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Review

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Using *in Vitro* Evolution and Whole Genome Analysis To Discover Next Generation Targets for Antimalarial Drug Discovery

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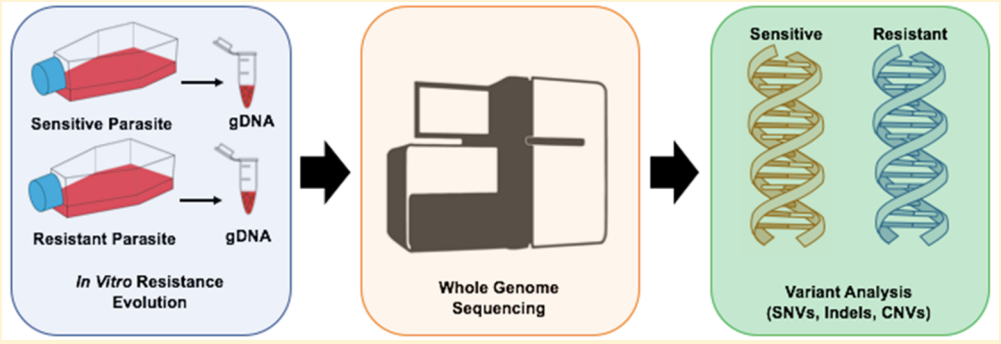
Supporting Information



ABSTRACT: Although many new anti-infectives have been discovered and developed solely using phenotypic cellular screening and assay optimization, most researchers recognize that structure-guided drug design is more practical and less costly. In addition, a greater chemical space can be interrogated with structure-guided drug design. The practicality of structure-guided drug design has launched a search for the targets of compounds discovered in phenotypic screens. One method that has been used extensively in malaria parasites for target discovery and chemical validation is *in vitro* evolution and whole genome analysis (IVIEWGA). Here, small molecules from phenotypic screens with demonstrated antiparasitic activity are used in genome-based target discovery methods. In this Review, we discuss the newest, most promising druggable targets discovered or further validated by evolution-based methods, as well as some exceptions.

KEYWORDS: *malaria, phenotypic screening, selections, resistance*

TARGET DISCOVERY: ON THE ROAD TO MALARIA ERADICATION

Malaria is the most prevalent parasitic disease in man. The World Health Organization (WHO) estimated 216 million cases and 445 000 deaths in 2016 globally, out of which 91% were in Africa alone and 99% were due to *Plasmodium falciparum* infections (WHO, World Malaria Report 2017). Most deaths occur in children under the age of five.

Malaria in humans is caused by 6 different species of *Plasmodium*, where *P. falciparum* causes the deadliest form of infection and *P. vivax* is the most widespread (WHO, World Malaria Report 2017). *Plasmodium* infection of the human host begins with the transmission of the parasite through the bite of an infected mosquito (Figure 1). Parasites immediately travel through the bloodstream, invade hepatocytes, and replicate asexually, resulting in thousands of merozoites. In *P. vivax* and *P. ovale*, sporozoites might form "hypnozoites", a dormant form, which can be activated weeks to months later and cause relapse of infection. The exoerythrocytic cycle in liver transcends into the intraerythrocytic stage with asexual replication in red blood cells. The lysis of red blood cells to release more parasites causes the symptomatic disease and can be fatal.[1] Some intraerythrocytic stage parasites can develop into "gametocytes", the sexual stage, that can subsequently be transmitted into the mosquito to perpetuate the infection cycle.

Artemisinin combination therapy (ACT) is the first line of treatment for uncomplicated falciparum malaria. To mitigate issues with their short half-lives and help avoid development of drug resistance, artemisinin (ART) derivatives are administered with partner drugs such as amodiaquine, piperaquine, lumefantrine, mefloquine, sulphadoxine−pyrimethamine, and pyronaridine, which have separate mechanisms of action and potentially different half-lives. The emergence of parasites showing resistance to ACTs in the Greater Mekong region could bring about a reversal in malaria control and undermine the feasibility of elimination, as has been reviewed previously.[3,4] For this reason, the consensus in the medical and scientific community is that there is a great need for the development of new classes of antimalarial medicines if malaria elimination is to

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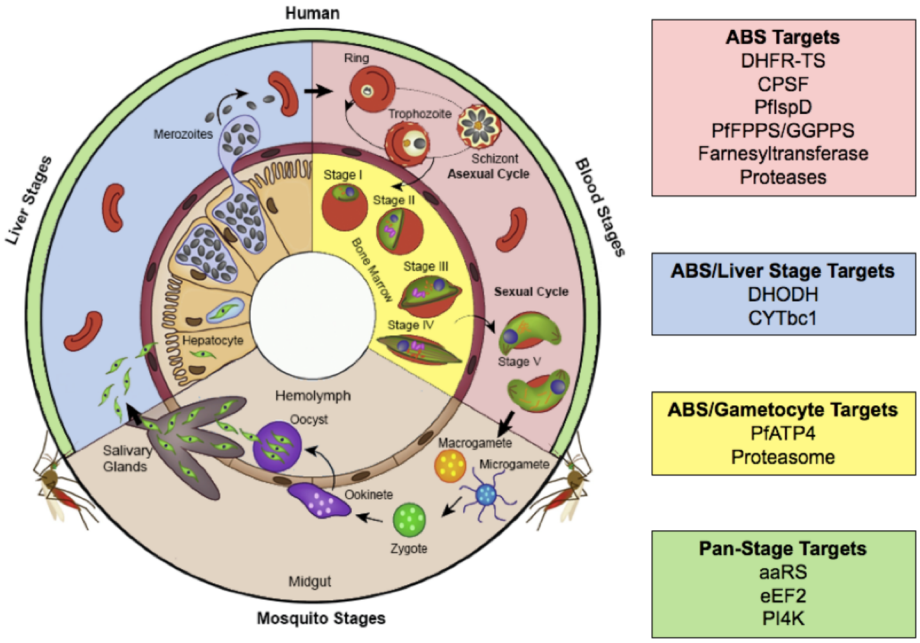

Figure 1. *Plasmodium* life cycle with chemically validated targets for chemotherapeutic intervention divided by the stages in which they have demonstrated antimalarial activity. Adapted with permission from Nilsson, S. K., Childs, L. M., Buckee, C., and Marti, M. (2015) Targeting Human Transmission Biology for Malaria Elimination. *PLoS Pathog. 11* (6), e1004871. DOI: 10.1371/journal.ppat.1004871 (ref 2). Copyright 2015 Nilsson et al.

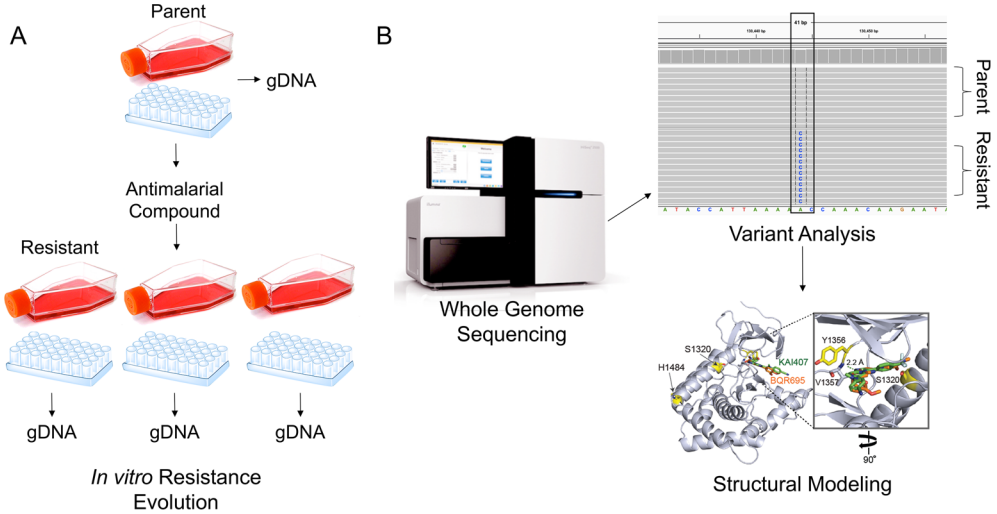

Figure 2. Overview of IVIEWGA process. (A) A clonal aliquot (obtained by limiting dilution in a microtiter plate) of a sensitive parent *P. falciparum* strain is cultured in triplicate and subjected to the selective pressure of an antimalarial compound using a slow ramping or pulse method. Upon successful generation of resistant parasite bulk cultures, clones are isolated using limiting dilution and retested for resistance. (B) Whole genome sequencing is performed using gDNA extracted from the parent and resistant clones. Bioinformatic analysis calls variants between the parent and resistant lines to determine which mutations confer resistance. Generally, mutations that arise in multiple, independently derived clones are prioritized for further validation, which may include structural modeling, molecular docking simulations, and/or reverse genetics techniques.

be achieved.[5] The ideal drug would target multiple stages of the parasite's life cycle, block or prevent transmission, or act against *Plasmodium vivax* liver hypnozoites. Because there are very few known drug targets, a major focus of the past decade has been the search for novel, chemically validated targets that can be starting points for development of next-generation antimalarials.

A method that has been used with great success to find new drug targets is *in vitro* evolution and whole genome analysis (IVIEWGA). In this method (Figure 2), parasites are exposed to sublethal concentrations of compounds that have shown antiparasitic activity. These compounds typically are identified in phenotypic screens, and some are active throughout the parasite lifecycle. To identify the genetic basis of their resistance, the genomes of the resistant clones are analyzed using tiling microarrays or more typically using whole genome sequencing (WGS) and are compared to the sensitive parent clone. In many cases, newly emerged genomic lesions are found in genes that are predicted to encode the targets. Modeling, crystallography, and further functional studies can confirm that the gene product is the actual target and resistance is not conferred nonspecifically by a multidrug resistance gene (e.g., a

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transporter that is frequently observed). The advantage of targets discovered through this method is that they are, by default, chemically validated and druggable, though complementary studies may be needed to further validate the targets and their clinical relevance. This Review discusses some of the many new drug targets (Table 1) that have been discovered or reconfirmed using this method.

Table 1. Chemically Validated Targets for *Plasmodium falciparum*

drug target	inhibitor	references
PfATP4	KAE609 (Cipargamin), GNF-Pf-4492, PA21A092, SJ733	7, 9, 12, 13
aminoacyl-tRNA sythetases		19−21, 23, 28, 30
prolyl-tRNA synthetase	febrifugine, halofuginone	
lysyl-tRNA sythetase	cladosporin	
isoleucyl-tRNA synthetase	mupirocin, thiaisoleucine	
phenylalynyl-tRNA sythetase	BRD1095	
threonyl-tRNA sythetase	borrelidin	
tryptophanyl-tRNA synthetase	indolmycin	
translation elongation factor 2	DDD107498	39
dihydroorotate dehydrogenase	DSM265, DSM421, DSM1, DSM74, Genz-669178, Genz-666136, BRD7539, BRD9185	21, 43, 44, 46, 51
cytochrome bc1	atovaquone, decoquinate, tetracyclic benzothiazepine, GW844520, GSK932121	52, 54
dihydrofolate reductase-thymidylate synthase	pyrimethamine, cycloguanil, MMV027634	59
phosphatidylinositol 4-kinase	KDU691, MMV390048, KAI407, BQR695, BRD73842	9, 57, 63, 100
cleavage and polyadenylation specificity factor	AN3661	64
isoprenoid biosynthesis	fosmidomycin, MMV008138, MMV019313	68, 70, 71
farnesyltransferase	BMS-388891, MMV019066	59
proteasome inhibition	bortezomib, carfilzomib, carmaphycin B, WLL-vs	85, 87
proteases		102, 103, 105
aspartic proteases		
plasmepsin I, II, IV	2-aminoquinazolin-4(3*H*)-ones	
plasmepsin V	aminohydantoins	
cysteine protease	WEHI-842, WEHI-916	
metalloprotease	actinonin	

P. FALCIPARUM P-TYPE CATION ATPase

One of the first novel targets that was discovered with IVIEWGA was PfATP4, a *P. falciparum* p-type cation ATPase. It was first identified as the target of the spiroindolone compound, KAE609 (also called NITD609), which is now commercially called Cipargamin.[6] KAE609 is highly active against both blood-stage *P. falciparum* and *P. vivax* field isolates and culture-adapted isolates with single-digit nanomolar IC50s.[7] It is also active against gametocyte and oocyst development in mosquitoes which would enable this compound to block transmission. Parasites generated from *in vitro* resistance evolution using KAE609 and analyzed with microarrays display both copy number variants (CNVs) and single nucleotide variants (SNVs) in various positions of PfATP4 (Table 2). These mutations were later proven to confer resistance in *P. falciparum* and result in at least a 10-fold increase in IC50 values.[7,8] KAE609 has progressed through Phase I and IIa clinical trials,[6] and it remains to be seen how far it will advance in drug development.

Although there are concerns that PfATP4 could be involved in multidrug resistance,[9] it is generally accepted to be a good drug target. All cells must maintain low cytosolic Na^+ concentration to survive, and PfATP4 mediates active extrusion of Na^+ from the parasite to maintain low-[Na^+]/high-[K^+] in the host cell cytosol upon infection.[10] PfATP4 is found in the *Plasmodium* plasma membrane, and the gene is expressed in all asexual blood stages of *Plasmodium*.[8] PfATP4 has been suggested to be the mechanism of action for multiple potential antimalarial candidates which have been shown to disturb Na^+ regulation and homeostasis in the parasite.[8,11]

Shortly after the discovery that PfATP4 was the target of the spiroindolones, evolution studies showed that it was involved in resistance to several other compound classes, including the aminopyrazoles, pyrazoleamides, and dihydroisoquinolones, all compounds identified by phenotypic screening.[9,12,13] Like spiroindolones, aminopyrazoles target both the asexual blood stages of the parasite and oocyst formation in mosquitoes, thus preventing transmission of the parasite. Mutations in PfATP4 acquired after treatment with the aminopyrazole GNF-Pf-4492 also confer resistance to spiroindolones. PfATP4 mutations found in resistant parasite lines treated with either small molecule are present in the region of the predicted transmembrane (TM) domain, suggesting they might exhibit a shared structure−function relationship. One of the selections also yielded a point mutation in the putative ADP/ATP transporter protein (PF3D7_1037300) localized to the inner mitochondrial membrane, which may be a compensatory mutation.[6]

Like all other PfATP4 inhibitors, pyrazoleamides kill both *P. falciparum* asexual laboratory isolates and *P. falciparum* and *P. vivax* field isolates, at very low nanomolar IC50 values. In addition, the lead compound PA21A092 inhibits gametocyte development similar to KAE609.[13] PA21A092 enables rapid increase in cytosolic Na^+, and since it does not affect resting cytosolic Ca^{2+} levels, the parasites swell due to the osmotic uptake of water.[13]

The dihydroisoquinolone SJ733 also originated with a cellular screen. Selections with SJ733 resulted in resistant parasite lines that displayed an IC50 increase of 2- to 750-fold, and WGS detected mutations in PfATP4 not seen previously in KAE609-resistant lines. Despite the different location of the mutations, SJ733-resistant lines are cross-resistant to spiroindolones.[12] SJ733 causes an increase in the parasite's cytosolic Na^+ level and concurrent alkalinization of the cytosol which seems in line with the loss of PfATP4 activity.[12] The overrepresentation of PfATP4 inhibitors in many phenotypic screens may be because PfATP4 is a high-value target whose inhibition results in rapid death, even at low inhibitor concentrations.[12] Additionally, its localization to the parasite plasma membrane may make it a more accessible target since membrane permeability of inhibitors would not be necessary for drug efficacy. Despite its significance, no crystal structure of PfATP4 or biochemical assay is available, and thus, there are still many open questions about how these compounds act and whether *pfatp4* may also function as a resistance gene.

AMINOACYL-tRNA SYNTHETASES

A well-known class of targets in other species, which has become increasingly relevant due to multiple discoveries using IVIEWGA in *Plasmodium*, is the tRNA synthetase family.

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Table 2. Drug Target Mutations Observed in *P. falciparum* IVIEWGA Studies[a]

drug target	inhibitor	gene ID	mutations
PfATP4	KAE609	PF3D7_1211900	Asp1247Tyr; Pro990Arg; Thr418Asn; Ile398Phe
	GNF-Pf-4492		Ala211Thr; Ile203Leu; Pro990Arg; Ala187Val
	PA21A092		Val178Ile
	SJ733		Val415Asp; Leu350His; Pro412Thr; Pro966Ser; Pro966Thr
aaRs	thiaisoleucine	PF3D7_1332900	Leu810Phe
	mupirocin	PF3D7_1225100	Pro1233Ser
	cladosporin	PF3D7_1350100	amplification
	halofuginone	PF3D7_1213800	Leu482His; Leu482Phe
	BRD1095	PF3D7_0109800	Leu550Val; Met316Ile; Gly512Glu; Val545Ile
eEF2	DDD107498	PF3D7_1451100	Glu134Gly; Glu134Asp; Tyr186Asn; Ala482Thr; Ile183Thr; Thr185Ile; Pro754Ala; Pro754Ser; Leu755Phe; Ser474Arg
DHODH	DSM1	PF3D7_0603300	amplification
	DSM74		Glu182Asp; Leu531Phe
	DSM265		Gly181Cys
	Genz-669178		Phe188Ile; Phe188Leu
	Genz-666136		Glu182Asp; Phe227Ile
Cytbc1	atovaquone	mal_mito_3	Met133Val; Met133Ile; Leu144Ser; Phe267Val
	decoquinate		Ala122Thr; Tyr126Cys
	tetracyclic benzothiazepine		Gly131Ser; Phe264Leu
DHFR-TS	MMV027634	PF3D7_0417200	Gly378Glu; Ile403Leu; His551Asn
PI4K	KAI407	PF3D7_0509800	His1484Tyr
	BQR695		Ser1320Leu; Tyr1356Phe
CPSF	AN3661	PF3D7_1438500	Thr406Ile; Tyr408Ser; Thr409Ala; Asp470Asn; His36Tyr
PfDXR	fosmidomycin	PF3D7_1467300	amplification
PfIspD	MMV008138	PF3D7_0106900	Glu688Gln; Leu244Ile
PfFPPS/GGPPS	MMV019313	PF3D7_1128400	Ser228Thr
farnesyltransferase	BMS-388891	PF3D7_1147500	Asn315Y; Gly612Ala
	MMV019066		Ala515Val; Ala515Thr

[a]References are as given in Table 1.

During protein synthesis, aminoacyl-tRNA synthetases (aaRS) accurately pair the cognate tRNAs with their corresponding amino acids hence defining the genetic code.[14] Translation fidelity is attained by two events: correct pairing of an amino acid with the matching tRNA and the accurate selection of the charged aminoacyl-tRNAs on the ribosome. Functional aspects of aaRSs and their role in keeping translational errors in check have been reviewed previously.[15,16]

Apicomplexan parasites have nuclear, apicoplast, and mitochondrial genomes which each require charged tRNAs for translation. To synthesize its proteome, the malaria parasite utilizes a combined array of 36 aaRSs (instead of the theoretical 60 aaRSs for the three compartments).[17] Of these, 16 aaRSs are present exclusively for cytoplasm and 15 nuclear-encoded aaRSs are present exclusively for the apicoplast. The mitochondria is speculated to import charged tRNAs from the cytoplasmic tRNA pool and only harbors an enzymatically active mitochondrial phenylalanyl-tRNA synthetase, a feature unique to *Plasmodium*.[18] There are multiple sites that can provide binding sites for small molecule inhibitors, including the editing site, aminoacylation pocket, and the tRNA binding region.[14,19−21]

■ ISOLEUCYL-tRNA SYNTHETASE

The first hints that aaRSs might be important drug targets for antimalarials came from IVIEWGA studies with two known inhibitors of isoleucyl-tRNA synthetase, thiaisoleucine and mupirocin.[19] *P. falciparum* contains two isoleucyl-tRNA synthetases, one in the cytoplasm and the other in the apicoplast.[22] Mupirocin, a natural product that is clinically used as an antibiotic, was found to inhibit the *P. falciparum* apicoplastic isoleucyl-tRNA synthetase, and the isoleucine analog, thiaisoleucine, was shown to target cytoplasmic isoleucyl-tRNA synthetase. Both compounds kill asexual blood stage parasites at mid-nanomolar and low-micromolar concentrations, respectively.[19]

■ LYSYL-tRNA SYNTHETASE

Lysyl-tRNA synthetase was discovered as another potential important target using array-based IVIEWGA. The *P. falciparum* cytosolic lysyl-tRNA synthetase is selectively and specifically inhibited by a fungal secondary metabolite, cladosporin, which has potent, nanomolar activity against both blood and liver stages of the parasite.[23] Cladosporin has >100-fold more potency against the parasite's lysyl-tRNA synthetase as compared to the human ortholog. This was corroborated by other studies that observed the dissociation constant for *P. falciparum* lysyl-tRNA synthetase with cladosporin to be ∼14 nM, while the human lysyl-tRNA synthetase was ∼4 μM.[24,25] Another study designed and tested inhibitors against apicoplast lysyl-tRNA synthetase and confirmed that the *Plasmodium* apicoplastic lysyl-tRNA synthetase can be specifically inhibited with compounds that show antimalarial activity in the micromolar range.[26]

■ PROLYL-tRNA SYNTHETASE

Cytoplasmic glutamyl-prolyl tRNA synthetase activity in mammalian cells is inhibited by febrifugine, a compound

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derived from a Chinese herb, and its synthetic derivative halofuginone.[27] However, these inhibitors could not be developed clinically because of their high toxicity. Herman and co-workers developed an analog called halofuginol that is active against both liver and asexual blood stages of the rodent malaria parasite *P. berghei* and is much better tolerated.[20] IVIEWGA was used with halofuginone to identify prolyl-tRNA synthetase as the target.[20] The group also showed that febrifugine analogs induce eukaryotic initiation factor 2α phosphorylation in both *Plasmodium* and transgenic yeast that express cytoplasmic prolyl-tRNA synthetase, making them important chemical tools to study the amino acid starvation pathway in both species. The importance of prolyl-tRNA synthetases as targets in multiple human eukaryotic pathogens (*Plasmodium*, *Leishmania*, *Toxoplasma*, *Cryptosporidium*, and *Eimeria*) is supported by the development of quinazolinone inhibitors using structure-guided drug design.[28]

PHENYLALANYL-tRNA SYNTHETASE

Three structurally and functionally different phenylalanyl-tRNA synthetases are present in the malaria parasite, each essential to the parasite's translational machinery in each of its compartments.[17,18] The cytoplasmic enzyme is an $(\alpha\beta)_2$ hetrotetramer while its apicoplast and mitochondrial counterparts are monomeric.[18] Kato et al. screened ~100 000 compounds from the diversity-oriented synthesis (DOS) library against *P. falciparum* and identified a series of bicyclic azetidines, which were then shown to inhibit the phenylalanyl-tRNA synthetase following *in vitro* evolution. These compounds showed activity at a single low-concentration dose in mice against all life cycle stages of the parasite.[21] This seems to be a very promising new drug target due to its presence in all three translational compartments in the parasite.

DUAL tRNA SYNTHETASE TARGETS

Although *in vitro* evolution has been often used to assign specific inhibitors to tRNA synthetase targets in an unbiased manner, data from other species can create testable hypotheses about which tRNA synthetase might be inhibited. Biochemical evidence from *E. coli* has indicated that indolmycin inhibits tryptophanyl-tRNA synthetase,[29] leading to the testable hypothesis that it behaves similarly in *P. falciparum*.[30] Likewise, borrelidin is a bacterial secondary metabolite with known antimalarial properties.[31] Characterization of borrelidin-resistant mutants in *Escherichia coli* showed that tolerance resulted from ThrRS (encoding threonyl-tRNA synthetase) overexpression.[32] Threonyl-tRNA (as with alanyl-, glycyl-, and cysteinyl-tRNA) synthetase is a very interesting potential target due to its dual localization in both cytosol and apicoplast. Targeting this enzyme would theoretically stall translation in all three compartments.[22,33,34] On the other hand, borrelidin also inhibits human threonyl-tRNA synthetase, making it highly toxic. To overcome this, a library of borrelidin analogs was synthesized and tested for toxicity and antimalarial activity. Some analogs lost their toxicity to human cells and still maintained antiparasitic activity both *in vitro* and *in vivo* when tested in mice against *P. yoelii*.[35] Although several of these analogs inhibited *P. falciparum* threonyl-tRNA synthetase in aminoacylation assays, it is theoretically feasible that these compounds exert their antiparasitic activity through interaction with a different target. The use of *in vitro* evolution studies can provide unbiased confirmation of on-target activity for threonyl and tryptophanyl tRNA synthetase.

TRANSLATION ELONGATION FACTOR 2

Previous studies have implicated translation elongation factor 2 (eEF2) as an important drug target in *Saccharomyces cerevisiae* for the antifungal compound sordarin.[36,37] Eukaryotic protein synthesis requires several elongation factors, and eEF2 is responsible for the translocation of the ribosome along the mRNA. Yeast studies have demonstrated that selective inhibitors for eEF2 can be synthesized that would not inhibit protein synthesis in human cells.[37,38]

IVIEWGA was used to show that *P. falciparum* eEF2 is inhibited by compound DDD107498. This compound has high potency and, like the tRNA synthetase inhibitors, antimalarial activity against multiple stages of the parasite's life cycle.[39] In a recent study, DDD107498 was confirmed to have transmission-blocking activity in experimental mosquito models using parasite densities observed in natural infections, supporting the decision to move this compound into early stage clinical trials.[40] The identification of inhibitors for tRNA synthetases and translation elongation factors clearly demonstrate that targeting protein synthesis in *Plasmodium* is a very effective way for attaining multistage antiparasitic activity.

DIHYDROOROTATE DEHYDROGENASE

Dihydroorotate dehydrogenase (DHODH) was already known to be a promising target for the development of antimalarial chemotherapy but has been rediscovered using evolution-based methods. DHODH is an enzyme localized to the mitochondrial electron transport chain (mETC), which provides oxidized ubiquinone as an electron acceptor for DHODH to synthesize pyrimidines *de novo*.[41,42] The parasite cannot salvage pyrimidines from its host, so the activity of the mETC is essential.[43] DHODH can be considered a good potential drug target with low toxicity due to the differences in the active site across species. Selective inhibitor binding with triazolopyrimidines in *Plasmodium* was first shown by Phillips and Rathod.[43]

A later triazolopyrimidine derivative, DSM265, has now advanced to Phase II clinical trials and is active against both liver and asexual blood stages of the parasite.[44] It has promising efficacy with single-dose regimens in human trials.[45] A secondary derivative, DSM421, has a similar selectivity and potent antimalarial activity.[46] In addition, 5-benzimidazolyl-theophene-2-carboxamides[47] and 7-arylaminopyrazolo[1,5-α]-pyrimidines[48] have also been shown to inhibit DHODH. *In vitro* evolution with DSM1, a scaffold related to DSM265, has shown that *P. falciparum* acquires copy number variants that encompass the DHODH loci, providing further *in vivo* validation of the target.[49]

Growth inhibition phenotypic screening identified many compounds with multistage activity against *Plasmodium* using diversity oriented synthesis.[21] BRD7539, an azetidine-2-carbonitrile, has been shown to inhibit DHODH in *P. falciparum* selectively over human DHODH and is potent against the parasite's asexual blood and liver stages. An optimized compound, BRD9185, has *in vitro* activity against blood and liver stage parasites and can clear infection in mouse models in just three doses.[50] This class of compounds is another promising group of potential new antimalarials that target DHODH.

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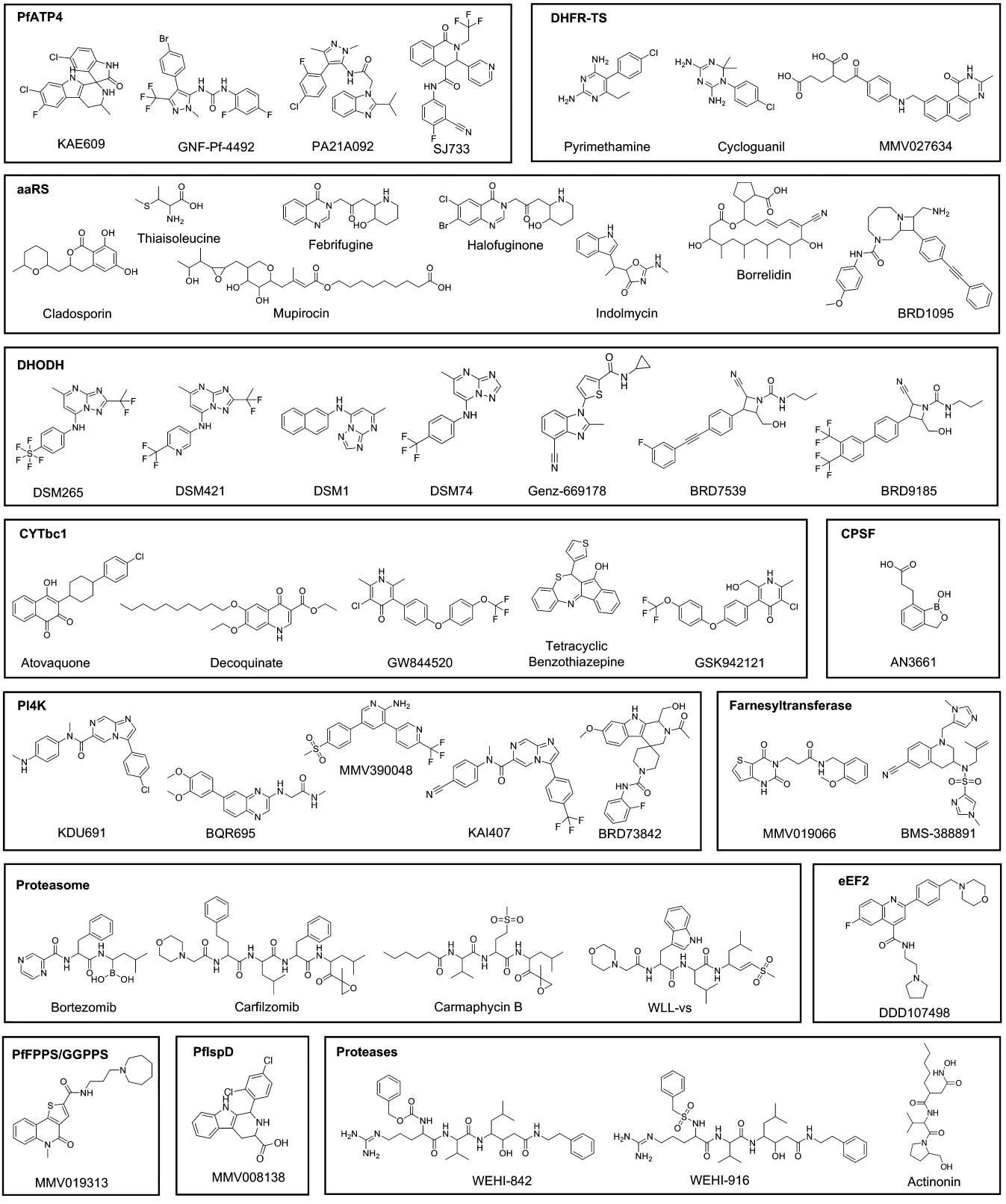

Figure 3. Antimalarial compounds grouped by targets as determined or supported by IVIEWGA studies. References are given in Table 1. SMILES for each compound are given in Table S1.

Ross and co-workers showed that resistance to PfDHODH inhibitors is primarily due to mutations and amplifications of the target gene DHODH. Several point mutations were found along with gene amplification that contributed to resistance. They also showed that the mutant parasites were sensitive to other PfDHODH inhibitors (Genz-669178, Genz-666136, and DSM74) which suggests a novel combination therapy approach to prevent resistance.[51]

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CYTOCHROME bc1 COMPLEX

The cytochrome bc1 complex (ubiquinol/cytochrome c oxidoreductase; complex III) is another target localized in the mETC and was previously known to be inhibited by drugs used in the treatment of malaria and toxoplasmosis. The dimeric cytochrome bc1 protein is comprised of three catalytic subunits: cytochrome b, cytochrome c1, and the Rieske iron−sulfur protein. The cytochrome bc1 complex catalyzes the transfer of electrons from ubiquinol to cytochrome c and, in *P. falciparum*, plays a very important role in pyrimidine biosynthesis by providing oxidized ubiquinone to DHODH.[41] Cytochrome bc1 is the target of atovaquone (which is combined with proguanil in the antimalarial drug, Malarone), a hydroxynapthoquinone which is a competitive inhibitor of the Q_o site.

The importance of cytochrome bc1 as an antimalarial target was first reconfirmed using IVIEWGA and decoquinate, a compound with low nanomolar activity against *P. yoelii* liver stage and *P. falciparum* blood stages.[52] Analysis of decoquinate-resistant lines revealed two new cytochrome bc1 mutations, A122T and Y126C, were present in the evolved lines and that these mutations mapped to the Q_o binding pocket of cytochrome bc1.[52] Although both atovaquone and decoquinate interact in the ubiquinol binding site of the cytochrome bc1 complex, each compound adopts a different mode of binding as has been shown by molecular docking studies and identification of different mutations in the respective resistant lines.[53] There is very limited cross-resistance with decoquinate in atovaquone-resistant lines.[52] At about the same time, a G131S mutation in the Q_o quinol binding site in resistant evolved lines was detected after selection with tetracyclic benzothiazepines, a compound class discovered in a phenotypic screen. In this case, however, only the cytochrome bc1-encoding gene was amplified and sequenced.[54]

Not much is known about the Qi binding site of cytochrome bc1 with respect to the interaction of its inhibitors. Recent studies have suggested that antimalarial compounds like 1-hydroxy-2-dodecyl-4(1*H*)quinolone bind to this site and are even effective against atovaquone-resistant strains with known Q_o mutations.[55] X-ray crystallography studies later provided evidence that 4(1*H*)-pyridone compounds GW844520 and GSK932121 bind to the Qi site of cytochrome bc1 complex and overcome atovaquone resistance.[56] IVIEWGA also established that the quinolone reduction site (Qi site) of cytochrome bc1 is a druggable target using an antimalarial probe from the DOS library that lacks cross-resistance with Q_o site inhibitors like atovaquone.[57] The presence of two different active sites in the same enzyme indicates that cytochrome bc1 is a very promising drug target. Furthermore, the absence of cross-resistance between inhibitors targeting these two sites can be harnessed for development of effective combination therapies.

THYMIDYLATE SYNTHASE PORTION OF BIFUNCTIONAL DIHYDROFOLATE REDUCTASE-THYMIDYLATE SYNTHASE

Dihydrofolate reductase-thymidylate synthase (DHFR-TS) is a very well-established drug target in *P. falciparum* for antifolates such as pyrimethamine and cycloguanil. This bifunctional enzyme is essential in the production of folates and thymidylate, which are crucial for DNA synthesis.[58] Although many antimalarials inhibit the DHFR portion of this bifunctional enzyme, a recent study evolved resistant parasites against the benzoquinazolinone MMV027634 and identified 3 non-synonymous mutations in the TS portion. A previously published crystal structure allowed the mapping of these novel mutations to the active TS binding site of the protein, revealing that each mutation occurs on the fringe of the 2′-deoxyuridylic acid (dUMP) binding site.[59]

Understanding the role of the junctional region of DHFR-TS could prove useful in antimalarial drug development, since it has been shown that a critical length of this junctional region (at least 44 amino acids) is essential for the *P. falciparum* TS domain to be catalytically active.[60] Exploiting the junctional region of this multifaced enzyme would simultaneously inhibit other portions of DHFR-TS enzyme to overcome drug resistance.

PHOSPHATIDYLINOSITOL 4-KINASE

Another target discovered using IVIEWGA is phosphatidylinositol 4-kinase (PI4K). This important new antimalarial drug target operates in all stages of the *Plasmodium* life cycle. PI4K is an essential cellular regulatory molecule, conserved in all eukaryotes that phosphorylate lipids (phosphatidylinositol to PI4-phosphate) to regulate intracellular signaling and trafficking.[61] In *Plasmodium*, PI4-P recruits lipid-binding effector proteins that are driven by Rab11A for the late-stage membrane ingression during merozoite biogenesis.[62]

PI4K was first discovered as the target of imidazopyrazines, a new class of antimalarial molecule that shows potency against blood-stage field isolates of *P. falciparum* and *P. vivax*. Imidazopyrazines such as KDU691 demonstrate therapeutic, preventive, and transmission-blocking activity in the malaria parasite by altering PI4-phosphate levels and membrane trafficking.[62] Selections with analogs of KDU691 (KAI407, KAI715, and BQR695) yielded both CNVs and SNVs in PI4K, confirming it as the target of this compound class.[59]

Subsequent phenotypic screens have identified other PI4K inhibitors. MMV390048, a 2-aminopyridine, also inhibits the PI4K enzyme, although this was discovered using affinity chromatography. Strains resistant to other antimalarials are sensitive to MMV390048, and it has activity against all stages of the parasite life cycle except hypnozoites.[63] Another compound, BRD73842, has activity in all stages of the parasite life cycle and was discovered in the DOS library discussed above.[21] It is notable that all three are structurally quite different from one another (Figure 3).

CLEAVAGE AND POLYADENYLATION SPECIFICITY FACTOR SUBUNIT 3

Cleavage and polyadenylation specificity factor (CPSF) is a multiprotein complex present in all eukaryotic cells that plays an important role in processing pre-mRNA to mRNA by cleavage and polyadenylation at the 3′ end of pre-mRNA.[64] CPSF was discovered to be a promising antimalarial drug target when point mutations were found in *P. falciparum* lines resistant to the small molecule AN3661, a benzoxaborole. Homology modeling studies showed the mutations were located in the active site where AN3661 binds to the protein. This compound was found to be highly potent against multiple stages of the parasite's life cycle *in vitro*, indicating further efforts should be made to design inhibitors that target CPSF.[64]

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ISOPRENOID PATHWAY

The isoprenoid biosynthesis pathway is essential in *Plasmodium* and occurs via the methyl-D-erythritol-4-phosphate (MEP) pathway. Isoprenoids are essential for protein prenylation and the synthesis of ubiquinone, dolichols, carotenoid, and vitamin E; tRNA prenylation has also been shown to be dependent on the isoprenoid pathway, making this pathway an attractive target for the development of new antimalarial therapies.[65−67] The first successful application of the IVIEWGA method to identify an antimalarial target attributed acquired fosmidomycin resistance to copy number variation in the *pfdxr* gene, which is involved in the isoprenoid biosynthesis pathway.[68] More recently, IVIEWGA using small molecules from the MMV Malaria Box, a library of 400 diverse drug- and probe-like molecules,[69] has identified multiple targets of this pathway, which will be discussed below.

P. FALCIPARUM 2-C METHYL-D-ERYTHRITOL 4-PHOSPHATE CYTIDYLYLTRANSFERASE

P. falciparum 2-C methyl-D-erythritol 4-phosphate cytidylyltransferase (PfIspD) is a key enzyme in the MEP isoprenoid biosynthesis pathway and was identified as the target of MMV008138 from the MMV Malaria Box. *In vitro* selections with MMV008138 followed by whole genome sequencing of drug-resistant parasites identified E688Q and L244I mutations to confer resistance.[70] The MEP pathway is not functionally conserved in humans, but an orthologous enzyme is expressed. The human ISPD shows only very weak sequence similarity to PfIspD, so antimalarials developed for this target should display low toxicity.

BIFUNCTIONAL FARNESYL/GERANYLGERANYL DIPHOSPHATE SYNTHASE

IVIEWGA using MMV019313 from the MMV Malaria Box[69] identified a SNV, S228T, in the *P. falciparum* bifunctional farnesyl and geranylgeranyl diphosphate synthase (PfFPPS/GGPPS) that conferred resistance.[71] PfFPPS/GGPPS synthesizes C15 and C20 prenyl chains for multiple downstream enzymes in the isoprenoid biosynthesis pathway. Bisphosphonates used for the treatment of osteoporosis also target PfFPPS/GGPPS and were shown previously to clear *Plasmodium* parasites in a mouse model, validating this enzyme as an antimalarial drug target. MMV019313 represents the first nonbisphosphonate inhibitor of PfFPPS/GGPPS and demonstrates superior physicochemical properties. It is highly selective for the *Plasmodium* FPPS/GGPPS and showed no activity against human FPPS or GGPPS.

Notably, Gisselberg et al. had to employ chemical mutagenesis with the alkylating agent ethylmethanesulfonate (EMS) in their selections to facilitate resistance evolution after multiple unsuccessful attempts to identify resistant strains using a standard protocol.[71] This represents a useful alternative strategy for resistance evolution when traditional IVIEWGA methods fail.

FARNESYLTRANSFERASE

An important post-translational event in eukaryotic organisms is the prenylation of proteins, which allows for protein−protein interactions and binding of intracellular proteins to membranes. One key process is the farnesylation of proteins, driven by farnesyltransferase. This enzyme catalyzes the transfer of the farnesyl group, a 15-carbon isoprenoid lipid unit, from farnesyl pyrophosphate to the C-terminus of proteins containing the CaaX motif.[72] Farnesyltransferase is an interesting known potential drug target in *P. falciparum* since the inhibition of this enzyme is fatal to the parasite.[73,74] Inhibitors of the protein have also been developed into anticancer therapeutics.[75,76]

Previously, resistance evolution of the parasite with the farnesyltransferase inhibitor BMS-388891, a tetrahydroquinoline, showed mutations in the protein at the peptide substrate binding domain. In selections with BMS-339941, another tetrahydroquinoline, a mutation was identified in the farnesyl pyrophosphate binding pocket.[77]

In a very recent IVIEWGA study, two different mutations in amino acid 515 (A515 V and A515T) were found in the beta subunit of the farnesyltransferase in *P. falciparum* strains resistant to MMV019066, a pyrimidinedione from the MMV Malaria Box. Modeling studies revealed that the mutations distort the critical interaction site of the small molecule with the farnesylation active site, thereby conferring resistance.[59]

USING IVIEWGA IN OTHER SPECIES TO INFORM ANTIMALARIAL DRUG DEVELOPMENT AND TARGET IDENTIFICATION

IVIEWGA data from other species can also be used to confirm likely on-target activity or to suggest new targets. *In vitro* evolution with *Saccharomyces cerevisiae* has shown that cladosporin, mentioned above, also inhibits yeast lysyl-tRNA synthase, just as it inhibits *P. falciparum* lysyl-tRNA synthetase.[23] IVIEWGA using KAE609 showed that mutations in the orthologous yeast protein PMA1 confer resistance.[78] In addition, yeast selections have been used to confirm on-target activity of proteasome inhibitors.

The proteasome is a well-known drug target important in the treatment of both infectious diseases[79] and cancer.[80] For example, the boronic acid inhibitor bortezomib has cytotoxic activity and is very effective in treating hematopoietic malignancies by targeting the β5 subunit.[81,82] On the basis of its success in cancer drug development, researchers suspected that proteasome inhibitors could also be antimalarials if problems with specificity could be overcome. A study using affinity probes showed that, when the β2 and β5 activities are inhibited in the *P. falciparum* proteasome, parasite growth is inhibited in all asexual blood stages even after short treatment.[83] Carfilzomib is an α',β'-epoxyketone inhibitor used to treat refractory or relapsed multiple myeloma that also kills *P. falciparum*, including artemisinin-resistant strains.[83,84] Later work identified WLL-vs (Trp-Leu-Leu-vinylsulfone) as a molecule that inhibits multiple subunits of the *Plasmodium* proteasome simultaneously while having lower activity against the human proteasome.[85] When tested in a *P. chabaudi* infection model, WLL-vs caused a drastic reduction in the parasite burden and was well-tolerated by host cells.[85] Carmaphycin B is a natural product derived from cyanobacterium *Symploca sp.* that targets the β5 subunit of the proteasome but exerts a cytotoxic effect on human lung adenocarcinoma and a colon cancer cell line.[86] Analogs have been designed that display high antimalarial activity against both asexual blood stages and gametocytes but also low human cytotoxicity.[87]

Although proteasome inhibitors bind to the proteasome in *in vitro* assays, questions remain as to whether the proteasome is their primary target. Employing IVIEWGA in *S. cerevisiae* has shown that carmaphycin B targets the β5 subunit as predicted, confirming affinity profiling data and the usefulness of

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IVIEWGA studies across species to identify the mechanism of action of small molecule compounds.

PROTEASE INHIBITION

Although many important targets have been discovered or confirmed with IVIEWGA, several new targets have been identified using conventional reverse genetic approaches. The best examples are the proteases. Malaria parasites utilize highly evolved and specific proteolytic pathways to invade and replicate in host cells while simultaneously avoiding the host immune response. Proteases play both regulatory and effector roles in parasite egress and invasion of red blood cells (RBCs). Proteases have well-characterized active site structures and are considered good drug targets. Protease inhibitors are already used to treat various human diseases like cancer, cardiovascular disorders such as hypertension and congestive heart failure, HIV, diabetes, and osteoporosis.[88,89]

In malaria parasites, serine proteases are implicated in RBC deformability and cytoadhesion[90] as well as in egress and RBC/hepatocyte invasion.[91,92] Aspartyl proteases (plasmepsins I–V),[93] cysteine proteases (falcipains 2, 2′, and 3; DPAP1),[94−98] and the metalloprotease falcilysin[99,100] all play an important role in hemoglobin degradation. Plasmepsin V is an essential aspartyl protease with a major role in the export of effector proteins to parasite-infected erythrocytes by cleaving proteins within the *Plasmodium* export element (PEXEL) in the parasite's endoplasmic reticulum.[101,102] Recent studies have shown that plasmepsin V might be an important drug target for both *P. falciparum* and *P. vivax*. Plasmepsin V is inhibited by WEHI-842, a compound designed to mimic the parasite PEXEL motif,[102] and by WEHI-916, which blocks PEXEL cleavage and kills the parasite at the ring-trophozoite transition.[103]

Plasmepsins IX and X are another class of aspartyl proteases that have remained unexplored until recently. Plasmepsin IX is functionally involved in erythrocyte invasion, targeting the biogenesis of the rhoptry secretory organelle. Plasmepsin X is paramount in both invasion and egress and for the final processing step carried out by subtilisin-like serine protease (SUB1) in the exoneme secretory vesicles.[104] Treatment with inhibitors in the aminohydantoin class resulted in an accumulation of schizonts and loss of SUB1 and SERA5 (a cysteine protease) maturation. Because inhibitors of plasmepsin X impair egress events downstream, aspartyl protease is a very promising antimalarial target.

The membrane metalloprotease FtsH1 was recently discovered as the target of the antibiotic actinonin using resistance evolution in *Toxoplasma gondii* after attempts to generate resistance in *P. falciparum* failed.[105] Treatment of *P. falciparum* with actinonin resulted in apicoplast biogenesis defects, establishing it as a promising target for a couple of reasons. Because apicoplast biogenesis is essential in proliferative stages of the *Plasmodium* life cycle, inhibitors of FtsH1 may have multistage efficacy. Additionally, there may be a decreased risk of host−cell toxicity since the organelle is unique to apicomplexan parasites.[106,107]

The attention that proteases have received leads to questions about why they have not been identified frequently using IVIEWGA. One possibility is that protease inhibitors lack specificity and/or there is genetic redundancy in the protease family. If a compound inhibits multiple proteases simultaneously, it is possible that specific mutations would not be identified. Alternatively, mutations in protease catalytic sites could lead to nonviable parasites given the many important biological functions they carry out in the parasite life cycle.

CONCLUSIONS

The recent dramatic increase in novel druggable pathways in *Plasmodium* parasites has been facilitated largely by the utilization of *in vitro* resistance evolution combined with mutation identification through a variety of genomic techniques. The increased efficiency and declining cost of WGS has only accelerated the rate of target discovery by this approach. Nonetheless, certain challenges remain in WGS analysis. The extreme A−T richness of the *P. falciparum* genome presents a unique problem for standard bioinformatic analysis protocols, for example, especially within intergenic regions where polymerase chain reaction (PCR) amplification bias can severely distort depth of coverage. Additionally, alignment confidences decrease across the highly variable *Plasmodium* genes involved in antigenic variation. Lack of complete functional annotation across the genome is another challenge for IVIEWGA studies because potentially interesting mutations may occur in uncharacterized genes. One example of this was the identification of mutations in the uncharacterized *P. falciparum* cyclic amine resistance locus (PfCARL) in selections with the clinical candidate KAF156.[108] As next-generation sequencing technology advances and methods of SNV and CNV detection become more sensitive, however, target discovery will become a more streamlined process. To date, the IVIEWGA method has been successfully applied to identify a number of novel antimalarial targets to expedite drug discovery. This Review highlights core druggable targets identified or further confirmed via IVIEWGA that should be integrated into the antimalarial drug development pipeline.

Genes that are identified using IVIEWGA are already chemically validated but must be further validated through structural docking studies and reverse genetics to prove their direct role in resistance. When a compound lacks strong on-target activity, mutation calls may be inconclusive or resistance may be conferred by generalized resistance mechanisms rather than a mutation in the target itself. Additionally, background and compensatory mutations that play an indirect role in resistance acquisition must be filtered out from genes that are truly relevant and druggable. While sequencing of resistant clones leads to more robust results, deep-coverage sequencing of bulk populations instead could make IVIEWGA more efficient. In a sample sequenced to 500× coverage, alleles with 1% frequency might be robustly detected and the additional time required to generate clones would be unnecessary. Nevertheless, if there is ambiguity, whole genome sequencing of singular parasite clones may be needed. The most promising mutations are ones that occur in multiple, independently derived resistant lines using the same compound. Even in cases where IVIEWGA identifies resistance genes rather than druggable targets, those genes are valuable for epidemiological tracking of antimalarial resistance when compounds enter clinical use. For example, presence of the Kelch13 C580Y mutation is used to track resistance to artemisinin and its derivatives in field isolates.

At present, no intergenic alleles have led to the identification of a target, although in theory mutations in promoter sequences could yield transcriptional upregulation of a target. Better annotations of promoter regions in *Plasmodium*, which has been accomplished for the human ENCODE projects, could be useful for defining regulatory regions.

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Occasionally, IVIEWGA methods fail during the *in vitro* resistance evolution process. Chemical mutagenesis has been shown to assist in resistance generation,[71] but alternative methods may still need to be developed. Despite several challenges, IVIEWGA has proven to be a very successful and robust method for target identification studies. The discovery of these druggable pathways will facilitate the creation of an ideal antimalarial that will be orally bioavailable, be cheap to synthesize, be easily stored at room temperature, and demonstrate low host cytotoxicity. It remains to be seen whether this same method will be adopted successfully for target identification in other pathogens.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00276.

Table S1: Simplified molecular-input line-entry system (SMILES) and structures of antimalarial compounds (XLSX)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

IVIEWGA, *in vitro* evolution and whole genome analysis; WHO, World Health Organization; ACT, artemisinin combination therapy; ART, artemisinin; WGS, whole genome sequencing; PfATP4, *P. falciparum* p-type cation ATPase; CNV, copy number variant; SNV, single nucleotide variant; aaRS, aminoacyl-tRNA synthetases; eEF2, translation elongation factor 2; TM, transmembrane; DHODH, dihydoorotate dehydrogenase; mETC, mitochondrial electron transport chain; DHFR-TS, dihydrofolate reductase-thymidylate synthase; dUMP, 2′-deoxyuridylic acid; PI4K, phosphatidylinositol 4-kinase; CPSF, cleavage and polyadenylation specificity factor; MEP, methyl-D-erythritol-4-phosphate; PfIspD, *P. falciparum* 2-C methyl-D-erythritol 4-phosphate cytidylyltransferase; PfFPPS/GGPPS, *P. falciparum* bifunctional farnesyl and geranylgeranyl diphosphate synthase; EMS, ethylmethanesulfonate; RBC, red blood cell; PEXEL, *Plasmodium* export element; SUB1, subtilisin-like serine protease; PCR, polymerase chain reaction; PfCARL, *P. falciparum* cyclic amine resistance locus; SMILES, simplified molecular-input line-entry system

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