Advances in Malaria Pharmacology and the online Guide to MALARIA PHARMACOLOGY: IUPHAR Review X

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Abstract

Antimalarial drug discovery has until recently been driven by high-throughput phenotypic cellular screening, allowing millions of compounds to be assayed and delivering clinical drug candidates. In this review, we will focus on target-based approaches, describing recent advances in our understanding of druggable targets in the malaria parasite. Targeting multiple stages of the *Plasmodium* lifecycle, rather than just the clinically symptomatic asexual blood stage, has become a requirement for new antimalarial medicines, and we link pharmacological data clearly to the parasite stages to which it applies. Finally, we highlight the IUPHAR/MMV Guide to MALARIA PHARMACOLOGY, a web resource developed for the malaria research community that provides open and optimized access to published data on malaria pharmacology.

1. Introduction

Malaria is a major global health challenge, with a disproportionate impact on pregnant women and children under the age of five. In 2020, there were an estimated 241 million cases, leading to 627,000 deaths (World malaria report, World Health Organization, 2021). Malaria is caused by *Plasmodium* protozoan parasites, which have a complex lifecycle involving several distinct stages in both their human host and their *Anopheles* mosquito vector (Figure 1). Of the five *Plasmodium* species that infect humans, *P. falciparum* is responsible for the most deaths, especially on the African continent, while *P. vivax* is geographically widespread and endemic in both Southeast Asia and Latin America. Efforts to eliminate malaria will require the development of new antimalarial medicines that target multiple lifecycle stages (Poonam et al., 2018), with the aim of relieving clinical symptoms, preventing transmission and achieving radical cure (including the elimination of the dormant liver form of *P. vivax* that can reactivate, weeks, months or even years after the primary infection).

Effective innovation requires effective communication. Where primary literature is extensive, expert-curated, easy-to-use, open-access data portals improve the efficiency of finding information. For this reason, the Nomenclature Committee of the International Union of Basic and Clinical Pharmacology (NC-IUPHAR) has partnered with the Medicines for Malaria Venture (MMV) to develop the IUPHAR/MMV Guide to MALARIA PHARMACOLOGY (GtoMPdb: Armstrong et al., 2020). This is an extension of the IUPHAR/British Pharmacological Society (BPS) Guide to PHARMACOLOGY (GtoPdb), an open-access, expert-curated database of molecular interactions between ligands and their targets (Harding et al., 2021). Quality control and peer review of GtoPdb, containing fully-searchable, quantitative interaction data on nearly 1,600 human protein targets and 8,000 ligands is maintained through NC-IUPHAR's 109 subcommittees. The new GtoMPdb resource benefits from similar oversight by a specific committee, drawn from IUPHAR, MMV and the Malaria Drug Accelerator (MalDA; Yang et al., 2021), and provides a purpose-built portal for access to malaria-relevant pharmacological content,

including molecular targets, interaction data for antimalarials, and curation of data from whole-cell screening.

2. A Brief History of Antimalarial Drug Discovery

2.1 The herbal medicine era

Malaria has been treated with therapeutic substances since ancient times: two of which led to families of modern synthetic drugs. One is the leaves of *Artemisia annua,* used in Chinese medicine for 1500 years; the other is the bark of *Cinchona*, chewed by Peruvians in pre-Columbian times, but which was probably introduced as a cure for malaria in the Americas by Europeans (Meshnick & Dobson, 2001). It was found to treat malaria by around 1630 and was later produced on a mass scale: by the 1930s, the Dutch *Cinchona* plantations in Java, produced 10 Gg of bark per year (Meshnick & Dobson, 2001).

2.2 The synthetic era

Pelletier and Caventou isolated quinine as the active ingredient of *Cinchona* in 1820 and, in an early example of open science, declined to patent it. Within three years, purified quinine was being used to treat malaria at scale (Taylor, 1943) and was the first example of therapeutic use of any pure organic substance. Demand for quinine stimulated attempts to make synthetic antimalarials. The first was methylene blue (1891), though it carried problems of serious off-target effects now known to include inhibition of monoamine oxidase (Gillman, 2011): inhibition of this enzyme causes high blood pressure in conjunction with dietary tyramine. It was followed by pamaquine (1924) and primaquine (1946), the latter with a much lower risk of causing methaemoglobinaemia. Parallel developments resulted in mepacrine (1932), which is still used against Giardia although it causes yellowness of skin and eyes (distinct from jaundice), and resochin, which was withdrawn due to toxicity after early clinical trials in Germany, but subsequently licenced in the USA as chloroquine. Despite a low therapeutic index (2x the therapeutic dose is toxic: Taylor and White, 2004), because chloroquine is cheap and easily stored, it became the drug of choice in the WHO's

global eradication programme (Schlitzer, 2007). The development of resistant strains, first discovered at the Thai-Cambodian border in the late 1950s (Farooq & Mahajan, 2004), has since limited its utility, particularly as monotherapy for the treatment of *P. falciparum*. It is still in use in certain countries to treat *P. vivax* and *P. ovale* malaria.

Synthetic quinine was first produced by William Doering and Robert Woodward in 1944. In the 1960s, the derivative mefloquine was introduced and used widely since the 1980s to chloroquine-resistant parasites and as a prophylactic for travellers despite mental health side-effects experienced by some (Croft, 2007; Tickell-Painter et al., 2017). Resistance is now a problem in many areas. The broadly similar molecule halofantrine emerged from the same programme (Croft, 2007), and lumefantrine from a military programme in China. The latter is currently used as a partner drug in combination therapy alongside artemether, an artemisinin derivative (see below).

Around the same time that the first synthetic antimalarials were being produced, broad spectrum antimicrobials that target folate metabolism, and therefore cell proliferation, were tried (reviewed by Anderson, 2005; Nzila, 2006). Initial antimalarial efficacy of compounds such as pyrimethamine (targeting *Plasmodium* dihydrofolate reductase-thymidylate synthase) and proguanil (the prodrug for cycloguanil), both introduced in the 1940s, were soon lost due to resistance (Yuvaniyama et al., 2003). Combinations of drugs that target different elements of the folate pathway, e.g. Fansidar® (pyrimethamine with sulfadoxine, targeting *Plasmodium* hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase), continue to be of great use in prophylaxis (Schlitzer, 2007). Antimicrobials such as rifampicin, tetracyclines and macrolides also show some antiparasitic activity, and have become relatively common in combination therapies (Schlitzer, 2007).

In 1972, the active ingredient from *Artemisia annua*, the sesquiterpene lactone, artemisinin, was isolated (Wang et al., 2019). Its later biosynthesis by yeast was one of the first true successes of synthetic biology, which in this case consisted of transferring genes of plant-derived enzymes into yeast to build a new metabolic pathway (Ro et al., 2006). Semisynthetic derivatives of artemisinin have been made, to address solubility

and other shortcomings of the natural molecule: they include dihydroartemisinin, artemether and arteether (reviewed by Woodrow et al., 2005). Artesunate is a prodrug that can be administered intravenously, allowing use in severe malaria patients too sick to eat or drink. This compound has proven to be highly effective in malaria control and performs well compared to quinine (see Maka et al., 2015 for an example trial). However, artemisinin partial resistance has now arisen (van der Pluijm et al., 2021) and is the subject of close monitoring worldwide.

2.3 Resistance and the rise of combination therapies

An important development in antimalarial therapy is the transition from using one drug to the use of several given together. This is driven mainly by the rise of resistance. If a given *Plasmodium* cell has a probability 1/n of gaining a resistance mutation to one agent, and 1/m to a second agent against an unrelated target, then the overall probability of resistance developing will be 1/(n x m) (White, 1999). The argument can be extended to any number of agents, but the problem of side-effects needs careful attention when combinations are developed (van der Pluijm et al., 2021). Once a parasite has acquired resistance to one of a pair of drugs used in combination, the other effectively becomes a monotherapy and resistance can be acquired quickly. The combination therapies of artesunate-mefloquine, dihydroartemisinin-piperaquine and artesunate-amodiaquine have all failed in the Thailand-Cambodia-Vietnam area because of parasites acquiring artemisinin partial resistance and subsequently acquiring resistance to the other agent. At least some of this resistance did not involve known mutations (Mairet-Khedim et al., 2020), suggesting that they are evolving *de novo* rather than travelling from other areas.

A critical element of the parasite's ability to escape a drug or vaccine is its overall population, both within a patient and within a whole population (White, 1999). Attempts to eradicate malaria by chemotherapy alone, even with the triple-combinations now being proposed and evaluated (van der Pluijm et al., 2021), are unlikely to work unless the populations of parasites are reduced and isolated by environmental controls. These measures may be joined by novel vector control strategies such as genetic engineering

of mosquitoes using 'gene drive' to replace wild-type populations with those engineered to be resistant to the parasite (e.g. Hoermann et al., 2021).

2.4 Vaccines

Although vaccines are not within the scope of this review, brief mention is warranted here. The greatest obstacles to malaria vaccine development are: absence of reliable and clearly-defined/reproducible correlates of protection, diverse and polymorphic parasite antigens and the induction of long-term immunity is challenging (Tuju et al., 2017). Nevertheless Mosquirix (synonym: RTS,S/AS01), developed in 1987, is now available to the community as a new weapon to fight against malaria. Its improved derivative R21, which is still early in its development, has shown promise in trials (Laurens, 2020) with an observed efficacy of 77% in African children in a 12-month trial.

3. Progress Towards the Discovery of New Antimalarial Therapies and Advances in Target-based Drug Development

Antimalarial compounds are identified and developed in two broad strategies. The 'classical' approach involves screening compound libraries and uses parasite survival as the main assay. It requires no specific knowledge of potential molecular targets and can result in effective drugs whose mechanism of action remains unknown, such as the clinical candidates ganaplacide (previously KAF156) and INE963 (Kuhen et al., 2014; Taft et al., 2022). Though the molecular targets of such drugs may be unknown, the target lifecycle-stages usually are known, for example ganaplacide is active against liver-stage, blood-stage and gametocyte stages (the latter important in controlling onward transmission from patient to mosquito) (Kuhen et al., 2014). Alternatively, the 'target-based' approach begins with a discrete target and identifies compounds that interact specifically with the selected target to modulate its function. Both can be effective, but the target-based approach is best placed to benefit from gains in knowledge about the parasite and its molecular constituents. This section concentrates on the target-based approach, describing key targets that are known to be essential for parasite development and that have been the focus of recent drug discovery efforts.

Table 1 provides a summary of these targets with examples of key inhibitors and the lifecycle stages in which notable anti-*Plasmodium* activity has been reported and attributed to the target in question. This organization by lifecycle stages does not necessarily imply that an inhibitor has no activity in other stages. Table 2, presents key information for each of these targets including validation, resistance potential and druggability. For readers interested in *Plasmodium* targets that have been studied in the past, but are now considered a lower priority, the GtoMPdb provides a source of further information (see section 4 for details of this resource). The focus of this manuscript is to describe the most recent targets identified.

3.1 Asexual Blood Stage (ABS) Targets

The targets described below have been classified as 'ABS only' based on the activity of compounds hitting these targets and being not active either in the liver stage or against gametocytes. This classification may change as new compounds, with better properties, emerge in the future. This is particularly true since some of these targets seem essential across the various lifecycle stages, based on their known biological functions.

3.1.1 *P. falciparum* hexose transporter 1 (PfHT1; PF3D7_0204700)

Plasmodium parasites, like all cells, require selective transport channels in their membranes to maintain homeostasis. Encoded by genes distinct from human orthologues, these are potential targets for pharmacological inhibition. Malaria parasites rely on glucose as their sole energy source during the blood stage, so inhibition of glucose uptake via PfHT1, the only hexose transporter in the *Plasmodium* genome (Huang et al., 2021; Qureshi et al., 2020) has shown that this target is essential to parasite survival. Structure-guided drug discovery (Jiang et al., 2020) resulted in small molecules that bind the orthosteric and allosteric pockets of PfHT1 simultaneously and selectively over human orthologues, and kill multiple strains of the blood-stage *P. falciparum in vitro* as rapidly as dihydroartemisinin. However, the current PfHT1 inhibitors are unsuitable for oral administration due to their poor permeability and

metabolic stability *in vivo* and require further development. New library screening campaigns and chemical starting points are therefore needed in the future.

3.1.2 *P. falciparum* bifunctional farnesyl/geranylgeranyl diphosphate synthase (PfFPPS/GGPPS; PF3D7_1128400)

Isoprenoids are a large group of natural products composed of two or more hydrocarbons. In *P. falciparum*, the products of isoprenoid synthesis are metabolized by farnesyl diphosphate synthase (FPPS), an enzyme that also catalyses the synthesis of geranylgeranyl diphosphate (GGPP), making it a bifunctional FPPS/GGPPS enzyme (Jordão et al., 2013). PfFPPS/GGPPS synthesizes C15 and C20 prenyl chains in the isoprenoid biosynthetic pathway (Jordão et al., 2013), and these are cyclized and/or conjugated to small molecules and protein scaffolds by various prenyl-transferases to biosynthesize final isoprenoid products. Because of its central role in the isoprenoid biosynthesis in *Plasmodium,* FPPS/GGPPS represents a key target for antimalarial development.

Nitrogen-containing bisphosphonate drugs can inhibit PfFPPS/GGPPS (No et al., 2012; Singh et al., 2010), but lack selectivity and bioavailability (Sinigaglia et al., 2007). Using a quantitative high-throughput screen (qHTS) of the Malaria box, Gisselberg et al. identified MMV019313 a selective, non-bisphosphonate inhibitor of PfFPPS/GGPPS (Gisselberg et al., 2018). Systematic SAR study of MMV019313 generated a novel class of thiazole-containing amides with higher potency against PfFPPS/GGPPS and pharmacokinetic studies identified AIM-1290 as having the best *in vitro* ADME profile and lipophilic efficiency (Kabeche et al., 2021). Further studies will be needed for the evaluation of this series as antimalarial drug candidates.

3.1.3 *P. falciparum* cleavage and polyadenylation specificity factor subunit 3 (PfCPSF3; PF3D7_1438500)

In most eukaryotes, pre-mRNA 3'-end processing involves endonucleolytic cleavage of precursor RNA at a cleavage site usually ~15 nucleotides downstream of the AAUAAA signal, followed by addition of a poly(A) tail to the upstream (5') cleavage product. This

is mediated by the cleavage and polyadenylation specificity factor (CPSF), consisting of four subunits: CPSF160 (also known as CPSF1, 160 kDa), CPSF100 (CPSF2, 100 kDa), CPSF73 (CPSF3, 73 kDa) and CPSF30 (CPSF4, 30 kDa) (Bienroth et al., 1991). CPSF3 has been identified as a potential drug target in trypanosomes (Wall et al., 2018) as well as in *Toxoplasma* (Palencia et al., 2017; Swale et al., 2019) and *Cryptosporidium* (Swale et al., 2019) where forward- and reverse-genetics, transcriptome sequencing and inhibition of the metal-dependent mRNase activity have identified this protein as a common target of benzoxaborole compounds.

PfCPSF3 is a 101kDa close homologue of human CPSF73, with 61% similarity and 39% identity (Sonoiki et al., 2017). The feasibility of leveraging PfCPSF3 as an antimalarial target was demonstrated using a benzoxaborole derivative with potent *in vitro* and *ex vivo* activity as well as efficacy against *P. berghei* infections *in vivo* (Sonoiki et al., 2017). Whole-genome sequence analysis of mutant parasites obtained from *in vitro* selection of resistance using AN3661 revealed a clustering of resistance-conferring mutations around the enzyme active site and predicted to be near the AN3661 binding site, suggesting functional relevance of the mutations and supporting the genetic evidence implicating PfCPSF3 as the antimalarial target of AN3661. Further evidence of direct and selective inhibition of PfCPSF3 by AN3661 was demonstrated in experiments showing altered parasite mRNA processing and stability in AN3661-treated wild-type PfCPSF3 trophozoites in contrast to the resistant PfCPSF3-mutant lines (Sonoiki et al., 2017). While the development of AN3661 itself has been halted, identification of PfCPSF3 as its target will lead to rationally designed inhibitors with improved drug-like properties.

3.2 Dual Stage Targets (i): ABS and liver stage

3.2.1 *P. falciparum* dihydroorotate dehydrogenase (PfDHODH; PF3D7_0603300)

PfDHODH is involved in *de novo* synthesis of pyrimidines, precursors for DNA and RNA. PfDHODH functions by catalyzing the oxidation of dihydroorotate to orotate and utilize ubiquinone as an electron acceptor in the fourth step of pyrimidine de novo

biosynthesis. A hydrophobic pocket, located at the N-terminus where ubiquinone binds, is structurally divergent from the mammalian orthologue, which enables the design of specific inhibitors against PfDHODH (Hurt et al., 2006; Liu et al., 2000), such as the lead triazolopyrimidine-class compound DSM265 (Booker et al., 2010; Coteron et al., 2011; Maetani et al., 2017). DSM265 has good pharmacokinetic properties and activity against PfDHODH in *in vitro* assays and in a *P. berghei* mouse model (Phillips et al., 2015). A Phase 2a study also suggests that it could be used for single-dose treatment, or chemoprevention with partner drugs (Llanos-Cuentas et al., 2018).

However, resistance to DSM265 associated with PfDHODH mutations was observed in recurring patients from the Phase 2a study (Llanos-Cuentas et al., 2018), confirming the previous results obtained in *in vitro* DSM265 resistance selections and in an *in vivo* mouse model (Mandt et al., 2019; White et al., 2019). Similarly, selections using other thiophene-2-carboxamides also selected for mutations in DHODH (Ross et al., 2014). More recently, a biochemical screen of a 40,400 compound chemical library led to the identification of 3,4-dihydro-2H,6H-pyrimido[1,2-c][1,3]benzothiazin-6-imine and its derivatives as a new class of PfDHODH inhibitor (Hartuti et al., 2021). As for previous DHODH inhibitors, understanding the ease of generating resistance as has been demonstrated through minimum inoculum of resistance (MIR) studies will be key before embarking on drug optimization.

3.2.2 *P. falciparum* N-myristoyltransferase (PfNMT; PF3D7_1412800)

N-myristoyltransferase (NMT: synonym: glycylpeptide N-tetradecanoyltransferase) is a monomeric enzyme catalysing irreversible co-translational transfer of myristate from myristoyl coenzyme A to an N-terminal glycine (Boutin, 1997). Myristoylation primes proteins for subsequent palmitoylation and a stable protein-membrane association (Wright et al., 2010) and regulates protein complex assembly and stability (reviewed in Schlott et al., 2018). This range of cellular consequences has led to the proposal of NMT as a drug target in several pathogens including *Toxoplasma* and *Leishmania*.

The *P. falciparum* genome encodes a single NMT isoform with peak expression early in the asexual erythrocytic cycle. A mimetic-based chemoproteomic analysis of the myristoylated and glycosylphosphatidylinositol-anchored proteome identified over 30 NMT substrates involved in motility, protein transport, parasite development and phosphorylation (Wright et al., 2014). The essentiality of PfNMT (Zhang et al., 2018) and its chemical tractability, demonstrated via selective inhibition of *N*-myristoylation using a myristoylate mimetic (Wright et al., 2014), underscores its attractiveness as a target.

PfNMT is 50% identical to, and shares key catalytic residues with, human NMT, although the recombinant versions of the two enzymes are distinguishable upon *in vitro* chemical modification (Giang & Cravatt, 1998). The design of selective PfNMT inhibitors has thus focused on exploiting the differential substrate specificities within the peptide-binding pocket. Selective inhibition of recombinant PfNMT and PvNMT by molecules containing benzothiazole (Bowyer et al., 2007), nitrophenol (Crowther et al., 2011), benzofuran (Yu et al., 2012), pyrimidines or methylindazoles (Bell et al., 2012), benzothiophene (Rackham et al., 2013), quinoline (Goncalves et al., 2012) or methoxyphenyl (Yu et al., 2015) scaffolds has been reported in early studies. Molecular docking analyses have further demonstrated the potential for targeting PfNMT with bepotastine-based sulfonamides (Anusha et al., 2015) and sulfonamide-substituted carboxamides (Onoabedje et al., 2021). High-throughput screening of a GlaxoSmithKline library established 23 selective chemical classes including 5 with novel scaffolds (Harupa et al., 2020) while a structure-guided analysis identified an imipramine-based PfNMT inhibitor, IMP-1002 (Schlott et al., 2019). Resistance selection studies with this compound yielded a G386E mutation in PfNMT that was associated with a 14-fold higher IC_{50} against *P. falciparum* ABS parasites. The causal role of this mutation was confirmed using CRISPR/Cas9-based edited parasites. Further studies identified an analogue, DDD85646, whose different binding properties enabled it to retain potent activity against the G386E mutant (Schlott et al., 2019). This compound is currently under further exploration.

3.2.3 *P. falciparum* histone acetyltransferase GCN5 (PfGCN5; PF3D7_0823300)

PfGCN5 belongs to the Gcn5-related N-acetyltransferase (GNAT) family of histone acetyltransferases and is the most studied histone acetyltransferase (HAT) in *Plasmodium*. It contains both a GNAT (HAT) domain and a bromodomain (Fan et al., 2004; Kanyal et al., 2018). The HAT domain acetylates conserved histone lysine amino acids by transferring an acetyl group from acetyl-CoA, whereas the bromodomain recognises acetylated histone tails and assists acetylation-dependent chromatin remodelling. PfGCN5 acetylates histone H3 at K9 and K14 residues (Fan et al., 2004). PfGCN5 regulates diverse functions including transcription/ translation, signalling, transport, DNA damage and repair (Cui et al., 2007a), with a role in upregulating stress induced genes during unfavourable conditions (Rawat et al., 2021), and for the bromodomain specifically in parasite virulence and erythrocyte invasion (Miao et al., 2021). Inhibition of PfGCN5 HAT activity by anacardic acid resulted in downregulation of several genes during the trophozoite stage due to hypoacetylation at H3K9 and H3K14 residues (Cui et al., 2008). Several other inhibitors (e.g. curcumin, garcinol and embelin) inhibit PfGCN5 acetyltransferase and dysregulate transcription (Cui et al., 2007b; Srivastava et al., 2014; Jeffers et al., 2016). Molecular dynamic simulations of different inhibitors identified C14, a compound with higher specificity for PfGCN5 than HsGCN5 and potent activity against *P. falciparum* with an IC_{50} of ~200 nM (Kumar et al., 2017). Identification of bromodomain inhibitors led to the discovery of the triazolopthalazine-based L-45 (Moustakim et al., 2017). A crystal structure of PfGCN5 bound to L-45 was obtained and showed L-45 in the acetyl-lysine binding site of PfGCN5 (Moustakim et al., 2017). Inhibition of parasite growth using bromodomain-specific inhibitors resulted in other work to identify more inhibitors specific to *Plasmodium.* With the help of *in silico* docking studies, Chua *et al*. identified compounds that can form a typical hydrogen bond interaction with the conserved asparagine (N1436) of the PfGCN5 bromodomain, with SGC-CBP30 as most potent inhibitor in their study (Chua et al., 2018).

3.3 Dual Stage Targets (ii): ABS and Gametocytes

3.3.1 *P. falciparum* non-SERCA-type Ca2+ -transporting P-ATPase (PfATP4; PF3D7_1211900)

PfATP4 is a validated antimalarial drug target explored in numerous independent drug screening efforts. Activation of parasite-induced permeability pathways within the erythrocyte membrane leads to an influx of low molecular weight solutes, including Na^+, that is thought to freely permeate the parasitophorous vacuole (Desai et al., 1993). Despite the high Na^+ concentration within the erythrocyte cytosol and potentially the parasitophorous vacuole, a low cytosolic Na^+ is maintained through the activity of the P-type cation-transporter, PfATP4. This plasma membrane-residing protein regulates intracellular Na^+ homeostasis through extrusion of cytosolic Na^+ coupled with influx of H^+ ions (Dyer et al., 1996; Rottmann et al., 2010; Spillman et al., 2013), which are counter-transported by the vacuolar-type H^+-ATPase to maintain a neutral cytosolic pH (Saliba & Kirk, 1999).

A high-throughput screen of a 12,000 compound library followed by further SAR optimization, led to the identification of a spiroindolone NITD609 (rebranded as KAE609 and now cipargamin). The compound was found to inhibit growth of *P. falciparum* and *P. vivax* with low nanomolar range IC_{50}, block gametocyte development *in vitro* and oocyst development in mosquitoes (Rottmann et al., 2010; van Pelt-Koops et al., 2012). NITD609 clears *P. berghei* from mice very rapidly (faster than artemisinin derivatives), has a favourable safety profile (Rottmann et al., 2010), and is effective in several human clinical trials (e.g. Ndayisaba et al., 2021). Mutations in *PfATP4* have been identified in parasites resistant to NITD609 through gradual exposure to increasing concentrations of the compound, and overexpression of the mutated PfATP4 protein in parasites confirmed the resistance phenotype. In a series of cellular phenotypic assays, treatment with spiroindolones resulted in an increase in cytosolic Na^+ concentration and induced alkalinization (Spillman et al., 2013), pointing to inhibition of PfATP4 as the mechanism of action.

The compound (+)-SJ733, derived from the dihydroisoquinolone series, is another PfATP4 inhibitor and potent antimalarial (Jiménez-Díaz et al., 2014). (+)-SJ733 is fast acting and effective *in vitro* and in a NOD/SCID/IL2Rγ^{null} (NSG) mouse model. *In vitro* resistance selection and whole genome sequencing revealed mutations in *PfATP4* confer resistance to (+)-SJ733, with mutant parasite lines exhibiting a higher resting cytosolic Na^+ concentration. Treatment with (+)-SJ733 invoked rapid cytosolic Na^+ increase and alkalinization, leading to osmotic swelling of the parasites and premature eryptosis (Jiménez-Díaz et al., 2014) consistent with inhibition of *Pf*ATP4. This compound is now being characterized in clinical trials (Gaur et al., 2020).

Additional PfATP4 inhibitors have been identified, including pyrazoleamides (PA21A092) (Vaidya et al., 2014), and molecules from the MMV Malaria Box and Pathogen Box collections (Lehane et al., 2014). Its overall attractive biological profile supports this target as one of high interest for next-line antimalarial therapeutics.

3.3.2 *P. falciparum* plasmepsin V (PfPMV; PF3D7_1323500)

Protein export from *Plasmodium* to the host erythrocyte is key to parasite survival and immune evasion (Maier et al., 2008). Most exported proteins contain a signal sequence for entry into the endoplasmic reticulum (ER) as well as the *Plasmodium* Export Element (PEXEL) motif (Maier et al., 2009). PfPMV is an ER-localized aspartic acid protease that cleaves the PEXEL motif and reveals the export signal for subsequent trafficking to the host erythrocyte (Boddey et al., 2010; Russo et al., 2010). PMV is essential for both asexual blood-stage survival (Boonyalai et al., 2018) as well as gametocytogenesis (Jennison et al., 2019), making it an attractive drug target for chemotherapeutic and transmission-blocking purposes.

WEHI-842 is a molecule that inhibits PMV protease activity by mimicking the RVL amino acid sequence in the PEXEL motif (Hodder et al., 2015; Sleebs et al., 2014). Compared to its earlier analogue WEHI-916, WEHI-842 shows improved pharmacological properties including reduced polarity, better membrane permeability, and increased

binding affinity to PMV. While a recent crystal structure of WEHI-842 bound to *P. vivax* PMV shows strong interactions that account for its improved binding affinity, it also identified regions in the binding site unoccupied by the inhibitor, highlighting potential for additional analogues (Hodder et al., 2015). Another peptidomimetic inhibitor, LG20, demonstrates picomolar activity against the PMV enzyme, but performs poorly against parasites likely due to membrane permeability and stability issues (Gambini et al., 2015). Although development of peptidomimetic PMV inhibitors is still at an early stage, drop-off in potency observed between isolated enzyme and cultured parasites is a major concern because it could translate to unacceptable dosing levels *in vivo*. This is largely believed to be a cellular permeability issue, but functional genetics studies also suggest that PMV is maintained at levels of functional excess in ABS parasites and nearly all cellular PMV must be inhibited before parasite growth is affected, which could contribute to this observed loss of potency (Polino et al., 2020). These data suggest that for PMV-targeting peptidomimetics to be clinically useful, improvements to their biochemical and pharmacokinetic properties are necessary. The resistibility potential is currently unknown and will need to be explored with new chemical scaffolds before embarking into drug development programmes.

3.4 Targeting Multiple Lifecycle Stages

3.4.1 *P. falciparum* cytochrome b (PfCYTB; PF3D7_MIT02300)

PfCYTB is a component of the cytochrome bc1 complex (complex III) in the *Plasmodium* mitochondrial electron transport chain (mETC). It is a well-established *P. falciparum* drug target, inhibited by atovaquone (used clinically in conjunction with proguanil under the trade name Malarone®), and a large number of additional small molecules. Although the mETC's canonical role in most eukaryotes is the generation of ATP via oxidative phosphorylation, in *Plasmodium* ABS parasites, its primary function is to oxidize ubiquinol to ubiquinone, which serves as an electron acceptor for PfDHODH, which is essential for the downstream pyrimidine biosynthesis (Painter et al., 2007). In ABS parasites, ATP is primarily generated via glycolysis, and multiple components of

the mETC are dispensable at this stage, including complex II, the type II NADH:ubiquinone oxidoreductase, and ATP synthase (Boysen & Matuschewski, 2011; Hino et al., 2012; MacRae et al., 2013; Sturm et al., 2015).

PfCYTB is a particularly intriguing target because it is critical in multiple stages of the *P. falciparum* lifecycle. In addition to ABS parasites, liver stage parasites are acutely susceptible to PfCYTB inhibitors (Barata et al., 2016). Atovaquone is most commonly used for prophylaxis, where it targets liver stage parasites before they can reach the erythrocytic stage and cause disease. There is also evidence that PfCYTB is essential during some mosquito stages. *P. falciparum* parasites with certain mutations in PfCYTB do not appear to be transmissible to mosquitoes and do not develop oocysts (Goodman et al., 2016), although these transmissibility defects are not observed in *P. berghei* (Blake et al., 2017). Moreover, treating mosquitoes with atovaquone disrupts the early parasite zygote to ookinete transition, completely blocking successful development (Paton et al., 2019). The vulnerability of PfCYTB to inhibition across multiple stages of the lifecycle makes it an attractive drug target for both treatment and prophylaxis as well as transmission-blocking strategies. On the other hand, inhibitors that target asexual reproduction, which involves rapid proliferation, may carry much higher risk of rapid development of and selection for drug resistance, compared to inhibitors that target only sexual reproduction.

Many compounds targeting PfCYTB have been identified. These inhibitors are frequently ligand analogues of ubiquinone and ubiquinol and act by interfering with the PfCYTB quinol oxidation (Q_o) and quinone reduction (Q_i) sites, respectively, disrupting the redox cycling necessary to regenerate ubiquinone. Atovaquone, decoquinate, tetracyclic benzothiazepenes, some endochin-like quinolones including ELQ-400, pyrimidine azepines, thiadiazines, and pyrazolo[3,4-b]pyridines, target the Q_o site while 4(1H)-pyridones, 2-pyrazolyl quinolones, and some endochin-like quinolones such as ELQ-300 target the Q_i site (Capper et al., 2015; Dong et al., 2011; Dorjsuren et al., 2021; Eagon et al., 2020; Hong et al., 2018; Korsinczky et al., 2000; Nam et al., 2011; Nilsen et al., 2013, 2014; Stickles et al., 2015). These compounds show selectivity for parasite CYTB over human, atovaquone being over 200-fold selective while the

preclinical candidate ELQ-300 demonstrates selectivity greater than 18,000-fold (Fry & Pudney, 1992; Nilsen et al., 2013). Conversely, insufficient selectivity and potential inhibition of mammalian CYTB may explain toxicity observed with the Q_i CYTB 4(1H)-pyridone inhibitors in preclinical studies (Bueno et al., 2012; Capper et al., 2015).

Resistance threatens the use of PfCYTB inhibitors. In early clinical trials, when patients were treated with atovaquone alone, approximately one third experienced parasite recrudescence and treatment failure (Looareesuwan et al., 1996). Co-administration of proguanil—which synergistically potentiates the ability of atovaquone to collapse the parasite mitochondrial membrane potential—in tandem with atovaquone dramatically improved treatment success and is now the clinical standard (Looareesuwan et al., 1996; Srivastava & Vaidya, 1999). Resistance is typically mediated by point mutations in the compound binding pocket, (Fisher et al., 2012; Fivelman et al., 2002; Korsinczky et al., 2000; Vaidya & Mather, 2000), so there is often some cross-resistance between PfCYTB inhibitors of varying chemical scaffolds targeting the same binding pocket. On the other hand, only limited cross-resistance between Q_o and Q_i inhibitors has been observed. Candidates for drug development should avoid cross-resistance with atovaquone given that it is already in clinical use. Little to no cross-resistance has been successfully demonstrated with PfCYTB inhibitors such as the tetracyclic benzothiazepenes, the substituted quinolones CK-2-68 and RYL-552 (previously described as NDH2 inhibitors), Q_i inhibitors such as ELQ-300, and dual Q_o and Q_i site inhibitors such as ELQ-400 (Dong et al., 2011; Ke et al., 2019; Lane et al., 2018; Nilsen et al., 2013, 2014; Song et al., 2018). Using both Q_o and Q_i site inhibitors as a combination therapy may thus be a promising method to reduce likelihood of resistance arising (Stickles et al., 2016).

3.4.2 *P. falciparum* bifunctional dihydrofolate reductase-thymidylate (PfDHFR-TS; PF3D7_0417200)

PfDHFR-TS is a bifunctional enzyme that catalyses folate recycling in thymidylate synthesis. Anti-folate drugs (targeting PfDHFR), such as Fansidar® (pyrimethamine in combination with sulfadoxine) were once extensively used for *P. falciparum* malaria

treatment before widespread pyrimethamine resistance arose due to mutations in PfDHFR (Mbugi et al., 2006). Cycloguanil is another PfDHFR inhibitor for malaria chemoprophylaxis and treatment and is formed *in vivo* through metabolism of proguanil. WR99210, a selective potent PfDHFR inhibitor, has limitations as an antimalarial drug candidate due to poor tolerance (Canfield et al., 1993; Rieckmann et al., 1996).

Unlike its host DHFR, which is a monofunctional enzyme, *Plasmodium* DHFR-TS has individual DHFR and TS domains. Quadruple mutations in the DHFR domain of the PfDHFR-TS lead to a reduced binding affinity of pyrimethamine and reduced drug efficacy (Peterson et al., 1990; Yuthavong et al., 2005; Yuvaniyama et al., 2003). A target-based drug discovery programme identified a selective potent *Plasmodium* DHFR-TS inhibitor, P218, which also circumvents resistance to pyrimethamine. P218 employed a slow-on/slow-off tight-binding mode as well as a tight binding to wildtype and quadruple *Pf*DHFR within the DHFR substrate, which altogether could reduce emergence of resistance (Yuthavong et al., 2012). In a Phase 1b study, P218 demonstrated excellent chemoprotective potency with favourable safety and tolerability (Chughlay et al., 2021). Transmission-blocking efficacy for P218 was also identified in a DHFR quadruple mutant gametocyte-producing *P. falciparum* strain (Posayapisit et al., 2021).

3.4.3 *P. falciparum* elongation factor 2 (PfeEF2; PF3D7_1451100)

The eukaryotic conserved PfeEF2 is a GTPase enzyme from the ribosomal factor family that catalyses the GTP-dependent translocation of the ribosome along the mRNA, by translocating the peptidyl-tRNA from the A site to the P site on the ribosome (Baragaña et al., 2015; Spahn et al., 2004). This enzyme plays a role in protein synthesis in eukaryotes and in *Plasmodium*, Zhang et al. demonstrated its essentiality for optimal growth, thus making it an attractive target (Jørgensen et al., 2006; Zhang et al., 2018). Interestingly, studies in *Saccharomyces cerevisiae* highlight the importance of this highly conserved protein as a drug target for antifungal compounds such as sordarin (Justice et al., 1998; Shastry et al., 2001).

Recent phenotypic studies show that M5717 (formerly DDD107498, proposed INN cabamiquine) targets PfeEF2 with nanomolar potency, while being highly selective vs mammalian targets (Baragaña et al., 2015). This compound has antimalarial activity against the liver-stage and asexual and sexual blood-stages. M5717 has become a very prominent candidate for antimalarial therapies as it advances from preclinical to clinical development, and has demonstrated that inhibiting PfeEF2 is a viable strategy for antimalarial drug development (Baragaña et al., 2015). Its moderate resistance profile *in vitro* will need to be monitored in clinical studies.

3.4.4 Kinases

Kinases, a superfamily of enzymes with a conserved kinase domain containing a highly druggable ATP-binding site, have been successfully targeted for cancer treatment and other indications (Roskoski, 2020). Typically, these enzymes catalyse the transfer of γ-phosphate from ATP to protein or lipid substrates. The *Plasmodium* kinome encodes between 60 and 105 putative protein kinases and ~7 putative phosphoinositide kinases, displaying significant divergence from the kinomes of humans and other eukaryotes (Adderley & Doerig, 2022; Hassett & Roepe, 2018; Miranda-Saavedra et al., 2012; Ward et al., 2004). Many *Plasmodium* kinases are essential for parasite development based on reverse genetic studies (Alam et al., 2019; Tewari et al., 2010) and are being explored as potential targets for malaria. Several *Plasmodium* kinases have been phenotypically validated where anti-*Plasmodium* activity has clearly been attributed to chemical inhibition of the kinase target in question using a range of orthogonal approaches, and inhibitors have demonstrated *in vivo* proof of concept (e.g. Alam et al., 2019; Baker et al., 2017; McNamara et al., 2013). The validation of *Plasmodium* kinases as targets is discussed in more detail in Arendse et al., 2021. In addition, *P. falciparum* has been shown to rely on the activity of human kinases within the infected host cell environment and several human kinases have been proposed as potential targets for host-directed therapy (Adderley et al., 2021).

Plasmodium kinase targets with the potential to deliver multistage antimalarials include phosphatidylinositol 4-kinase type III beta (PfPI4Kβ, PF3D7_0509800), cyclic guanosine

monophosphate (cGMP)-dependent protein kinase (PfPKG, PF3D7_1436600) and cyclin-dependent–like kinase 3 (PfCLK3, PF3D7_1114700). Miniaturized biochemical assays making use of purified recombinant protein, suitable for high-throughput screening and routine profiling, have been developed for these kinases (Alam et al., 2019; Paquet et al., 2017; Penzo et al., 2019). Together with structural information (homology models/ high-resolution crystal structures) (El Bakkouri et al., 2019; Mahindra et al., 2020), these facilitate target-based drug discovery programmes. Understanding the selectivity requirements for *Plasmodium* kinase inhibitors in relation to the required compound dose and duration of treatment is a key challenge for drug discovery due to the highly conserved nature of the ATP-binding site across this enzyme superfamily.

3.4.4.1 Phosphoinositide kinase targets

PfPI4Kβ was first reported as a promising multistage antimalarial target after it was identified as the primary target of a novel class of imidazopyrazine compounds (KDU691 and KAI407) displaying potent prophylactic liver stage, ABS and transmission blocking anti-*Plasmodium* activity in experimental models (McNamara et al., 2013). The most advanced *Plasmodium* PI4Kβ inhibitor, the 2-aminopyridine MMV390048, was developed from a whole-cell, phenotypic screening approach without prior knowledge of the target (Paquet et al., 2017). MMV390048 progressed to Phase 2a clinical trials where a single oral of 120 mg rapidly cleared asexual parasites and gametocytes in eight patients with *P. vivax* malaria and was well tolerated (Mohammed et al., 2023). However, clinical development was stopped due to teratoxicity concerns based on data emerging from studies in rats (Demarta-Gatsi et al., 2022). A number of other scaffolds potently inhibiting this kinase have also been reported, including the quinoxaline BQR695 (McNamara et al., 2013), BRD73842 (Kato et al., 2016), imidazopyridazines (Cheuka et al., 2021; McNamara et al., 2013), naphthyridines (MMV024101) (Kandepedu et al., 2018), bipyridine-sulfonamides (CHMFL-PI4K-127) (Liang et al., 2020), Torin 2 derivatives (NCATS-SM3710) (Krishnan et al., 2020) and the anticancer mTOR inhibitor sapanisertib (Arendse et al., 2022). PI4Kβ inhibition leads to a

moderately slow rate of parasite kill *in vitro* (Paquet et al., 2017), although it should be noted that MMV390048 displayed fast killing kinetics in the clinic (Mohammed et al., 2023; Sinxadi et al., 2020). MMV390048 and other PI4Kβ ATP-competitive inhibitors show a moderate propensity to generate *de novo* resistance as demonstrated *in vitro,* where a higher number of parasites in culture is needed to raise resistance compared to other drugs known to be highly mutable such as atovaquone (Brunschwig et al., 2018; Paquet et al., 2017). *In vitro* resistant selections carried out with different PI4Kβ inhibitors have resulted in resistant lines showing low to moderate EC_{50} shifts between 3- and 22-fold, with single point mutations in the PI4Kβ kinase domain, Rab11a and/or PI4Kβ copy number variations (Arendse et al., 2022; Brunschwig et al., 2018; Kato et al., 2016; Krishnan et al., 2020; McNamara et al., 2013; Paquet et al., 2017). The success of *Plasmodium* PI4Kβ inhibitors has led to an interest in targeting *Plasmodium* phosphatidylinositol 3-kinase (PfPI3K, PF3D7_0515300). Although genetic essentiality has been demonstrated for ABS development (Tawk et al., 2010; Zhang et al., 2018) and several compounds showing activity against *Pf*PI3K *in vitro* have been identified (Mott et al., 2015; Sternberg & Roepe, 2020), selective on-target whole cell anti-*Plasmodium* activity has not been conclusively demonstrated. The value of *Pf*PI3K as a target is therefore unclear.

3.4.4.2 *Protein kinase targets.*

The extensively studied *Plasmodium* serine/threonine protein kinase PfPKG acts as a signalling hub, playing a key role in a wide range of cellular processes at multiple stages of the parasite lifecycle (Alam et al., 2015). PKG inhibition results in multistage anti-*Plasmodium* activity, disrupting hepatocyte invasion by sporozoites, merozoite egress from ABS schizonts, and male gamete exflagellation, as demonstrated by ATP-competitive inhibitors imidazopyridine ML10 and trisubstituted imidazole MMV030084 in various experimental models (Baker et al., 2017; Vanaerschot et al., 2020). Oral dosing of ML10 was shown to clear parasites from the blood in a humanised mouse model of *P. falciparum* (Baker et al., 2017). *Plasmodium* PKG has a small threonine gatekeeper residue, allowing inhibitors to extend into the so-called back pocket, a small

hydrophobic pocket adjacent to the ATP binding site, that can be exploited for selectivity (El Bakkouri et al., 2019) because this pocket is inaccessible in both human PKG orthologues and most other human serine/threonine kinases. Engineered parasite lines with a PKG gatekeeper mutation (T618Q) renders the parasites insensitive to *Plasmodium* PKG inhibitors. Combined chemogenetics, conditional knockdown, and conditional knockout approaches have provided useful tools for studying PKG function and testing for on-target anti-*Plasmodium* activity (Alam et al., 2015; Baker et al., 2017; Brochet et al., 2014; Koussis et al., 2020; McRobert et al., 2008; Taylor et al., 2010; Vanaerschot et al., 2020). Importantly, *in vitro* resistance selections with both ML10 and MMV030084 did not yield mutations in the PKG sequence, indicating that this target may be refractory to resistance, although caution is needed, as neither compound is completely selective for PKG (Baker et al., 2017; Vanaerschot et al., 2020). The imidazole war-head in MMV030084 is not shared by any of the antimalarials in current clinical use, thus minimizing the likelihood of cross-resistance to this target. A disadvantage of PKG inhibitors is their slow killing rate *in vitro*, probably due to the narrow window of PKG activity in the asexual blood stage (Baker et al., 2020; Matralis et al., 2019). Despite this, the other favourable attributes of PKG as a target, together with the availability of high-resolution crystal structures (El Bakkouri et al., 2019), biochemical assays and genetic tools, make it an attractive target for malaria drug discovery. A high-throughput screen of GlaxoSmithKline (GSK) Full Diversity Collection against recombinant PKG has identified additional chemotypes as starting points for drug discovery (Penzo et al., 2019; Tsagris et al., 2018).

PfCLK3 is another promising protein kinase target. There are four CLKs in *Plasmodium*; CLKs 1- 3 have been shown to be genetically essential for ABS development (Alam et al., 2015; Bushell et al., 2017; Tewari et al., 2010; Ward et al., 2004; Zhang et al., 2018). TCMDC-135051 was identified as a specific CLK3 inhibitor in a target-based screen of kinase-focused libraries against recombinant PfCLK1 and PfCLK3 (Alam et al., 2019). This compound displays prophylactic liver stage activity, ABS activity and transmission reducing potential. *In vitro* resistance selections with TCMDC-135051 gave rise to resistant parasites with single point mutations in genes encoding for CLK3 and putative RNA processing protein PfUSP39 (PF3D7_1317000) resulting in low to

moderate EC_{50} shifts between 4- and 11-fold. CLK3 was further validated as the primary target of TCMDC-135051 using an engineered parasite line, wherein a CLK3 specific active site residue G499 was mutated to proline, the corresponding residue in CLK1, leading to reduced sensitivity to TCMDC-135051 (Alam et al., 2019). TCMDC-135051 displayed a rapid rate of kill in the parasite reduction ratio (PRR) assay (Alam et al., 2019). Encouragingly, TCMDC-135051 displayed selectivity for *Plasmodium* CLK3 relative to human orthologue PRPF4B and human CLK1 and medicinal chemistry optimisation efforts on TCMDC-135051 have been reported (Mahindra et al., 2020).

3.4.5 Aminoacyl-tRNA synthetases (aaRSs)

These enzymes play essential roles in the ATP-dependent aminoacylation of cognate tRNAs with their respective amino acids for use in protein synthesis (Bhatt et al., 2009; Ibba & Soll, 2000). The *P. falciparum* genome encodes 36 different aaRSs, which participate in both cytoplasmic and organellar protein synthesis. There is compelling evidence for the druggability of this target class in bacteria and *Plasmodium* (Istvan et al., 2011; Pasaje et al., 2016; Xie et al., 2022).

3.4.5.1 *P. falciparum* cytoplasmic phenylalanyl-tRNA synthetase (PfcFRS)

The *Plasmodium* genome contains four genes that translate into three FRSs, cytosolic FRS (cFRS; α-subunit, PF3D7_0109800; β-subunit, PF3D7_1104000), mitochondrial FRS (PF3D7_0603700), and apicoplast FRS (PF3D7_1232000). The three FRSs are expressed in all lifecycle stages and belong to the class IIc aaRSs family, with distinct signatures in their architecture and functional adaptations (Manickam et al., 2018). BRD7929, a bicyclic azetidine compound that selectively targets PfcFRS, was developed from a hit identified from a phenotypic screen of a diversity-oriented synthesis library with three-dimensional features reminiscent of natural products (Kato et al., 2016). Oral BRD7929 at low dose rapidly kills liver-, blood- and sexual stages of parasites in multiple *in vivo* efficacy models, validating PfcFRS as a druggable

antimalarial target. BRD7929 shows a low propensity for *de novo* resistance selection but displays relatively high cardiotoxicity, thus requiring further optimization to eliminate these liabilities and/or discovery of alternative inhibitory scaffolds.

3.4.5.2 *P. falciparum* cytoplasmic prolyl-tRNA synthetase (PfcPRS; PF3D7_1213800)

The herb *Dichroa febrifuga,* used in Chinese Traditional Medicine to treat malaria, is the source of febrifugine, a potent PfcPRS inhibitor. This and the synthetic derivative halofuginone, established PfcPRS as a promising antimalarial target *in vivo* (Herman et al., 2015). Halofuginone attracted attention as one of the most potent antimalarials discovered (Dd2 asexual blood stage IC_{50} = 0.5 nM) and halofuginone-analogues were shown to cure liver stage *P. berghei* mouse models in a single dose (Derbyshire et al., 2012; Herman et al., 2015). However, halofuginone suffers from development of rapid tolerance mediated by elevated intracellular proline levels, a halofuginone-competitive PfcPRS substrate, and sustained treatment with halofuginone for >6 months resulted in halofuginone-resistant parasites with both elevated proline and halofuginone-resistance mutations in PfcPRS proline-binding site (Fagbami et al., 2019; Herman et al., 2014, 2015). Recently, several classes of ATP-competitive PfcPRS inhibitors have been disclosed (e.g. T-3767758, NCP26, T-3833261, (*S*)-4-(3-cyano-3-(1-methylcyclopropyl)-2-oxopyrrolidin-1-yl)-*N*-(3-fluoro-5-(1-methyl-1*H*-pyrazol-4-yl)benzyl)-6-methylpicolinamide), several of which are proline-uncompetitive (proline increases binding affinity), suggesting they may not be cross-resistant with halofuginone and could form a synergistic combination (Adachi et al., 2017; Okaniwa et al., 2021; Shibata et al., 2017; Tye et al., 2022). ATP-competitive PfcPRS inhibitors are promising for improved species selectivity because all non-conserved active site residues are found in the ATP-binding site (Jain et al., 2015).

3.4.5.3 *P. falciparum* isoleucyl-tRNA synthetase (PfIRS)

There are cytoplasmic (PfcIRS; PF3D7_1332900) and apicoplast (PfaIRS; PF3D7_1225100) localized IRS activities (Bhatt et al., 2009; Istvan et al., 2011). A

transposon mutagenesis screen revealed PfcIRS to be essential in asexual blood stages (Zhang et al., 2018). PfcIRS is a class I aaRS, with an N-terminal Rossman fold in its catalytic domain, and a C-terminal anticodon binding domain (Bhatt et al., 2009; Chaliotis et al., 2017; Nyamai & Tastan Bishop, 2019). Additionally, it bears an insertion in its Rossman fold called the connective polypeptide (CP1) which functions as an editing pocket. This editing domain provides proofreading activity capable of correcting amino acid-tRNA mismatches either pre- or post-transfer (Eldred & Schimmel, 1972; Fersht, 1977).

In 2011, Istvan *et al*. reported both cytoplasmic and apicoplast IRSs to be druggable targets. A missense mutation in the gene encoding PfcIRS was identified in malaria parasites resistant to thiaisoleucine, an analogue of isoleucine (Istvan et al., 2011). Resistance to thiaisoleucine was obtained with a parasite inoculum around 10^7 suggesting a moderate resistibility for such targets. Targeting of PfaIRS by mupirocin was also revealed through *in vitro* evolution; mupirocin-resistant parasites generated through drug selection acquired a missense mutation in the highly conserved Rossman fold of PfaIRS (Istvan et al., 2011). Mupirocin is a widely used, clinically approved antibiotic, that has long been known to inhibit bacterial IRS and is active against gram-positive bacteria such as staphylococci and most streptococci, and moderately active against gram-negative bacteria (Hurdle et al., 2004; Yanagisawa et al., 1994). The compound also shows potent antimalarial activity against *P. falciparum* (IC_{50} <100 nM) and has been shown to target PfaIRS within the parasite, which is not unexpected due to the similarity between PfaIRS and the prokaryotic IRS (Istvan et al., 2011). Like other drugs that target the apicoplast, mupirocin causes a “delayed death” phenotype, where the growth of blood-stage parasites is unaffected until the second intraerythrocytic development cycle following drug treatment (Istvan et al., 2011). Additionally, mupirocin has been known to be unstable *in vivo* and is highly bound by serum (Casewell & Hill, 1987). Unfortunately, mupirocin’s slow mode of action and poor serum stability limit its use as a first-line therapeutic for clinical malaria.

3.4.5.4 *P. falciparum* tryptophanyl-tRNA synthetase (PfWRS)

There are cytosolic (PfcWRS; PF3D7_1336900) and apicoplast-localized (PfaWRS; PF3D7_1251700) WRS activities. PfcWRS preferentially aminoacylates tRNA from a eukaryotic source, while PfaWRS preferentially charges tRNA from a bacterial source (Pasaje et al., 2016). Inhibition of the apicoplast enzyme by the natural product and tryptophan analogue, indolmycin, supports druggability of malaria WRSs (Pasaje et al., 2016). Indolmycin inhibits growth of intraerythrocytic *P. falciparum* with a “delayed death” phenotype characteristic of inhibiting apicoplast housekeeping machinery (Kennedy et al., 2019), and ablates the apicoplast (Koh et al., 2013) in parasites supplemented with the essential product of apicoplast metabolism, isopentenyl pyrophosphate (IPP). Biochemical assays revealed that indolmycin inhibits aminoacylation of PfaWRS, and *in silico* analysis using a homology model of the protein showed that the compound can bind to the amino acid-binding pocket. Indeed, increasing levels of tryptophan in culture medium lowered sensitivity to indolmycin, implicating tryptophan utilization as the target of the compound. While selective inhibition of the apicoplast WRS protein can be further explored using other bacterial-type inhibitors of the enzyme as a starting point, the inherently slow rate of kill by these inhibitors may limit utility as primary treatment, though use in prophylaxis regimens could be useful to evaluate.

Despite the lack of inhibitors against the cytosolic WRS protein, insights into the structural differences between the *P. falciparum* and human proteins during substrate binding (Koh et al., 2013) can be exploited for future antimalarial drug discovery programmes. So far the resistibility profile of this protein has not been explored.

3.4.6 Histone deacetylases (HDACs)

Histone deacetylases are chromatin-modifiers that remove acetyl groups from the ε-amino group of lysine residues in histones. Deacetylation causes chromatin compaction and transcriptional repression. Several HDACs target non-histone proteins. In

Plasmodium, there are five HDAC enzymes divided into three classes: Class I (PfHDAC1; PF3D7_0925700), Class II (HDA1; PF3D7_1472200 and HDA2; PF3D7_1008000) and Class III (Sir2A; PF3D7_1328800 and Sir2B; PF3D7_1451400) (Kanyal et al., 2018). HDACs regulate schizogony, gametocytogenesis, antigenic variation and hepatocyte invasion (Chaal et al., 2010; Coleman et al., 2014; Tonkin et al., 2009). A wide range of HDAC inhibitors exhibit activity in the range of 10-200 nM against *P. falciparum;* these include apicidin, SAHA (suberoylanilide hydroxamic acid, or vorinostat), romidepsin, belinostat and panobinostat (Chua et al., 2017; Darkin-Rattray et al., 1996; Marfurt et al., 2011). These compounds have activity against multiple stages of the *P. falciparum* lifecycle. Peptoid-based HDAC inhibitors also have antimalarial activity against the liver stage and asexual blood stage (Koehne et al., 2019). The PfHDAC1-specific inhibitor JX21108 (modified quisinostat) was also found to be active against multiple stages, including liver and gametocyte (Huang et al., 2020). Potluri *et al*. discovered FNDR-20123, a pan HDAC inhibitor with an IC_{50} value of 31 nM against *P. falciparum* (Potluri et al., 2020). This compound was active against both drug-sensitive and multidrug resistant strains (Potluri et al., 2020). Li *et al*. synthesised novel spirocyclic hydroxamic acid derivatives with potent activity against several multidrug resistant parasites including artemisinin resistant isolates (Li et al., 2021). PfHDAC1-specific activity for these compounds was confirmed using gene knockdown and *in vitro* enzymatic inhibition experiments. Recently, an inhibitor screen on 350 diverse epigenetic modifiers against different stages of *P. falciparum* parasites (Vanheer et al., 2020) identified active compounds, seven of which were HDAC inhibitors, with quisinostat being the most potent. Wang *et al.* modified quisinostat by replacing the 4-aminomethylpiperidine moiety with a 2,6 diazaspiro[3.4]octane moiety (Wang et al., 2022). Out of 35 derivatives (JX1-JX35), JX35 showed highest inhibitory activity against different stages (blood, liver and gametocyte) of *P. falciparum* with IC_{50} values of <2 nM against both 3D7 and Dd2 strains. One key property that will be needed is exquisite selectivity for PfHDAC over human orthologues. Finally, the resistibility profile of this protein has not been explored.

3.4.7 *P. falciparum* proteasome subunit beta type-5 (PF3D7_1011400)

The eukaryotic 26S proteasome is a highly conserved, multi-subunit enzymatic complex that degrades proteins and is involved in diverse cellular processes such as cell cycle regulation and oxidative stress response (Coux et al., 1996). The 20S catalytic core, recently elucidated in *P. falciparum*, is comprised of the proteolytic β1 (caspase-like), β2 (trypsin-like), and β5 (chymotrypsin-like) subunits (Li et al., 2016). Functional studies performed in *S. cerevisiae* suggest that β5 shows highest involvement in protein degradation and is therefore most attractive as a drug target (Arendt & Hochstrasser, 1997; Heinemeyer et al., 1997). Proteasome inhibitors are considered promising antimalarial agents due to their potent activity against all human-relevant parasite stages (Gantt et al., 1998) and demonstrated synergy with first-line artemisinin-based therapies (Dogovski et al., 2015; Stokes et al., 2019). However, host-parasite selectivity remains a key hurdle impeding further clinical development of the proteasome as a target.

Bortezomib, a peptide boronate that targets the human proteasome and is used to treat multiple myeloma (Orlowski et al., 2002), exhibits nanomolar activity against *P. falciparum* (Reynolds et al., 2007). Specific activity against the β5 subunit has been validated for bortezomib and several of its analogues, but toxicity remains a problem (Xie et al., 2018). Better species selectivity has been documented following medicinal chemistry optimization efforts on Carmaphycin B, a tripeptide natural product derived from cyanobacteria (analog 18) (LaMonte et al., 2017), as well as on the vinyl sulfone WLL-vs (Stokes et al., 2019). Despite these improvements, both classes retain micromolar potency against the human proteasome (Yoo et al., 2018).

3.5 Targeting the Mosquito Stage

Extensive research has focused on drug targets for malaria treatment in humans, but prevention is also a critical goal of malaria control programmes. Such efforts have often focused on the malaria vector, *Anopheles* mosquitoes, and vector control methods have had the greatest impact on the reduction of malaria cases in recent decades. Insecticide

treated nets (ITN) and indoor residual spraying (IRS) of insecticides were responsible for approximately 68% and 10% of these averted cases, respectively (Bhatt et al., 2015). Despite these advances, vector control strategies have limitations, including the rise of mosquito insecticide resistance (Ranson et al., 2011; Toé et al., 2014).

Halting the initial transmission of *Plasmodium* parasites from humans to mosquitoes, inhibiting their mosquito-stage development, or preventing transmission from mosquitoes back to humans, could all disrupt the spread of malaria. The mosquito stages of *Plasmodium* development also represent a particularly enticing time to target because parasites undergo a population bottleneck: an infected mosquito may harbour only a handful of oocysts; a miniscule number relative to the potentially trillions of circulating asexual blood stage parasites (Graumans et al., 2020). Substantial work has thus been done to identify compounds with transmission-blocking potential (Burrows et al., 2017; Yahiya et al., 2019).

Current clinically used antimalarials do not have transmission blocking activity, with the notable exception of primaquine, an 8-aminoquinoline, which clears all stages of gametocytes, thus blocking transmission to the mosquito vector (Munro & McMorran, 2022). However, primaquine use is limited due to its contraindication in patients with G6PD deficiency (Kheng et al., 2015). Thus, although standard artemisinin combination therapies successfully target circulating ABS parasites, mature gametocytes can persist and infect new mosquitoes for weeks after ABS parasites have been cleared (Delves et al., 2012; Plouffe et al., 2016). Phenotypic screens have been undertaken that directly assay gametocyte readouts, such as exflagellation or macrogamete formation (Buchholz et al., 2011; D'Alessandro et al., 2013; Ruecker et al., 2014), or transmission blocking activity following standard membrane feeding assays (SMFA; Vos et al., 2015; Delves et al., 2016). These screens have identified a number of inhibitors of gametocyte development and/or transmission blocking compounds with a range of targets (Bolscher et al., 2015; Bowman et al., 2014; Buchholz et al., 2011; Delves et al., 2018; Duffy & Avery, 2013; Lucantoni et al., 2013; Plouffe et al., 2016; Reader et al., 2021; Ruecker et al., 2014; Sanders et al., 2014; Sun et al., 2014).

Compounds have also been identified that target the mosquito-stage of *Plasmodium* during its ookinete and oocyst stages. 13,533 compounds in the Tres Cantos Antimalarial Set (TCAMS) library with known ABS activity were screened in a *P. berghei* ookinete development assay and 437 (3.2%) were found to be inhibitory (Delves et al., 2019). The most potent and selective compounds from the six chemical families tested were validated by SMFA and found to be transmission blocking in both *P. berghei* and *P. falciparum*. These representative compounds included inhibitors of cytochrome b, dihydrofolate reductase (DHFR), protein translation, and coenzyme A biosynthesis (Delves et al., 2019). Ideally, mosquito-stage specific targets would be identified that were unique to parasite biology during the vector portion of its lifecycle (Burrows et al., 2019), but such target identification is hindered by the inability to perform techniques commonly used in ABS parasites, such as *in vitro* evolution and whole genome analysis (IVIEWGA) (Luth et al., 2018) during these stages. However, a number of studies have identified areas of *Plasmodium* biology that may be highly susceptible to mosquito-stage targeting, such as TCA cycle metabolism, mitochondrial oxidative phosphorylation, heme biosynthesis, and fatty acid synthesis (Hino et al., 2012; Ke et al., 2014, 2015; Nagaraj et al., 2013; Sturm et al., 2015; van Schaijk et al., 2014).

It has been proposed that such transmission-blocking compounds could feasibly be administered to mosquito-stage parasites via mosquito ingestion of blood meal with circulating compound at effective concentrations (Birkholtz et al., 2016; Burrows et al., 2013, 2017). This was demonstrated with the primaquine-derived 8-aminoquinoline analogue NPC1161B, wherein a second blood meal containing NPC1161B was provided to mosquitoes four days after their initial infectious blood meal. At this time point, oocysts have already established on the midgut basal lamina and begun growing. Although the NPC1161B blood meal did not alter the number of oocysts in the established infection, the oocysts did appear to be smaller (potentially indicating slowed or stalled growth) and there was a significant reduction in the number of sporozoites that ultimately developed (Hamerly et al., 2019). Even when mosquito-stage sporogony is not completely disrupted, reducing the total number of sporozoites can have a substantial impact on malarial transmission as greater mosquito salivary gland

sporozoite numbers correlate with successful transmissibility to the mammalian host (Aleshnick et al., 2020; Churcher et al., 2017).

Mosquito-stage parasites could also be exposed to mosquito stage active compounds through the mosquito vector itself, rather than via the human blood meal. It was recently demonstrated that *P. falciparum* development within its vector can be completely inhibited by allowing *Anopheles* mosquitoes to rest on a surface coated with the potent cytochrome bc1 (PfCYTB) inhibitor atovaquone prior to infection, in a manner analogous to typical routes of mosquito insecticide exposure (Paton et al., 2019). Such an approach could represent a novel method for targeting mosquito stage *Plasmodium* (Burrows et al., 2019). There has also been limited research on sporozoite targeted compounds, although most of this work has focused on inhibiting sporozoite motility in the human host rather than targeting it specifically in the mosquito. Such efforts have identified monensin sodium, gramicidin S, and the plant derivatives epigallocatechin gallate and digitonin as inhibitors of sporozoite motility or viability (Douglas et al., 2018; Hellmann et al., 2010).

There is burgeoning interest in the promise of mosquito-stage *Plasmodium* drug targets as a strategy for halting malaria transmission. Improved i*n vitro* gametocyte culture and screening assays have enabled the identification of compounds which uniquely target these sexual stages. Continued research into mosquito-stage drug targets with transmission blocking activity will be key for a successful, multi-pronged malaria eradication programme.

3.6 Mechanisms of multidrug resistance

Transporters can also be the means through which antimalarials enter or leave the cell, and transporter mutation is a common route to multiple drug resistance. *P. falciparum* Cyclic Amine Resistance Locus protein (PfCARL; PF3D7_0321900) is a seven-transmembrane domain transport protein, localized to the Golgi apparatus in asexual blood-stage parasites and gametocytes (LaMonte et al., 2016). Using *in vitro* evolution and whole genome analysis, this gene has been shown to confer resistance against a

broad range of antimalarial compound classes that are likely to exert their parasiticidal activity through dissimilar targets and mechanisms (Kuhen et al., 2014; LaMonte et al., 2016; Lim et al., 2016; Magistrado et al., 2016; Meister et al., 2011). Of these, the pan-active imidazolopiperazine class is perhaps the most well-studied, given that its member ganaplacide is currently in Phase 2 clinical trials. Mutations in PfCARL also decrease sensitivity to structurally diverse molecules with parasite-killing properties such as: MMV007564, a benzimidazolyl piperidine (Magistrado et al., 2016); tyroscherin, an antitumor antibiotic that inhibits insulin-like growth factor 1 (Hayakawa et al., 2004; LaMonte et al., 2016); an asymmetric adolylmaleimide (LaMonte et al., 2016) and oxazole-4-carboxamide (LaMonte et al., 2016).

3.7 Host-direct therapy and host targets

Targeting the *Plasmodium,* with its short life-cycle and rapid reproduction, always runs a strong risk of selecting for resistance. The alternative strategy, of targeting host factors/pathways that favour parasite growth, runs a much lower risk: there is no selection pressure for host mutation to accommodate the parasite. Human CD68 and CD81 are required for parasite invasion of liver cells, and blocking either with antibodies reduces parasite burden (Cha et al., 2015; Silvie et al., 2003). Human peroxiredoxin 6 is essential for parasite survival in erythrocytes (Wagner et al., 2022). Inhibiting the kinase MEK reduces parasite proliferation in liver and erythrocytes (Sicard et al., 2011). Reduction of proinflammatory signalling in infected hosts may also reduce the severity of disease (for review see Matteucci et al., 2022). Vaccination is, of course, another important area for host-directed intervention.

4. The GtoMPdb: accessing and viewing the data

The GtoMPdb home page (https://www.guidetomalariapharmacology.org) is a dedicated web-portal, designed in consultation with malaria researchers to give optimized access to antimalarial data. The portal allows users to browse the data via targeted searches, searches for targets or ligands, or by parasite lifecycle stage and *Plasmodium* species.

The malaria-relevant data themselves currently includes 40 *Plasmodium* targets and more than 130 antimalarial ligands (release version 2023.1). The selection of targets and ligands for inclusion in the GtoMPdb is guided by a group of scientific advisors, drawn from IUPHAR, MMV and MalDA. Our focus has been on the curation of validated *Plasmodium* targets, including those in the MalDA pipeline (Forte et al., 2021; Yang et al., 2021), and the approved medicines, clinical candidates and research leads that act on them. Readers interested in more details of the process and criteria for selection of data are referred to Armstrong et al., 2020.

4.1 Viewing target data in GtoMPdb

The ‘Targets’ panel on the home page provides a direct link to the ‘Antimalarial targets’ page. From here, users can find an overview of this target family, browse target subfamilies, navigate to individual target pages and find detailed information about each target and its interactions (Figure 2). A ‘Malaria Pharmacology Comments’ section explains why a target is of particular relevance to malaria. These expert-reviewed comments include information about the role of the target in essential cellular processes and during the *Plasmodium* lifecycle. In addition, they indicate where possible, the current status of a target in the drug discovery process. Reference to additional compounds, not selected for inclusion in the GtoMPdb, may also be found in the comments section. Examples include, compounds where selectivity at the *Plasmodium* target is an issue (e.g. the PI3K inhibitors wortmannin and LY 294002) and compounds where we have been unable to identify activity data (e.g. a series of *P. falciparum* GPI-anchored wall transfer protein 1 inhibitors, extracted from a patent).

Data on the interactions of ligands and targets are organized, as in the general pharmacology GtoPdb, into sub-sections and arranged by ligand type, including a section specifically for 'whole organism data' linked to the target. Interaction tables list the ligand, species, assay descriptions, activity values, other parameters, and cite references. Each interaction can be expanded to view more details, including the relevant lifecycle stage of the interaction/target and assay description. A general feature of GtoPdb is the inclusion of icons to provide at-a-glance summaries of ligand properties

(https://www.guidetomalariapharmacology.org/helpPage.jsp#ligandTables), for example, whether a ligand is an approved drug. They also include a specific malaria logo, to indicate that the ligand is curated as part of GtoMPdb (everything in GtoMPdb can also be accessed via GtoPdb so the malaria link may not otherwise be obvious to someone finding a ligand by that route).

4.2 Viewing ligand data in GtoMPdb

The ‘Ligands’ panel on the home page provides direct access to the ligand lists page which gives a simple tabular view of all antimalarial ligands in the database. Selection tabs can restrict the view to approved antimalarial drugs only, or to antimalarial ligands with a Target Candidate Profile (TCP), which is a description of the key attributes of a compound from the MMV global malaria portfolio as it enters clinical development (Burrows et al., 2013, 2017).

Clicking on a ligand name takes the user to the individual ligand summary page, which includes synonyms, drug approval status, icons to indicate key properties and curatorial comments with information on a ligand's pharmacology. The 2D ligand structure is displayed on the right-hand side of the top-section, where an expandable section contains the ligand's physico-chemical properties and SMILES/InChI/InChI Keys (i.e., computer-friendly structural information for use with other databases and software).

Under the top section, a tabbed panel provides extensive information about the ligand. The summary tab contains IUPAC names and external database identifiers (such as PubChem) for the ligand. Interaction data are found under the ‘Biological activity’ tab along with bioactivity comments and the ‘Clinical data’ tab contains information about the ligand’s clinical use, mechanisms of actions, pharmacokinetics and a tabular view of any associated clinical trials. The ‘Malaria’ tab contains additional curatorial comments of relevance to malaria pharmacology.

4.3 Viewing *Plasmodium* lifecycle and target species data in GtoMPdb

The GtoMPdb provides information about the lifecycle of the *Plasmodium* parasite on a dedicated summary page, that can be accessed from the 'Parasite Lifecycle Stages' panel on the home page. The panel also includes a list of our top-level lifecycle stage categories against which interactions in the database have been annotated and which form the basis of organising, navigating and searching for parasitic lifecycle activity. Each of these categories links to a new page providing a description of the lifecycle stage and all interactions contained in the database that are associated with it.

The ‘Target Species’ panel provides a link to a summary page that gives information about *Plasmodium* species that are of clinical or experimental importance, together with links to external resources. The panel also includes links to individual pages for *Plasmodium* species, for which GtoMPdb has available data.

4.4 GtoMPdb connectivity

GtoMdb, like GtoPdb, is connected richly to other databases, including PubChem and UniProt. Readers interested in these links and in the architecture and development of the database, its technical details and the basis of its nomenclature and data selection, are referred to Armstrong et al., 2020 and to explanatory pages on the database itself.

5. Concluding Remarks

Antimalarial drug discovery has a long history: starting with natural products and their semisynthetic derivatives it is now making rapid progress, with the emphasis on using chemically validated targets in biochemical screens to aid rational drug design. In this review, we have discussed advances in our knowledge of key *Plasmodium* molecular targets and the progression of new antimalarial drug candidates suitable for preclinical development and clinical testing. Target-based approaches enable compounds with different mechanisms of action and with activity at different stages of the parasite lifecycle to be identified. This is crucial to combat the rise in drug-resistant malaria and

for the goal of eliminating the disease. The public-private partnerships MMV as well as MalDA, with their collaborative approach, will continue to play a critical role in the acceleration of the antimalarial drug discovery process. In addition, expert-curated databases, such as the GtoMPdb, have an important function in supporting the open exchange of data.

6. Nomenclature of Targets and Ligand

Key protein targets and ligands in this review article are hyperlinked to corresponding entries in the IUPHAR/MMV Guide to MALARIA PHARMACOLOGY, the portal for antimalarial data from the IUPHAR/BPS Guide to PHARMACOLOGY (Armstrong et al., 2020; Harding et al., 2021). The nomenclature adopted for *Plasmodium* targets is derived from information available from PlasmoDB (Aurrecoechea et al., 2009).

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8. Author contributions

JFA, BC and JAD coordinated writing of the manuscript. JAD is PI of the database described here and drafted the history section. All authors contributed text and checked the manuscript. All authors agreed to submission of the manuscript.

9. Conflict of Interests

BC is an employee of MMV. No competing interests have been declared by the remaining authors.

10. Abbreviations

ABS asexual blood stage

BPS British Pharmacological Society

GtoPdb IUPHAR/BPS Guide to PHARMACOLOGY database

GtoMPdb IUPHAR/MMV Guide to MALARIA PHARMACOLOGY database

IUPHAR International Union of Basic and Clinical Pharmacology

mETC mitochondrial electron transport chain

MIR minimum inoculum of resistance

MMV Medicines for Malaria Venture

NC-IUPHAR Nomenclature Committee of the International Union of Basic and Clinical Pharmacology

qHTS quantitative high-throughput screen

SAR Structure-Activity Relationship

SMFA standard membrane feeding assay

TCP Target Candidate Profile

WHO World Health Organization

11. Figure and Table Legends

Figure 1. The *Plasmodium* lifecycle (timings in the image are specific to *P. falciparum*). Infection of the human host begins when sporozoites, a motile form of the parasite, are released into the bloodstream by an infected mosquito during its blood meal, before migrating to the liver and entering hepatocytes. The number of sporozoites introduced is very small (<100) and hepatic invasion is extremely rapid (<1 hour). Inside the hepatocyte, the parasite develops into a multinucleate form (schizont) by asexual reproduction with incomplete cytokinesis. Completion of cytokinesis produces merozoites, followed by rupture of the infected hepatocytes and release of large numbers of merozoites into the bloodstream between 2 and 16 days after the initial infection depending on *Plasmodium* species. *P. vivax* and *P. ovale* can remain in a dormant form (hypnozoite) in the liver and can be reactivated in the absence of a mosquito bite, causing the clinical symptoms of relapsing malaria. Once released into the bloodstream, the merozoites invade the red blood cells beginning the intraerythrocytic asexual blood stage (ABS) of the parasite's lifecycle. This is the symptomatic stage of the disease, with the repeated cycles (lasting between 24 and 72 hours depending on *Plasmodium* species) of growth, asexual replication, rupture and reinfection of erythrocytes leading to the clinical manifestations of malaria. A small number of merozoites commit to a cycle of sexual reproduction and differentiate into circulating sexual forms called gametocytes. When a mosquito takes a blood meal from an infected human, gametocytes are taken up and quickly transform into gametes. Male gametocytes divide, undergo exflagellation, and become numerous microgametes, while the female gametocyte converts to one large macrogamete. These fuse to form a diploid zygote that differentiates into a tetraploid, motile ookinete which can travel from the blood meal in the lumen of the midgut to the midgut epithelium and encyst on the basal lamina of the mosquito midgut. The ookinete then becomes an oocyst, which undergoes sporogonic asexual replication within the oocyst over the course of days before rupturing approximately 10-14 days after the initial blood meal and releasing thousands of fully formed sporozoites into the mosquito haemolymph. Some of these sporozoites reach the mosquito salivary glands, where they can repeat the parasite's lifecycle by infecting the next human host when the mosquito takes another blood meal. Image is copyright of MMV and used with permission.

Figure 2. A GtoMPdb detailed target page, here showing PfPKG. Associated external database identifiers, such as those to UniProtKB and PlasmoDb are included in the sections on 'Gene and Protein Information' and 'Database Links'. The page also includes, where available, selected 3D structures and links to the ChEMBL target page. The interaction tables list the ligands, species, assay descriptions, activity values, other parameters, and cite references. Each interaction can be expanded to view more details, including the relevant lifecycle stage of the interaction/target and assay description (example expanded rows are marked with an arrow).

Table 1. *P. falciparum* drug targets and key antimalarial ligands described in this article and included in the GtoMPdb, divided by lifecycle stage activity. Inhibitors that have advanced to clinical evaluation are indicated with an asterisk (*).

Table 2. *P. falciparum* targets, described in this article, with a summary of key information which includes level of validation, resistibility profile and druggability.

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Plasmodium Target	Validation	Resistance Potential	Druggability
Hexose transporter (PfHT1)	Phenotypic validation achieved using TH-PF01 and its derivatives and chemo-genetics approach (Jiang et al., 2020; Huang et al., 2021; Qureshi et al., 2020).	Unknown	Unknown
Bifunctional farnesyl/geranylgeranyl diphosphate synthase (PfFPPS/GGPPS)	Phenotypic validation achieved using MMV019313. and chemo-genetics approach (Gisselberg et al., 2018).	Resistible with an *in vitro* MIR ~10^8 suggesting moderate to low resistance risk (Gisselberg et al., 2018).	Druggable
Cleavage and polyadenylation specificity factor subunit 3 (PfCPSF3)	No evidence of chemical or genetic validation of PfCPSF3 as a target but molecular docking studies have demonstrated direct binding of an inhibitor (oxaborole) to this protein (Sonoiki et al., 2017).	Resistible with an *in vitro* MIR ~10^6 suggesting low to high resistance risk (Sonoiki et al., 2017).	Unknown because of no direct interaction shown between PfCPSF3 and the oxaborole compound
Dihydroorotate dehydrogenase (PfDHODH)	Clinically validated (DSM265 was shown efficacious in a Phase 2a clinical trial). Phenotypic validation achieved using DSM265 and other DHODH inhibitors and chemo-genetics approach (Ross et al., 2014).	Resistible with an *in vitro* MIR ~10^6 suggesting low to high resistance risk (Ross et al., 2014).	Highly druggable
N-Myristoyltransferase (PfNMT)	Chemical validation has been shown via selective inhibition of N-myristoylation using a myristoylate mimetic (Wright et al., 2014).	Resistible with an *in vitro* MIR ~10^6 suggesting low to high resistance risk (Schlott et al., 2019).	Highly druggable
Histone acetyltransferase GCN5 (PfGCN5)	Phenotypic validation achieved using acetyltransferase and bromodomain inhibitors such as L-45 and chemo-genetics approach (Cui et al., 2008; Srivastava et al., 2014; Jeffers et al., 2016; Moustakim et al., 2017).	Unknown	Highly druggable
non-SERCA-type Ca2+ -transporting P-ATPase (PfATP4)	Clinically validated (cipargamin progressed up to Phase 2a clinical trials). Phenotypic validation achieved using cipargamin and cell-based phenotypic screen for Na(+) homeostasis perturbation and intracellular alkalinization as well as chemo-genetics approach (Rottmann et al., 2010; Vaidya et al., 2014; Spillman et al., 2013; Lehane et al., 2014; Jiménez-Díaz et al., 2014).	Low level of resistance generated In vitro with mutation identified in the PfATP4 protein (Rottmann et al., 2010; Vaidya et al., 2014).	Highly druggable

Plasmepsin V (PfPMV)	Phenotypic validation achieved using WEHI-842 and chemo-genetics approach (Sleebs et al., 2014; Hodder et al., 2015).	Unknown	Druggable
Cytochrome b (PfCYTB)	Clinically validated (atovaquone is currently on the market). Phenotypic validation achieved using atovaquone and several others Cytb inhibitors and chemo-genetics approach. (Fry & Pudney, 1992; Painter et al., 2007; Srivastava et al., 1997). Structural validation of atovaquone bound to S. cerevisiae Cyt bc1 complex (Birth et al., 2014).	Highly Resistible. Resistance arose rapidly in atovaquone clinical trials and *in vitro* with an MIR ~10^{5-6} (Chiodini et al., 1995; Fisher et al., 2012; Fivelman et al., 2002; Goodman et al., 2016; Korsinczky et al., 2000; Looareesuwan et al., 1996).	Highly druggable
Bifunctional dihydrofolate reductase-thymidylate synthase (PfDHFR-TS)	Clinically validated (pyrimethamine and cycloguanil used in the field, P218 progressed to Phase 2a clinical trials). Phenotypic validation achieved using pyrimethamine, cycloguanil and P218 and chemo-genetics approach (Peterson et al., 1990; Yuthavong et al., 2005; Yuvaniyama et al., 2003).	*In vitro* resistance studies show mutations in several areas of DHFR. Resistance also arose rapidly when DHFR inhibitors were used in the field (Peterson et al., 1990; Yuthavong et al., 2005; Yuvaniyama et al., 2003).	Highly druggable
Elongation factor 2 (PfeEF2)	Phenotypic validation achieved using M5717 and chemo-genetics approach (Baragaña et al., 2015).	Resistible with an *in vitro* MIR ~10^{7} suggesting moderate resistance risk (Baragaña et al., 2015).	Highly druggable
Phosphatidylinositol 4-kinase beta (PfPI4Kbeta)	Clinically validated (MMV390048 progressed up to Phase 2a clinical trials) (Sinxadi et al., 2020; McCarthy et al., 2020). Phenotypic validation achieved using MMV390048 and chemo-genetics approach. (Paquet et al., 2017).	Resistible with an *in vitro* MIR ~10^{6} suggesting moderate resistance risk. Resistance-mediating mutations identified within kinase domain (McNamara et al., 2013; Paquet et al., 2017; Brunschwig et al., 2018).	Highly druggable
cGMP-dependent protein kinase (PfPKG)	Phenotypic validation achieved using ML-10 and other imidazopyridine analogues as well as MMV030084 and chemo-genetics approaches (Baker et al., 2017; Vanaerschot et al., 2020). *In vivo* validation achieved in humanized mouse model of *P. falciparum* infection (Baker et al., 2017).	Resistible with an in vitro MIR ~10^{9} suggesting low resistance risk.	Highly druggable
Cyclin-dependent-like kinase CLK3 (PfCLK3)	Phenotypic validation achieved using TCMDC-135051 and	Resistible with an *in vitro* MIR ~10^{9} suggesting low resistance risk.	Highly druggable

	chemo-genetics approach. *In vivo* validation achieved in mouse model of *P. Berghei* infection (Alam et al., 2019).	Resistance-mediating mutations identified in CLK3 outside of kinase domain (Alam et al., 2019).	
Phenylalanyl-tRNA synthetase alpha subunit (PfcFRS)	Phenotypic validation achieved using BRD7929 and chemo-genetics approaches (Kato et al., 2016).	No resistance has been raised so far. MIR >10^9 suggesting low resistance risk (Kato et al., 2016).	Druggable
Prolyl-tRNA synthetase (PfcPRS)	Phenotypic validation achieved using halofuginone and several other inhibitors as well as chemo-genetics approaches (Herman et al., 2015; Adachi et al., 2017; Okaniwa et al., 2021; Shibata et al., 2017; Tye et al., 2022).	Rapid selection of resistance was observed *in vitro* using halofuginone drug pressure, but no MIR has been characterized (Fagbami et al., 2019; Herman et al., 2014, 2015).	Highly druggable
Isoleucyl-tRNA synthetase (PfaIRS) and (PfcIRS)	Phenotypic validation achieved using mupirocin and thiaisoleucine and chemo-genetics approaches (Istvan et al., 2011).	Resistible with an *in vitro* MIR ~10^7 suggesting low resistance risk (Istvan et al., 2011).	Druggable
Tryptophanyl-tRNA synthetase (PfaWRS)	Phenotypic validation achieved using indolmycin and analogues and chemical approaches (Pasaje et al., 2016).	Unknown	Druggable
Histone deacetylase 1 (PfHDAC1)	Phenotypic validation achieved using several diverse chemotypes and chemo-genetics approaches (Huang et al., 2020; Li et al., 2021).	Unknown	Highly druggable
Proteasome subunit beta type-5	Phenotypic validation achieved using bortezomib and several of its analogues and chemo-genetics approaches (Li et al., 2016; Xie et al., 2018; Stokes et al., 2019).	*In vitro* resistance studies show mutations in the beta type-5 subunit confer 5-20-fold resistance to inhibitors (Xie et al., 2018; Stokes et al., 2019).	Highly druggable