to confirm their in vitro activity against *P. falciparum* 3D7, as published in the TCAMS database [7], and to determine IC₅₀s. Six of the TCAMS PS compounds contained a known antimalarial pharmacophore, either a diaminopyrimidine (GSK-7, 12, 14, 22 and 23) or a 4-aminoquinoline group (GSK-10 and 18). The diaminopyrimidine containing compounds (GSK-7, 12, 14, 22 and 23) displayed IC₅₀s in the range of 0.26 - 1.31 µM and the two 4-aminoquinoline containing compounds (GSK-10 and 18) displayed IC₅₀s of 0.57 µM and 1.57 µM, respectively.

The remaining compounds that did not possess a known antimalarial pharmacophore (Figure 3.5) were grouped according to structural similarity. Group 1 compounds (GSK- 1, 3, 4, 5 and 6) showed IC₅₀s ranging from 0.46 – 1.89 µM. Group 2 compounds GSK-16 and 17 had IC₅₀s of 1.09 µM and 3.04 µM, respectively and Group 3 compounds (GSK-24, 25 and 30) displayed IC₅₀s in the range of 0.79 – 3.21 µM. The remaining eight TCAMS PS GSK compounds (Group 4 GSK- 2, 9, 11, 15, 19, 20, 27 and 31) did not share enough similarity to any other compounds to allow SAR be discussed as a group. These compounds displayed IC₅₀s in the range of 0.23 – 3.02 µM.
Figure 3.4: Structures of GSK PS compounds with antimalarial pharmacophore. Diaminopyrimidine highlighted in blue; 4-aminoquinoline highlighted in red.
Figure 3.5: Structures of GSK PS compounds with no antimalarial pharmacophore. Group 1 - red; Group 2 – Blue; Group 3 – Green; Group 4 – black.
Table 3.4: GSK PS compound screening data

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<th>CPD #</th>
<th>CHEMBL ID</th>
<th>MW</th>
<th>cLogP</th>
<th>% inh @ 2µM</th>
<th>3D7 IC50 µM</th>
<th>3D7% inh @ 10µM</th>
<th>HepG2% inh @ 2µM</th>
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</tr>
<tr>
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</table>

*HepG2% inhibition at 10 µM data derived from ChEMBL GSK TCAM data [251] bMean% inhibition against *P. falciparum* line 3D7 (± standard deviation) for three independent experiments, each performed in triplicate (this study); cCompounds contain a known antimalarial pharmacophore; dCompounds have serine/threonine protein kinase e data derived from Table 3.2; All other data derived from ChEMBL GSK TCAM data [251]. DAP/PS PS compounds with a diaminopyrimidine group; AQ/PS PS compounds with a 4-aminoquinoline group.
3.3.4.1 Antimalarial structure-activity relationships analysis of GSK PS Diaminopyrimidines

Diaminopyrimidines are a class of compound that comprise two amine groups directly attached to a pyrimidine ring (Figure 3.4; highlighted in blue), and include the antimalarial drug pyrimethamine - a dihydrofolate reductase inhibitor [67, 78]. Five of the GSK compounds (GSK-7, 12, 14, 22 and 23) contained a diaminopyrimidine group as part of their structure as well as a PS group. Of these, GSK-7 and 22 displayed the best IC\textsubscript{50}s against the drug sensitive parasite line \textit{P. falciparum} 3D7 (Pf3D7; IC\textsubscript{50} 0.28 and 0.26 µM, respectively) and the best selectivity (SI >35) for \textit{P. falciparum} parasites with versus mammalian cell line HepG2 (Table 3.4). It should be noted that GSK-7 and GSK-22 do have opposing cLogP values (4.5 and -0.5, respectively) indicating that there may be potential to improve the potency and selectivity of GSK-22 via rational medicinal chemistry approaches to improve membrane permeability. Compound GSK-12 (Pf3D7 IC\textsubscript{50} 0.62 µM) and GSK-23 (Pf3D7 IC\textsubscript{50} 0.84 µM), although having moderate antimalarial activity, showed lower selectivity (SI <20; HepG2/Pf3D7). Compound GSK-14 had the poorest antimalarial activity and selectivity of the diaminopyrimidines (Pf3D7 IC\textsubscript{50} 1.3 µM; HepG2, 74% inhibition @10 µM) and would probably not be considered a good candidate for further investigation. All of the GSK PS diaminopyrimidines displayed good antimalarial activity against the multi-drug resistant \textit{P. falciparum} parasite line Dd2 (>85% inhibition @ 2µM). These data suggest that the GSK PS diaminopyrimidines GSK-7, 12, 22 and 23 warrant further investigations into their potential as antimalarials.
3.3.4.2 Antimalarial structure-activity relationship analysis of GSK PS 4-aminoquinolines

The 4-aminoquinoline is another known antimalarial pharmacophore (Figure 3.4; highlighted in red), with the antimalarial drug chloroquine being a well-known 4-aminoquinoline derivative [44]. Compounds GSK-10 and GSK-18 contain a 4-aminoquinoline and a PS group. Although GSK-10 had good antimalarial activity against Pf3D7 (IC$_{50}$ 0.57 µM) it only had moderate selectivity for the parasite versus the HepG2 mammalian cell line (56% inhibition @ 10 µM), and lower activity against the parasite drug resistant line (Dd2 35% inhibition @ 2µM). Therefore the predicted resistance index (Ri), which is the ratio of the IC$_{50}$s of the resistant line Dd2 to the sensitive line 3D7, for compounds GSK-10 is likely to be >3.5 which is approaching the Ri of chloroquine (Ri = 5). The P. falciparum multi-drug resistant line Dd2 is resistant to chloroquine, pyrimethamine and sulfadoxine [242] and therefore GSK-10 is likely to have issues of cross resistance. GSK-18 had ~3 fold lower antimalarial activity (Pf3D7 IC$_{50}$ 1.57 µM) than GSK-10 and low selectivity (SI >6.3 HepG2/Pf3D7) and, like GSK-10, lower sensitivity when tested against drug resistant parasites (Dd2 21% inhibition @ 2 µM). Overall these data suggest that the GSK PS 4-aminoquinolines are not ideal candidates as lead compounds due to potential cross resistance with chloroquine and poor selectivity for the parasite.
3.3.4.3 Structure activity relationship analysis of GSK PS compounds with no known antimalarial pharmacophore

The remaining GSK PS compounds did not contain a known antimalarial pharmacophore but did possess a primary benzenesulfonamide as part of their structure (Figure 3.5). Where possible these compounds were categorised into groups according to structure similarity. Group 1 compounds GSK-1, 3, 4, 5 and 6 are all variably substituted phenyl/pyridinyl imidazo[1,2-b]pyridazines with the PS attached to a common benzyl amine substituent. Compounds GSK-3 (trifluoromethoxyphenyl substituent), 5 (nitrilephenyl substituent), and 6 (methoxypyridinyl substituent) displayed the best antimalarial activity (Pf3D7 IC_{50} 0.46-0.47 µM) and good parasite-specific selectivity (SI >20 HepG2/Pf3D7) indicating that the variable substituents of these structures did not impact on antimalarial activity. However there is a two-fold increase in cLogP values for GSK-3 with respect to compound GSK-5 indicating that the trifluoromethoxy group may aid in improving biopharmaceutical properties. GSK-1 (chlorophenyl substituent) has reduced antimalarial activity (Pf3D7 IC_{50} 0.84 µM) with respect to GSK-5 (Pf3D7 IC_{50} 0.46 µM). GSK-4 (methoxyphenyl substituent) had the lowest antimalarial activity (Pf3D7 IC_{50} 1.89 µM) and selectivity (SI >5 HepG2/Pf3D7) of group 1 compounds. GSK-4 is identical to GSK-6 except for pyridine versus phenyl group, suggesting that this modification is responsible for the four-fold increase seen in antimalarial activity of GSK-6 compared to GSK-4.

Group 2 comprises two compounds, GSK-16 and 17 (Pf3D7 IC_{50} 1.09 and 3.04 µM, respectively) which are 2-pyrimidinyl pyrazolopyridines that differ only in the position
of the PS group attachment to the benzene ring. It appears that position 4 (GSK-16) on the benzene ring confers better antimalarial activity with respect to position 5 (GSK-17) for the placement of the PS group for these two compounds. However, both these compounds have poor selectivity (HepG2 >80% inhibition @ 10 µM).

Group 3 consists of compounds GSK-24, 25 and 30. Of this group GSK-25, a 3-(4-oxo-1H-quinazolin-7-yl)benzenesulfonamide, and GSK-30, a 3-[4-(3-sulfamoylphenyl)quinolin-6-yl]benzenesulfonamide, showed similar antimalarial activity (Pf3D7 IC50 0.81 and 0.79 µM, respectively), moderate selectivity (SI >10 HepG2/Pf3D7) and similar cLogP values indicating that the addition of a second benzenesulfonamide group and/or the orientation of the benzenesulfonamide group to the core of these compounds confers no improvement in antimalarial activity or selectivity. GSK-24, a 3-[4-[3-(sulfamoylmethyl)phenyl]quinolin-6-yl]benzenesulfonamide, has a different core group with two benzenesulfonamides attached and displays a fourfold decrease in antimalarial activity (Pf3D7 IC50: 3.2 µM) and poor selectivity (SI~3: HepG2/Pf3D7). In addition this compound is the only compound other than the 4-aminoquinoline PS compounds mentioned earlier to have reduced sensitivity to drug resistant parasites (PfDd2 42% inhibition @ 2 µM).

The remaining eight compounds (Group 4 GSK-2, 9, 11, 15, 19, 20, 27 and 31) from the GSK non-antimalarial pharmacophore group (Figure 3.5) did not share structural similarity to any other compounds to allow informative SAR analysis. Of these, GSK-15, a 3-[(4-pyrazolo[1,5-b]pyridazin-3-ylpyrimidin-2-yl)amino]benzenesulfonamide
had the best antimalarial activity (Pf3D7 IC50 0.23 µM), highest selectivity (SI >44: HepG2/Pf3D7) and showed no evidence of cross resistance (PfDd2 89% inhibition @ 2 µM). **GSK-2**, a 2-[2-amino-5-(3-cyanophenyl)pyridin-3-yl]-3H-benzimidazole-5-sulfonamide, and **GSK-20**, a N-methyl-5-[(E)-2-(2-sulfamoylphenyl)ethenyl]pyridine-3-carboxamide, displayed 3-4 fold lower antimalarial activity (Pf3D7 IC50 0.69-0.85 µM) and selectivity (SI >12: HepG2/Pf3D7) than **GSK-15**. **GSK-2** is a novel benzimidazolyl-pyridine that has been implicated in the inhibition of Serum and Glucocorticoid-Regulated Kinase 1 (SGK-1) activity [252]. The remaining compounds **GSK-9, 11, 19, 27** and **31** showed only moderate antimalarial activity (Pf3D7 IC50 1.55-3.02 µM) and low selectivity (SI >3 to >7 HepG2/Pf3D7). Compounds **GSK-2, 9, 11, 19, 20, 27** and **30** also demonstrated some antimalarial activity against the drug resistant *P. falciparum* parasites (PfDd2 >72% inhibition @ 2 µM), although IC50s were not determined.

In summary the GSK compounds with no antimalarial drug pharmacophore that may warrant further investigations are: Group 1: **GSK-1, 3, 5 and 6** (Pf3D7 IC50 0.46-0.84 µM; PfDd2 >98% inhibition @ 2µM; SI >20 HepG2/Pf3D7); Group 3: **GSK-25 and 30** (Pf3D7 IC50 0.79-0.81 µM; PfDd2 >98% inhibition @ 2 µM; SI >10 HepG2/Pf3D7) and, Group 4, **GSK-2, 15** and **20** (Pf3D7 IC50 0.23-0.85 µM; PfDd2 >77% inhibition @ 2 µM; SI >12- >44 HepG2/Pf3D7).
3.3.5 Evaluation of the antimalarial activity and selectivity of novel 4-aminoquinoline hybrids

To further address the need for safe and effective new antimalarial drugs that can overcome parasite resistance the antimalarial activity of a panel of novel hybrid compounds that comprise the 7-chloro-4-aminoquinoline moiety of the antimalarial drug chloroquine and a primary benzenesulfonamide were investigated (Figure 3.6). Benefits of utilising chloroquine as a hybrid molecule partner are that it is inexpensive, easily administered, and safe for use in infants and pregnancy [237]. Although *P. falciparum*, the most lethal human malaria parasite species, has developed resistance to chloroquine in most areas of the world there is evidence of re-emergence of chloroquine sensitive parasites in Africa [235]. Furthermore this drug remains effective for the treatment of other malaria parasite species such as *P. vivax* [236, 237]. While the antimalarial target of the primary sulfonamide group is still under investigation, data generated to date for the primary sulfonamide glycosides investigated in this study (Section 3.3.2 and Table 3.2) indicates that there is no cross-resistance with the Dd2 *P. falciparum* line which is resistant to several antimalarial drugs, including chloroquine. Thus, as proof of concept, six hybrid compounds (H4, H5, H7, H8, H10 and H11) comprising the 7-chloro-4-aminoquinoline scaffold of chloroquine and a primary benzenesulfonamide, and three control compounds (H6, H9 and H12) lacking the PS group were synthesised by Assoc. Prof. Sally-Ann Poulsen, Griffith University (Figure 3.6). These compounds contain a 1,2,3-triazole moiety as the linker group as this group is synthetically suited to covalently combining two scaffolds and is also stable to
environments encountered by small molecules when added to cells and tissues such as acidic, basic, reductive and oxidative conditions as well as to enzymatic degradation [253]. Three different linker spacings with one, two or three methylene groups directly attached to the 1,2,3-triazole, were investigated. The sulfonamide group is either meta- or para- to the 1,2,3-triazole group while in the control compounds (6, 9 and 12) the sulfonamide is replaced by a hydrogen. The in vitro antimalarial activity and mammalian cell cytotoxicity of these compounds was evaluated as follows.

![Figure 3.6: Structural components of 7-chloro-4-aminoquinoline: primary sulfonamide hybrid compounds](image)

Figure 3.6: Structural components of 7-chloro-4-aminoquinoline: primary sulfonamide hybrid compounds
3.3.5.1 In vitro antimalarial activity of PS hybrid compounds

The in vitro growth inhibitory activity of PS hybrid compounds was tested against a drug-sensitive (3D7) and drug-resistant (Dd2) *P. falciparum* line using a standard $^3$H-Hx uptake growth inhibition assay (Section 2.5). Of the compounds tested, $\text{H6}$ (IC$_{50}$ ~1 µM), $\text{H9}$ (IC$_{50}$ ~0.2-0.3 µM) and $\text{H12}$ (IC$_{50}$ ~0.6 µM) displayed the most potent inhibitory activity; however all were at least 10-fold less active than chloroquine alone (Table 3.5). Unexpectedly, all of these compounds were the control analogues that do not contain a PS, suggesting that the sulfonamide moiety may reduce antimalarial potency when combined with the 7-chloro-4-aminoquinoline scaffold in hybrid compounds. Interestingly the resistance index (Ri), which is the ratio of the IC$_{50}$’s of the resistant line Dd2 to the sensitive line 3D7, for compounds $\text{H6}$, $\text{H9}$ and $\text{H12}$ was ≤ 1.4. This Ri value is substantially lower than that obtained for chloroquine (Ri = 4.3), indicating that that the addition of the phenyl-1,2,3-triazole to the aminoquinoline scaffold overcomes the change in sensitivity seen for these two lines when exposed to chloroquine.

3.3.5.2 Antimalarial structure-activity relationship analysis of PS hybrid compounds

Of the hybrid compounds with a PS group either meta- or para- to the 1,2,3-triazole group, the position of the primary sulfonamide substituent had an effect on antimalarial potency. The meta- regiosomers ($\text{H5}$, $\text{H8}$ and $\text{H11}$) were more potent than the corresponding para- regiosomers ($\text{H4}$, $\text{H7}$ and $\text{H10}$) against both sensitive and resistant parasite lines. This finding suggests that the sulfonamide group has the potential to be
optimized further for antimalarial potency using this hybrid compound approach. The meta- sulfonamide H11 was the most potent sulfonamide, with similar activity against the drug sensitive and resistant *P. falciparum* lines (IC$_{50}$ ~2 µM; Ri = 0.8). In contrast, H10, the para- regioisomer of H11, had poorer activity for the Dd2 line resulting in a higher Ri (Ri = 3.7) than H10. This Ri value is similar to that for chloroquine (Ri = 4.3). The structure-activity relationship between the meta- and para- sulfonamide regioisomers and the Ri is reversed for regioisomers H4 and H5. In this compound pair

<table>
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<th>Cpd</th>
<th>cLogP$^a$</th>
<th><em>P. falciparum</em> IC$_{50}$ (µM)</th>
<th>Ri$^b$</th>
<th>NFF IC$_{50}$ (µM)</th>
<th>SI$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3D7</td>
<td>Dd2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>+2.68</td>
<td>15.2 (±2.9)</td>
<td>18.0 (±0.3)</td>
<td>1.2</td>
<td>&gt;250</td>
</tr>
<tr>
<td>H5</td>
<td>+2.68</td>
<td>4.0 (±0.3)</td>
<td>13.2 (±2.0)</td>
<td>3.2</td>
<td>&gt;250</td>
</tr>
<tr>
<td>H6</td>
<td>+3.93</td>
<td>1.0 (±0.2)</td>
<td>1.1 (±0.1)</td>
<td>1.1</td>
<td>&gt;250</td>
</tr>
<tr>
<td>H7</td>
<td>+2.79</td>
<td>3.9 (±0.8)</td>
<td>18.7 (±1.4)</td>
<td>4.8</td>
<td>&gt;250</td>
</tr>
<tr>
<td>H8</td>
<td>+2.79</td>
<td>1.4 (±0.5)</td>
<td>7.0 (±1.2)</td>
<td>5.1</td>
<td>&gt;250</td>
</tr>
<tr>
<td>H9</td>
<td>+4.04</td>
<td>0.2 (±0.04)</td>
<td>0.3 (±0.03)</td>
<td>1.4</td>
<td>&gt;250</td>
</tr>
<tr>
<td>H10</td>
<td>+2.85</td>
<td>4.0 (±0.5)</td>
<td>14.8 (±0.2)</td>
<td>3.7</td>
<td>&gt;250</td>
</tr>
<tr>
<td>H11</td>
<td>+2.85</td>
<td>2.0 (±0.1)</td>
<td>1.7 (±0.2)</td>
<td>0.8</td>
<td>&gt;250</td>
</tr>
<tr>
<td>H12</td>
<td>+4.11</td>
<td>0.6 (±0.1)</td>
<td>0.6 (±0.02)</td>
<td>0.9</td>
<td>62.4 (±1.2)</td>
</tr>
<tr>
<td>CQ</td>
<td>+3.73</td>
<td>0.02 (±0.001)</td>
<td>0.08 (±0.004)</td>
<td>4.3</td>
<td>24.1 (±4.8)</td>
</tr>
</tbody>
</table>

$^a$Calculated LogP (cLogP) values using CS ChemDraw Ultra. $^b$Resistance Index (Ri) - IC$_{50}$ chloroquine resistant line (Dd2)/IC$_{50}$ chloroquine sensitive line (3D7). The higher the Ri the higher the level of resistance. $^c$Selectivity index (SI) - mammalian cell (NFF) IC$_{50}$/*P. falciparum* IC$_{50}$. Range shown for 3D7 and Dd2 where IC$_{50}$ achieved or minimum SI for compounds where NFF IC$_{50}$ > 250 µM. Higher SI indicated greater parasite-specific selectivity; CQ: Chloroquine.
the change in position of the PS group from para- (4) to meta- (5) results in Ri values of 1.2 and 3.2, respectively. Finally, for regioisomers H7 and H8, the Ri is high (Ri = 4.8 and 5.1, respectively) irrespective of the position of the primary sulfonamide substituent. For the three different linker spacings, with one (H4 and H5), two (H7 and H8), or three (H10 and H11) methylene groups directly attached to the 1,2,3-triazole, the SAR was flat (i.e. no obvious activity differences based on structural features).

3.3.5.3 Mammalian cell cytotoxicity

In order to examine the selectivity of 7-chloro-4-aminoquinoline primary sulfonamide hybrids and controls for \textit{P. falciparum} parasites versus normal mammalian cells, \textit{in vitro} cytotoxicity assays (Section 2.6) were carried out with neonatal foreskin fibroblast (NFF) cells and the results compared to that obtained for chloroquine. In agreement with previous reports by ourselves and others, chloroquine has a high selectivity index (SI) for parasites versus this normal mammalian cell line (SI = 301-1220) [254-256]. Of the 7-chloro-4-aminoquinoline PS hybrid and control analogues, control H9 had the highest SI (>833) and was also the most potent in terms of its activity \textit{P. falciparum} (IC$_{50}$ 0.2-0.3 µM; Table 3.5). The other control compounds, H6 and H12, also had good selectivity (SI >227 and 104, respectively) for parasites versus NFF cells (Table 3.5). All PS hybrids were relatively non-toxic to NFF cells with no inhibition observed at the highest concentration tested (250 µM).
3.4 Discussion

Discovery of new antimalarial drug leads with the potential to combat drug resistance requires the use of a variety of drug discovery approaches including high throughput cell based screening, target-based screening, drug repurposing, and the use of complementary drug combinations, either as combined single entities or possibly as hybrid compounds combining two pharmacophores. In this chapter some of these approaches were utilised to investigate the antimalarial potential of primary sulfonamide (PS) containing compounds, no example of which is currently clinically used for malaria. A drug repurposing approach was used to profile the antimalarial activity of a group of clinically used sulfonamides and a panel of novel PS glycosides, the latter having been previously tested for in vitro anticancer activity [201, 238, 257]. PS compounds identified from a recent GSK high throughput antimalarial screening campaign of over 2 million compounds [7] were also further profiled. Finally, novel 4-aminoquinoline PS hybrid compounds were investigated.

Drug repurposing can potentially reduce the time and cost associated with the development of a new drug and has played a role in the discovery of several antiparasitic agents [258]. An example is the secondary sulfonamide sulfadoxine, an antibacterial which is used in combination therapies to treat *P. falciparum* infections [69]. There are over 100 FDA approved sulfonamide containing drugs used to treat a variety of human diseases [181] however most have not been investigated for their antimalarial activity. In this chapter the in vitro antimalarial activity of a selection of 14 clinically used PS containing compounds was investigated (Table 3.1). All of these
compounds were found to have poor in vitro activity against *P. falciparum* parasites (IC$_{50}$ >12.5 µM), with only one compound, celecoxib, having an IC$_{50}$ below 10 µM (IC$_{50}$ ~6 µM). Celecoxib is a nonsteroidal anti-inflammatory drug (NSAID) and a selective COX-2 inhibitor used in the treatment of osteoarthritis and rheumatoid arthritis. Celecoxib also has several off target effects including inhibition of several isoforms of human CAs [259]. Although celecoxib displayed better in vitro antimalarial activity than the other clinically available PS compounds, the activity was still not within the range of an early phase hit for malaria (Appendix 2), and more importantly the compound kills human cells at a similar concentration (SI~4). Thus, overall the clinically available compounds tested in this study do not warrant pursuing further as antimalarials. Given that CA may be a potential target in *Plasmodium* parasites for these types of compounds, the lack of activity observed may be explained by the low amino acid sequence identity seen between the *Plasmodium* and human CAs (Table 1.2; 3-10%). This is supported by recent findings by Professor C. Supuran in collaboration with our group, that the *Plasmodium* CA (*Pf*CA) belongs in a putatively novel and distinct class of its own (namely the η-CAs [260]). This finding is discussed in more detail in Chapter 4.

In addition to testing clinically used PS compounds, the antimalarial activity of a panel of 16 novel experimental PS glycosides (some of which have shown anti-cancer activity in vitro [201, 238, 257] were also investigated in this study. Of these, PS-3 and PS-4 displayed the best antimalarial activity and selectivity and no evidence of cross resistance with the antimalarial drugs chloroquine and mefloquine, as determined using
drug-sensitive and resistant *P. falciparum* parasite lines (Pf3D7 and PfDd2, respectively, IC\textsubscript{50}~1 µM; SI >50 NFF/Pf3D7). Structure-activity relationship studies may support a role for the PS moiety contributing to the antimalarial activity of some of these compounds ([PS-3, 4, 9 and 10](#)), as derivatives lacking the PS displayed reduced antimalarial activity (Pf3D7 and PfDd2 IC\textsubscript{50}~5 µM). However, no change in activity or an increase in activity (~7.5 fold) was seen for PS-7/PS-7′ and PS-8/PS-8′ respectively. Therefore it is unclear at this stage of the exact role of the PS group in these compounds. To date only one of the PS glycosides examined in this study has been tested *in vivo* for antimalarial efficacy. **PS-3** was tested in a mouse malaria model by another member of the lab (Dulangi Sumanadasa; unpublished data), however no attenuation of parasitemia observed following oral administration for three days to *P. berghei* ANKA infected mice at 50 mg/kg/day (given as a split dose at 25 mg/kg with a 4h interval between doses). This poor *in vivo* efficacy may be explained by the relatively low amino acid sequence identity seen between the *P. falciparum* and *P. berghei* CAs (47% amino sequence identity; **Table 1.2**). If PfCA is proven to be a target of **PS-3**, the low homology between the human and mouse *Plasmodium* species may require a more biologically relevant *in vivo* test model, such as a humanised mouse malaria model [261]. Alternatively the poor human oral absorption properties predicted for **PS-3** (**Table 3.3**) may explain the lack of *in vivo* efficacy. Pharmacokinetic profiling of this compound would be required to confirm this *in silico* prediction. Overall, the antimalarial activity of **PS-3** (IC\textsubscript{50}~1 µM) fits the ‘hit’ criteria for an antimalarial but is not yet at the level required for a lead-like antimalarial compound (**Appendix 2** [8]), and would require further structural modifications to improve both its *in vitro* and
probably *in vivo* efficacy if pursued in the future. Structural modifications of the core structure to identify structure-activity and structure-property relationships are necessarily part of an iterative workflow cycle between compound synthesis and biological evaluation (phenotypic assays – parasite death, cytotoxicity) and enzyme assays if target enzyme is available). The results of each cycle inform decisions for the next cycle. It is standard to explore different moieties to identify and optimise steric, hydrophobic and electrostatic properties. Elucidation of the mode of action of this compound, which is investigated in subsequent chapters of this thesis, would also be helpful for development of next generation derivatives with improved potency and selectivity.

In the past decade, advances in high throughput, whole-cell based, antimalarial screening platforms has facilitated the screening of millions of compounds to identify hits for antimalarial drug development. As mentioned previously (Section 3.1), screening of a GlaxoSmithKline (GSK) library of approximately two million compounds identified approximately eight thousand compounds that inhibited the *in vitro* growth of drug-sensitive (3D7) and resistant (Dd2) *P. falciparum* parasites by ≥80% and ≥50% at 2 µM, respectively [7]. In this project, these data were exploited, with a panel of 26 PS compounds identified in this library via a sub-structure search. As existing data on these compounds was limited to % Inhibition at 2 µM, these data were extended and their *in vitro* IC₅₀ against *P. falciparum* 3D7 parasites determined. Thirteen potential lead-like compounds (Pf3D7 IC₅₀ <1 µM; SI >10 HepG2/Pf3D7) were identified. In particular **GSK-7**, **GSK-15** and **GSK-22** displayed the best
antimalarial activity against both drug sensitive and resistant parasites (Pf3D7 IC\textsubscript{50} 0.2-0.3 µM; PfDd2 89-100% inhibition @ 2 µM) and good selectivity (SI >40 HepG2/Pf3D7). However it should be noted that GSK-7 and GSK-22 contain a known antimalarial pharmacophore (a diaminopyrimidine, which is a component of the antimalarial drug pyrimethamine, a DHFR inhibitor) and therefore should be screened against a pyrimethamine resistant line (e.g. HB3 [241]) before considering future studies. The next step will be to test GSK-7, GSK-15 and GSK-22 in a mouse malaria model to determine \textit{in vivo} efficacy, however this work will be dependent on future compound availability. Though it is possible that the PS group may contribute to the antimalarial activity associated with the GSK compounds, further studies will be needed to confirm this.

Finally the antimalarial activity of a group of novel 7-chloro-4-aminoquinoline PS hybrids was assessed in an attempt to develop compounds with improved efficacy and safety and to reduce the potential for parasite resistance. Chloroquine, a safe and well tolerated antimalarial developed in the 1930s is still used widely to treat \textit{P. vivax}, and although \textit{P. falciparum} chloroquine resistance is widespread, there is some evidence of re-emergence of \textit{P. falciparum} sensitivity to chloroquine in Africa [235]. This has led to a renewed interest in the 4-aminoquinoline scaffold for developing hybrid drugs [137, 144, 145, 149-151, 232-234]. Although the hybrid compounds investigated in this chapter all had reduced antimalarial activity when compared to chloroquine, an interesting feature was that they demonstrated the potential to overcome the resistance profile of chloroquine. This was reflected in the resistance indices of the three control
compounds H6, H9 and H12 (Ri ≤ 1.4) compared to that of chloroquine (Ri = 4.3), indicating that the addition of the phenyl-1,2,3-triazole to the aminoquinoline scaffold overcomes the resistance profile observed with chloroquine only. However it should also be noted that none of these control compounds contain a PS group - all were non-PS control compounds. Nonetheless H9 had the highest SI (>833) and was also the most potent in terms of its activity (P. falciparum IC₅₀ 0.2-0.3 µM) and therefore will be considered for future in vivo investigations given that these compounds may have the potential to avoid the resistance issues associated when using chloroquine alone.

While the P. falciparum target/s for the PS compounds examined in this study is not yet known, the malaria parasite CA is a possible candidate. As discussed in the next Chapter (and Chapter 1), many PS compounds are known to target CA enzyme activity [184-186] and therefore this was a logical starting point for target identification studies.
4 Investigating PfCA as a primary sulfonamide target

Publications arising from this chapter


Contribution: I developed the PfCA protein expression plasmid used to generate the recombinant PfCA protein for work in this paper and contributed to editing of the final drafts. Where relevant, work carried out by others is acknowledged in this chapter.


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4.1 Introduction

It is not essential to identify the mode of action of an antimalarial drug to progress a new compound to the clinic, and this is the case with many currently used malaria therapies, including artemisinin and primaquine [95, 98]. However, identifying an antimalarial drug target may help prioritize compounds for further investigation by excluding those that may be cross-resistant to existing targets or pathways and providing better tools to develop new drug-leads. For example, if a target is known and recombinant protein able to be produced, it may be possible to utilize enzyme assays or generate crystal structures for use in inhibitor docking studies, both of which can facilitate target-based rational medicinal chemistry approaches to develop new compounds with improved biological activity and selectivity [262]. In the previous chapter several novel primary sulfonamides (PS) glycosides were identified as possessing some of the criteria required for antimalarial drug development (IC$_{50}$ ~1µM against drug sensitive and resistant $P. falciparum$ lines; Selectivity Indexes >50), however further work is required to improve the potency and selectivity of these compounds. Determining the target(s) of these compounds in the malaria parasite may aid in improving the lead-like properties of this chemical class and may provide a way to screen for new chemotypes with the same target.

The PS moiety is in the structure of several drugs where the mechanism of action is attributed to CA inhibition [186]. The CAs from Helicobacter pylori, Candida albicans, Candida glabrata, Cryptococcus neoformans and Brucella suis are essential for
pathogen growth and have proven susceptible to inhibition with several compound classes including PS compounds [225]. CAs from these pathogens are now recognised as potential anti-infective drug targets [225]. A recombinantly expressed form of the \textit{P. falciparum} CA (\textit{PfCA}) has been shown to be inhibited by one PS type compound, 4-(3,4-dichlorophenylureido)thiourea-benzenesulfonamide ($K_i = 0.18 \text{ µM}$, Table 1.3), although the structure of this compound is different to those described in Chapter 3, they share the benzenesulfonamide fragment/pharmacophore. [215]. This compound was also shown to have \textit{in vitro} IC$_{50}$ of $\sim 1 \text{ µM}$ against \textit{Plasmodium falciparum} malaria parasites [215]. Despite this moderate \textit{in vitro} activity against whole parasites the same thiourea-benzenesulfonamide compound displayed promising efficacy in a mouse malaria model (\textit{P. berghei} infected BALB/c mice) with a 50% inhibitory dose (ID$_{50}$) of 10 mg/kg compared to the antimalarial drug chloroquine with an ID$_{50}$ of 5 mg/kg [215]. This, together with the current dogma that \textit{PfCA} is likely to be essential for parasite survival as it is believed to be involved in the first step of the crucial pyrimidine synthesis pathway in \textit{P. falciparum} (Figure 1.4) [263], led to the working hypothesis that \textit{PfCA} may also be a target of the PS glycoside compounds identified in Chapter 3.

The gene \textit{PfCA} (PF3D7_1140000; aa 1-600), is predicted to encode a 72kDa carbonic anhydrase protein containing a transmembrane domain (TM) at amino acids (aa) 577-599 [113], however the purpose of the TM domain has not yet been investigated. To date four orthologues have been identified in the \textit{Plasmodium} rodent species, \textit{P. berghei} (PBANKA_090900), \textit{P. chabaudi} (PCHAS_071030) and \textit{P. yoelii} species (PYYM_0909900; PY00744), which share 40-47% amino acid sequence identity with
*Pf*CA [113]. *Pf*CA shares low homology with human CAs (3-10% amino acid sequence identity (Table 1.2), suggesting that specific *Pf*CA inhibitors may be developed that exploit these differences to minimise the potential for off target effects. *Pf*CA transcriptome data shows that *Pf*CA mRNA is expressed over the *P. falciparum* asexual and gametocyte lifecycle stages but is more highly expressed in ookinetes, a lifecycle stage of the mosquito vector [113].

Although a previous report from a Thai group utilized recombinant *Pf*CA in enzyme studies (as discussed above), we were unsuccessful in attempts to obtain either purified protein or the expression plasmid clone from that group. Thus to investigate *Pf*CA as a possible target of the PS glycoside compounds identified in Chapter 3, work presented in this Chapter focused on generating recombinant *Pf*CA from an *E. coli* codon optimised synthetic gene to allow testing of compounds for inhibition of the activity of this enzyme. The work described in this Chapter was carried out in collaboration with two of the world’s leaders in CA research, Dr Clemente Capasso from Institute of Bioscience and Bioresources (IBBR), Napoli, Italy and Prof Claudiu Supuran, from the University of Florence, Florence, Italy. As discussed below, an active form of *Pf*CA was expressed in *Escherichia coli* and tested against a selection of the PS glycoside antimalarial compounds identified in Chapter 3.
4.2 Materials and Methods

4.2.1 Recombinant human CAII

Recombinant human CAII (hCA II) was supplied by Associate Professor Andreas Hofmann, Eskitis Institute for Drug Discovery, Griffith University. hCA II was supplied at 13mg/mL in 100 mM NaCl, 20 mM HEPES at pH 8.0 and stored at 4°C.

4.2.2 Cloning of codon optimised PfCA fragments

The putative coding sequence of the PfCA gene (gene ID: PF3D7_1140000) was retrieved from PlasmoDB [113]. An E. coli codon optimised version of this gene (Appendix 4), flanked by restriction enzyme sites BamHI and NheI was purchased commercially (DNA 2.0, USA). The codon optimised gene was supplied in the pJexpress 404 vector. Seven oligonucleotides (Table 4.1) were designed to generate four truncated forms of the PfCA gene with the same restriction enzyme sites as the codon optimised gene. Four gene fragments (Table 4.1) were amplified using PCR (Section 2.9), checked via 1% agarose gel electrophoresis (Section 2.13) to ensure they were the correct size, and then purified using a QIAquick® Gel extraction kit, QIAGEN (Section 2.14). Each PfCA gene fragment was then ligated into the pENTR™5’-TOPO® vector, Invitrogen (Section 2.15) and transformed into Top10 E. coli cells using the pENTR™5’-TOPO® TA cloning kit (Invitrogen, USA) as per manufacturer’s instructions. Plasmid DNA was purified using a QIAprep Spin Miniprep Kit
(QIAGEN®, USA) and checked to ensure it was the correct size via 1% agarose gel electrophoresis (Section 2.13). The four fragments were then excised from the pENTR™5'-TOPO® vector using BamHI and NheI restriction enzymes (Section 2.12), ligated into a modified pET43.1aNHIS protein expression vector (supplied by Dr Tom Peat CSIRO Melbourne; Appendix 6) and then transformed into DH5α cells for long term storage (Section 2.15). Following this, the pET43.1aNHIS vector containing the PfCA1 inserts 1, 2 and 3 were transformed into E. coli BL21 cells for protein expression studies.

Table 4.1: PfCA gene fragments amplified from codon optimised PfCA

<table>
<thead>
<tr>
<th>Frag #</th>
<th>Frag Namea</th>
<th>Oligonucleotide pair</th>
<th>NT</th>
<th>MWb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PfCA1-600</td>
<td><strong>Forward:</strong> 5’-ATGggatccAAACTGCTCTACTTGCTC-3’&lt;br&gt;<strong>Reverse:</strong> 5’-TTAgctagcAAACAGAAACAGAGATG-3’</td>
<td>1815</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>PfCA211-445</td>
<td><strong>Forward:</strong> 5’-ATGggatccATGAAAGACCTGAAGGAGC-3’&lt;br&gt;<strong>Reverse:</strong> 5’-TTAgctagcGGTCGGGTCGGACCCAC-3’</td>
<td>717</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>PfCA174-538</td>
<td><strong>Forward:</strong> 5’-ATGggatccATCAACAGACCTTCTTGCTC-3’&lt;br&gt;<strong>Reverse:</strong> 5’-TTAgctagcCTTGGCTAGTGACGC-3’</td>
<td>1107</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>PfCA1-577</td>
<td><strong>Forward:</strong> 5’-ATGggatccAAACTGCTCTACTTGCTC-3’&lt;br&gt;<strong>Reverse:</strong> 5’-TTAgctagcGCTCATATTCTCAGGAGATGTC-3’</td>
<td>1743</td>
<td>68</td>
</tr>
</tbody>
</table>

aSuperscript numbers designate amino acids on PfCA open reading frame (Appendix 5) spanned by primer pairs. bPredicted molecular weight (kDa) of protein encoded by cloned insert. BamHI and NheI restriction sites underlined. Frag = fragment; NT = nucleotides.
4.2.3  PfCA protein expression

Luria broth (LB) 5 mL (Appendix 1) containing 50 µg/mL ampicillin (Sigma - Aldrich®, USA) was inoculated with a single colony of E. coli BL21 containing the pET43.1a-NHIS plasmid containing PfCA fragments 1, 2 or 3 (Table 4.1) and incubated overnight at 37°C at 200 rpm in an orbital shaker incubator. The following day the 5mL overnight culture was added to 100 mL of LB media with 50 µg/mL ampicillin (Sigma - Aldrich®, USA) and incubated at 37°C and 200 rpm in an orbital shaker incubator until OD600 reading of 0.4-0.8 was reached. Cultures were then induced with IPTG (Sigma - Aldrich®, USA, 0.2-0.5 mM) for 2-4 hrs at 37°C or overnight at 16°C at 200 rpm on an orbital shaker incubator. Cultures were then centrifuged at 4000 x g for 20 min at 4°C and each pellet resuspended in 20 mL of ice cold PBS and centrifuged as above. The pellet was then resuspended in 10 mL of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole pH 8.0) containing 1mg/mL lysozyme (Sigma - Aldrich®, USA) and incubated at RT for 30 min. One cOmplete™ mini EDTA free protease inhibitor cocktail tablet (Roche, Germany) was added at this point and the sample was sonicated on ice 4 x 30 sec bursts with 10 sec cooling period in between each burst. The lysate was then diluted 1 : 4 in lysis buffer and 1 mL sample taken for SDS PAGE analysis. The lysate was then centrifuged at 10,000 x g for 30 min at 4°C and 1 mL sample collected for SDS PAGE analysis (Section 2.16). This method was also up scaled proportionately to allow large scale production of protein where mentioned.
4.2.4 Purification of protein lysates under native conditions

Purification of protein lysates (Section 4.2.3) under native conditions was carried out using the QIAexpressionist™ System (QIAGEN®, USA), as described by the manufacturer (Protocol 12 of the handbook; fifth edition). Briefly, 1 mL of a 50% Ni-NTA (QIAGEN®, USA) and 50% ethanol slurry was added to 4 mL of protein lysate (Section 4.2.3) and mixed gently on a rotary shaker at 4°C for 60 min. The lysate-Ni-NTA mixture was then loaded onto a 10 mL polypropylene chromatography column (Bio-Rad®, USA) and the flow-through collected and stored on ice, until required for SDS PAGE analysis. The column was then washed twice with 4 mL wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole pH 8.0) and wash fractions collected and stored on ice, until required for SDS PAGE analysis. Bound protein eluted from the column 4 times using 0.5 mL elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole pH 8.0). Six 1 mL fractions were collected and analysed using SDS PAGE (Section 2.16).

4.2.5 Purification of protein lysates under denaturing conditions

Purification of protein lysates (Section 4.2.3) under denaturing conditions was carried out using the QIAexpressionist™ System (QIAGEN®, USA), as described by the manufacturer (Protocol 17 of the handbook; fifth edition). Briefly, protein lysates were resuspended in 5 mL denaturing lysis buffer (100 mM NaH$_2$PO$_4$, 10 mM Tris-Cl, 8 M urea pH 8.0) and incubated at RT for 60 min. The lysate was then centrifuged at 10,000
x g for 30 min at RT and supernatant saved and purification carried out as per Protocol 17 of the QIAexpressionist™ handbook (fifth edition). Briefly 1 mL of a 50% Ni-NTA (QIAGEN®, USA) and 50% ethanol slurry was added to 5 mL of protein lysate (Section 4.2.3) and mixed gently on a rotary shaker at 4°C for 60 min. The lysate-Ni-NTA mixture was then loaded onto a 10 mL polypropylene chromatography column (Bio-Rad®, USA) and the flow-through collected and stored on ice, until required for SDS PAGE analysis. The column was then washed twice with 4 mL wash buffer (100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M urea pH 6.3) and wash fractions collected and stored on ice, until required for SDS PAGE analysis. Bound protein was then eluted from the column 4 times using 0.5 mL elution buffer (100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M urea) at pH 5.9 and 4 times at pH 4.5. The eight 0.5 mL fractions were collected and analysed using SDS PAGE (Section 2.16).

4.2.6 Protein refolding via step-wise dialysis

Protein fractions (5-20 mL) obtained by purification under denaturing conditions (Section 4.2.5) were pooled and added to Snakeskin™ Dialysis Tubing, 10 MWCO (Thermoscientific™, USA) and dialysed overnight at 4°C in 500 mL refolding buffer (50 mM Tris, 50 mM NaCl, 10% glycerol; pH 8.0) containing 6 M urea. Following this the buffer was changed every 8 h and the concentration of urea reduced to 4 M, 2 M and finally 1 M urea over each 8 h change. The protein was then dialysed 3x for 8 h in 1x refolding buffer without urea. The refolded protein was concentrated using Amicon
Ultra-15 centrifugal filter units (Merck Millipore, Germany) and the final protein concentration determined using via a Bradford assay (Section 2.17).

4.2.7 Esterase activity assays

The esterase activity of PfCA was measured as previously reported [216]. Briefly, enzymatic reactions were set up in a total final volume of 50 µL in wells of Corning® 3596 clear 96 well plates (Corning, USA) containing 12.5 mM Tris, 75 mM NaCl at pH 7.5-8.0 and recombinant PfCA (or hCA II as a control) at various concentrations. Assays were carried out with and without inhibitors and the control inhibitor acetazolamide (AAZ; 0.1 mM -1 mM). Prior to measuring absorbance, 50 µL 2 mM p-nitrophenylacetate (Sigma, USA) was added to each well and absorbance monitored over 10 min at 37°C at 405 nM using a FLUOStar Optima plate reader (BMG LABTECH, Germany) in kinetic mode. Esterase activity was measured without inhibitors (vehicle only) and with inhibitors (0.1mM -1mM), including the control inhibitor AAZ.

4.2.8 PfCA^{211-445-GNK} expression and purification

[Carried out in Dr Clemente Capasso’s Laboratory at the Institute of Bioscience and Bioresources (IBBR), Napoli, Italy].

ArcticExpress (DE3) chemically competent cells (Agilent, USA) were transformed with the PfCA^{211-445-GNK} pET43.1aNHS vector as per the ArcticExpress manual instructions. 5mL LB media (Appendix 1) with ampicillin (50 µg/mL) and gentamicin (20 µg/mL;...
Sigma - Aldrich®, USA) was inoculated with a single colony from ArcticExpress cells containing the $PfCA^{211-445}$ vector and incubated overnight at 37°C at 200 rpm in an orbital shaker incubator. The following day the 5 mL overnight culture was added to 100 mL of LB media with no selection antibiotics and incubated at 30°C with shaking at 200 rpm until the OD600 reading of 0.4-0.8 was reached. The cultures were then incubated with shaking at 200 rpm until the culture had equilibrated to 20°C and then induced with 1 mM IPTG (Sigma - Aldrich®, USA). After a further 30 min, 0.5 mM ZnSO$_4$ (Sigma - Aldrich®, USA) was added and the cultures were incubated for a further 16 h at 20°C with shaking at 200 rpm. At 16 h post induction, cells were harvested and disrupted by sonication at 4°C as previously described. Following centrifugation (10,000 x g for 30 min at 4°C), the supernatant was loaded onto HIS-Select HF Nickel Affinity Gel (Sigma - Aldrich®, USA) and the protein was eluted with 250 mM imidazole (Section 4.2.4).

4.2.9 CO$_2$ hydration assay

[Carried out in Professor Claudiu Supuran’s Laboratory at the University of Florence, Italy].

A stopped-flow CO$_2$ hydration assay with an Applied Photophysics instrument was used for measuring the catalytic activity and inhibition of the $PfCA^{211-445}$ [264]. Phenol red (0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.4) buffer and 20 mM NaClO$_4$ for maintaining constant ionic strength. The initial rates of the CA-catalyzed CO$_2$ hydration reaction were followed for a period of 10-100 sec [264]. The concentrations of substrate (CO$_2$) ranged from 1.7 to
17 mM for the determination of the kinetic parameters and inhibition constants, with at least six traces of the initial 5-10% of the reaction being used for determining the initial velocity, for each inhibitor. The uncatalyzed rates were determined, subtracted from the total observed rates. Stock solutions of inhibitors (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were used in the assay buffer. Enzyme and inhibitor solutions were preincubated prior to assay for 15 min RT, in order to allow the formation of the enzyme-inhibitor (E-I) complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation. The kinetic parameters for the uninhibited enzymes were derived from Lineweaver-Burk plots, as reported earlier [260, 265, 266] and represent the mean from at least three different determinations.

4.3 Results

4.3.1 Cloning of codon optimised PfCA

The pET43.1aNHIS vector containing the PfCA PCR (Section 2.9) amplified and BamHI/NheI ligated inserts: 1 (amino acids (aa) 1-600; PfCA<sup>1-600</sup>; full gene), 2 (aa 211-445; PfCA<sup>211-445</sup>; published predicted active site [216]) and 3 (aa 174-538; PfCA<sup>174-538</sup>; PlasmoDb [113] predicted active site), Table 4.1, were transformed (Section 2.15) into E. coli BL21 cells. The DNA sequences of PfCA<sup>211-445</sup> and PfCA<sup>174-538</sup> clones, including the presence of an in frame N-terminal His tag were confirmed by DNA sequencing (Section 2.10). The plasmid containing Insert 4 (aa 1-577; Table 4.1; PfCA<sup>1-577</sup>; full
gene lacking the transmembrane domain) was not successfully transformed into BL21 cells and therefore was excluded from the study.

4.3.2 Expression of \( P f CA^{174-538} \) in \( E. coli \) and purification under native conditions

\( E. coli \) BL21 cells containing pET43.1a-NHIS-\( P f CA^{174-538} \) (Table 4.1) were grown at 37°C and 16°C and expression induced using IPTG (Section 4.2.3). Samples were taken at different time points, protein lysates prepared and analysed using SDS PAGE (Section 2.16), and western blot using anti-His G-HRP Antibody (Section 2.18). This antibody recognises the His epitope tag present on the N-terminal of all expressed fragments. A band of \(~45\) kDa, corresponding to the predicted MW of \( P f CA^{174-538} \), was observed on the Coomassie stained gel and western blot (representative clone shown in Figure 4.1). No significant bands of similar size were observed in lysates prepared from \( E. coli \) BL21 cells transformed with pET43.1aNHIS vector (vector control). Based on these pilot studies, the best expression was obtained after 4 h induction at a 37°C growth temperature. Purification of the \( P f CA^{174-538} \) protein fragment was next attempted using NiNTA agarose under native conditions (Section 4.2.4) and samples analysed using SDS PAGE (Section 2.16). Unfortunately no soluble protein was obtained with the majority of the protein remaining in the protein pellet for both the 37°C and the 16°C samples (Figure 4.2).
Figure 4.1: SDS-PAGE and western blot showing expression of \( PfCA^{174-538} \) in \( E.\ coli \) under different conditions.

(A) Coomassie stained 12% SDS PAGE gel showing expression of \( PfCA^{174-538} \) in BL21 cells at 37°C and 16°C for the vector only and a representative clone. For 37°C incubation samples were taken before induction with 0.5 mM IPTG (0 h) and following induction with 0.5 mM IPTG for 1 h, 2 h and 4 h. For 16°C incubation samples were taken before induction with 0.2 mM IPTG (0 h) and following induction with 0.2 mM IPTG 16 h. (B) Western blot of the same protein samples from (A) probed using anti-His G-HRP Antibody (1:1000, Novex® by Life Technologies, USA), some low MW bands were observed on the membrane (not shown) which may be due to anti-His antibody background or degraded His tagged protein. Arrow denotes the 45kDa bands corresponding to the MW of PfCA\(^{174-538}\). Approximate molecular weight in kDa is indicated.
**Figure 4.2: Purification of PfCA^{174-538} under native conditions**

Coomassie stained 12% SDS PAGE gel showing expression of PfCA^{174-538} in BL21 cells at 37°C (A) after 4 h induction with 0.5 mM IPTG and at 16°C (B) after 16 h induction with 0.2 mM IPTG. For each induction temperature cells were centrifuged and the supernatant purified using NiNTA agarose (Section 4.2.4). P = pellet; S = supernatant; FT = flow through; W = wash; E = elution. Red arrow denotes the ~45kDa band corresponding to the expected MW of PfCA^{174-538}. A 1Kb PageRuler™ (Thermoscientific, USA) prestained protein ladder is loaded to indicate approximate molecular weights (Std).
4.3.3 Expression of PfCA^{211-445} in *E. coli* and purification under native conditions

*E. coli* BL21 cells containing pET43.1a-NHIS-PfCA^{211-445} (Table 4.1) were grown at 37°C (4 h) for and 16°C (16 h) and expression induced using IPTG (Section 4.2.3). Samples were taken and analysed using SDS PAGE (Section 2.16) and western blot using anti-His G-HRP Antibody (Section 2.18). A band of ~30 kDa, corresponding to the predicted MW of *PfCA*^{211-445} was observed on the Coomassie stained gel and western blot (Figure 4.3). For each induction temperature, cell pellets were solubilised via lysozyme treatment and sonication (Section 4.2.4) and a sample of the remaining pellet and resulting supernatant analysed by SDS PAGE (Section 2.16). Comparing the presence of the ~30 kDa band corresponding to the MW of *PfCA*^{211-445} showed that no band corresponding to ~30 kDa was seen in the soluble supernatant fraction (Figure 4.3). Because of the lack of soluble protein no purification was able to be carried out for this fragment under native conditions.

4.3.4 Refolding of PfCA protein using dialysis

In addition to the work described above a number of different approaches were employed to try and produce soluble protein from the *PfCA*^{211-445} and *PfCA*^{174-538} containing clones including: (i) using different IPTG concentrations with variations in incubation times and temperatures in conjunction with alternative culture media such as the Enpresso™ (BioSilta™, UK) expression system designed to improve yield of soluble recombinant proteins [267], and (ii) inclusion of the metal ion Zn(II) during the
induction and purification process (Zn(II) is essential for catalysis of α-CAs [189] and has been shown to improve the yield of soluble protein in studies using bovine carbonic anhydrase (BCA) [268]). Unfortunately all of these efforts to generate soluble recombinant PfCA protein were unsuccessful, therefore subsequent efforts focused on investigating protein solubilisation and refolding methods to try and produce active PfCA from the expressed, but insoluble material.

Figure 4.3: SDS-PAGE and Western blot showing expression of PfCA\textsuperscript{211-445} in E. coli under different conditions.

(A) Coomassie stained 12\% SDS PAGE gel showing expression of a representative clone of \textit{PfCA}\textsuperscript{211-445} in BL21 cells at 16°C and 37°C. For 16°C incubation, samples were taken before induction with 0.2 mM IPTG (0 h) and following induction with 0.2 mM IPTG 16 h. For 37°C incubation, samples were taken before induction with 0.5 mM IPTG (0 h) and following induction with 0.5 mM IPTG at 4 h. For each induction temperature, cell pellets were solubilised via lysozyme treatment and sonication and the remaining pellet (P) and resulting supernatant (S) loaded. Arrow shows the ~30 kDa bands corresponding with the expected MW of the \textit{PfCA}\textsuperscript{211-445} fragment. (B) Western blot of same protein samples from (A) probed using Anti-His G-HRP Antibody 1:2500 (novex\textsuperscript{®} by Life Technologies, USA). Some low MW bands were observed on the membrane (not shown) which may be due to anti-His antibody background or degraded His tagged protein. Approximate molecular weight in kDa is indicated.
4.3.4.1 Refolding of PfCA<sup>174-538</sup> using step-wise dialysis

Recombinant PfCA<sup>174-538</sup> was expressed as per section Section 4.2.3 and purified under denaturing conditions (Section 4.2.5). The resulting protein was then refolded using a step-wise dialysis method (Section 4.2.6), samples checked by SDS PAGE (Appendix 7), and protein concentration determined using the Bradford method (Section 2.17). A total of ~ 500 µg was obtained from a 1 L culture volume. Next esterase activity was assessed, as in Section 4.2.7. Esterase activity was detected using 20 µg of PfCA<sup>174-538</sup> per assay, but not in lower amounts (not shown), however no inhibition was observed using the CA inhibitors AAZ, sulphamamide (SFA) or furosemide (FUR) all at 1 mM (Figure 4.4 A-C). In contrast at this concentration, all three compounds inhibited the esterase activity of the control hCA II (Figure 4.4 F-H). Unexpectedly, when the inhibitors celecoxib (CLX) and PS-3 (the most active antimalarial PS glycoside compound from Table 3.2 in Chapter 3) were assessed in the esterase assay against PfCA<sup>174-538</sup>, the activity of the refolded protein increased 1.5 to 2 fold (Figure 4.4 D and E). As this effect was not observed for the hCA II control protein (Figure 4.4 I and J), the increased absorbance seen with PfCA in the presence of PS-3 and CLX is not likely to be due to an optical effect associated with these compounds. This suggests that the effect observed may not be compound specific and that CLX and PS-3 may be behaving as agonists of the observed PfCA esterase activity. However, given the high concentrations of protein required to produce the PfCA<sup>174-538</sup> esterase activity (20 µg protein per assay well), it is difficult to determine if this is due to correctly refolded PfCA protein or possible low level E. coli contaminants (a limitation of using this type of assay). In addition, the low amounts of refolded PfCA able to be obtained limited the
Figure 4.4: Analysis of recombinant PfCA\textsuperscript{174-538} esterase activity in the presence and absence of different control compounds. The esterase activity of PfCA\textsuperscript{174-538} (20 µg per assay well; refolded by stepwise dialysis) and recombinant h CAII (1 µg per assay well) was assessed with and without 1 mM AAZ, sulphanilamide (SFA), furosemide (FUR), celecoxib (CLX) or PS-3. Esterase activity was measured by monitoring the change in absorbance at 405 nM of \textit{p}-nitrophenylacetate (Sigma, USA) to \textit{p}-nitrophenol over a period of 10 min using a FLUOStar Optima plate reader (BMG LABTECH, Germany). Due to limited amounts of protein obtained, mean RFU (±SD) from only two independent experiments are shown.
number of assays able to be carried out, and thus to ascertain if this result is reproducible and statistically significant additional studies would be required.

Other methods investigated as part of this thesis project included slow and rapid dilution as well as column refolding (data not shown) using $PfCA^{174-538}$ and $PfCA^{211-445}$. However in all of these cases the purity of the protein was poor or protein yields were too low to allow esterase activity assays to be carried out.

4.3.5 Expression of recombinant $PfCA$ in the Capasso Lab at the Institute of Bioscience and Bioresources (IBBR), Napoli, Italy

While a significant amount of time was spent trying to generate correctly refolded $PfCA$ protein, as discussed above, very poor yields of protein were obtained with limited evidence of correct folding. To try to address this issue, three $PfCA$ fragments $PfCA^{211-445}$, $PfCA^{174-538}$ and the full gene $PfCA^{1-600}$ (poor expression for the full gene was observed in our lab; data not shown) cloned into the pET43.1aNHIS expression vectors were sent to Dr Clemente Capasso (Institute of Biosciences and BioResources, Napoli, Italy) and Professor Claudiu Supuran (University of Florence, Florence, Italy). In addition, a modified version of $PfCA^{211-445}$ ($PfCA^{211-445}_{GNK}$, was expressed in BL21 cells in our lab but no soluble protein obtained, data not shown) was also sent in the pET43.1aNHIS protein expression vector. This $E. coli$ codon optimised fragment was identical to the published amino acid sequence used to generate recombinant $PfCA$ by the Thai group (discussed in Section 4.1) and differed from the PlasmoDB derived
sequence in the last three C terminal amino acids (DPT to GNK). Dr Capasso and Professor Supuran are world leading experts in CA enzyme research and have the expertise and equipment needed to determine native CA activity via the CO₂ hydration assay, which is far more specific and robust than the esterase activity assay [269]. Recombinant PfCA²¹¹-⁴⁴⁵-GNK was successfully expressed in Dr Clemente Capasso’s laboratory using ArcticExpress (DE3) competent cells (Agilent, USA) and purified as per Section 4.3.5. Arctic cell Expression of PfCA²¹¹-⁴⁴⁵ and PfCA¹⁷⁴-⁵³⁸ was also attempted but a better result was obtained with PfCA²¹¹-⁴⁴⁵-GNK and thus this fragment was used in inhibitor studies (Section 4.3.6). No expression was attempted with the full gene PfCA¹-⁶⁰⁰. ArticExpress competent cells are derived from the high performance Stratagene BL21-Gold competent cells and are engineered to address protein insolubility [270]. Unlike BL21 cells (as used in the initial attempts in this study to obtain soluble PfCA- Section 4.3.2 and 4.3.3), these ArticExpress cells co-express the cold adapted chaperonins Cpn10 and Cpn60 from the psychrophilic bacterium, Oliespira antarctica [271]. When expressed in ArcticExpress cells these chaperonins confer improved protein processing at lower temperatures, potentially increasing the yield of active, soluble recombinant protein[271].

Purified PfCA²¹¹-⁴⁴⁵-GNK was tested for CA activity by Professor Claudiu Supuran using the CO₂ hydration assay (Section 4.2.9) and then tested against a selection of PS compounds as described below.
4.3.6 Inhibition of PfCA\textsuperscript{211-445-GNK} activity with a panel of PS glycosides and clinically used PS compounds

[Carried out in Professor Claudiu Supuran’s Laboratory at the University of Florence, Italy].

Due to the low protein yield (~ 20µg from 2 L of culture) only a selection of the PS glycosides and clinically used CA inhibitors from Chapter 3 were able to be assessed for their PfCA activity during this project. Of the PS glycoside compounds tested (Table 4.2), PS-3 and PS-4 displayed the best inhibition of PfCA\textsuperscript{211-445-GNK} (Ki 85.2 nM and 83.8 nM, respectively). These two compounds were also previously shown to have the best in vitro antimalarial activity (Pf3D7 IC\textsubscript{50}: 0.9 µM and 1.0 µM), however, there appeared to be no clear correlation between PfCA\textsuperscript{211-445-GNK} inhibition and antimalarial in vitro activity with the remaining compounds. For example the PfCA\textsuperscript{211-445-GNK} activity was ~8 fold lower for PS-11 (Ki: 708 nM) with respect to compound PS-3 (Ki: 85.2 nM) but similar antimalarial activities (Pf3D7 IC\textsubscript{50}: 0.9 µM and 1.4 µM). PS-11 differs only in its sugar group with respect to compound PS-3 (PS-3: Glucose; PS-11: Galactose), which may suggest that the sugar component of these compounds is also important for their in vitro antimalarial activity and may also impact on their PfCA activity. Interestingly the substitution of an acetate for a hydrogen in the R group positions of compounds PS-7 and PS-8 results in very little loss of PfCA\textsuperscript{211-445-GNK} activity (Ki 529 nM and 680 nM) but does result in an ~ 6 fold decrease in antimalarial activity (Pf3D7 IC\textsubscript{50}: 5.3 µM and 28.6 µM). In addition compounds PS-1 and PS-2 have similar in vitro antimalarial activity (Pf3D7 IC\textsubscript{50}: 31 µM and >50 µM) but PS-2 has an ~ 3 fold increase in PfCA\textsuperscript{211-445-GNK} activity.
Table 4.2: Inhibition of \( P/CA^{211-445-GNK} \), hCA I and hCA II with PS Glycosides

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Structural features</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Ki (nM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( P/3D7^a )</td>
<td>( P/CA )</td>
<td>h CA I</td>
</tr>
<tr>
<td>PS-3</td>
<td>Glc Ac I S</td>
<td>0.9 (±0.3)</td>
<td>85</td>
<td>112</td>
</tr>
<tr>
<td>PS-4</td>
<td>Glc H I S</td>
<td>1.0 (±0.5)</td>
<td>84</td>
<td>119</td>
</tr>
<tr>
<td>PS-11</td>
<td>Gal Ac I S</td>
<td>1.4 (±0.5)</td>
<td>708</td>
<td>115</td>
</tr>
<tr>
<td>PS-7</td>
<td>Glc Ac I SO₂</td>
<td>5.3 (±4.6)</td>
<td>529</td>
<td>103</td>
</tr>
<tr>
<td>PS-8</td>
<td>Glc H I SO₂</td>
<td>28.6 (±19)</td>
<td>680</td>
<td>3920</td>
</tr>
<tr>
<td>PS-1</td>
<td>Glc Ac H S</td>
<td>31.0 (±10.7)</td>
<td>562</td>
<td>91</td>
</tr>
<tr>
<td>PS-2</td>
<td>Glc H H S</td>
<td>&gt;50</td>
<td>189</td>
<td>114</td>
</tr>
<tr>
<td>PS-5</td>
<td>Glc Ac H SO₂</td>
<td>&gt;50</td>
<td>724</td>
<td>97</td>
</tr>
<tr>
<td>PS-6</td>
<td>Glc H H SO₂</td>
<td>&gt;50</td>
<td>746</td>
<td>101</td>
</tr>
<tr>
<td>AAZ</td>
<td></td>
<td>&gt;200</td>
<td>170</td>
<td>250</td>
</tr>
</tbody>
</table>

\(^aP/3D7\) (data derived from Chapter 3, Table 3.2). \(^b\)Ki values determined using CO₂ hydration assay Section 4.2.9 (carried out in Professor Supuran’s laboratory, University of Florence, Italy).

None of the above anomalies are able to be explained by differences in LogP. LogP < 0 indicates better compound solubility whereas LogP > 0 indicates better compound membrane permeability. For example PS-3 and PS-4 have opposing LogP values (1.37 and -1.48) but display similar inhibition of \( P/CA^{211-445-GNK} \) activity (Ki 85.2 nM and 83.8 nM) and similar \textit{in vitro} antimalarial activity (\( P/3D7 \) IC<sub>50</sub>: 0.9 µM and 1.4 µM).

This is also the case for PS-1 and PS-8 (LogP: 0.93 and -2.40; Ki 562 nM and 680 nM; \( P/3D7 \) IC<sub>50</sub>: 31 µM and 28.6 µM respectively). Furthermore, the lack of selectivity for the parasite enzyme is concerning, with the majority of the compounds being 2-17 times more selective for hCA I and 1-110 times more selective to hCA II (Table 4.2).

PS-8 was the only compound to display a 6 fold increase in sensitivity to \( P/CA^{211-445-} \).
over h CAI (Table 4.2), however PS-8 also displayed very poor antimalarial activity in vitro (Pf3D7 IC₅₀: 28.6 µM).

Eight clinically used PS compounds (HCT: Hydrochlorothiazide; SAC: Saccharin; MZA: Methazolamide; CLX: Celecoxib; ZNS: Zonisamide; VLX: Valdecoxib; SLT: Sulthiam; AAZ: Acetazolamide) were also evaluated for their PfCA²¹¹-⁴⁴⁵-GNK activity. Of the eight compounds tested, all displayed moderate PfCA²¹¹-⁴⁴⁵-GNK inhibition (Ki 132-246 nM; Table 4.3) with the exception of VLX which displayed very poor PfCA inhibition (Ki 54,000 nM; Table 4.3). However six of these compounds (MZA, CLX, ZNS, VLX, SLT and AAZ) were also more selective to h CAII (SI; h CAII Ki/PfCA Ki = 0.004-0.140). Additionally MZA, ZNS and VLX were also more selective to h CAI (SI; h CAI Ki/PfCA Ki = 0.001-0.250). All of the eight compounds, with the exception of CLX (Pf3D7 IC₅₀ 6.88±2.97 µM), were previously shown in work carried out for this thesis to have very poor activity in vitro (Pf3D7 0-7.4% inhibition @ 12.5 µM; Chapter 3, Table 3.1). This may be attributed to the LogP of these compounds conferring poor membrane permeability (LogP < 0.91) in all cases except for CLX and VLX (LogP 3.47 and 2.67, respectively). However VLX did show very poor PfCA²¹¹-⁴⁴⁵-GNK inhibition (Ki 50,000 nM) indicating that PfCA is not likely a target of VLX.

Notably CLX displayed good selectivity to PfCA²¹¹-⁴⁴⁵-GNK over h CAI (SI; h CAII Ki/PfCA Ki = 230) but was more selective to h CAII than PfCA²¹¹-⁴⁴⁵-GNK (SI; h CAII Ki/PfCA Ki = 0.1). Interestingly SAC (Table 4.3), the only secondary sulfonamide tested, displayed good selectivity for PfCA²¹¹-⁴⁴⁵-GNK over both of the human CA
isoforms tested (SI; h CAI Ki/PfCA Ki = 102; SI; h CAII Ki/PfCA Ki = 33) and may be
worth investigating further to determine whether SAC analogues can be developed with
improved in vitro antimalarial activity, without compromising the selectivity for PfCA
over human CAs.

Table 4.3: Inhibition of PfCA<sup>211-445-GNK</sup>, hCA I and hCA II with clinically used CA
inhibitors

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Pf3D7&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PfCA Ki (nM)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>h CAI Ki (nM)</th>
<th>h CAII Ki (nM)</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT</td>
<td>0.95 (±1.65)</td>
<td>153</td>
<td>328</td>
<td>290</td>
<td>-0.07</td>
</tr>
<tr>
<td>SAC</td>
<td>7.42 (±15.18)</td>
<td>181</td>
<td>18,540</td>
<td>5,959</td>
<td>0.91</td>
</tr>
<tr>
<td>MZA</td>
<td>2.80 (±2.77)</td>
<td>198</td>
<td>50</td>
<td>14</td>
<td>0.13</td>
</tr>
<tr>
<td>CLX</td>
<td>6.88&lt;sup&gt;b&lt;/sup&gt; (±2.97)</td>
<td>217</td>
<td>50,000</td>
<td>21</td>
<td>3.47</td>
</tr>
<tr>
<td>ZNS</td>
<td>0.00</td>
<td>246</td>
<td>56</td>
<td>35</td>
<td>0.36</td>
</tr>
<tr>
<td>VLX</td>
<td>3.93 (±19.04)</td>
<td>54,000</td>
<td>43</td>
<td>226</td>
<td>2.67</td>
</tr>
<tr>
<td>SLT</td>
<td>4.63 (±11.17)</td>
<td>132</td>
<td>374</td>
<td>9</td>
<td>-0.27</td>
</tr>
<tr>
<td>AAZ</td>
<td>0.85 (±1.47)</td>
<td>170</td>
<td>250</td>
<td>12</td>
<td>-0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup>Pf3D7 %Inhibition @ 12.5µM. <sup>b</sup>Pf3D7 IC<sub>50</sub> (° and <sup>b</sup> data derived from Chapter 3, Table 3.1).<sup>c</sup>Ki values determined using CO<sub>2</sub> hydration assay Section 4.2.9 (carried out in Professor Supuran’s laboratory, University of Florence, Italy). HCT: Hydrochlorothiazide; SAC: Saccharin; MZA: Methazolamide; CLX: Celecoxib; ZNS: Zonisamide; VLX: Valdecoxib; SLT: Sulthiam; AAZ: Acetazolamide.
4.4 Discussion

Determining the biological target of potential antimalarial drug leads can greatly aid in advancing compounds through the drug discovery pipeline. Several PS glycosidic compounds with antimalarial activity were identified in Chapter 3, however further optimisation would be required to progress this class of compound to antimalarial lead status, and information on their molecular target would aid this work. The PS pharmacophore is present in many drugs where the mechanism of action is attributed to CA inhibition [183-185]. Very limited data is available on PS compounds and Plasmodium, however one experimental aromatic PS compound has been shown to inhibit activity of a PfCA protein fragment (amino acids 211-445; PfCA 1; Table 1.1) in an esterase activity assay (Ki 180 nM) [215]. Unfortunately attempts to obtain the recombinant PfCA plasmid clone used in that single published study were unsuccessful. Therefore work in this Chapter first focused on generating recombinant PfCA enzyme for target-based assays. Two clones, PfCA<sup>211-445</sup> and PfCA<sup>174-538</sup>, generated using DNA sequences codon optimized for expression in E. coli to overcome potential expression problems commonly associated with the high AT content of P. falciparum genes [272, 273], were successfully expressed in E. coli cells. Unfortunately no soluble protein was obtained, with all protein apparently in inclusion bodies within E. coli cells. While subsequent purification under denaturing conditions combined with protein refolding generated material, albeit in low yields, that was positive in an esterase enzymatic assay, this activity was not able to be inhibited by various CA inhibitors, including the most potent antimalarial PS glycoside identified in Chapter 3 (PS-3). Various reasons may explain the lack of inhibition obtained with these recombinant protein fragments,
including incorrect folding and/or non-specific esterase activity from contaminating *E. coli* proteins remaining in the protein preparations. The esterase assay itself suffers from several limitations and has been shown to be unreliable when determining the Ki values for several inhibitors tested on human CA I [269].

Due to the low yields of recombinant *Pf*CA able to be obtained and the limitations of the esterase assay available in the laboratory where this work was carried out, collaboration was initiated with Dr Clemente Capasso at the Institute of Biosciences and BioResources, Napoli, Italy, and Professor Claudiu Supuran at the University of Florence, Florence, Italy. Both are world leaders in the study of CA enzymes from various organisms [208, 266, 274, 275] and routinely utilize a CA-specific assay for testing of enzymes and inhibitors [208, 266, 274-277]. This assay uses a native substrate (CO$_2$) as opposed to the non-native substrate (4-NPA) used in the esterase assay, and the reaction rate for human CA I or CA II catalysed hydrolysis of 4-NPA is ~100,000 times slower than the catalysed hydration of CO$_2$ [278, 279]. Of the four plasmid clones sent to Italy, only a modified version of clone *Pf*CA$^{211-445}$ (*Pf*CA$^{211-445}$-GNK; DNA insert codon optimized for expression in *E.coli* and identical to that reported in the previously published study discussed above [214]), yielded sufficient soluble protein (albeit still very low amounts). This expression was achieved using ArcticExpress (DE3) cells, which are a low temperature expression system designed to improve the yield of soluble protein [270]. Protein generated in this expression system was shown to have CA activity and was inhibited by several of the antimalarial PS glycoside compounds identified in Chapter 3 (**Table 4.2**), as well as the human CA inhibitor AAZ. Although
PS glycosides PS-3 and PS-4 displayed the best inhibition of recombinant PfCA activity in this chapter and the most potent in vitro antimalarial activity (Chapter 3), there was no correlation between enzyme activity and in vitro antimalarial activity with the remaining compounds. Furthermore, assuming PfCA is confirmed as a target of antimalarial PS glycosides in situ, the lack of selectivity of these compounds for the P. falciparum enzyme over the human CA I and CA II isoforms is worrying and needs to be addressed, potentially via design of compounds that more specifically target PfCA versus the human enzymes. As discussed in Chapter 1 and 3, PfCA is thought to be the first enzyme in the essential P. falciparum pyrimidine synthesis pathway (Section 1.6.1), however the essentiality of PfCA has yet to be confirmed in asexual intraerythrocytic stage P. falciparum parasites, so alternative targets of antimalarial PS compounds need to be considered. In particular it is worrying that no human Plasmodium orthologues are reported for the CA gene suggesting that this gene is not conserved among the Plasmodium species and therefore not important for survival. Therefore it is crucial that the essentiality of PfCA is determined before further work is carried out in relation to this target. While the scope of this thesis project did not allow for reverse genetic studies to determine if PfCA is essential in P. falciparum asexual or other life cycle stages, it is an ongoing focus of this laboratory in collaboration with Dr Christopher Goodman at the School of Botany, Melbourne University. Future studies will also examine whether P. falciparum asexual intraerythrocytic parasites are able to utilise host erythrocytic CA enzymes to survive. This will be achieved by using specific hCAI and hCAII antibodies to assess the localisation of human hCAI and hCAII in the parasite. In a second approach, uninfected red blood cells will be depleted of human
hCAI and hCAII using Valdecoxib (a specific hCAI and hCAII inhibitor identified in Chapter 3). These hCA depleted red blood cells will then be infected with the malaria parasite, and invasion and growth will be monitored to determine if hCAs are essential for parasite survival in vitro. If parasites cannot survive in the absence of hCAs this may also support the existence of a functioning PfCA.

Possibly the most interesting result to emerge from enzyme inhibition studies with recombinant PfCA was the finding that the commercially available compound saccharin (SAC), a cyclic secondary sulfonamide included in these studies, had better selectivity for PfCA over the human CA I and II isoforms (SI; hCA I Ki/PfCA Ki = 102; SI; hCA II Ki/PfCA Ki = 33). Although SAC does have poor in vitro antimalarial activity (Pf3D7 7.42% inhibition @ 12.5 µM) its lack of human CA I and II activity may warrant investigation into the development of analogues with improved potency without compromising the selectivity and the safety profile of this FDA approved artificial sweetener. It may also be of interest to analyse the activity of SAC, and potentially other moderately potent compounds, against other Plasmodium life cycle stages. For example, interrogation of the PlasmoDB database [113] shows that in some microarray studies (e.g.[280]) PfCA has greater expression in ookinetes than asexual intraerythrocytic or gametocyte stage parasites which may mean that PfCA plays a more important role in this lifecycle stage. Again, it will be important to determine if PfCA is essential to any of these life cycle stages in future work.
During this thesis project timeframe, our Italian collaborators discovered that PfCA belongs to a new genetic family of CAs [260]. Previously PfCA was reported by others to be considered as belonging to the α-class of CAs [214], however a more detailed examination of the PfCA amino acid sequence by Professor Claudiu Supuran’s laboratory in Italy (phylogeny and anion inhibition studies utilizing a plasmid clone (PfCA^{211-445-GNK}) generated as part of this PhD project) led to the conclusion that PfCA belongs to a novel CA class - namely the η-CAs [260]. If this hypothesis is able to be validated using protein crystallography, these differences may be exploited in the design of specific PfCA inhibitors in the future and to generate new biochemical information on a potentially novel CA family. To that end, plans are currently underway to scale up the production of PfCA^{211-445-GNK} protein for crystal structure generation in collaboration with Dr Tom Peat from the Protein Expression and Modelling and Structure facility at CSIRO in Melbourne.

Although data presented in this Chapter suggests that some of the antimalarial PS glycosides identified in Chapter 3 can inhibit PfCA in enzyme activity, this may not be the target/only target of these compounds in vitro. In particular PS-3 and PS-11, which only differ with respect to their sugar groups (PS-3: glucose; PS-11: galactose), have opposing PfCA activity but similar in vitro activity, suggesting that the sugar component may play an important role in the in vitro antimalarial activity of these compounds. Furthermore the poor in vitro activity of the human CA inhibitor AAZ (Pf3D7 IC_{50} >200 μM) yet good PfCA activity (Ki: 170 nM) suggests that this compound may not be able to target PfCA in vitro. Within an erythrocyte the asexual
form of the *Plasmodium* parasite resides within a parasitophorous vacuole membrane (PVM) and therefore the uptake of a compound into the parasite may require crossing three distinct membranes (erythrocyte membrane, PVM and parasite plasma membrane), depending on its target. AAZ is known to possess poor membrane permeability [281] and this may account for its poor *in vitro* antimalarial activity. Therefore to investigate other possible targets of antimalarial PS glycoside compounds, the following two Chapters focus on unbiased target identification strategies. In **Chapter 5** a biolabelling probe based on the PS pharmacophore was designed with the aim of trying to identify PS protein targets in *P. falciparum* protein lysates using photaffinity labelling (**Section 1.5.4**). In **Chapter 6** *in vitro* resistance selection (**Section 1.5.5**) was utilised to develop a PS-resistant *P. falciparum* line using **PS-3**. This line was then used to profile the activity of a selection of clinically used antimalarial compounds and a selection of PS compounds from Chapter 3, to further identify potential biological targets.
5 Investigation of Photoaffinity Labelling as a Target Identification Tool for Primary Sulfonamide Compounds
5.1 Introduction

While target based drug discovery using proteomic-based approaches has had some success in different settings [166], application of proteomic-based approaches for antimalarial target identification has been limited to date (Section 1.5.4). This includes the use of activity based probes and photoaffinity labelling (PAL), the focus of this Chapter. One of the crucial aspects of PAL is the design of highly specific small molecule probes that are capable of covalent binding to their active enzyme targets [9]. Following probe-target binding, photoactivation allows covalent cross-linking of the small molecule probe to its protein target and, once cross-linked, the covalently bound protein-probe complexes are amenable to downstream biochemical analysis. A typical PAL probe consists of a target specific ligand (pharmacophore), a photoreactive group (eg. benzophenone) and a reporter tag (eg. biotin or fluorophore) (Figure 5.1A) [282]. As the reporter tags can interfere with protein binding owing to their large size [166] a two-step based PAL method has been developed that replaces the reporter tag with a small ‘handle’ to the probe to allow conjugation to a complimentary functionalised reporter tag (eg. biotin or a fluorophore) following the covalent cross-linking step. The most commonly used handles are the azide and alkyne groups that conjugate to the reporter tags using click chemistry, these are small groups (just three atoms each) and are bioorthogonal (i.e. cannot react with cellular components). The fluorescent protein/probe product can then be visualised by in-gel fluorescence or, in the case of biotin, by western blot. The proteins of interest can then be pulled down using avidin
enrichment to characterise probe labelled proteins via mass spectrometry (Figure 5.1B) [166, 169, 283-286].

**Figure 5.1: General schematic of a typical PAL approach**

A) A typical PAL probe consists of a target specific ligand (pharmacophore), a photoreactive group (eg. benzophenone) to stabilise covalent binding to the target of interest, and a reporter tag (eg. biotin or fluorophore for one-step PAL) or handle (azide or alkyne for two-step PAL) for visualisation of probe/target binding. B) A typical PAL method involves incubating the PAL probe (A) with cell lysates to allow the target specific ligand to bind to their respective protein target. The protein/probe complex is then stabilised by UV crosslinking of the benzophenone group to proximal amino acid acid residues. The protein/probe complex can then be visualised via reaction with a fluorophore azide/alkyne in a click chemistry reaction and in-gel fluorescence. Alternatively if a biotin reporter group is pre-installed, the protein/probe complex can be visualised using Western blot and subsequently pulled down for further protein analysis by mass spectrometry.
The PAL strategy has been used successfully in the design of probes for several different enzyme families [287]. Hydroxamic acid-based probes were utilised to profile enzyme activity across the metalloprotease family of enzymes [288, 289]. A similar strategy was used to generate PAL probes based on the reversible HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) to target histone deacetylases (HDACs) in human cancer cell lines [167]. Additionally, PAL probes have been used to aid in target identification in drug discovery. For example photo-reactive γ-secretase modulators (GSMs) and γ-secretase inhibitors were developed to better understand their mechanism of action in binding to neurotoxic Aβ42 peptides in Alzheimer’s [290-292]. PAL probes have also been used to investigate the target of a potent antiviral agent PD404182 against the HIV virus [282], and antiproliferative activity of a library of compounds in the human breast cancer cell line MDA-MB-231 [293]. Recently several benzenesulfonamide based PAL probes were validated using recombinant human carbonic anhydrase II (rhCA II) as the target. As mentioned previously, benzenesulfonamide is a component of several of the PS inhibitors studied in Chapter 3. Benzenesulfonamide is known to be a potent inhibitor of rhCA II (Ki 0.27 nM) and protein ligand binding for this group when further functionalised has been well characterised both biochemically and via protein X-ray crystallography [294-298].

In this Chapter, PAL probes were employed to investigate whether CA and/or other protein/s are the target of antimalarial PS compounds. Several PAL probes based on benzenesulfonamide, or the clinically used PS acetazolamide (AAZ), were investigated (Table 5.1). These PAL probes consisted of a PS pharmacophore group
(benzenesulfonamide or AAZ), a photoreactive benzophenone group and an alkyne handle to allow linkage to a fluorescent azide reporter group via click chemistry (Figure 5.2). In the first part of work presented in this Chapter probes were validated as binding to CA protein using rhCA II, which is known to bind to benzenesulfonamides and AAZ [297, 299]. Therefore it was predicted that rhCA II would specifically bind to the PAL probes. Next, the ability of the PAL probes to bind to proteins in cell lysates was investigated using HeLa, erythrocyte and P. falciparum cells.

**Figure 5.2: General design of PAL probe.**
PAL probes comprise: a target specific ligand (PS pharmacophore, AAZ shown) to allow binding to specific target proteins (left); a photoreactive group (benzophenone) for UV crosslinking to covalently attach to bound protein (middle); an alkyne handle that can react with an azide fluorophore using click chemistry to allow visualisation of the complete protein/probe complex (right).
5.2 Materials and Methods

5.2.1 Photoaffinity labelling (PAL) Probes

All of the PAL probes used in this chapter (Table 5.1) were synthesised in Assoc. Prof Sally-Ann Poulsen’s laboratory at the Eskitis Institute for Drug Discovery, Griffith University. The compounds were supplied as solids and diluted in 100% DMSO (Sigma-Aldrich®, USA) as required.

Table 5.1: Structures of PAL probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Structure</th>
<th>MW</th>
<th>SO₂NH₂*</th>
</tr>
</thead>
<tbody>
<tr>
<td>662</td>
<td>![Structure 662]</td>
<td>560.6</td>
<td>+</td>
</tr>
<tr>
<td>663</td>
<td>![Structure 663]</td>
<td>1201.4</td>
<td>+</td>
</tr>
<tr>
<td>665</td>
<td>![Structure 665]</td>
<td>588.7</td>
<td>+</td>
</tr>
<tr>
<td>666</td>
<td>![Structure 666]</td>
<td>509.6</td>
<td>-</td>
</tr>
<tr>
<td>671</td>
<td>![Structure 671]</td>
<td>582.7</td>
<td>+</td>
</tr>
</tbody>
</table>

*active probes (+ SO₂NH₂); inactive probe (- SO₂NH₂)
5.2.2 Azide Fluorophores

Lissamine rhodamine B azide was synthesised in Assoc. Prof Sally-Ann Poulsen’s laboratory at the Eskitis Institute for Drug Discovery, Griffith University and Alexafluor®594 azide (Molecular Probes®, USA) was purchased from Invitrogen. Both compounds were supplied as solids and diluted in 100% DMSO (Sigma - Aldrich®, USA), as required.

5.2.3 Recombinant hCA II

Recombinant human carbonic anhydrase II (rhCA II) was supplied by Dr Tom Peat, CSIRO Protein Expression, Modelling and Structure (PEMS) program in Melbourne. rhCA II was supplied at 14 mg/mL in 30 mM Tris pH 8.0, 20 mM NaCl, 0.1 mM ZnSO₄, 1 mM DTT. The protein was stored at -80°C until needed.

5.2.4 Red blood cell (RBC) lysate preparation

100 µL of O positive packed red blood cells (RBCs) were washed 3x in PBS and centrifuged for 4 min at 1000 x g at RT. The samples were then resuspended in 5 volumes of PBS with 1X cOmplete, EDTA free protease inhibitor (Roche, Germany) and frozen overnight at -20°C. The samples were then thawed and centrifuged at 17,000 x g for 20 min at 4°C and the supernatant kept on ice. The protein concentration of each
sample was determined using the Bradford method (Section 2.17) and the lysates were then used in PAL experiments (Section 5.2.7) or stored at -20°C until needed.

5.2.5 HeLa cell lysate preparation

HeLa cells were cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (CSL Biosciences, Australia), 1% streptomycin (Life Technologies, Inc., Rockville, MD; complete medium) at 37°C and 5% CO₂. Cells were harvested when 80% confluent using Trypsin-EDTA 0.25% (Gibco®, USA). After harvesting, the cell pellet was resuspended in 1 mL PBS with 1x cOmplete, EDTA free protease inhibitor (Roche, Germany) and lysed by 4 cycles of freeze/thawing and centrifuged at 17,000 x g for 20 min at 4°C. The supernatant was then collected and the protein concentration of each sample was determined by the Bradford method (Section 2.17). The lysates were then used in PAL experiments (Section 5.2.7) or stored at -80°C until needed.

5.2.6 Parasite lysate preparation

*P. falciparum* 3D7 parasites were cultured in human RBCs (Section 2.1) and isolated using saponin lysis (Section 2.7). The parasites were then resuspended in 100 µl of high salt buffer (Error! Reference source not found.) per 500 µL pre-lysis pellet and incubated on ice for 30 min with vortexing every 10 min. Following this the samples were then centrifuged at 17,000 x g for 20 min at 4°C and the supernatant collected. A
buffer exchange in to PBS was performed using Amicon Ultra-0.5 10K centrifugal filter units (Merck Millipore, Germany) and the protein concentration quantified using the Bradford method (Section 2.17). The lysates were then used in PAL experiments (Section 5.2.7) or stored at -80°C until needed.

5.2.7 PAL for 1D in-gel fluorescence analysis

Purified rhCA II (Section 5.2.3) and/or protein lysates (Sections 5.2.4, 5.2.5 and 5.2.6) at various concentrations were resuspended in PBS and 50 µL samples dispensed into 96 well clear plastic plates (Corning, USA). The samples were then incubated at RT for 15 min with/without inhibitors or DMSO vehicle control. The samples were then cross-linked on ice for 1 h using a spectrolite UV lamp (Spectronics®, USA) at 365 nM. After UV exposure the crosslinked samples were transferred to Eppendorf tubes and Alexafluor®594 azide or Lissamine rhodamine B azide (final concentration 40 µM) was added followed by addition of 1 part 20 mM CuSO₄ (Sigma - Aldrich®, USA) to 2 parts 50 mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (final concentration 100 µM and 500 µM, respectively), aminoguanadine (Sigma - Aldrich®, USA, 5 mM final concentration) and sodium ascorbate (Sigma - Aldrich®, USA, 5 mM final concentration) added. The samples were then incubated for 1.5 h at RT protected from light. The samples were then resuspended in SDS loading buffer (Error! Reference source not found.), heated to 94°C for 3 min, separated by 1D SDS PAGE (12%; Section 2.16) and visualised by in-gel fluorescent scanning using a FLA5000 flatbed scanner.
Immediately following visualisation the gels were stained with coomassie blue (Appendix 1) and total protein loading was assessed.

5.3 Results

5.3.1 Investigating PAL probes for labelling rhCA II

As the PS moiety is known to bind to human CA isoforms, initial validation of the novel PAL probes (Section 5.2.1) was investigated using rhCA II (Section 5.2.3). PAL probes 662, 665, 666 and 671 (Table 5.1) were the first probes to be synthesised and tested in click reactions (Section 5.2.7) to determine if they were able to bind to rhCA II. 662 and 665 differed by the length of the linker group between the benzophenone and benzene sulfonamide groups (662 ethyl linker; 665 butyl linker). Probe 666, a control partner of 665, lacks the PS group of 665 and allows the contribution of the PS group to rhCA II binding to be assessed. Preliminary testing of all PS PAL probes in in vitro whole cell parasite growth inhibition assays (including the inactive probe 666), showed all had IC_{50}s ~1-3\mu M (n=1). However all of these probes contained pharmacophores other than the primary sulfonamide moiety so it is not known how these other components might contribute to the in vitro antimalarial activity. Given that PS PAL probe 666 was also active in in vitro whole cell growth inhibition assays it would seem that the non-PS moieties do have some anti-parasitic effect. However, it should be noted that all of the PAL probe labelling experiments conducted in this study were carried out...
ex vivo with protein lysates so parasite growth inhibition activity is not directly relevant in this case.

PAL probes 662, 665 and 666 (40 µM) were incubated with rhCA II (50 µg/mL) with and without UV cross-linking and the human CA II inhibitor AAZ (1 mM), followed by reaction with a fluorescent azide using click chemistry and 1D in-gel fluorescence analysis (Section 5.2.7). Changes in band intensity were quantitated by densitometry (Section 5.2.8) using Image Studio Lite Version 4.0 (Li-COR Biosciences, USA). A slight decrease (~42%) in the intensity of the ~29kDa band corresponding to the MW of rhCA II was seen for 665 compared to 662 (Figure 5.3A; lanes 1 and 4). In addition,
Figure 5.3: Comparison of PAL probes 662, 665 and 671 binding to rhCA II.

(A) PAL probes 662, 665 and 666 (40 µM) were incubated with rhCA II (50 µg/mL) with and without UV cross-linking and/or AAZ (1 mM). Samples were then reacted with an Alexafluor® 594 azide (40 µM) using click chemistry, separated by 1D SDS PAGE and visualised using an FLA5000 (GE Lifesciences) flatbed scanner at 750v. Control: rhCA II without probe.

(B) PAL probes 662 and 671 were tested in PAL 1D SDS PAGE analysis as per the conditions outlined in (A). In each case, immediately following in-gel fluorescence, the same gel is stained with coomassie to demonstrate loading levels and graphs show relative density of each band normalised to loading control, as determined by densitometry using Image Studio Lite Version 4.0 (Li-COR Biosciences, USA)
the non-PS control probe 666 displayed a reduction in band intensity (~72%) with respect to its PS-containing active partner 665 (Figure 5.3A; lanes 4 and 7) indicating that the PS component of 665 is likely to play a role in binding to rhCA II. Probe 666 does show minimal binding, which is expected with these types of probes as the benzophenone component is prone to some non-specific binding [9], hence the background observed (Figure 5.3A; lanes 7). Probe 666 was used initially to demonstrate that its partner probe 665 did have increased binding to rhCA II but was not used in subsequent experiments as the aim was to compare the binding affinities of the active probes. Furthermore, the other active probes did not have partner control probes for comparison with the inactive probe 666.

Substantially decreased band intensity was observed in the presence of the competing CA binding compound AAZ and in the absence of UV cross-linking for 662 (~91% and ~80%, respectively; Figure 5.3A lanes 2 and 3) and 665 (71% and 28%, respectively; Figure 5.3A lanes 5 and 6), suggesting that bands in lane 1 and 4 are due to specific binding of PAL probes to rhCA II. However more background was observed using 665 (Figure 5.3A; lanes 4-6) possibly due to probe saturation at 40 µM. Steps to reduce the background associated with PAL probe 665 are further addressed in Section 5.3.2.

As previously discussed, AAZ is a potent human CA inhibitor used to treat various human diseases [186] and has been shown to inhibit PfCA in enzyme assays by ourselves (Section 4.3.6) and others [216, 260]. Thus the AAZ structure was next incorporated into PAL probe 671 (Table 5.1) to enable a direct comparison of the
effectiveness of this heterocyclic sulfonamide with the probes comprising a benzene sulfonamide group. PAL probes 662 and 671 (Table 5.1) were compared for binding to rhCA II, as above. PAL probe 662 and 671 showed similar intensity of the ∼29kDa band corresponding to the MW of rhCA II (relative density 15,558 and 16,770 respectively; Figure 5.3B lanes 1 and 4), however higher background was observed for 671 (Figure 5.3B lanes 5 and 6). As mentioned above, this background may be due to over saturation with probe and in the next section PAL probes 665 and 671 were titrated against rhCA II to determine if this background could be reduced.

5.3.2 Optimisation of PAL probe concentration for 665 and 671

To determine if the background associated with the binding of PAL probes 665 and 671 to rhCA II could be reduced, 665 and 671 were incubated with 50 µg/mL rhCA II at concentrations ranging from 0.625 to 80 µM, in the presence or absence of the CA inhibitor AAZ then UV cross-linked, treated with Alexafluor®594 azide (Molecular Probes®, US) and visualised using in-gel fluorescence, as described previously. Similar band intensity for either 665-rhCA II (relative density 13,735-22,884; Figure 5.4A lanes 1, 3, 5, 7, 9, 11, 13, 15) or 671-rhCA II (relative density 14,108-21,218; Figure 5.4B lanes 1, 3, 5, 7, 9, 11, 13, 15) conjugates was observed for any of the different concentrations tested. Background intensity and non-specific binding decreased for both probes at concentrations of 1.25 and 0.625 µM. Based on these findings PAL probes 665 and 671 were used in subsequent studies at 0.625 µM.
Figure 5.4: Optimisation of PAL probe concentrations for 665 and 671.
PAL probes 665 (A) and 671 (B) were incubated at concentrations ranging from 0.625 µM-80 µM with rhCA II (50 µg/mL) with and without AAZ (1 mM). Samples were then reacted with an Alexafluor® 594 azide (40 µM) using click chemistry, separated by 1D SDS PAGE and visualised using an FLA5000 (GE Lifesciences) flatbed scanner at 750v. rhCA II without probe served as a negative control (control). In each case, immediately following in-gel fluorescence, the same gel is stained with coomassie to demonstrate loading levels and graphs show relative density of each band normalised to loading control, as determined by densitometry using Image Studio Lite Version 4.0 (Li-COR Biosciences, USA)
5.3.3 Comparison of PAL probes 662, 665, 671 and 663 for labelling of rhCA II

Following optimization of PAL probes 665 and 671 to reduce background (Section 5.3.2), results with these probes were compared to that with ethyl linked PAL probe 662 at a concentration of 0.625 µM. Probes were incubated with 50 µg/mL rhCA II with and without the inhibitor AAZ followed by UV treatment, reaction with Alexafluor®594 azide and in-gel fluorescence, as previously described (Section 5.2.7). PAL Probes 662, 665 and 671 displayed similar binding to rhCA II (relative density 12,311, 17330, 12,854; Figure 5.5; lanes 1, 3 and 5). In addition, another probe, 663, was designed to compare the effectiveness of “one-step PAL” (probe includes a pre-installed reporter group) with “two-step PAL” (probe includes a handle moiety to react with a reporter group in a second step). The one-step PAL probe 663 is a direct analogue of the ethyl linked two-step PAL probe 662 modified to incorporate a triazole linked rhodamine reporter tag (Table 5.1) and does not require the click reaction step following incubation with the protein. Although the need for a second step adds time and complexity to the PAL method, probes with pre-installed fluorophores are much bulkier molecules and so can potentially interfere with binding of the probe to its target protein and/or increase the level of nonspecific protein binding and hence increased background intensity [9]. PAL probe 663 was tested at 0.625 and 5 µM (as it was expected that this probe may be required at a higher concentration due to the limitations mentioned above) in the presence of rhCA II (50 µg/mL). PAL probe 663 exhibited poor labelling of rhCA II (band intensity ~59-71% lower; Figure 5.5 lane 7) when compared to that of 662, 665 and 671 at 0.625 µM (Figure 5.5; lanes 1, 3 and 5), but similar band intensity at
5µM (relative density 13,044; **Figure 5.5** lane 9). However, it should be noted that the in house rhodamine fluorophore signal is not as intense as the commercially available Alexafluor®594 azide signal (A/Prof S. Poulsen, personal communication), which may also account for lower signal in the 0.625 µM reaction. In summary, PAL probes 662, 665 and 671 are similarly effective at binding to rhCA II (50 µg/mL) at 0.625 µM while probe 663, with the pre-installed lissamine fluorophore, requires higher probe concentrations to achieve similar intensity.

**Figure 5.5**: Sensitivity test of PAL probes
PAL probes 662, 665 and 671 (0.625 µM) and 663 (0.625 µM and 5 µM) were incubated with rhCA II (50 µg/mL) with and without AAZ (1 mM). Samples were then reacted with an Alexafluor®594 azide (40 µM) using click chemistry (except in the case of 663), then separated by 1D SDS PAGE and visualised using an FLA5000 (GE Lifesciences) flatbed scanner at 750v. rhCA II without probe served as a negative control (control). Immediately following in-gel fluorescence, the same gel is stained with coomassie to demonstrate loading levels and graph shows relative density of each band normalised to loading control, as determined by densitometry using Image Studio Lite Version 4.0 (Li-COR Biosciences, USA)
5.3.4 Investigating the ability of PAL probes to bind to native CA in red blood cells

Optimisation of the PAL probes for use with rhCA II provided a positive control to validate investigations using cell lysates. Red blood cells (RBCs) contain a relatively high abundance of human CA I and CA II protein (~1%) and were therefore chosen as a starting point to investigate the ability of the PAL probes to bind to CA in cell lysates. RBC lysates (10 mg/mL total protein) were prepared (Section 5.2.4) and incubated with PAL probes 662 (ethyl linked), 663 (one step PAL) and 665 (butyl linked) at 40 µM with and without AAZ. Samples were UV cross-linked, reacted with an Alexafluor® 594 azide in a click reaction (662 and 665 only) and then analysed using in-gel fluorescence, as described previously (Section 5.2.7). Negative controls included RBC lysates treated with (i) PAL probe 666 (non-PS control for 665) and (ii) no added probe. rhCA II reacted with probe 662 was used as a positive control. No difference in the intensity of the expected protein size of ~ 29kDa (MW of native human CA I and CA II and rhCA II) was observed for PAL probes 662, 663 or 665 (relative density 9,445-12,840; Figure 5.6A; lanes 1-6) compared to the negative controls or in the presence of the inhibitor AAZ (relative density 8,228 and 12,734; Figure 5.6A; lanes 7 and 8). However, the lack of inhibition by AAZ and signal in the negative controls indicates the presence of non-specific binding. As expected the positive control rhCA II displayed an intense band at the correct size (relative density 26,273; Figure 5.6A; lane 9). To investigate whether something in the RBC lysate inhibited the click reaction, PAL probes were incubated with RBC lysates spiked with 1% rhCA II. RBCs, RBCs + 1% rhCA II and
rhCA II alone were incubated with and without PAL probe 662 and/or AAZ. The samples were then UV cross-linked, reacted with an Alexafluor®594 azide and analysed using in-gel fluorescence, as above. No difference in band intensity was seen for the RBC samples (relative intensity 2,013, 1,882 and 2,248; **Figure 5.6B; lanes 1-3**) and RBC + 1% rhCA II samples (relative intensity 2,995, 3,471 and 3,003; **Figure 5.6B; lanes 4-6**). The expected specific labelling was seen for the rhCA II positive control (**Figure 5.6B; lanes 7-9**). Given that over 95% of RBC protein is haemoglobin and haemoglobin subunits (30 kDa) may be present at the same MW as human CA I and CAII (29kDa), it is possible that haemoglobin inhibits the binding of the PS probes to human CA I and CA II or quenches the signal that may result from any probe bound to CA. Therefore it may be necessary to take steps to reduce the haemoglobin content of the lysates prior to incubation with the PAL probes. Given these observations, and time constraints, future investigations focussed on mammalian cell lysates and on lysates from parasites released from host erythrocytes, with haemoglobin removed.
Figure 5.6: Investigating PAL probe binding in RBC lysates.

(A) PAL probes 662, 663 and 665 (40 µM) were incubated with RBC lysates (10 mg/mL) with and without AAZ (1 mM). Samples were then reacted with an Alexafluor® 594 azide (40 µM) using click chemistry (except in the case of 663), then separated by 1D SDS PAGE and visualised using an FLA5000 (GE Lifesciences) flatbed scanner at 750v. Negative Controls included, non PS probe 666, RBC lysate only and no probe. The positive control was rhCA II (50 µg/mL) with 662. (B) PAL probe 662 (40 µM) was incubated with RBC lysate (10 mg/mL) only; RBC lysate and 1% w/w rhCA II; rhCA II (50 µg/mL) only, with and without AAZ (1 mM). The samples were then analysed by PAL 1D SDS PAGE analysis as per the conditions outlined in (A). All lysate combinations were run without probe for negative controls. In each case, immediately following in-gel fluorescence, the same gel is stained with coomassie to demonstrate loading levels and graphs show relative density of each band normalised to loading control, as determined by densitometry using Image Studio Lite Version 4.0 (Li-COR Biosciences, USA) The ~29kDa band seen in RBC lysates may represent human CA I/II or haemoglobin subunits (2 x 15kDa). No specific bands of other sizes were observed on gel.
5.3.5 Investigating the ability of PAL probes to bind to native CA in mammalian cell lysates

Mammalian cell lysates were utilised to further investigate the efficiency of the PAL probes against complex protein mixtures to ascertain their efficiency in a system lacking haemoglobin. As mentioned previously (Section 5.1) PAL probes have been used previously to identify and characterise protein targets of HDAC, metalloprotease and tubulin inhibitors in cancer cells [167, 293], and therefore in theory our PS probes could potentially be used to detect carbonic anhydrases in mammalian cell lysates. Although the abundance of CAs in mammalian cells is likely to be less than that of RBCs, haemoglobin interference is not a problem with this system. HeLa cell lysates were chosen to demonstrate the potential binding of proteins to the PAL probes and were prepared as per Section 5.2.5. Lysates (3 mg/mL) were incubated with 40 µM PAL probes 662 (ethyl linked), 665 (butyl linked) and 671 (AAZ based) with and without AAZ (1 mM). HeLa cell lysates with no probe served as a negative control and rhCA II, with and without AAZ (1 mM), as a positive control. The samples were then UV cross-linked, reacted with an Alexafluor®594 azide and analysed using in-gel fluorescence, as described previously (Section 5.2.7). The protein profiles observed were similar for all three PAL probes (Figure 5.7A; lanes 1-6) both in the presence or absence of AAZ. Specific binding of PAL probe 662 to rhCA II positive control was observed (Figure 5.7A; lanes 8 and 9) and equivalent loading of all proteins was confirmed via coomassie stained gels (Figure 5.7B). Whilst it is possible that the benzene sulfonamide based probes 662 and 663 may not be inhibited by AAZ due to potentially binding to targets
other than CA, it would be expected that the AAZ based probe would be inhibited by excess AAZ.

Lower concentrations of probe were also investigated by incubating 662 at 5, 10 and 20 µM with HeLa lysates as above and binding analysed as previously mentioned (Section 5.2.7). When compared to the loading in the coomassie stained gel there was no difference observed in the HeLa lysate profiles at all three concentrations (Figure 5.7; C and D). Given that CA proteins are in low abundance in mammalian cells it is possible that the current method is not sensitive enough to discriminate between specific and non-specific binding of our PAL probes to CA proteins in mammalian cell lysates. To investigate this further HeLa cell lysates spikd with 1% rhCA II were investigated. Firstly PAL probe 662 at 40 µM (higher concentrations were trialled but did not result in any difference in signal) was incubated with HeLa alone or HeLa + 1% rhCA II (w/w), with and without AAZ (1 mM). Samples were UV cross-linked, reacted with an Alexafluor® 594 azide and analysed using in-gel fluorescence, as described previously (Section 5.2.7). While there was an apparent decrease in the intensity of the ~29 kDa band corresponding to the MW of human CA I and II in the HeLa only samples treated with AAZ (Figure 5.8A; lanes 1 and 2), this was not observed in the previous experiment (Figure 5.7A lanes 1 and 2) and is likely due to slight loading differences. As seen for HeLa lysates alone (Figure 5.7A), there was a substantial amount of background observed. No difference was seen in the HeLa lysates spiked with 1% rhCA II, with similar intensity seen at ~29 kDa for both the AAZ treated and untreated samples (Figure 5.8A; lanes 5 and 6). This all points towards background problems in
Figure 5.7: Investigating PAL probe binding in HeLa cell lysates.

(A) Lanes 1-6: PAL probes 662, 665 and 671 (40 µM) were incubated with HeLa cell lysates (3 mg/mL) with and without AAZ (1 mM). HeLa cell lysates with no probe served as a negative control (HC; lane 7) and rhCA II 50 µg/mL (+/− AAZ) as a positive control (lanes 8 and 9). Following this the samples were reacted with an Alexafluor®594 azide (40 µM) using click chemistry, separated by 1D SDS PAGE and visualised using an FLA5000 (GE Lifesciences) flatbed scanner at 750v. (C) PAL probe 662 was incubated at (5-20 µM) with HeLa lysates as above and binding analysed per the protocol outlined in (A). In each case, immediately following in-gel fluorescence, the same gel is stained with coomassie to demonstrate loading levels (B) and (D).
Figure 5.8: Investigating PAL probe binding in HeLa lysates spiked with rhCA II.

(A) PAL probe 662 (40 µM) was incubated with HeLa cell lysates (3 mg/mL) or HeLa cell lysates with 1% w/w rhCA II, with and without AAZ (1 mM). Samples were reacted with an Alexafluor® 594 azide (40 µM) using click chemistry, separated by 1D SDS PAGE and visualised using an FLA5000 (GE Lifesciences) flatbed scanner at 750v. Positive control: rhCA II 50 µg/mL (lane 9).

(B) PAL probe 662 (40 µM) was incubated with HeLa cell lysates (3 mg/mL) or HeLa cell lysates with 1% w/w rhCA II, with and without AAZ (1 mM) and analysed by PAL 1D SDS PAGE as per (A). A difference in intensity seen for the 29kDa band with respect to the HeLa spiked with 5% rhCA II samples with a decrease seen in the intensity of the AAZ treated band as denoted by the red arrow (lanes 4 and 5). Negative controls (lanes 3 and 6) HeLa no probe/+ click reaction. NB. rhCA II was run as a positive control at a concentration corresponding to the 5% w/w HeLa/rhCA II sample but the gel was cropped (rhCA II shown in separate panel) to allow for visualisation of the much less intense bands to be analysed at maximum intensity (1000V). In each case, immediately following in-gel fluorescence, the same gel is stained with coomassie to demonstrate loading levels (B) and (D).
cell lysates or inhibition of the click reaction. To try to address this issue the amount of rhCA II was next increased to 5% (w/w) in HeLa lysates and a different probe (AAZ based probe) was used. PAL probe at 40 µM was incubated with HeLa lysates alone or HeLa lysates spiked with 5% rhCA II, in the presence and absence of competitive CA inhibitor AAZ, and in-gel fluorescence assays carried out as above. While no difference in intensity of the ~29 kDa band was seen in the HeLa only samples a difference in intensity was observed for the HeLa samples spiked with 5% rhCA II and treated with AAZ (Figure 5.8C; lanes 4 and 5 red arrow). Therefore it may be that for PAL probes to be able to detect recombinant protein in a complex mixture of proteins, concentrations of the recombinant protein need to be 5% w/w or greater. This concurs with what others have seen when using 1D in-gel fluorescence PAL methods [300, 301]. This also suggests that detection of low abundance proteins in cell lysates may be challenging. Given that most protein targets are in much lower abundance than 5% in cell lysates improvement of the sensitivity of our PAL probes may be needed. Recently the use of blocking probes and 2D gel methods have evolved and have shown improvement in these areas and this is discussed further in Section 5.4.

5.3.6 Investigating P. falciparum protein lysates using PAL probes

Ultimately the goal of this chapter was to probe the P. falciparum proteome for primary sulfonamide targets using PAL. To commence investigations into PAL of P. falciparum proteins we first examined the ability of the PAL probes (ethyl linked) and (one step PAL) to bind to protein targets in P. falciparum cell lysates prepared from parasites.
released from host RBC to remove haemoglobin. PAL probes 662 and 663 were incubated at 40 μM with *P. falciparum* 3D7 lysates (0.5 mg/mL; **Section 5.2.6**), with and without crosslinking and analysed using in-gel fluorescence, as previously described (**Section 5.2.7**). In parallel, rhCA II at 0.25 mg/mL was also used as a positive control. PAL probes 662 and 663 displayed different protein profiles in the presence of crosslinking versus no crosslinking, indicating covalent binding had occurred (**Figure 5.9A**; lanes 3, 4, 7 and 8). However to determine if any of these bands are due to the specific binding it would be necessary to repeat the assay using PS inhibitors. To confirm the presence of PS-specific binding, further experiments were carried out using the inhibitors AAZ (a human carbonic anhydrase inhibitor; *Pf*CA Ki 170 nM; **Table 4.2**), PS-3 (PS glycoside; *Pf*CA Ki 85 nM; **Table 4.2**) and an irrelevant inhibitor, the antimalarial drug pyrimethamine (a DHFR inhibitor). In addition to this the parasite lysate concentrations were increased to 2 mg/mL to try and improve the detection of low abundant proteins. PAL probe 665 (butyl linked) became available at this time and was used alongside PAL probe 662 (ethyl linked). Briefly PAL probes 662 and 665 at 40 μM were incubated with *P. falciparum* parasite lysates (2 mg/mL) with and without inhibitor (1 mM). The samples were then UV cross-linked, reacted with an Alexafluor®594 azide, analysed using in-gel fluorescence, as described previously (**Section 5.2.7**). The non-PS probe 666 was also used as a control to assess specific versus non-specific binding. A similar protein profile was seen for PAL probes 662, 665 and 666 under all conditions (**Figure 5.9C**) with even protein loading observed on the coomassie stained gel (**Figure 5.9D**). These results indicate the presence of non-specific binding as seen previously. Again, there appear to be issues relating to non-
specific binding, with these probes and as discussed in Section 5.4 work is underway to address this problem.

Figure 5.9: Investigating *P. falciparum* protein lysates using PAL probes. (A) PAL probes 662 and 663 (40 µM) were incubated with *P. falciparum* 3D7 lysates 0.5 mg/mL and rhCA II control (250 µg/mL) with and without crosslinking. Samples were then reacted with an Alexafluor®594 azide (40 µM) using click chemistry, separated by 1D SDS PAGE and visualised using an FLA5000 (GE Lifesciences) flatbed scanner at 750v. C = rhCA II; P = *P. falciparum* 3D7 lysates. (C) Lanes 1-8: PAL probes 662 and 665 (40 µM) were incubated with *P. falciparum* 3D7 lysates 2 mg/mL with and without inhibitors (1 mM) and analysed by PAL 1D SDS PAGE as per (A). The non PAL probe 666 was used as a negative control (lane 9). In each case, immediately following in-gel fluorescence, the same gel is stained with coomassie to demonstrate loading levels (B) and (D). PS-3 = PS glycoside 3; AAZ = acetazolamide; PYR = pyrimethamine.
5.4 Discussion

Photoaffinity labelling (PAL) is an approach that may be useful in identifying the biological targets of bioactive compounds from complex mixtures of cellular proteins [167, 169, 302]. In this Chapter, several novel PAL probes designed around the primary sulfonamide (PS) chemical class were investigated for their applicability as chemical tools to detect protein targets using different approaches. First, and in the absence of a recombinant form of *P. falciparum* CA enzyme at the time studies were carried out, the PAL probes were validated using recombinant human CA II (rhCA II), a well characterised target of PS-based compounds. This provided a robust positive control for use in subsequent investigations of probes against the protein lysates of human erythrocytes (RBCs) and the HeLa human cell line.

While the probes were successfully validated as binding to rhCA II using in-gel analysis, the data obtained using RBC and HeLa protein lysates was less successful. RBC lysates were initially investigated because they contain a high abundance of human CA I and CA II protein (~1%) and because human CA I and II are known to be targeted by PS compounds [299]. Unfortunately, the PAL probes were not able to specifically label proteins in RBC lysates, even when spiked with the control protein rhCA II at 1% w/w. This finding was unexpected, and we hypothesise that haemoglobin, making up 95% of RBC protein [303], may inhibit binding to CAs in RBC lysates and/or quench the signal that may result from any probe bound to CA. To the best of our knowledge, despite the plethora of cell-based click chemistry
applications reported, none have been carried out in RBCs during the time frame of this thesis. However a very recent report describes the use of solid phase affinity labelling to detect hCA I and hCA II in RBC lysates [304], although it is difficult to ascertain if haemoglobin depleted lysates were used as no information was given regarding the preparation of the RBC lysates in that study. High intracellular levels of haemoglobin [303] represent a major challenge concerning the characterisation of lower abundant RBC cytosolic proteins [303, 305] and may limit the application of PAL probes for asexual intraerythrocytic stage *Plasmodium* parasites. Therefore, future work should focus on steps to reduce the haemoglobin content of the lysates prior to incubation with the PAL probes.

To try to bypass potential complications of haemoglobin interfering with the PAL reaction, HeLa cell lysates were utilised as a model to validate the PAL probes with complex protein mixtures. This cell line has previously been reported to have been used successfully with PAL methodology in identifying specific protein targets of bioactive small molecules, namely HDACs, metalloproteases and tubulin in cancer cell lysates [167, 302, 306]. When using HeLa lysates no specific labelling of the PAL probes to proteins corresponding to the MW of native CAs (~29KDa) or to any other HeLa proteins was observed. Additionally, when using rhCA II-spiked HeLa lysates the acetazolamide based PAL probe 671 was only effective at detecting the recombinant protein when it was used at concentrations equivalent to 5% of the total protein which is a much higher proportion of protein than would be present endogenously. In this respect
our findings are consistent with what others have observed when using related PAL methodology coupled with 1D in-gel fluorescence. [300, 301].

As the main aim of work in this Chapter was to ultimately use the novel PAL probes to mine *P. falciparum* protein lysates for potential targets of antimalarial PS compounds, a pilot study using *P. falciparum* parasite lysates (i.e. parasites released from RBC and washed extensively to remove haemoglobin) was carried out. Unfortunately, similar outcomes were experienced as for HeLa lysates, with problems of non-specific binding needing to be addressed in future work. It should also be noted that the relative amount of *Pf*CA present in *P. falciparum* parasites, versus *Pf*CA proteins present in the infected RBC cytosol, is still not known and would need to be confirmed in future studies with free parasites.

The use of inactive blocking probes has recently been used with success to reduce background in PAL experiments. For example Sakurai and others [300] used a dual affinity labelling approach to detect rhCA II at 1% w/w in HeLa cell lysates. This was achieved utilising a biologically inactive blocking probe (active PS group replaced with a methoxy group and no reporter tag) in parallel to the active probe to reduce non-specific binding and enhance the active probe selectivity to rhCA II [300]. Work has commenced on synthesising an inactive probe partner for our active PAL probe 671. This probe will be identical in every respect to 671 but will not contain the PS active group or the alkyne handle. In theory this “inactive” probe should block non-specific binding whilst avoiding detection by the azide fluorophore in the click reaction,
allowing discrimination of the active probe binding with lower background. Additionally 2D gel electrophoresis PAL methods will be employed to further differentiate actual binding events from non-specific binding. This method has recently been used successfully to detect rhCA II at 0.1% w/w in HeLa lysates and also to identify tubulin as a target to novel anticancer agents [301, 302]. In this approach probe-labelled and non-probe-labelled proteomes are cross-linked with two different fluorescent dyes, combined, and then separated by two-dimensional gel electrophoresis (2DGE). The desired proteins can then be excised and subjected to mass spectrometry analysis for target protein identification [302]. Solid phase photoaffinity labelling is another strategy emerging recently that appears to overcome issues with non-specific binding [304, 307] and should be considered for future studies. This method uses a solid resin-based support instead of solution based biotin and so allows the use of stringent washing to remove irrelevant proteins without the loss of target proteins [304, 307]. As mentioned above this method was used to detect CAs in red blood cell lysates [304] and in the identification of proteins used in lipid metabolism as possible targets to the clinical antimalarial candidate albitiazolium [307].

Overall work in this Chapter introduces the use of photoaffinity labelling using novel PAL probes as a potential target identification strategy for antimalarial drug discovery. Although preliminary data is showing that there appears to be no specific protein target to the PS PAL probes, this approach is notoriously challenging [9] and further work is under way to address the limitations of non-specific binding encountered with this approach. In the next Chapter another unbiased target identification approach – *in vitro*
selection of resistant *P. falciparum* parasites combined with phenotypic analysis – is utilized as an alternative approach to try to identify novel *Plasmodium* targets of the PS class of compounds.
6 Investigating the mode of action of antimalarial primary sulfonamides using \textit{in vitro} resistance selection
6.1 Introduction

Recent reductions in the cost of whole genome sequencing have begun to yield some exciting progress in the antimalarial target identification arena. When combined with selection of compound resistant \textit{P. falciparum} lines, this approach has resulted in the identification of new antimalarial targets in \textit{P. falciparum} [5, 170, 171]. This approach involves generating parasite lines that are resistant to a compound and then comparing the genomes of the resistant lines to that of their sensitive parental lines (Figure 6.1). Selection typically begins with a \textit{P. falciparum} clone (parent) being subjected to increasing compound pressure (step-wise selection) or a higher initial compound concentration (one-step selection) and then monitoring for parasite survival over time [173]. When healthy parasites are observed growing in the presence of compound (generally 3-10 times IC$_{50}$) the parasite lines can then be tested in growth inhibition assays to assess for a change in phenotype, such as an increased IC$_{50}$ to the selection compound [173]. Once a resistant phenotype is established the lines are then re-cloned and analysed using cross resistance studies, whole genome sequencing [175] or microarrays [172, 174] to identify any genetic variations between the resistant clones and their parental sensitive clones. Candidate targets discovered in this manner can be confirmed using reverse genetic approaches [173].

Several novel and previously known antimalarial targets have been identified using \textit{in vitro} resistance selection combined with whole genome sequencing or microarrays [5, 170, 171, 177, 178]. This was first demonstrated using fosmidomycin-resistant parasites
Figure 6.1: Identification of targets of novel compounds using *in vitro* resistance selection

A *P. falciparum* parasite line is cloned and then grown in the presence of the compound of interest using either a one-step (high initial compound concentration (3 – 10 times IC$_{50}$; until recrudescence occurs) or in a step-wise (gradual increase of compound concentration (1-10 times IC$_{50}$ over time; Section 6.2.4). When healthy parasites are observed growing at 3 - 10 times IC$_{50}$ of the selection compound, the phenotype of the resistant parasites is compared to the wild type control parasites using growth inhibition assays. When an increase in IC$_{50}$ (3 – 10 times) is confirmed for the resistant parasites over the wild type lines, the resistant clone is re-cloned, DNA extracted and compared to the parental clones for genomic variations using whole genome sequencing or microarrays.
whereby the putative drug target deoxyxylulose-5-phosphate reductoisomerase (PfDXR) was identified [172]. Recently the mechanism of action of mupirocin and thiaisoleucine was revealed through mutations in apicoplast and cytoplasmic isoleucyl-tRNA synthetases respectively [171]. Furthermore a P-type ATPase cation transporter (PfATP4) was linked to spiroindolone resistance using this technique [5]. Most importantly in vitro resistance selection has led to a better understanding of artemisinin (a component of our currently used frontline drugs the artemisinin combination therapies) resistance, whereby a kelch protein (K13) was found to be linked to in vitro resistance in an artemisinin selected P. falciparum line that took over five years to establish [102, 103]. This association was also confirmed in clinical measures of artemisinin resistant and sensitive parasites in Cambodia [102, 103]. Furthermore a cell cycle regulator (PfE415w) was linked to artemisinin dormant parasites using the same in vitro artemisinin resistant line [105].

An important consideration when using in vitro selection as a target identification tool is to be aware that P. falciparum often acquires mutations in drug transport as a means of resistance [11, 81, 308-312]. For example, chloroquine (CQ) resistance is related to polymorphisms in the gene encoding the CQ transporter protein (PfCRT) [309] and mefloquine, artemisinin and halofantrine resistance is associated with amplification of a gene that encodes a drug efflux pump (pfmdr) [11, 308]. Thus investigation of these other resistant mechanisms needs to be considered in parallel to other results.
In this Chapter, *P. falciparum* parasites were selected for *in vitro* resistance to PS glycoside PS-3 (Chapter 3; Table 3.2) in order to try to identify the molecular target(s) of this compound. The phenotype of PS-3 resistant clones were assessed by determining sensitivity to various antimalarial compounds, including a panel of PS containing compounds and analogues lacking the PS moiety from Chapter 3. This phenotypic analysis led to the hypothesis that resistance was related to hexose transport, which was tested using glucose rescue assays in our laboratory, and by assessing the activity of PS-3 to a *P. falciparum* hexokinase over-expressing line carried out in the laboratory of Associate Professor Kevin Saliba, Australian National University, Canberra. Lastly genomic DNA was extracted from both the PS-3 resistant and sensitive clones to be sent to Professor Elizabeth Winzeler’s laboratory, University of California, US, for whole genome sequencing to characterise the PS-3 resistant phenotype. These studies are currently being undertaken.

### 6.2 Materials and Methods

#### 6.2.1 Compounds

PS glycosides, GSK PS compounds and celecoxib were sourced as described in Section 3.2.1. Artesunate, pyrimethamine and cytochalsin B were purchased from (Sigma-Aldrich®, USA). DSM161 was supplied by Professor Margaret Philips from UT Southwestern, Dallas, Texas, USA. Stock solutions were prepared in 100% DMSO (Sigma-Aldrich®, USA), stored at -20°C and diluted when required.
6.2.2 Cloning of *P. falciparum* 3D7 parasites by single cell sorting using flow cytometry

*P. falciparum* 3D7 parasites were cultured as per Section 2.1 and synchronised to ring stage (Section 2.2). Synchronous ring stage parasites were cultured in suspension for two cycles (96 h) on a plate shaker (Gyro mini, labnet, USA) under standard culture conditions (Section 2.1) until multiple infections were less than 1%, as determined by microscopy. Parasite cultures were then diluted to $\sim 9 \times 10^6$ infected red blood cells/mL (15% ring stage parasites at 0.1% haematocrit) in filter sterilised culture media and sorted to one cell per well in sterile 96 well clear plastic plates (Corning®, USA). Each well of the 96 well plate contained 200 µL of parasite culture media at 1% hematocrit prior to the addition of single cells. Flow cytometry sorting was performed at 37°C under aseptic conditions on a BD FACSAría™ (BD Biosciences, USA) cell sorter at the QIMR Berghofer Medical Research Institute by Grace Chojnowski. The plates containing sorted cells were then incubated under standard culture conditions (Section 2.1) with media replaced weekly. On day 16 post sorting, geimsa-stained thin blood smears were prepared and examined. Parasite-positive cultures were transferred into 6 well sterile culture plates (Corning®, USA), for culture scale up and subsequently into 100 mm petri dishes (Corning®, USA) for expansion and cryopreservation (Section 2.3).
6.2.3 Cloning of *P. falciparum* parasites by limiting dilution

*P. falciparum* infected RBCs were cultured as per Section 2.1 and synchronised to ring stage (Section 2.2). Synchronous ring stage parasites were cultured for two cycles (96 h) on a plate shaker (Gyro mini, labnet, US) under standard culture conditions (Section 2.1) until multiple infections were less than 1%, as determined by microscopy. Cultures were then diluted to 0.5 and 0.1 parasites per 200 µL of standard culture media at 2% hematocrit and dispensed into sterile 96 well plates (Corning®, USA). The plates were then incubated under standard culture conditions (Section 2.1) and media replaced weekly. On day 16 geimsa stained thin blood smears were taken and examined and parasite-positive cultures were transferred into 6 well sterile culture plates (Corning®, USA) and subsequently into 100 mm petri dishes (Corning®, USA), for expansion and cryopreservation (Section 2.3).

6.2.4 *In vitro* resistance selection of *P. falciparum*

*P. falciparum* 3D7 clones generated in Section 6.3.1 were cultured with and without selection compound at 1 x IC$_{50}$ concentrations. When compound treated parasites were observed to be replicating at a similar rate to the untreated controls, compound pressure was gradually increased in a step-wise manner over several weeks until the parasites were surviving in 10 x IC$_{50}$ concentrations. At this point the selected clones and wild type clones were assessed in growth inhibition assays (Section 2.5).
6.2.5 *In vitro* Growth rate analysis of *P.falciparum*

Highly synchronous (Section 2.2) ring stage *P. falciparum* cultures starting at 0.25% rings and 2.5% hematocrit were cultured under standard conditions (Section 2.1) for 96hr. Geimsa stained blood smears were prepared every 24 h and >1000 red blood cells counted per smear, to determine the number of parasites infecting RBCs at each time point. Multiple infected cells were scored based on the number of infecting parasites. The stage of each parasite was noted (ring, trophozoite and schizont). Three independent assays were assessed per cell line.

6.2.6 Determination of the *pfmdr1* gene copy number by quantitative real-time PCR

The copy number (CN) of the *pfmdr1* gene was estimated by quantitative real-time PCR in an Mx4000 multiplex quantitative PCR system (Stratagene, USA) using a SYBR green-based assay as previously described [308]. The single-copy-number lactate dehydrogenase 1 gene (*pfldh1*) was used as a reference (normalizer) gene for estimating the copy number of *pfmdr1* (the target). Primers MDR1-T1F and MDR1-T1R and primers LDH-T1F and LDHT1R [308] were used to amplify fragments of the *pfmdr1* and *pfldh1* genes, respectively. PCRs were performed in triplicate in 25 µL PCR mixtures containing 1 µL Absolute QPCR SYBR green mix (ABgene), 1 µL of DNA template, and 400 nM each primer for either *pfmdr1* or *pfldh1* amplification. Cycling conditions were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 30 s,
57°C for 1 min, and 72°C for 30 s. Fluorescence data were collected at the end of each annealing and extension step three times and were averaged. Following the amplification cycles, melting curve analysis was performed to confirm that the correct products were synthesized. The text report, containing the threshold cycle (CT) values for every well, was exported into the Excel program (Microsoft Office XP) and analyzed. The results are presented as population mean CNs ± SD from two independent experiments.

6.2.7 Statistical analysis

For comparing in vitro antimalarial activities and growth rates a two-tailed $t$ test was used using GraphPad Prism® data analysis software.

6.3 Results

6.3.1 In vitro generation of P. falciparum line 3D7 clones

Eight P. falciparum 3D7 clones (3D7-C1 to C8) were generated as per the method outlined in Section 6.2.2. Eight parasite-positive wells were identified on day 16 from 60 wells total (13.3%) which correlated well with the expected starting culture of 15% rings that was used for single cell sorting. The parasite negative wells were checked one week later by microscopic analysis of geimsa stained blood smears and no further parasite positive wells were identified. All eight clones were expanded and
cryopreserved as per Section 2.3. Clones 3D7C-3 and 3D7C-4 were maintained in
culture and expanded to collect parasite pellets which were stored at -20°C for later
DNA extraction (Section 2.8). Clones 3D7C-3 and 3D7C-4 were then subjected to in
vitro resistance selection using PS-3 as described in the following Section.

6.3.2 Generation of P. falciparum parasites resistant to PS glycoside 3

In order to begin to determine the mode of action of the PS glycoside compounds
studied in Chapter 3, the PS compound with the most potent and selective antimalarial
activity (PS glycoside 3 (PS-3); Section 3.3.2) was chosen for resistance selection
against two clones of P. falciparum line 3D7 (Section 6.3.1; clones 3D7-C3 and 3D7-
C4). Clones 3D7-C3 and 3D7-C4 were independently cultured with increasing amounts
of PS-3 (Section 6.2.4), beginning at 1 x IC_{50} (~1 µM; Table 3.2), until parasites
survived in the presence of 10 x IC_{50} (10 µM), indicating a potentially resistant or
tolerant phenotype. Following four months culture of 3D7-C3 or 3D7-C4 with PS-3
(termed 3D7-C3^{PS3} and 3D7-C4^{PS3}, respectively) both lines were able to replicate in 10
x IC_{50} PS-3, however in contrast to 3D7-C3^{PS3}, the survival of 3D7-C4^{PS3} under these
conditions was not robust and a resistance phenotype was not confirmed. 3D7-C4^{PS3} is
currently being subjected to further compound pressure and was not further analysed as
part of this project. All work presented below focuses on 3D7-C3^{PS3}.

The inhibitory activity of PS-3 against 3D7-C3^{PS3} versus wild type 3D7-C3 parasites
was assessed in vitro in dose response growth inhibition assays (Section 2.5). The
antimalarial drug chloroquine was included as a positive control. 3D7-C3<sup>PS3</sup> displayed a significant (P<0.05) reduction in sensitivity to PS-3 compared to wild type parasites, with a > 6-fold higher IC<sub>50</sub> (IC<sub>50</sub> 10.41 µM versus 1.58 µM, respectively; Figure 6.2A). Furthermore, the 3D7-C3<sup>PS3</sup> selected line retained its PS-3 resistant phenotype following cryopreservation, thawing and re-culture (IC<sub>50</sub>; 10.8±0.7 µM) and also after the removal of compound pressure for >10 weeks (IC<sub>50</sub>; 11.8±0.7 µM; data not shown). In contrast, no significant difference in IC<sub>50</sub> was obtained for the antimalarial drug chloroquine (IC<sub>50</sub> 0.010 µM and 0.013 µM, respectively; Figure 6.2B) indicating that 3D7-C3<sup>PS3</sup> is not cross-resistant with this clinically used antimalarial drug. Selecting 3D7-C3<sup>PS3</sup> parasites on higher concentrations of PS-3 (~20 x IC<sub>50</sub> (20µM)) did not result in any further decrease in susceptibility to PS-3 (IC<sub>50</sub> 12.0 ± 2.4 µM; growth curves not shown) beyond that obtained at 10 x IC<sub>50</sub>. 

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Figure 6.2: Phenotypic analysis of the *P. falciparum* 3D7-C3<sup>PS3</sup> parasite line. The sensitivity of *P. falciparum* 3D7-C3<sup>PS3</sup> (black lines) versus wild type clone 3D7-C3 (red lines) against compound PS-3 (A) and chloroquine (B) was assessed using <sup>3</sup>H-Hypoxanthine growth inhibition assays. In each case the mean (±SD) % inhibition is shown. A significant difference in IC<sub>50</sub> was observed for 3D7-C3<sup>PS3</sup> for PS-3 (P <0.05) but not for CQ (P >0.05). Results are from three independent assays, each in triplicate wells, and 50% Inhibitory concentrations (IC<sub>50</sub>) determined using non - linear regression analysis in GraphPad prism<sup>®</sup>.

Figure 6.3: *In vitro* growth analysis of *P. falciparum* 3D7-C3<sup>PS3</sup> versus 3D7-C3 wild type parasites.
Parasite growth rate analysis for 3D7-C3<sup>PS3</sup> and 3D7-C3 *P. falciparum* parasites over 96hrs was determined by analysis of giemsa stained blood smears taken every 24hrs and >1000 infected red blood cells counted to determine the mean number of parasites infecting cells (A). In each case differential life cycle stages were examined (B). Results are mean (±SD) from three independent experiments.
6.3.3 Analysis of the in vitro growth rate of *P. falciparum* 3D7-C3<sup>PS3</sup> versus 3D7-C3 wild type parasites

To ascertain whether the differences in PS-3 IC<sub>50</sub> for 3D7-C3<sup>PS3</sup> versus 3D7-C3 wild type parasites was due to differences in parasite growth rates, parasite growth over two intraerythrocytic asexual stage cycles (~96h) was assessed via microscopy (Figure 6.3). Although there was a slight reduction in total parasitaemia at 72hrs and 96hrs for 3D7-C3<sup>PS3</sup> versus 3D7-C3 parasites, this was not statistically significant (Figure 6.3A; P >0.05). Additionally, when different parasite life cycle stages were assessed over the same period, no significant change was observed in the percentage of rings, trophozoites or schizonts at each time point (Figure 6.3B; P >0.05). These data support a mechanism independent of alterations to parasite growth and reinvasion rates as contributing to the resistance phenotype observed in the 3D7-C3<sup>PS3</sup> parasite line.

6.3.4 Analysis of *P. falciparum* 3D7-C3<sup>PS3</sup> in vitro susceptibility to a panel of antimalarial compounds

To rule out possible cross resistance of 3D7-C3<sup>PS3</sup> to other antimalarials, the in vitro growth inhibitory activity (Section 2.5) of a panel of four antimalarial compounds, including chloroquine, was assessed. In each case data for 3D7-C3<sup>PS3</sup> were compared to the 3D7-C3 wild type clone. Antimalarial compounds chosen were based on their apparent differences with respect to their known/putative parasite targets. These included the clinically used antimalarial drugs pyrimethamine (a dihydrofolate
reductase (DHFR) inhibitor [78]), the artemisinin derivative artesunate (thought to exert its effect via the generation of reactive oxygen species [88], disrupting mitochondrial function [313] or by inhibiting a calcium dependent ATPase (PfATP6) [314]), and DSM161 an analogue of DSM265 a dihyroorotate reductase (DHOD) inhibitor currently in phase 1 clinical trials [315]. No significant change in parasite sensitivity was seen for any of the compounds tested. This was reflected in the calculated resistance indices (Ri; 3D7-C3<sup>PS3</sup> IC<sub>50</sub>/3D7-C3 wild type IC<sub>50</sub>) which ranged from 0.8 – 1.6. In contrast, the Ri of PS-3 was 6.6 (Table 6.1). These data suggest there is no evidence cross resistance with the antimalarials compounds tested herein.

**Table 6.1: Activity of a panel of antimalarials against* P. falciparum* 3D7-C3<sup>PS3</sup> and 3D7-C3 wild type lines**

<table>
<thead>
<tr>
<th>Cpd</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Ri</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3D7-C3</td>
<td>3D7-C3&lt;sup&gt;PS3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>0.033 (±0.010)</td>
<td>0.054 (±0.028)</td>
</tr>
<tr>
<td>Artesunate</td>
<td>0.004 (±0.002)</td>
<td>0.004 (±0.001)</td>
</tr>
<tr>
<td>DSM 161</td>
<td>0.345 (±0.065)</td>
<td>0.359 (±0.027)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.014 (±0.003)</td>
<td>0.011 (±0.004)</td>
</tr>
<tr>
<td>PS-3</td>
<td>1.580 (±0.480)</td>
<td>10.410 (±1.380)</td>
</tr>
</tbody>
</table>

*Mean IC<sub>50</sub> (± SD) for three independent experiments, each in triplicate wells; Resistance Index (Ri) - IC<sub>50</sub> resistant line (3D7-C3<sup>PS3</sup>) /IC<sub>50</sub> wild type line (3D7-C3). The higher the Ri the higher the level of resistance.
6.3.5  *Pfmdr1* gene copy number is not amplified in the *P. falciparum* 3D7-C3<sup>PS3</sup> selected line

One of the mechanisms *Plasmodium* parasites have developed to facilitate resistance to antimalarial drugs is via mutation or amplification of genes involved in drug transport [10, 53, 59]. In particular the *P. falciparum* multidrug resistance transporter 1 (*Pfmdr1*) has been associated with resistance in several structurally diverse antimalarial compounds [10, 11]. To rule out *Pfmdr1* amplification as a possible cause of PS-3 resistance to 3D7-C3<sup>PS3</sup>, the copy number of the *Pfmdr1* gene was evaluated using quantitative real-time PCR, as per Section 6.2.6. No increase in the copy number of *Pfmdr1* was observed for the 3D7-C3<sup>PS3</sup> PS-3 selected and 3D7-C3 wild type lines with respect to the control *P. falciparum* line W2 (Figure 6.4). W2 is known to contain one copy of *Pfmdr1*. *P. falciparum* line W2AL80 was also included as a control and is known to have three copies of *Pfmdr1* (Figure 6.4). These data indicate that alterations to *Pfmdr1* copy number are not associated with the resistance phenotype of 3D7-C3<sup>PS3</sup>. This correlates well with the lack of cross resistance observed with respect to artemisunate for 3D7-C3<sup>PS3</sup> versus 3D7-C3 (Table 6.1; Section 6.3.4), as *Pfmdr1* amplification has been associated with artemisinin resistance [308].
Figure 6.4: Amplification of the \textit{Pfmdr1} gene is not associated with the resistant phenotype of 3D7-C3\textsuperscript{PS3}.

The copy number of the \textit{Pfmdr1} gene was assessed in DNA samples extracted from 3D7-C3\textsuperscript{PS3} and 3D7-C3 clones using quantitative real-time PCR in an Mx4000 multiplex quantitative PCR system (Stratagene, USA). A single copy of the \textit{Pfmdr1} gene was detected for both the wild type (3D7-C3; CN 0.9±0.4) and the selected (3D7-C3\textsuperscript{PS3}; CN 1.1±0.05) clones. DNA from control lines W2 and W2AL80, contained one (CN 1.0±0.00) and three (CN 3.2±0.07) copies of the \textit{Pfmdr1} gene, respectively, as previously reported [308]. Results are from 2 independent experiments performed in triplicate. CN: copy number mean ±SD.

6.3.6 Investigating the sensitivity of \textit{P. falciparum} 3D7-C3\textsuperscript{PS3} parasites to a panel of primary sulfonamide containing compounds

PfCA has been validated as a target of PS-3 in enzyme assays (Table 4.2) and it is known that the PS group binds to CAs in many different organisms [299]. Therefore a logical starting point in investigating the possible resistance mechanism for 3D7-C3\textsuperscript{PS3} was to investigate whether other PS containing compounds, including those known to target CAs were also cross resistant with the PS-3-selected 3D7-C3\textsuperscript{PS3} phenotype. Compounds examined included: six of the most potent PS compounds present in the
GlaxoSmithKline (GSK) TCAMs library (GSK - 3, 5, 7, 15, 18 and 22), as discussed in Section 3.3.4, three of which also contain a known antimalarial pharmacophore; and the clinically used human CA inhibitor celecoxib (Table 3.1). None of these compounds exhibited a significantly different IC$_{50}$ against 3D7-C3$^{\text{PS3}}$ versus 3D7-C3 (P >0.05), which is reflected in the calculated Ri’s of 0.8 – 1.6. This result was surprising and prompted us to evaluate the activity of an analogue of PS-3 that is lacking the PS moiety (PS-3'; reported in Chapter 3). Surprisingly, PS-3' was found to have an Ri of 6, similar to that of PS-3 (Table 6.2). These data indicate that the PS group may not be responsible for the resistance phenotype of 3D7-C3$^{\text{PS3}}$ prompting the investigation of other components of the PS-3 structure, as discussed in the following Section.

Table 6.2: Activity of a panel of PS compounds against *P. falciparum* 3D7-C3$^{\text{PS3}}$ versus 3D7-C3 parasites

<table>
<thead>
<tr>
<th>Compd</th>
<th>$^a$IC$_{50}$ (µM)</th>
<th>$^b$Ri</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3D7-C3</td>
<td>3D7-C3$^{\text{PS3}}$</td>
</tr>
<tr>
<td>*GSK-7</td>
<td>0.33 (±0.09)</td>
<td>0.35 (±0.12)</td>
</tr>
<tr>
<td>*GSK-18</td>
<td>1.46 (±0.47)</td>
<td>1.16 (±0.46)</td>
</tr>
<tr>
<td>*GSK-22</td>
<td>0.32 (±0.05)</td>
<td>0.41 (±0.07)</td>
</tr>
<tr>
<td>GSK-3</td>
<td>0.43 (±0.05)</td>
<td>0.43 (±0.06)</td>
</tr>
<tr>
<td>GSK-5</td>
<td>0.49 (±0.04)</td>
<td>0.48 (±0.08)</td>
</tr>
<tr>
<td>GSK-15</td>
<td>0.32 (±0.08)</td>
<td>0.51 (±0.26)</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>6.88 (±2.97)</td>
<td>6.18 (±2.39)</td>
</tr>
<tr>
<td>PS-3</td>
<td>1.58 (±0.48)</td>
<td>10.41 (±1.38)</td>
</tr>
<tr>
<td>PS-3'</td>
<td>6.42 (±1.43)</td>
<td>38.73 (±2.40)</td>
</tr>
</tbody>
</table>

*a Mean IC$_{50}$ (± SD) for three independent experiments, each in triplicate wells; *Resistance Index (Ri) = IC$_{50}$ resistant line (3D7-C3$^{\text{PS3}}$)/IC$_{50}$ wild type line (3D7-C3). The higher the Ri the higher the level of resistance. *compounds contain a known antimalarial pharmacophore (Section 3.3.4).
6.3.7 Investigating the sensitivity of *P. falciparum* 3D7-C3<sup>PS3</sup> parasites to a panel of analogues of PS-3

To begin to dissect which structural group(s) of PS-3 may contribute to the resistance phenotype of 3D7-C3<sup>PS3</sup>, analogues of PS-3 (Section 3.3.2) were tested in growth inhibition assays (Section 2.5) against 3D7-C3<sup>PS3</sup> and 3D7-C3 wild type clones. All compounds contain the PS moiety and a glucose type sugar with an acetyl R group and a triazole substituent (Y=I) but vary with respect to their glycosidic linker (X = S, O, or SO<sub>2</sub>). PS-7 and PS-10 showed a reduction in activity against 3D7-C3<sup>PS3</sup> versus 3D7-C3 with similar Ri values (Table 6.3; Ri 6.1 and 7.2, respectively) to the selection compound PS-3 (Table 6.3; Ri 6.6). This indicates that the glycosidic linker is not likely to be the selection target of PS-3. PS-1, which is identical to PS-3 except for the triazole substituent (Table 6.3; Y = H) showed >2.3 fold reduced activity against 3D7-C3<sup>PS3</sup> versus 3D7-C3 suggesting that the triazole substituent is also not contributing to the resistance phenotype of 3D7-C3<sup>PS3</sup> parasites. PS-4, the free sugar (Table 6.3; R = H) partner of PS-3 also displayed an increase in resistance/tolerance against 3D7-C3<sup>PS3</sup> versus 3D7-C3 (Table 6.3; Ri 10.4) with respect to the selection compound PS-3 (Table 6.3; Ri 6.6). Overall these data indicate that the glucose molecule in the PS-3 selection compound may be the structural group responsible for the antimalarial resistance phenotype of 3D7-C3<sup>PS3</sup> parasites. This hypothesis was tested in the following Section via investigation of PS-3 derivatives with varying sugar groups.
Table 6.3: In vitro antimalarial activity of a panel of PS glycoside glucose analogues against *P. falciparum* 3D7-C3<sup>PS3</sup> and 3D7-C3 parasites

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Structural features</th>
<th>&lt;sup&gt;a&lt;/sup&gt;IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>&lt;sup&gt;b&lt;/sup&gt;Ri</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sugar R Y X</td>
<td>3D7-C3</td>
<td>3D7-C3&lt;sup&gt;PS3&lt;/sup&gt;</td>
</tr>
<tr>
<td>PS-7</td>
<td>Glucose Ac I SO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>9.08 (±2.70)</td>
<td>65.53 (±4.90)</td>
</tr>
<tr>
<td>PS-10</td>
<td>Glucose Ac I O</td>
<td>5.15 (±1.42)</td>
<td>31.56 (±18.53)</td>
</tr>
<tr>
<td>PS-1</td>
<td>Glucose Ac H S</td>
<td>43.19 (±9.04)</td>
<td>&gt;100.00</td>
</tr>
<tr>
<td>PS-3</td>
<td>Glucose Ac I S</td>
<td>1.58 (±0.48)</td>
<td>10.41 (±1.38)</td>
</tr>
<tr>
<td>PS-4</td>
<td>Glucose H I S</td>
<td>2.12 (±0.71)</td>
<td>22.22 (±12.57)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean IC<sub>50</sub> ± SD for three independent experiments each in triplicate wells. <sup>b</sup>Resistance Index (Ri) = IC<sub>50</sub> resistant line (3D7-C3<sup>PS3</sup>) / IC<sub>50</sub> wild type line (3D7-C3). The higher the Ri the higher the level of resistance. Ac – acetate; H – hydrogen; I – iodine; S – sulphur; SO<sub>2</sub> – sulphur dioxide; O – oxygen.

6.3.8 Investigating the sensitivity of *P. falciparum* 3D7-C3<sup>PS3</sup> parasites to a panel of PS-3 derivatives containing various sugar groups

As mentioned previously (Section 3.3.2), the PS glycosides investigated in this project include compounds with a glucose, galactose, glucuronic acid or maltose group (Figure 3.3). The sugar group being included in order to improve compound permeability and solubility (Section 3.3.2). To determine if sugar fragments other than glucose play a
role in the resistance phenotype of 3D7-C3PS3 parasites, analogues of PS-3 that differed only with respect to the sugar group were tested in growth inhibition assays (Section 2.5) against 3D7-C3PS3 and 3D7-C3 wild type clones. PS-11 (galactose) and PS-12 (glucoronic acid) displayed cross-resistance with 3D7-C3PS3 with similar Ri values (Table 6.4; Ri 4.5 and 4.8, respectively) to the selection compound PS-3 (Table 6.4; Ri: 6.6). While no maltose analogue of PS-3 was available, PS-14 is an analogue of the glucose containing PS glycoside PS-7 which was previously shown to have increased resistance to 3D7-C3PS3 versus 3D7-C3 parasites (Table 6.3; Ri 7.2). Interestingly, PS-14 did not show any evidence of increased resistance (Table 6.4; Ri 1.1) to the 3D7-C3PS3. These data suggest that the sugars glucose, galactose and glucoronic acid are all

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Structural features</th>
<th>aIC50 (µM)</th>
<th>bRi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sugar</td>
<td>R</td>
<td>Y</td>
</tr>
<tr>
<td>PS-3</td>
<td>Glucose</td>
<td>Ac</td>
<td>I</td>
</tr>
<tr>
<td>PS-11</td>
<td>Gal</td>
<td>Ac</td>
<td>I</td>
</tr>
<tr>
<td>PS-12</td>
<td>GlcOMe</td>
<td>Ac</td>
<td>I</td>
</tr>
<tr>
<td>PS-7</td>
<td>Glucose</td>
<td>Ac</td>
<td>I</td>
</tr>
<tr>
<td>PS-14</td>
<td>Mal</td>
<td>Ac</td>
<td>I</td>
</tr>
</tbody>
</table>

aMean IC50 ± SD for three independent experiments, each in triplicate wells. bResistance Index (Ri) - IC50 resistant line (3D7-C3PS3) / IC50 wild type line (3D7-C3). The higher the Ri the higher the level of resistance. Gal – galactose; GlcOMe – glucoronic acid; Mal – maltose; Ac – acetate; H – hydrogen; I – iodine; S – sulphur; SO2 - sulphur dioxide; O – oxygen.
affected by the phenotypic change associated with the 3D7-C3\textsuperscript{PS3} phenotype, but maltose is not. Thus, the 3D7-C3\textsuperscript{PS3} phenotype may be associated with certain sugar types but not all.

6.3.9 Investigating the sensitivity of \textit{P. falciparum} 3D7-C3\textsuperscript{PS3} parasites to PS-3 in a low glucose environment

As discussed in the previous Section, our data suggest that the glucose moiety of PS-3 may be contributing to the resistance phenotype of the 3D7-C3\textsuperscript{PS3} parasites. This leads to the hypothesis that glucose transport may be associated with PS-3 resistance. To test this, both the PS-3-selected 3D7-C3\textsuperscript{PS3} clone and the 3D7-C3 wild type clone were examined in \textit{in vitro} growth inhibition assays (Section 2.5) with PS-3 at glucose concentrations of 2.5, 5 and 10 mM. These levels of glucose represent 0.5x, 1x and 2x physiological glucose levels \cite{316}, respectively. The generic transport inhibitor ctyochalasin B was used as a control inhibitor as it has been previously shown to inhibit the \textit{P. falciparum} hexose transporter (PfHT) activity in \textit{Xenopus laevis} oocytes \cite{317}. The antimalarial drug chloroquine was utilised as a negative control.

Under varying glucose concentrations (2.5, 5 and 10 mM), no significant change in the IC\textsubscript{50}’s of 3D7-C3 parasites against PS-3 was observed (Figure 6.5A; IC\textsubscript{50} 1.5 \textmu M, 1.9 \textmu M, 1.3 \textmu M for 2.5, 5 and 10 mM glucose, respectively; P >0.05). As expected, there was also no significant change in IC\textsubscript{50} for the negative control chloroquine (Figure 6.5C; IC\textsubscript{50} 0.014 \textmu M, 0.017 \textmu M, 0.018 \textmu M for 2.5, 5 and 10 mM glucose, respectively;
Figure 6.5: The effect of glucose concentrations on the sensitivity of 3D7-C3<sup>PS3</sup> and 3D7-C3 to PS-3, chloroquine and Cytochalasin B.

3D7-C3 (A, C and E) and 3D7-C3<sup>PS3</sup> (B, D and F) clones were tested in <sup>3</sup>H-Hypoxanthine growth inhibition assays in glucose concentrations of 2.5mM (red), 5mM (black) and 10mM (blue) in the presence of compound PS-3 (A and B), chloroquine (C and D) and cytochalasin B (E and F). In each case at least three independent assays, each in triplicate wells, were carried out and 50% Inhibitory concentrations (IC<sub>50</sub>) determined using non-linear regression analysis in GraphPad prism®.
P >0.05). Likewise, the generic transport inhibitor cytochalasin B did not result in altered activity under the different glucose concentrations (Figure 6.5E; IC\(_{50}\) 1.0 \(\mu\)M, 1.0 \(\mu\)M, 0.7 \(\mu\)M for 2.5, 5 and 10 mM glucose, respectively). Despite the finding that changing the glucose concentration did not significantly alter IC\(_{50}\) in 3D7-C3 parasites, when the activity of PS-3 against 3D7-C3\(^{PS3}\) was assessed, a significant decrease (P <0.05) in PS-3 activity was observed at 2.5 mM glucose compared to 5 mM and 10 mM for 3D7-C3\(^{PS3}\) (Figure 6.5B; IC\(_{50}\) 37.6 \(\mu\)M, 16.1 \(\mu\)M, 13.7 \(\mu\)M for 2.5, 5 and 10 mM glucose, respectively), but not for chloroquine or cytochalasin B (Figure 6.5 D and F). While the IC\(_{50}\) increased 8-10 fold compared to wild type 3D7-C3 at 5 mM or 10 mM glucose (similar to shown above under standard (~10mM) culture conditions; Section 6.3.3) the IC\(_{50}\) was >25 fold higher for 3D7-C3\(^{PS3}\) parasites versus 3D7-C3 parasites at 2.5mM glucose (Figure 6.6; P <0.05). It is likely that this effect is physiologically relevant as no similar significant change in activity was seen for either chloroquine or cytochalasin B (Figure 6.6; P >0.05). Possible reasons for this alteration are discussed in Section 6.4 below.
Figure 6.6: Comparison of glucose concentrations on the sensitivity of 3D7-C3<sub>PS3</sub> and 3D7-C3 to PS-3, chloroquine and Cytochalasin B. 3D7-C3<sub>PS3</sub> (black) and 3D7-C3 (red) parasite lines were tested in <sup>3</sup>H-Hypoxanthine growth inhibition assays in glucose concentrations of 2.5mM, 5mM and 10mM in the presence of compound PS-3 (top), chloroquine (middle) and cytochalasin B (bottom). In each case three independent assays, each in triplicate wells, were carried out and 50% Inhibitory concentrations (IC<sub>50</sub>) determined using non-linear regression analysis in GraphPad prism®. Fold change in IC<sub>50</sub> (3D7-C3<sub>PS3</sub> IC<sub>50</sub>/3D7-C3 IC<sub>50</sub>) for each case denoted in black box.
6.3.10 Generation of 3D7-C3\textsuperscript{PS3} and 3D7-C3 clones for whole genome sequencing

\textit{P. falciparum} 3D7-C3\textsuperscript{PS3} and 3D7-C3 were re-cloned by limiting dilution (Section 6.2.3). For the 3D7-C3\textsuperscript{PS3} cultures seeded at 0.5 parasites per well, 45 out of 96 wells (47\%) were parasite positive at day 16 from an expected 50\% and 55 out of the 96 wells (57\%) were parasite positive at day 16 from an expected 50\% for the 3D7-C3 cultures. Parasite negative wells were re-confirmed one week later by microscopic analysis of geimsa stained blood smears and no further parasite positive wells were identified. Eight clones from each line (3D7-C3\textsuperscript{PS3} A-H and 3D7-C3 A-H) were expanded and cryopreserved as per Section 2.3. Clones 3D7-C3\textsuperscript{PS3} A, E and G and 3D7-C3 A, B and E were examined in \textit{in vitro} growth inhibition assays (Section 2.5) with PS-3 and chloroquine (Figure 6.7). Collectively a 6.2 fold change in sensitivity to PS-3 was observed for the PS-3 selected clones compared to the wild type clones (3D7-C3\textsuperscript{PS3} clones IC\textsubscript{50} 10.7±1.5 \textmu M; 3D7-C3 clones IC\textsubscript{50} 1.7±0.7 \textmu M; P<0.05). No significant difference in sensitivity to chloroquine was seen for either the selected or wild type clones (3D7-C3\textsuperscript{PS3} clones IC\textsubscript{50} 0.013±0.003 \textmu M; 3D7-C3 clones IC\textsubscript{50} 0.012±0.000 \textmu M; P>0.05). Genomic DNA was extracted from clones 3D7-C3\textsuperscript{PS3} A, E and G and 3D7-C3 A, B (Section 2.8) for whole genome sequencing in Professor Elizabeth Winzeler’s laboratory at the University of California, USA. Sequencing is currently underway.
Figure 6.7: Phenotypic analysis of the *P. falciparum* 3D7-C3<sup>PS3</sup> and 3D7-C3 clones.
The sensitivity of *P. falciparum* 3D7-C3<sup>PS3</sup> clones A, E and G (top) and 3D7-C3 clones A, B and E (bottom) against compound PS-3 and chloroquine was assessed using <sup>3</sup>H-Hypoxanthine growth inhibition assays. In each case three independent assays, each in triplicate wells, were carried out and 50% Inhibitory concentrations (IC<sub>50</sub>) determined using non-linear regression analysis in GraphPad prism®.
6.4 Discussion

Using an *in vitro* resistance selection protocol, a PS glycoside-resistant *P. falciparum* clone was established (3D7-C3<sup>PS3</sup>). Compared to the wild type *P. falciparum* line, the resistant clone displayed a 6.6 fold decrease in sensitivity to the selection compound PS-3 and the loss of sensitivity was not attributed to a difference in parasite growth rate. The 3D7-C3<sup>PS3</sup> clone was also shown to retain its resistant phenotype following cryopreservation and after the removal of PS-3 for at least 10 weeks. This indicates that it is likely that a genetic change has occurred rather than just a metabolic change, which would usually result in the reversion of drug tolerance after drug withdrawal. When the antimalarial drugs chloroquine, artemisinin, and pyrimethamine were screened against the 3D7-C3<sup>PS3</sup> clone there was no change in *in vitro* growth inhibition activity (IC<sub>50</sub>) indicating a lack of cross resistance with these drugs. This kind of information is important as drug resistance is a major concern with currently used antimalarials and it is crucial that potential new antimalarial candidates are not susceptible to resistance via the same mechanisms. Likewise, mutations or amplification of genes involved in drug transport are a known mechanism associated with multi drug resistance in malaria parasites [10, 53, 59]. In particular the *P. falciparum* multidrug resistance transporter 1 (Pfmdr1) has been associated with resistance to several structurally different antimalarial compounds [10, 11]. However, in this case we were able to rule out an association between the 3D7-C3<sup>PS3</sup> clone and Pfmdr1, as no increase in copy number was observed in the DNA of the 3D7-C3<sup>PS3</sup> clone.
Given that the PS moiety is known to target CAs in many different organisms [299] and that \(Pf/CA\) has been validated as a target of \textbf{PS-3} in enzyme assays (Chapter 4; Table 4.2) it was hypothesised that the PS group may play a role in the resistance phenotype of the 3D7-C3\(^{PS3}\) clone. It was therefore surprising that when 3D7-C3\(^{PS3}\) parasites were tested in growth inhibition assays against a panel of PS-containing compounds, no change in activity was observed. Interestingly, however, when the non-PS partner (\textbf{PS-3'}) to \textbf{PS-3} was screened against 3D7-C3\(^{PS3}\) parasites, a 6 fold decrease in antimalarial activity was seen. This appears to suggest that the PS moiety of \textbf{PS-3} is not responsible for the 3D7-C3\(^{PS3}\) resistance phenotype. Further screening of the PS glycoside analogues revealed that the glucose component of \textbf{PS-3} may be contributing to the resistance of the 3D7-C3\(^{PS3}\) line but that the maltose analogue \textbf{PS-14} was not resistant to the 3D7-C3\(^{PS3}\) line. These data led to the working hypothesis that a sugar transporter may be linked to the resistance phenotype of the 3D7-C3\(^{PS3}\) line and that this transporter is independent of maltose transport. Unfortunately, there are no reports regarding maltose transport in \textit{Plasmodium} parasites and only very limited reports on maltose usage in general in this species [318, 319]. As discussed in greater detail below, there are several uncharacterised sugar transporters in \textit{P. falciparum} that may, if characterized in future studies, involve maltose transport.

Glucose is a vital energy source for \textit{P. falciparum} parasites [320] and glucose metabolism occurs anaerobically via the parasites glycolytic pathway [321]. The transport of glucose into the parasite is believed to start with movement of extracellular glucose across the host red blood cell plasma membrane via the sugar transporter...
GLUT1. Once inside the parasitised red blood cell, glucose can freely diffuse across the parasitophorous vacuole membrane (PVM) and is then transported into the parasite cytosol via the *P. falciparum* hexose transporter (*PfHT*) [322]. *PfHT* is a single copy gene with no close orthologues and has been shown to be essential for parasite survival and has also been validated as a potential drug target [323, 324]. An 0-3 hexose derivative (compound **3361**) has been shown to specifically inhibit *PfHT* in oocytes and the *P. falciparum in vitro* activity of **3361** decreases with increased glucose media concentrations [325]. Importantly inhibitors based on modifications to the position 1 and 3 carbons (C-1 and C-3) of the glucose molecule have resulted in more effective inhibition of *PfHT* [325]. The C1 position of **PS-3** is joined by a triazole linker to the benzenesulfonamide group. It is possible that this bulky addition to the C-1 glucose of **PS-3** is potentially leading to inhibition of *PfHT*. As a starting point to investigate if **PS-3** can inhibit glucose transport, glucose competition assays were carried out on 3D7-C3**PS3** and wild type parasites. No significant change in the activity of **PS-3** was observed when glucose was titrated against the 3D7-C3 wild type line. No change in activity was seen for what was considered a positive control, cytochalasin B (a known *PfHT* inhibitor). However, given that cytochalasin B is a general membrane inhibitor its lack of specificity may render it inappropriate as a reliable positive control for this application. As mentioned above the only other *PfHT* specific inhibitor known is **3361** however we were not able to obtain this compound for use in these assays. On the other hand this assay may not be sensitive enough to detect changes in compound activity at low micromolar concentrations. This method has only been previously validated using the 0-3 hexose derivative (compound **3361**) which has an IC₅₀ of ~90µM in standard
parasite media (~10mM glucose) whereas the IC\textsubscript{50} of PS-3 in the same glucose concentration is ~1-2 µM. A more sensitive way to see if PS-3 does inhibit glucose uptake would be to use \textsuperscript{14}C-glucose uptake in the presence/absence of PS-3 [326]. Despite these findings, a significant decrease in sensitivity (~25 fold) to PS-3 was seen for the 3D7-C3\textsuperscript{PS3} compared to wild type 3D7-C3 parasites in half the physiological glucose concentration (2.5 mM glucose). One possible explanation for this effect could be that the there is a switch in the parasites energy source to a glutamine-based generation via the parasites TCA cycle [161], and this may result in rescuing the effect of PS-3. This could be investigated by removing glutamine from the culture media to see if the sensitivity to PS-3 could be increased.

Three other putative sugar transporters have been identified in the \textit{P. falciparum} genome (PF3D7_0919500; PF3D7_0529200; PF3D7_0916000) which have < 21% amino acid sequence identity with \textit{PfHT} [110, 327]. Additionally there may be more putative sugar transporters not identified given that 50% of the \textit{P. falciparum} genome is made up of genes with no known function [110]. To further investigate the phenotype of the PS-3-resistant clone, both the 3D7-C3\textsuperscript{PS3} and 3D7-C3 wild type parasites were re-cloned (standard practice after resistance selection as long term culture may result in a mixed population) and genomic DNA extracted and sent to Professor Elizabeth Winzeler’s laboratory (University of California, USA) for whole genome sequencing. Unfortunately the results of this sequencing were not available at the time of submission of this thesis. In parallel, compounds PS-3 and its non-PS partner PS-3' were sent to Associate Professor Kevin Saliba’s laboratory (Australian National University,
Canberra) for testing against a *P. falciparum* hexokinase (*PfHK*) over expressing cell line [328]. *PfHK* is the first enzyme in the glycolytic pathway of *P. falciparum* and is also inhibited by glucose analogues [329], and therefore may be a potential target of PS-3. However, initial studies with the *PfHK* overexpressing line showed no difference in the activity of PS-3 versus PS-3′ (data not shown). There are certainly other avenues to investigate in relation to the involvement of PS-3 in *P. falciparum* hexose transport - for example, a *PfHT* overexpressing line is available [324] and also transport inhibition studies could be carried out using a *PfHT Xenopus laevis* oocyte expression system [317]. Additionally, more sensitive glucose rescue assays could be employed using radioactive forms of glucose [326, 330]. However, as whole genome sequencing of the PS-3-selected line is underway further investigations will be carried out when a putative phenotype is available to guide future studies.

While the main aim of this chapter was to use *in vitro* resistance selection to identify antimalarial targets of the PS chemotype, it appears that the resistance mechanism achieved following selections in this study may be linked to the glycosidic component of PS-3 and not the PS moiety. While this may lead to an interesting new drug target in its own right, to pursue this approach for PS containing compounds specifically it may be necessary to try selection with different, non-glycosidic, PS compounds. Nevertheless information forthcoming from whole genome sequencing in relation to the resistance phenotype of PS-3 may reveal novel antimalarial targets/pathways or resistance mechanisms not yet known.
7 General Discussion

Malaria remains a major global concern with efforts to control this disease being hampered by parasite drug resistance and the lack of an approved broadly effective vaccine [1]. Currently there are several drugs in various stages of preclinical or clinical development, however most of these are reformulations or new combinations of existing antimalarial drugs [109]. For example, rectal artesunate is under development for severe malaria and combination therapies such as sulfadoxine pyrimethamine and amodiaquine (SPAQ) are being rolled out as treatments for seasonal malaria chemoprevention (SMC) for children under the age of five [73]. It is alarming that only one new chemical class (chemotype) of antimalarial, the spiroindolones [5], has progressed to the point of clinical trials in the past decade. Likewise, the exciting progress that has recently been made in testing the RTS,S malaria vaccine in children Phase III trials in several African countries is tempered by its lack of complete protection (<46% efficacy) [331]. This, together with the current research agenda striving for elimination of this disease [109], is driving efforts to discover new therapies for both prevention and treatment of malaria, including new antimalarial chemotypes that have novel modes of action in order to prevent cross resistance with existing drugs.

While it is promising that recent antimalarial screening campaigns using whole cell assays have identified thousands of antimalarial hit compounds [7, 332], generally little is known about the target of these compounds. Focusing efforts on compounds that belong to novel chemotypes may help limit potential downstream issues of cross-
resistance with existing antimalarial chemotypes. Although antimalarial drug leads can be advanced through the drug discovery pipeline without any knowledge of their target, target information can aid in optimization of pharmacodynamic and pharmacokinetic properties and identify any potential off target effects. In this current study a chemotype not presently used clinically for malaria, primary sulfonamides (PS), was investigated for antimalarial potential using whole cell screening combined with target identification strategies utilizing both biased and un-biased approaches.

To try to broadly investigate the PS chemotype for malaria, compounds were selected based on various strategies. This included examining compounds already used clinically for other human diseases in a repurposing or repositioning strategy [133, 206, 254, 333], investigation of experimental compounds designed for cancer use in a “piggyback” strategy [334], investigation of PS compounds arising from a large antimalarial high throughput (HTS) screen of a GSK pharmaceutical library [7], and the investigation of “hybrid” compounds [335, 336] that combine the PS moiety with an existing antimalarial pharmacophore. As discussed below, each of these strategies has advantages and disadvantages.

The first approach was to examine a panel of 16 clinically used sulfonamide compounds, 15 of which contained a PS, in a drug repurposing strategy. Because this approach uses compounds that are already clinically approved drugs or that have been tested in clinical trials, if a suitable compound is identified there is the potential to reduce the time and cost associated with development for a new therapeutic application.
Unfortunately, none of the compounds investigated in this study displayed potent \textit{in vitro} antimalarial activity against \textit{P. falciparum} asexual stage parasites (Table 3.1) with the best compound, celecoxib, having an IC$_{50}$ of only 6 µM and poor selectivity for the parasite versus mammalian cells (Selectivity Index (IC$_{50}$ mammalian cell line/IC$_{50}$ \textit{P. falciparum} parasites) <4 [243]). The lack of inhibitory activity for compounds known to target human cells, cancerous or otherwise, is perhaps not surprising. For example, all the compounds tested are known to bind to human carbonic anhydrase (CA) isozymes [6, 183, 186, 189], and possibly other targets [259]. While \textit{P. falciparum} does contain at least one CA enzyme (PF3D7_1140000 [113]), as discussed in Chapter 1 there is very low amino acid sequence identity between this protein and the human CAs (<10%; Table 1.2) which may limit the use of inhibitors targeted specifically to human enzymes against malaria parasites. Indeed, seven of the clinically used compounds were tested against recombinant PfCA enzyme and no parasite specific selectivity was observed (Table 4.3). The low amino acid sequence identity between the parasite and human enzymes does, however, represents a potential advantage of targeting CA enzymes in malaria parasites, as it may mean that highly parasite-selective inhibitors can be developed that are not toxic to human cells. While none of the clinical PS compounds displayed \textit{P. falciparum} whole cell or PfCA specific selectivity, the secondary sulfonamide SAC, was found to display good PfCA selectivity (SI; hCA I Ki/PfCA Ki = 102; SI; hCA II Ki/PfCA Ki = 33). SAC is a cyclic secondary sulfonamide which is structurally different from the noncyclic antimalarial secondary sulfonamides such as sulfadoxine. Therefore SAC could also be considered a new chemical class with respect to malaria therapy and may not have issues of cross-resistance. Nonetheless, it would be
important to first develop antimalarial SAC analogues with improved potency and to
determine if PfCA is essential for parasite survival before pursuing this approach.

The next set of compounds was selected using a “piggyback” strategy. This strategy
involves utilising compounds that are in clinical use or have been assessed for other
disease targets, as a starting point for antimalarial drug discovery [133, 334, 337, 338].
The compounds selected were previously shown to selectively block CA IX-induced
survival in human fibroblast cells and reduced the activity of other membrane associated
CAs that have been implicated in hypoxic tumours [201, 238, 257]. In vitro growth
inhibition assays showed that of the 14 compounds tested, 6 had some antimalarial
activity (IC\textsubscript{50} 1-3 µM), with several having >40 fold selectivity for \textit{P. falciparum} versus
mammalian cells. In particular one compound, \textbf{PS-3}, displayed the potency and
selectivity required for antimalarial hit status (IC\textsubscript{50} <1 µM; SI >10 [8]), however the
level of potency is outside the preferred range that would be required for an early stage
lead antimalarial compound (IC\textsubscript{50} <0.1 µM; SI >100 [8]). This study did, however,
generate important structure activity relationship (SAR) information, with compounds
containing an iodine substituted triazole group displaying the best antimalarial activity
(Section 3.3.2.1). While the target of the PS glycosides remains to be definitively
elucidated, as a result of work on this thesis project a recombinant form of \textit{P. falciparum}
CA (PfCA) was able to be obtained via collaboration with Dr Clemente
Capasso from Institute of Bioscience and Bioresources (IBBR), Napoli, Italy and Dr
Claudiu Supuran, from the University of Florence, Florence, Italy. In enzyme studies
carried out with this group, the PS glycoside compounds were shown to inhibit the
activity of this protein fragment (9 compounds with Ki <750 nM), however the compounds were also found to generally have poor selectivity for PfCA versus human CA I and II. This suggests that the compounds have the potential for adverse side effects through targeting of human cells in a physiological setting. However, the generation of recombinant PfCA in this study does provide a starting point for future generation of a crystal structure which would enable inhibitor docking studies to be carried out and possibly aid in the development of antimalarial PS inhibitors with improved potency and selectivity. In addition, crystal structure determination will also test the hypothesis proposed by our Italian collaborators that PfCA belongs to a completely new class of CAs - the η class [339]. It should also be noted that only asexual stage P. falciparum parasites were investigated in this thesis project and other Plasmodium life cycle stages should also be tested. For example PfCA has shown greater expression in ookinetes than asexual intraerythrocytic or gametocyte stage parasites [113], which may mean that PfCA plays a more important role in ookinetes, a part of the Plasmodium mosquito stage life cycle. Targeting PfCA in ookinetes may, however, be challenging as vector control need only rely on a general toxic agent to eradicate the mosquito population and block transmission of P. falciparum. At present, it is not known whether PfCA is essential to any of the Plasmodium life cycle stages. With this in mind, its essentiality in asexual stage parasites is being investigated in our laboratory in collaboration with Dr Christopher Goodman at the School of Botany, Melbourne University. A PfCA knockout has been developed using the pCC-1 vector and transfection into P. falciparum will commence shortly.
It is also surprising that there is currently no evidence of a CA gene in human-infecting *Plasmodium* species other than *P. falciparum* [113]. One would expect that if *Pf*CA was essential this gene would be conserved amongst the different Plasmodium species. It could be hypothesised that the parasite may utilise host CA. Although CA has been shown to localise in the parasite cytosol and parasite membrane using electron microscopy [212], there is no evidence as to whether this is host or parasite derived. Along these lines, one of the clinical inhibitors investigated in this thesis, VLX, may prove useful for determining if the parasite is utilising its own CA. VLX was shown to be 240-1000 times more selective for the human CA I and CA II isoforms than for *Pf*CA and therefore could be used to “block” human CAs in erythrocytes. These hCA depleted cells could then be cultured with parasites to see if re-invasion and proliferation occurs in the absence of hCA, or if susceptibility to PS compounds alters.

In the past decade, advances in high throughput, whole-cell based, antimalarial screening platforms has facilitated the screening of millions of compounds to identify hits for antimalarial drug development (e.g. [7, 332]). As a result, information sharing of antimalarial hit data by the large pharmaceutical company GSK allowed us to source additional PS compounds for investigation. From a panel of 25 compounds examined, three compounds in particular displayed promising antimalarial activity and selectivity (*GSK-7, GSK-15* and *GSK-22; Table 3.4; IC$_{50}$ 0.2-0.3 µM and SI >40). However, to progress these compounds to early lead status it would be necessary to determine if they possess appropriate pharmacokinetic properties and *in vivo* efficacy [8]. Additionally, if *Pf*CA is shown to be essential for parasite survival, these compounds could also be
tested against recombinant PfCA to determine their specificity and selectivity for this enzyme.

The final approach used to investigate the antimalarial potential of PS compounds was to generate hybrid compounds. The theory behind hybrid compounds is that by chemically synthesizing a new molecule that combines antimalarial chemotypes with different modes of action it may be possible to generate a drug that has better pharmacokinetic properties, or that has the potential to delay the onset of drug-resistance in a clinical setting [139-141]. In this study a panel of novel 7-chloro-4-aminoquinoline primary sulfonamide hybrids were generated and tested for antimalarial activity compared to the 7-chloro-4-aminoquinoline drug chloroquine alone. Many hybrid compounds have focused on the antimalarial drug chloroquine, comprising a chlorinated 4-aminoquinoline scaffold [137, 144, 145, 149-151, 232-234]. Benefits of utilising chloroquine as a hybrid molecule partner are that it is inexpensive, easily administered, and safe for use in infants and pregnancy [237]. Although P. falciparum, the most lethal human malaria parasite species, has developed resistance to chloroquine in most areas of the world there is evidence of re-emergence of chloroquine sensitive parasites in Africa [235]. Furthermore this drug remains effective for the treatment of other malaria parasite species such as P. vivax [236, 237]. While the activity of these hybrids (Pf3D7 IC₅₀ 0.2 – 18.7 µM) was generally less potent than that of chloroquine (Pf3D7 IC₅₀ 0.02 µM), the most interesting finding was that some of the compounds appeared to lack cross-resistance with chloroquine, as determined using chloroquine sensitive versus resistant P. falciparum lines. In particular H9 displayed an in vitro
antimalarial activity (Pf3D7 IC\textsubscript{50} 0.2 µM; PfDd2 0.3 µM) and selectivity profile (SI >833) that is approaching that of an early lead antimalarial compound (IC\textsubscript{50} <0.1 µM; SI >100; [8]), with no evidence of cross resistance with chloroquine (resistance index (Ri) 1.4). Along these lines, there is published evidence from other SAR studies that show that altering the 4-aminoquinoline side chain can overcome chloroquine resistance [151]. Interestingly, this compound was one of the chemically modified control compounds lacking a PS group. Thus, the addition of the phenyl-1,2,3-triazole to the aminoquinoline scaffold in H9 is an interesting finding for this series of hybrids and will be further investigated in future studies.

In the second part of this project the target of some of the antimalarial PS compounds identified in Chapter 3 was investigated using target-biased and target-unbiased approaches. Based on the history of PS compounds in targeting CA enzymes in humans and other organisms [183-185], the initial hypothesis was that PfCA may be a target of antimalarial PS compounds. As discussed above, although PfCA was found to be inhibited by several of these compounds in \textit{in vitro} enzyme assays, the lack of correlation with the \textit{in vitro} antimalarial activity of these compounds means that other targets must also be considered. For example PS-11 had an ~ 8 fold decrease in PfCA enzyme inhibitory activity (Ki: 708 nM) with respect to compound PS-3 (Ki: 85.2 nM) yet the two compounds have similar antimalarial activities (Pf3D7 IC\textsubscript{50}: 0.9 µM and 1.4 µM). PS-11 differs only in its sugar group with respect to compound PS-3 (PS-3: Glucose; PS-11: Galactose), which may suggest that the sugar component of these compounds is also important for their \textit{in vitro} antimalarial activity. Evidence to further
support this hypothesis resulted from the assessment of a PS-3 resistant line, of which is discussed further below.

Next a proteomic approach was attempted using biolabelling probes based on the PS pharmacophore, to try and identify PS protein targets in protein lysates using photaffinity labelling (PAL). Whilst these probes were able to be validated using a recombinant human CA II protein, they were not successful for target identification in cell lysates due to issues with non-specific labelling. However, recent advancements in this field using blocking probes, 2D PAL and solid phase affinity labelling have been shown to eliminate the background issues associated with conventional PAL \([300, 301, 304, 307]\), providing scope to improve our current PS PAL method. Furthermore the novel PAL probes validated with recombinant human CA II protein in this study may have uses in other fields, such as biolabelling in cancer related CAs (CA IX and CA XII). Work on this approach is currently being undertaken by Ms Kanae Teruya, another PhD student at our institute.

Lastly, a PS resistant line, 3D7-C3^{PS3}, was generated using \textit{in vitro} resistance selection. 3D7-C3^{PS3} parasites appeared to have a phenotype that is independent of the PS component of the compound and led to the hypothesis that the resistance phenotype may be associated with the glycoside part of the compound, possibly related to hexose transport. Whilst this hypothesis was not able to be confirmed in the time frame of this thesis, whole genome sequencing is currently being carried out on clones of the original 3D7-C3^{PS3} clone and any putative targets will then be confirmed using reverse genetics.
and other approaches. Furthermore, the 3D7-C3PS3 clone provides a resistant line for screening next generation compounds of this type to prioritise hits based on their lack of resistance. If in vitro selection is to be used in the future to try and determine the target/s of the PS chemotype it would be necessary to consider compounds without the glycosidic component. For example the hit compound GSK-15 (IC\textsubscript{50} 0.2-0.3 µM and SI >40; Table 3.4) may be a better candidate given that it contains a PS group but no antimalarial pharmacophore. There are also many benzene sulfonamides with variable tail groups [340-346] which may be utilised in a HTS antimalarial screening approach to provide alternative candidates to take forward for in vitro selection studies.

Overall the main aim of this thesis was to investigate the PS pharmacophore as a novel antimalarial chemotype. In general, the majority of the PS compounds investigated in this study displayed poor antimalarial activity. At this stage only three GSK PS compounds, (GSK-7, GSK-15 and GSK-22; Table 3.4; IC\textsubscript{50} 0.2-0.3 µM and SI >40) and the hit hybrid H-9 compound (Table 3.5; IC\textsubscript{50} 0.2-0.3 µM and SI >833) possess some of the criteria needed for early stage lead antimalarials (Appendix 2). However, a possible limitation when developing any PS compound for therapy is the potential for off target CA effects, therefore, it would be necessary to rule out any potential human CA targeting of the GSK PS compounds before pursuing these compounds further. On the other hand, the hit hybrid compound H-9 (a non-PS compound) should avoid any CA targeting be it human or parasite and therefore warrants further investigation given its potential to overcome chloroquine resistance. Ultimately to decide if PS compounds are worth pursuing in the future it will be necessary to determine if this chemotype has
an essential therefore druggable target in the malaria parasite. As mentioned above the essentiality of PfCA, a putative target of PS compounds is currently being investigated in the asexual stage of P. falciparum. If PfCA is found to be essential for parasite survival the recombinant PfCA generated in this study will provide a useful tool for screening other potential PfCA inhibitors and for the generation of a crystal structure to aid in the design of novel PfCA inhibitors with improved potency and selectivity for malaria parasites.

Surprisingly, the most interesting finding to come out of this study was that it appears that the PS group is not linked to the in vitro antimalarial activity of the PS glycoside hit compound PS-3. The theory proposed was that the sugar component of PS-3 may be responsible for the resistance phenotype of the PS-3 resistant line and that glucose transport may be involved. However, initial experiments using glucose rescue assays were not able to confirm this. One explanation may be that the parasites TCA cycle may provide an alternative route for energy production [161] thus rescuing the effect of PS-3 on the parasites glycolytic pathway. If proven this must be considered when developing these types of compounds as it may be necessary to combine them with a parasite TCA inhibitor in a combination therapy to block this survival mechanism. At this stage there is little known about P. falciparum TCA inhibitors [161] and it would require further investigations to identify appropriate candidates. As mentioned previously the PS group may prove to be a liability due to its potential for targeting human CAs and therefore future efforts may be better spent on developing non PS glycosides for malaria therapy. Speculation aside, optimistically the whole genome sequencing of the PS resistant line
3D7-C3\textsuperscript{ps3} may lead to the discovery of novel antimalarial targets to the PS glycoside compounds which may be exploited in future studies or may reveal different parasite resistance mechanisms which will need to be considered when developing new antimalarials with a glycosidic moiety.
Appendices

Appendix 1 General materials

Parasite culture media

- RPMI-Hepes 1640 incomplete media 500 ml (Gibco, USA)
- Normal Human Serum (heat inactivated) 50 ml
- Gentamycin (10 mg/ml) 250 μl (Sigma-Aldrich, USA)

Aseptically combine all the components in RPMI media. Store at 4 °C.

Freezing solutions

- 3% Sorbitol (Sigma-Aldrich, USA)
- 0.65% NaCl (Ajax Chemicals, Australia)
- 28% Glycerol (Chem-Supply, Australia)

Combine all the components in ddH2O. Filter sterilize. Store at 4 °C.

Phosphate buffer saline

- 145 mM NaCl (Ajax Chemicals, Australia)
- 10 mM Na2HPO4
- 3 mM KH2PO4

Combine all the components in ddH2O. Adjust the pH. Store at RT.
**SDS Loading dye (6 x)**

- Beta-mercaptoethanol 1800 µl (Merck, Germany)
- Sodium dodecyl sulphate 1.8 g (Sigma-Aldrich, USA)
- Bromophenol Blue 0.18 g (Carl Roth, Germany)
- 20% Glycerol (Chem-Supply, Australia)

Combine all the components and bring the volume up to 10 ml with ddH2O. Store at -20 °C.

**10-12% SDS polyacrylamide separating gel**

- 1 M Tris-HCl pH 8.8 1.25 ml
- 0.05% SDS (Sigma-Aldrich, USA)
- 5% and 6% Acrylamide for 10% and 12% gels respectively
- 0.025% APS (Sigma-Aldrich, USA)
- TEMED 5 µl (Bio-Rad, USA)

Combine all the components and bring the volume up to 10 ml with ddH2O. Use fresh.

**SDS polyacrylamide stacking gel**

- 1M Tris-HCl pH 6.8 1.25 ml (AppliChem, Germany)
- 0.05% SDS (Sigma-Aldrich, USA)
- 1.95% Acrylamide 650 µl (Bio-Rad, USA)
- 0.5 M Tris-HCl pH 6.8 1.26 ml (AppliChem, Germany)
- 10% APS 25 µl (Sigma-Aldrich, USA)
- TEMED 5µl 170 (Bio-Rad, USA)
Combine all the components and bring the volume up to 10 ml with ddH2O. Use fresh.

**SDS Running Buffer (10 x)**

- 0.1% SDS (Sigma-Aldrich, USA)
- Glycine 14.4 g (Chem-Supply, Australia)
- Tris-base 3.03 g (AppliChem, Germany)
- Methanol 200 ml (Chem-Supply, Australia)

Combine all the components and bring the volume up to 1 l with ddH2O. Store at RT.

**Coomassie Blue Stain**

- 10% Acetic Acid (Chem-Supply, Australia)
- 0.25% Coomassie Brilliant Blue R250 (Bio-Rad, USA)
- 50% Methanol (Chem-Supply, Australia)

Combine all the components. Filter with Whatman number 1 filter paper. Store at RT.

**SDS-PAGE destaining solution**

- 80% H2O
- 10% Acetic acid (Chem-Supply, Australia)
- 10% Methanol (Chem-Supply, Australia)

Combine all the components. Store at RT.
1x Transfer Buffer

- Tris 3.03 g (AppliChem, Germany)
- Glycine 14.4 g (Chem-Supply, Australia)
- Methanol 200 ml (Chem-Supply, Australia)

Combine all the components bring the volume up to 1 l with ddH2O. Store at RT.

High salt lysis buffer

- 20 mM HEPES (Sigma-Aldrich, USA)
- 350 mM NaCl (Sigma-Aldrich, USA)
- 1 mM MgCl₂ (Sigma-Aldrich, USA)
- 0.5 mM EDTA (Sigma-Aldrich, USA)
- 20% glycerol (Chem-Supply, Australia)
- 0.5% Triton (Sigma-Aldrich, USA)
- 1X protease inhibitor (Roche, Germany)

Combine all the components with ddH2O and adjust pH to 7.9 at 4°C. Store at 4°C.
Appendix 2 MMV ‘Hit’ and ‘Early lead’ criteria

<table>
<thead>
<tr>
<th>MMV ‘hit’ criteria</th>
<th>MMV ‘early lead’ criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀&lt;1 µM for <em>P. falciparum</em> drug resistant &amp; sensitive lines; no cross-resistance apparent</td>
<td>IC₅₀&lt;0.1 µM for <em>P. falciparum</em> drug resistant &amp; sensitive lines; no cross-resistance apparent</td>
</tr>
<tr>
<td>IC₅₀ normal mammalian cell line /IC₅₀ <em>P. falciparum</em> &gt;10-fold</td>
<td>IC₅₀ normal mammalian cell line /IC₅₀ <em>P. falciparum</em> &gt;100-fold</td>
</tr>
<tr>
<td>Clear IP space/novel pharmacophore</td>
<td>Compound stable in mouse &amp; human plasma. Microsome CaCo-2 stability &gt;2x10⁻⁶ cm⁻¹/s</td>
</tr>
<tr>
<td>&quot;Druggable&quot; properties and some structure activity relationship (SAR) apparent</td>
<td>No known toxic reactive groups; no toxicity in mice</td>
</tr>
<tr>
<td>Compound stable as solid/in test media with good solubility in PBS</td>
<td>Oral efficacy (or protection) in mouse model of malaria</td>
</tr>
</tbody>
</table>
Appendix 3 Lipinski’s Rule of Five

Lipinski’s rule [347] states that, in general, an orally active drug has no more than one violation of the following criteria:

- No more than 5 hydrogen bond donors (the total number of nitrogen–hydrogen and oxygen–hydrogen bonds)
- Not more than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms)
- A molecular mass less than 500 daltons
- An octanol-water partition coefficient log P not greater than 5
Appendix 4  Codon optimised PfCA DNA sequence

**BamH1**

ATGGATCC

**Nhe1**

GCTAGC
Appendix 5 *Pf*CA protein sequence

*Pf*CA gene (gene ID: *PF3D7_1140000*) retrieved from PlasmoDB [113]

MKLLYYLLYPILLFYVNFINYKKSRMLEDIDKYNTHFVQTTKPYEYEFVNTLNTNSKK
KKKKKKRENHILGSGENMQKKEKNIKDFHINDYEIDGTKTQHNIKNSKDDSFKMKNKKNKNDNE
ELFYMDNILYSKPKKKKLEHTSFSENEGSEKEETLYNKKNMKNSVNNINIKKFLYNK
LKNVDYEHGNYNDIGGCTGKYQSPVDPKLKLKEREKLKISDVFYLNLFDDDDNYAWNHY
NKPWMKGGFYYEYFIEKIVINRQNNIFQIKRAARDGIIIPFGVLFTEQPAMFYADQIH
HAPSEHTQGSGNREIREMROIHESTNYFYDIQDDKSYKKKYLHGLHNLKKNKSESTK
SSRYHSLMSFLMNSLEQLNQNYKICKRKKYQKYEVISITFSAEINATSKF
KLPSEKFIRTIINVSSAVHVGSDFPLVELKDALNLDAKHLMNEMQFLSYESGSLPL
CDENVSXKVARQPLPVSTETILNFYYLLKHKKHPYSGSNDNYRSQNNVEDNTRHRKFS
LVQVFIQQLSSAISNIEDKKVINIKISPSKMSFTYYSKWDYPIFLFIFYN1VLEF

**Bold underline** = predicted active site domain retrieved from PlasmoDB [113]

Yellow = transmembrane domain retrieved from PlasmoDB [113]
Appendix 6 N-terminal His-tag construct vector sequence

[Supplied in a commercial pEt43.1a vector (Novagen, USA) by Dr Tom Peat, CSIRO Protein Expression, Modelling and Structure (PEMS) program, Melbourne].

\[
\begin{align*}
RsrII & \times NdeI \mid \text{Gly} \mid \text{His tag} \mid KpnI \mid \text{TEV cleavage} \mid \text{BamH1} \\
\text{cggwccg x catatg gga catcaccaccaccac} & \text{catcac ggtacc gaaaacctgtattttcag gqatcc} \\
\text{MET GLY HISHISHISHISHIS GLYTHR GLUASNLEUTYPHEGLN GLYSER} \\
\text{protein sequence} & \mid NheI \mid \text{stop stop} \mid XhoI \\
\text{ta cgatatccca} & \mid gctagc \mid \text{taataa gtaag ctcgag} \\
\text{PROTEIN SEQUENCE ALASER END}
\end{align*}
\]
Appendix 7 PfCA$^{174-538}$ purified protein

Coomassie stained 12% SDS PAGE gel showing $PfCA^{174-538}$ post purification using NiNTA agarose (Section 4.2.4) and refolding using step wise dialysis (Section 4.2.6). A 1Kb PageRuler™ (Thermoscientific, USA) prestained protein ladder is loaded to indicate approximate molecular weights (Std).
References

84. Kuhn, S., M.J. Gill, and K.C. Kain, Emergence of atovaquone-proguanil resistance during treatment of Plasmodium falciparum malaria acquired by a


