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New targets for antimalarial drug discovery

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Abstract

Phenotypic screening methods have placed numerous preclinical candidates into the antimalarial drug-discovery pipeline. As more chemically validated targets become available, efforts are shifting to target-based drug discovery. Here, we briefly review some of the most attractive targets that have been identified in recent years.

Introduction

Malaria remains a devastating disease, impacting 40% of the world's population. Although vaccines have been licensed, which reduce the frequency and severity of malarial infections, their efficacy in preventing clinical disease is less than 40% in the 48+-month long-term period and those who develop disease still need to receive small-molecule treatment that remains the gold-standard approach to saving lives [1]. In addition, it is increasingly recognized that malaria chemoprophylaxis, alone or in combination with a malaria vaccine, can be effective at preventing severe malaria and decreasing the burden of the disease [2].

History of antimalarial drug discovery

In the face of emerging resistance to frontline antimalarial drugs, great progress has been made in filling the anti-malarial drug-discovery pipeline with new candidate molecules. However, at this stage, most of the later candidates have come from modifications to existing scaffolds or from phenotypic screening approaches. Given the expected attrition, it is important to keep the early discovery pipeline full. As much of the low-hanging fruit has been plucked from libraries that have been tested for whole-cell antimalarial activity and researchers are looking to take advantage of new technologies, such as cryoEM, for solving structures and a host chemically validated drug targets have now been discovered and to further explore target-enabled drug discovery. Target-based drug discovery, which includes structure-enabled drug discovery, is attractive because it is possible to better refine compounds and to increase potency and selectivity.

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The importance of chemical validation

Although the power of structure-enabled drug discovery has long been known, discovering high-quality targets that lead directly to new therapies is not as easy as it may seem, even for single-celled organisms. Once the *P. falciparum* genome sequence was completed in 2002 [3], many anticipated that many new drug targets would be revealed. Importantly, effort was made to scan the genome for both pathways and known druggable genes such as kinases and to compare the collected pathways and genes to see if they were found in the human genome, or might be more similar to bacterial pathways or enzymes. Given that malaria is caused by eukaryotic pathogens that are genetically more similar to humans than are bacteria or viruses, it was predicted that the best drug targets would be those that were found only in Plasmodium species or other apicomplexan parasites because it would be easy to achieve selectivity — to engineer compounds that would potently kill parasites but would have little impact on humans because the target was missing in the human. Accordingly, there was much focus on the proteins that were predicted to play a role in the apicoplast, an apicomplexan parasite organelle that is only found the phyla Apicomplexa. It was noted, for example, that the parasites likely used a type-II fatty acid biosynthesis pathway and it was predicted that proteins in this pathways would be attractive targets [4].

However, although some drug-discovery work ensued, in the years following the release of the genome sequence, effort was made to determine essentiality. It soon became clear that many of some attractive, often bacteria-like targets, were not actually needed for cell viability. In fact, in a landmark paper, Yeh and coworkers showed that the parasite could be cured of its apicoplast organelle and not immediately die [5]. These data indicated that any drug that targeted the apicoplast was not likely to work quickly and might lead to less-desirable medicines, especially when drugs are needed to provide rapid symptomatic relief and prevent deaths. In other cases, attractive targets were demonstrated to be only essential in the stages of the malaria-parasite life-cycle that were not associated with symptomatic disease, and thus targeting these proteins would likely not reduce symptomatic infection [6,7]. Target-based drug discovery on anything other than very-well-validated targets, such as DHFR, the target of the antifolate, pyrimethamine, largely fell out favor.

An era of phenotypic screening

It was soon discovered that vast collections of chemical compounds could be readily tested for antimalarial activity and that new scaffolds and chemical matter could be discovered that both potently killed malaria parasites [8,9]. Drug discovery has been largely focused on the compounds from these screens over the next decade. Numerous attractive starting points were discovered, resulting in multiple new clinical candidates, some of which are likely to be eventually licensed. Although in most cases some understanding of their mechanism of action has been discovered, most of these candidates were optimized using a ‘black box’ approach that focused on making a large number of compounds looking for ones that gave an increase in activity against blood-stage parasites while minimizing the toxicity and improving pharmacokinetic properties (Figure 1). This laborious approach nevertheless has yielded multiple new candidates such as KAE609, KAF156, and SJ733, which are summarized on the Medicines for Malaria Venture website (https://www.mmv.org/).

Although some are likely to be licensed, questions about their mechanism of action remain (Figure 2).

A return to target-based drug discovery

Target-based drug discovery began to look more attractive once methods were developed for finding the targets of phenotypic screening hits. The most fruitful method for doing this was using *in vitro* evolution and whole-genome sequencing (reviewed in [10]). Compounds that rapidly killed malaria parasites, but which were relatively nontoxic, including the clinical candidates described above, were first chosen. Then parasites were slowly exposed to increasing concentrations of the compound, until resistance emerged. Then, the mutations that caused resistance were pinpointed by comparing the drug-resistant genome to the original genome, initially using scanning microarray methods but later, by whole-genome sequencing. A plethora of chemically validated targets were discovered using this method (Table 1) and assays and protein structures are available for many. These targets were considered more attractive because it was known that their inhibition would result in parasite killing, that they were by default, druggable, and were predicted to harbor pockets that would accommodate a small-molecule tool compound. Now, many of these targets are progressing into structure-guided drug-discovery activities. A list of some of the most intriguing targets are given in Table 1 and some are described below. Interestingly, and perhaps not unexpectedly, most targets that have been discovered with reverse chemical genetics have been shown to be attractive targets in other species.

The proteasome

The proteasome is a very-well-validated target in many species [11,12]. The *P. falciparum* proteasome has defined a very low risk of resistance with vinyl sulfone peptide inhibitors that bind the β2 or β5 subunits [13,14] and the natural product, carmaphycin [15]. One uniquely attractive feature is that these inhibitors synergize with artemisinin derivatives, in both sensitive and artemisinin-resistant parasites, including against artemisinin-resistant parasites [16]. Selectivity and antiplasmodial potency have also been excellent. Another attractive feature is the availability of cryoEM structures. Challenges associated with tackling the proteosome will be determining whether inexpensive selective molecules that are orally bioavailable and have a good half-life can be created. The proteosome is also essential in parasite liver stages, suggesting that proteosome-targeting medicines might be useful for malaria chemoprophylaxis.

P. falciparum acetyl-CoA synthetase

Two new classes of inhibitor that target acetyl-CoA synthetase (AcAS) have been discovered, validating this enzyme as another attractive antimalarial target. Depletion of acetyl-coA gives a variety of metabolic effects and also appears to impact histone acetylation. MMV019721 and MMV084978 were found to target *P. falciparum* AcAS [••17].

Proteases

Proteases have long been pursued as attractive drug targets. Proteases that were initially identified using functional genomic approaches have largely not yielded molecules that have potent on-target cellular activity. For example, efforts to drug the malaria falcipains were largely unsuccessful [18,19], despite excitement about this class early on. More recently, proteases that were discovered as targets from potent phenotypic hits have shown better druggability. There has been extensive interest in the plasmepsin family of Plasmodium aspartic proteases. WM382 is a dual inhibitor of plasmepsin IX and X that are essential aspartic proteases required for parasite invasion and release [20,••21].

Kinases

Kinases are one of the most well-known target classes in oncology. Protein kinase G (PKG) remains an attractive target for antimalarial drug discovery and while it was initially identified and explored using functional genomic approaches, recent work has identified potent tool compounds such as inhibitor MMV003084. This work shows that PKG had a minimal resistance liability and potent prophylactic, anti-asexual blood-stage and transmission-blocking activity [22]. PKG is a key mediator of parasite invasion of and egress from host cells [23,24]. A concern with kinases is that the noted low-resistance potential is because compounds bind in the catalytic site. *Pf*CLK3, a kinase that participates in pre-mRNA splicing, was identified by a functional genomic method and target-based screen of the Tres Cantos Anti-Malarial Set [9,25]. On-target activity was confirmed with subsequent *in vitro* evolution studies [26].

Aminoacyl tRNA synthetases

Aminoacyl tRNA synthetases (aaRSs) have long been known to be an attractive class of target for other infectious diseases. By 2022, inhibitors have been discovered or developed against all aaRS targets. In other species, there are generally 4 categories of aaRS inhibitors: 1) catalytic-site inhibitors, 2) editing-site inhibitors, 3) allosteric-site inhibitors, and 4) protein–protein-interaction inhibitors (Figure 1) [27,••28]. Various starting points have been pursued, such as mimicking one of the aaRS substrates (ATP, amino acid, and tRNA 3’-tail), creating an intermediate analog, adding a reactive species (e.g. boron in AN2690) to a small molecule, such that covalent bonds can be formed with protein or substrate nucleophiles (e.g. hydroxyl group on 3’-tRNA), and creating analogs of natural product inhibitors (e.g. pseudomonic acid and mupirocin). Intermediate-based inhibitors (IBIs) have been some of the most potent inhibitors developed with binding affinities in the picomolar-to- nanomolar range [29]. These IBIs, mostly in the form of aminoacyl sulfamates and aminoalkyl adenylates, have poor uptake by cells or are easily hydrolyzed and therefore have not been further developed [29]. An allosteric steric-inhibitor series MSR02 based on ATP analogs was discovered through X-ray crystallography and supported through biochemical analysis.

aaRSs can generally be categorized as either class-I (Rossman fold) or class-II (seven stranded β-sheet) enzymes based on the structures of their active sites [27,30,31]. Within

Plasmodium spp., there are a total of 37 aaRSs distributed between the cytoplasm, apicoplast, mitochondria, and nucleus [31,32]. In comparison, there are 38 aaRSs total within humans [32]. Four plasmodial aaRSs (ARS, GRS, TRS, and CRS) are dually localized to both the apicoplast and cytosol [31,33]. There are three different PRS enzymes separately localized to the mitochondria, apicoplast, and cytoplasm [31]. QRS is only found in the cytoplasm [31]. All the remaining other aaRSs have different enzymes in the apicoplast and cytoplasm [31]. Despite the similarities between *Homo sapiens* and *Plasmodium spp*. aaRSs, several compounds have been found to be specific for the parasite form, which opens the possibility for developing other selective inhibitors [27,34,35].

aaRSs have proven to be an abundant class of targets for malaria. The importance of aaRSs as antimalarial drug targets began to emerge when it was discovered that cladosporin, a compound with antimalarial activity, acted via inhibition of lysyl tRNA synthetase [35]. Since then, a host of other highly validated aaRSs have been discovered as the targets of phenotypic screening hits' *in vitro* evolution. The most well-validated set includes FRS, PRS, YRS, and cytoplasmic IRS. Structures are available for *Plasmodium* FRS, LRS, KRS, RRS, PRS, YRS, and WRS. Most, if not all aaRSs that have been matched to a chemical probe appear to be essential for liver-stage activity. A potential weakness is the perceived speed of action. A recent exciting development is the demonstration that YRS is the target of ML901, a compound with potent blood- and liver-stage activity. Structural studies show that ML901, a adenylate analog, acts via a reaction-hijacking method [••36]. More work will be needed to determine if any can be progressed into drug candidates.

Diversity of binding sites

Good targets can have multiple different compound-binding sites and each binding site may have different resistance liabilities. Within the aaRS family of inhibitor-target interactions, different compounds have been shown to bind to one (tavaborole), two (halofunginone), or three sites (borrelidin) on their target aaRS [37]. If lessons may be learned from the development of antibiotics, simultaneously inhibiting two or more aaRSs may decrease the resistance liability [38].

Drug resistance and structure-guided drug discovery

A criticism of structure-guided drug discovery is that there may be a higher tendency to develop resistance. Indeed, dihydroorotate reductase is often cited as a key example of the power of structure-guided drug discovery, but resistance to DHODH has been known to emerge rapidly and key alleles known to cause resistance in the laboratory were found in patients treated with DSM265 in Phase-II clinical trials [39]. More work will be needed to determine if this is a feature of DHODH or if the selectivity that can be achieved in target-based drug design comes at the expense of resistance. This concern however may be unfair, especially when a structure and genomes of resistant phenotypes are available. The crystal structure of DHFR was used to guide the development of the P218 inhibitor and to mitigate the DHFR-resistance issues [40,41]. Therefore, it is likely that newer-generation DHODH inhibitors can be designed that will not have the same resistance liabilities as DSM265.

Conclusions

Although work continues on essential targets that are druggable, but which are not associated with a mid-to-low nanomolar-tool compound, there is a danger that limits to potency may be met if the target is not as critically essential. Highly potent compounds are likely needed to achieve physiologically relevant exposure levels in humans. Ideally, orally bioavailable compounds with long half-lives are of interest, but with the advancement of newer drug-delivery vehicles, for example, amphiphilic nanodroplet injections, physiological levels can be more readily achieved over longer periods of time that delays first-pass metabolism in the liver and rapid clearance by glomerular filtration in the kidneys. Regardless of the methods employed to discover and develop a drug, the complexity of efficacy and toxicity may not be elucidated, until the drug reaches human clinical trials. Relatively few drugs that have come from target-enabled drug discovery have progressed into human trials and licensing. In addition, it is possible that the increased selectivity that can be engineered with structure-enabled drug discovery may come with increased resistance risk and that the dirty compounds that interact with multiple targets have the lowest risk. As a corollary, dirty compounds have also long been thought of as the driver for unforeseen off-target toxicity effects. However, perhaps, this will change with the recent progress in structural biology combined with genomic information from resistant-mutant phenotypes. Drawing lessons from HIV antiretroviral treatment, prolonged exposure to a single drug from a multidrug multitarget regimen can lead to resistance. The combination of drugs, particularly those that are composed with newer-generation integrase-strand inhibitors (INSTIs), has a much higher barrier to resistance. Stronger target interactions with newer-generation INSTIs demonstrate limited cross-resistance and select for alternative mutational phenotypes with only minimally to moderately reduced susceptibility [42–44]. Thus, combining resistant-mutant genomic and phenotypic results with essentiality and structure-based drug design may allow for the tailoring of next-generation antimalarial compounds that account for *in vivo* selective pressures of resistance.

Declaration of interest

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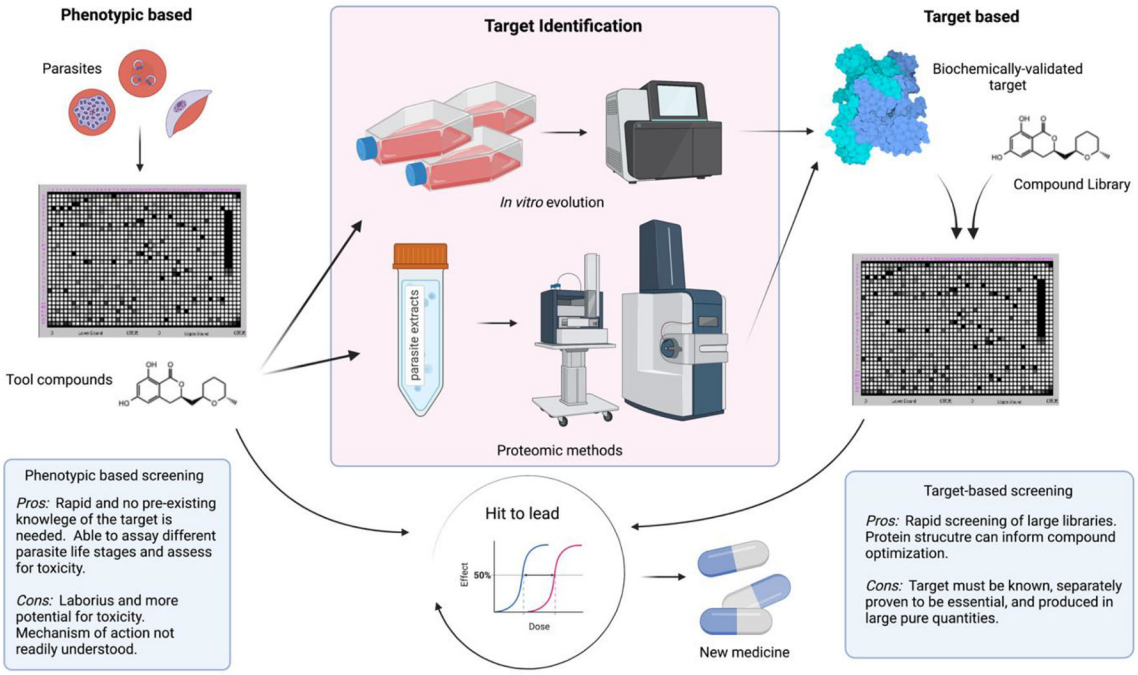

Figure 1.

Phenotypic and Target-based approaches to antimalarial drug discovery with advantages and disadvantages of different methods. Both phenotypic and target-based methods are able to screen large compound libraries rapidly. Potent inhibitory compounds identified through phenotypic-based screens can be used to identify targets through *in vitro* evolution with subsequent whole-genome sequencing analysis for mutants or through various proteomic methods, such as label-based or pull-down assays. Hits can be passed through both phenotypic and target-based methods for optimization.

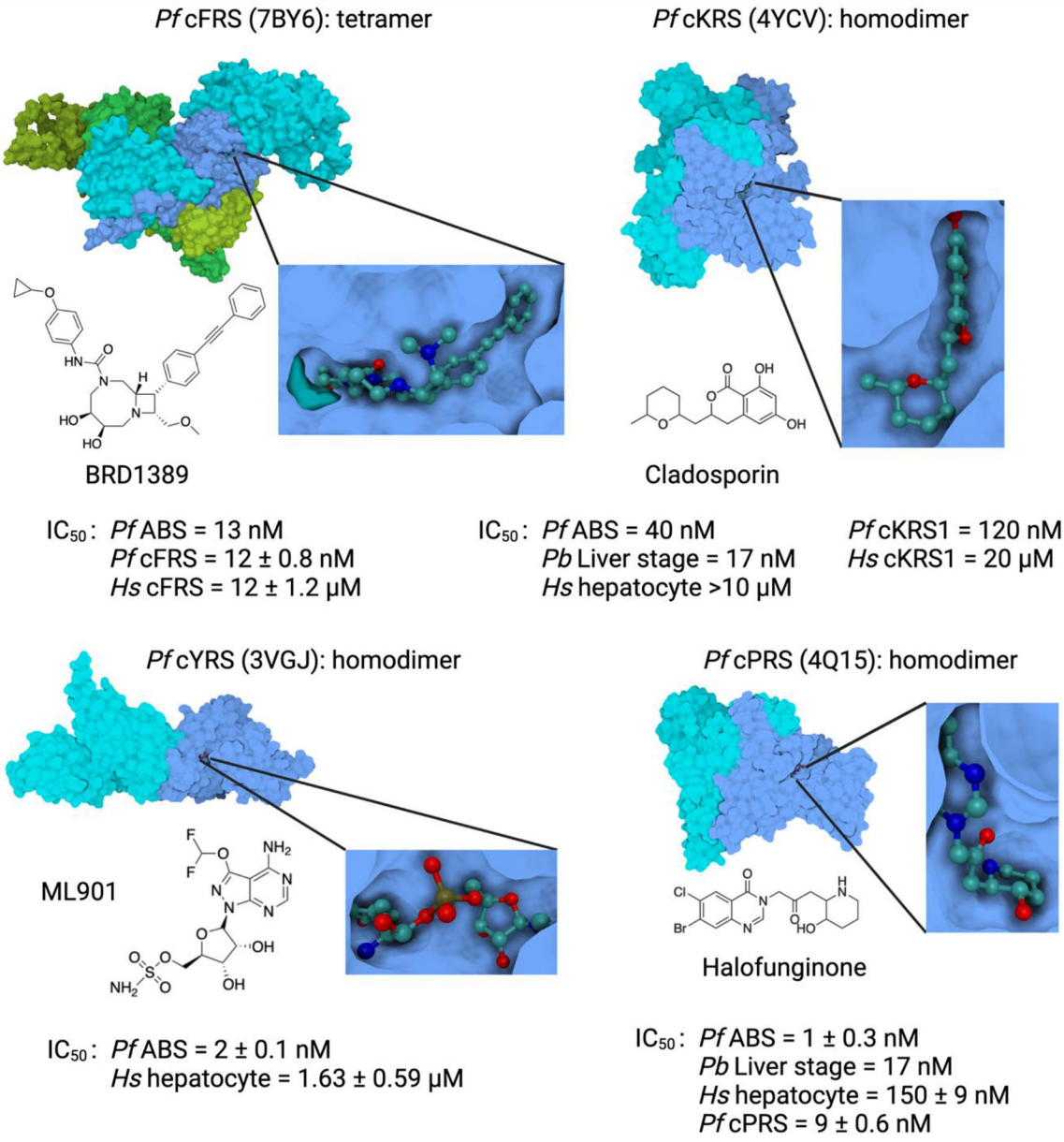

Figure 2.

X-ray crystallographic models of aaRSs complexed with inhibitors* that have biochemically validated each protein target through *in vitro* evolution. *P. falciparum* cFRS, cKRS, cYRS, cPRS are complexed with BRD1389, cladosporin, ML901, and halofunginone, respectively. Most x-ray structures solved without tRNA which may play a critical role. *PfYRS structure with ML901 to be released soon and 3VGJ shown in its place. *Pf* (*P. falciparum*), *Hs* (*Homo sapiens*), *Pb* (*Plasmodium* berghei), cFRS (cytoplasmic phenylalanine tRNA synthetase), cKRS1 (cytoplasmic lysine tRNA synthetase 1), cYRS (cytoplasmic tyrosine tRNA synthetase), cPRS (cytoplasmic proline tRNA synthetase) [••36, ••45–49].

Table 1

Select high-value, chemically validated targets for malaria. Listed tool compounds were used to establish on-target activity using reverse chemical genetics, usually using *in vitro* evolution and whole-genome sequencing.

Gene	Product Description	Tool Compound	Structure	Ref
AcAS (PF3D7_0627800)	Acetyl-CoA synthetase	MMV019721 MMV693183		[50–52]
ACS11 (PF3D7_1238800)	Acyl-CoA synthetase	MMV019719		[53]
ATP4 (PF3D7_1211900)	Non-SERCA-type Ca2+ -transporting P-ATPase	KAE609, SJ733		[10]
cIRS (PF3D7_1332900)	Isoleucine tRNA synthetase	MMV1081413, thiaisoleucine		[10,54]
CLK3 (PF3D7_1114700)	Cyclin-like kinase 3	TCMDC135051		[26]
CPSF3 (PF3D7_1438500)	Cleavage and polyadenylation specificity factor subunit 3, putative	AN13762		[••55]
CYTB (mal_mito_3)	Cytochrome bc1 complex	Atovaquone, DDD01061024, MMV008149, MMV1427995, MMV1432711	4PD4	[10,54]
DHFR-TS (PF3D7_0417200)	Bifunctional dihydrofolate reductase-thymidylate synthase	MMV027634(TS), pyrimethamine (DHFR), P218	3QGT	[56]
DHODH (PF3D7_0603300)	Dihydroorotate dehydrogenase	DSM265, BRD7539, BRD9185, DSM1	4CQ8	[10,57,58]
DPCK (PF3D7_1443700)	Dephospho-CoA kinase, putative	Amb3377585, STK740987		[59–61]
eEF2 (PF3D7_1451100)	Elongation factor 2	M5717 (DDD498)		[10]
Ftbeta (PF3D7_1147500)	Protein farnesyltransferase subunit beta	MMV019066, BMS-388891		[10]
GCN5 (PF3D7_0823300)	Histone acetyltransferase GCN5	SGC-CBP30	PQNS	[62]
GGPPS (PF3D7_1128400)	Geranylgeranyl pyrophosphate synthase, putative	GDI11–078A, MMV019313	3CC9	[63]
HT1 (PF3D7_0204700)	Hexose transporter	C3361	6M2L	[64,65]
KRS1 (PF3D7_1350100)	Lysine tRNA synthetase	Cladosporin	6KAB 4H02	[35,66]
NCR1 (PF3D7_0107500)	Lipid/sterol:H+ symporter	MMV009108, MMV019662, MMV028038		[67]
NMT (PF3D7_1412800)	Glycylpeptide N-tetradecanoyltransferase (NMT)	IMP-1002	6MB1	[68]
PFK9 (PF3D7_0915400)	6-phosphofructokinase	PS-3		[69]
PheRS (PF3D7_0109800)	Phenylalanine--tRNA synthetase (α subunit)	BRD1095, BRD3444	7BY6 7DPI	[70]
PI4K (PF3D7_0509800)	Phosphatidylinositol 4-kinase	BRD9685, MMV390048, KAI407		[71,72]
PKG (PF3D7_1436600)	cGMP-dependent protein kinase	ML10	5DYK	[24]
PMX (PF3D7_0808200)	Plasmepsin X	WM382	7RY7	[21]
PNP (PF3D7_0513300)	Purine nucleoside phosphorylase	DADMe-ImmG		[73]
PPPK-DHPS (PF3D7_0810800)	6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK)–dihydropteroate synthase (DHPS)	Pterin/pHBA, Sulfadoxine-dihydropteroate	6KCM 6JWX	[74]
Proteasome (PF3D7_1011400)	Proteasome subunit beta type-5	Bortezomib	7LXT	[34]
Proteasome (PF3D7_1328100)	Proteasome subunit beta type-7, putative	WLW	5FMG	[75]
Proteosome (PF3D7_0518300)	Proteasome subunit beta type-1, putative	WLL	5FMG	[75]
PRS (PF3D7_1213800)	Proline--tRNA synthetase	Compound 1S, Halofuginone	4TWA 4WI1	[10,76]

Gene	Product Description	Tool Compound	Structure	Ref
TyrRS (PF3D7_0807900)	Tyrosine--tRNA synthetase	ML901	7ROR 3VGJ	[••36]