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Tackling resistance: Emerging antimalarials and new parasite targets in the era of elimination [version 1; referees: 2 approved]

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REVIEW

Tackling resistance: emerging antimalarials and new parasite targets in the era of elimination [version 1; referees: 2 approved]

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Abstract
Malaria remains a significant contributor to global human mortality, and roughly half the world's population is at risk for infection with *Plasmodium* spp. parasites. Aggressive control measures have reduced the global prevalence of malaria significantly over the past decade. However, resistance to available antimalarials continues to spread, including resistance to the widely used artemisinin-based combination therapies. Novel antimalarial compounds and therapeutic targets are greatly needed. This review will briefly discuss several promising current antimalarial development projects, including artefenomel, ferroquine, cipargamin, SJ733, KAF156, MMV048, and tafenoquine. In addition, we describe recent large-scale genetic and resistance screens that have been instrumental in target discovery. Finally, we highlight new antimalarial targets, which include essential transporters and proteases. These emerging antimalarial compounds and therapeutic targets have the potential to overcome multi-drug resistance in ongoing efforts toward malaria elimination.

Keywords
Plasmodium, antimalarial, drug targets, drug discovery, resistance

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Introduction

Malaria has posed a risk to human life since the origin of our species. Despite this long history, it was not until 1880 that French army surgeon Charles Louis Alphonse Laveran discovered intraerythrocytic parasites in the blood of a patient with malaria[1]. Immediately following Laveran's discovery, crucial aspects of this infection, including species classification and the details of the human–mosquito transmission cycle, were revealed[2–6]. These early studies shaped our understanding of the protozoan parasites of genus *Plasmodium* that cause malaria. In the complex life-cycle of *Plasmodium* spp., human infection begins with the bloodmeal of a female *Anopheles* mosquito. Parasites migrate to the liver, where they undergo a large, asymptomatic expansion, emerging to invade red blood cells and initiate asexual replication. A small fraction of these blood-stage parasites terminally differentiate into gametocytes that are taken up by the mosquito to complete sexual replication and begin the infection cycle anew. Five different species of *Plasmodium* cause the majority of human malaria: *P. falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale*, and *P. vivax*. Malaria still accounts for an estimated 445,000 deaths and 216 million cases annually, and the majority of deaths result from infection with *P. falciparum*[7].

Malaria deaths have declined in large part because of the development of effective antimalarial medicines. Beginning in the late 1940s, chloroquine was the standard treatment for uncomplicated malaria[8]. However, by the late 1950s and early 1960s, chloroquine-resistant *P. falciparum* was observed throughout Southeast Asia, Oceania, and South America. Resistance to chloroquine has since spread to nearly all areas of the world[9]. Subsequent resistance has developed to antimalarials such as sulfadoxine/pyrimethamine, mefloquine, halofantrine, and quinine[10]. Most recently, resistance to artemisinin-based combination therapies emerged in 2008 in parts of Southeast Asia and continues to spread[11–19]. Clinical artemisinin resistance manifests as a delayed clearance phenotype; that is, infection eventually resolves with treatment with artemisinin-based combination therapy, but the time required for parasite clearance substantially increases[14,20,21]. This delayed clearance could contribute to the even more troubling rise in multi-drug resistance, as parasites have gained both reduced artemisinin sensitivity[22] and resistance against partner drugs, such as piperaquine[12,19]. As multi-drug resistance spreads, there is an urgent need for new antimalarial agents to control malaria infections. Optimally, new antimalarials will overcome multi-drug resistance, will be highly safe for use in vulnerable populations (such as infants and pregnant women), and will target more than one life-cycle stage in order to break the cycle of transmission. In this brief review, we highlight promising novel antimalarials currently in development and introduce emerging drug targets that may be key to ongoing efforts to eliminate malaria worldwide.

Promising new antimalarials in development

Global efforts to end malaria have led to the development of promising compounds. At the forefront of antimalarial development is the Medicines for Malaria Venture (MMV), which was established in 1999 as a not-for-profit, public–private partnership. The current MMV portfolio contains many promising compounds at various stages of development (research, translational, product development, and access; https://www.mmv.org/research-development/mmv-supported-projects). To illustrate the diversity of the current portfolio, a selection of the emerging antimalarials currently in development is discussed below (Table 1)[23–29].

Table 1. Selected promising antimalarial compounds.

Antimalarial compound	Alternative names	Protein target/predicted target	Target candidate profiles[a]	Current status in MMV pipeline[b]
Artefenomel	OZ439	Unknown	Asexual parasite clearance; transmission blocking	Combined artefenomel–ferroquine is in the patient-exploratory stage
Cipargamin	KAE609, NITD609	*Pf*ATP4, based on resistance screen mutations[23]	Asexual parasite clearance; transmission blocking	Patient-exploratory stage
DSM265	Not applicable	*Plasmodium* DHODH[24]	Asexual parasite clearance; targeting liver schizonts	Patient-exploratory stage
Ferroquine	SSR97193	Unknown	Asexual parasite clearance; transmission blocking	Combined artefenomel–ferroquine is in the patient-exploratory stage
KAF156	GNF156	*Pf*CARL[25], *Pf*ACT, and *Pf*UGT[26], based on resistance screen mutations	Asexual parasite clearance; transmission blocking; targeting liver schizonts	Combined KAF156–lumefantrine is in the patient-exploratory stage
MMV048	MMV390048	*Plasmodium* PI4K[27]	Asexual parasite clearance; transmission blocking; targeting liver schizonts	Patient-exploratory stage
SJ733	(+)-SJ000557733	*Pf*ATP4, based on resistance screen mutations[28,29]	Asexual parasite clearance; transmission blocking	Human volunteer stage
Tafenoquine	WR 238605, Etaquine	Unknown	Targeting *Plasmodium* hypnozoites	Regulatory review stage

DHODH, dihydroorotate dehydrogenase

PI4K, phosphatidylinositol 4-kinase

[a]According to Medicines for Malaria Venture (MMV) Target Candidate Profile classification.

[b]Status as of 31 July 2018.

Artefenomel and ferroquine

The current standard of care for malaria is combination therapy based on artemisinin, which is highly valued as a potent, rapidly active antiparasitic compound. Like artemisinin, synthetic ozonides contain an endoperoxide bond. A first-generation ozonide, arterolane (OZ277), has already been licensed for clinical use in India as a combination therapy with piperaquine. However, concern has arisen that there may be a loss of potency against kelch13 mutant parasites, which are artemisinin resistant[30–33]. Other synthetic ozonides, including artefenomel (OZ439), have been developed[34]. Artefenomel displays activity in transmission-blocking assays *in vitro*[35], and clinical studies support its use in a single-exposure combination therapy[36]. Unlike artemisinin's peroxide bond, artefenomel's peroxide bond is more stable and has an improved half-life in plasma: 23 hours compared with 0.5 hours[34]. Promisingly, artemisinin-resistant mutants do not appear to be cross-resistant to artefenomel[30,31], although some mutations in kelch13 may lead to partial cross-resistance[30–33]. Together, these properties support the continued efforts toward development and licensure of artefenomel for clinical use.

Ferroquine is a third-generation 4-aminoquinoline and a derivative of the antimalarial chloroquine. Although chloroquine resistance has spread to nearly all areas of the world, ferroquine efficacy is not impeded by chloroquine resistance mechanisms and resistance selection has not been observed in the laboratory[37]. Ferroquine also retains activity against parasites resistant to chloroquine, mefloquine, quinine, and piperaquine[38,39]. An initial clinical study with ferroquine in combination with artesunate showed a high malaria cure rate, and treatment with ferroquine also displayed post-treatment prophylaxis activity for at least 2 months[40]. Recent phase 2 trials replaced artesunate with the more effective artefenomel[40–42], and a combination of artefenomel and ferroquine therapy is in the patient-exploratory stage of the MMV portfolio.

DSM265

Plasmodium spp. depend on *de novo* pyrimidine synthesis because they lack pyrimidine salvage enzymes. An essential enzyme in the pyrimidine biosynthesis pathway is dihydroorotate dehydrogenase (DHODH). Large high-throughput screens were conducted to identify *Pf*DHODH inhibitors[43,44]. These screens identified several classes of molecules that target DHODH, such as triazolopyrimidines, phenylbenzamides, ureas, and naphthamides[44]. One triazolopyrimidine that has potent activity against asexual and liver-stage parasites is DSM265[45]. DSM265 selectively inhibits *Plasmodium* spp. DHODH enzymes over human orthologues[24]. DSM265 is currently in the patient-exploratory stage of the MMV pipeline and exhibits promising single-dose efficacy. Interestingly, DSM265 is predicted to remain at therapeutic concentrations in humans for more than a week after a single dose because of its favorable pharmacokinetic properties[45]. DSM265 also has promising prophylactic activity, providing some protection against infection with a single dose up to 7 days before parasite challenge[46,47]. The single-dose efficacy of DSM265 makes it exceptionally promising for both prophylaxis and treatment of disease. Furthermore, the potency of DSM265 supports future development of compounds that target *Plasmodium* DHODH.

Cipargamin and SJ733

Cipargamin (KAE609), a spiroindolone, also has potent activity against blood-stage malaria parasites[23,48]. A recent phase 2 study used once-daily dosing of cipargamin for 3 days on 21 adults with either uncomplicated *P. vivax* or *P. falciparum* malaria[49]. Parasite clearance rates in this clinical study and *in vitro* are among the fastest of any antimalarial yet characterized[23,49]. Cipargamin likely targets the P-type Na^+ ATPase, *Pf*ATP4, because resistance mutations have emerged[23]. *Pf*ATP4 appears to regulate parasite ion homeostasis, which is essential for survival, through active Na^+ export[50]. Inhibition of *Pf*ATP4, through cipargamin treatment, perturbs ion homeostasis in the parasite and increases host cell membrane rigidity, resulting in blocked blood-stage development and transmission to mosquitoes[28,29,50–52]. Cipargamin is currently in the patient-exploratory stage of the MMV portfolio; however, it is not the only *Pf*ATP4 inhibitor currently in development. A diverse range of compounds, including spiroindolones[23,51,53], pyrazoleamides[29], aminopyrazoles[53], dihydroisoquinolones[28], and other compounds[54], have been shown to target *Pf*ATP4. A dihydroisoquinolone that likely targets *Pf*ATP4, SJ733, is in the human volunteer stage of the MMV portfolio. Resistance selection with SJ733, like cipargamin, has generated point mutations, some unique to SJ733 and not induced by cipargamin, in the *pfatp4* gene[28,29]. The antimalarial properties of *Pf*ATP4 inhibitors, such as cipargamin and SJ733, are exceptionally promising and support future development of compounds with this mechanism of action.

KAF156

A novel class of antimalarials, imidazolopiperazines, has recently emerged and been found to have potent asexual blood-stage and liver-stage activity[55,56]. One such imidazolopiperazine, KAF156, is currently in the patient-exploratory stage of the MMV portfolio in combination with lumefantrine. Lumefantrine is a clinically approved partner agent; however, it has been modified to a new once-daily formulation for use with KAF156. In addition to displaying asexual blood- and liver-stage activity, KAF156 also inhibits the growth of sexual blood-stage parasites, including mature gametocytes[57]. Therefore, KAF156 may be effective in preventing parasite transmission from humans to mosquitoes. The antimalarial mechanism of KAF156 is still unclear because resistance *in vitro* is thought to be indirectly mediated through mutation of *pfcarl*, *pfact*, and *pfugt*, which encode a conserved protein of unknown function, an acetyl-CoA transporter, and a UDP-galactose transporter, respectively[25,26,55]. Future studies may illuminate the parasiticidal mechanism of imidazolopiperazines, but this class of drugs has great potential in the treatment of acute disease and reduction of parasite transmission.

MMV048

Another novel chemical class of antimalarials, 2-aminopyridines, was identified to have potent single-dose activity against *in vitro P. falciparum* and *in vivo P. berghei*[58]. From this initial screen of 2-aminopyridines, compound 15 (now known as MMV048) was identified with robust antimalarial activity. A follow-up study with MMV048 replicated the potent *in vitro* and *in vivo* activity of asexual blood-stage malaria parasites and

also confirmed transmission-blocking and liver-stage activity[27]. Genomic and chemoproteomic approaches identified *Plasmodium* phosphatidylinositol 4-kinase (PI4K) as the likely target of MMV048[27]. PI4K functions in membrane trafficking and membrane assembly during asexual blood-stages[59]. *Pf*PI4K is likely essential during the asexual blood-stage because attempts to insert an early stop codon were unsuccessful[59]. The multi-stage antimalarial activity, prolonged half-life, and single-dose efficacy of MMV048 make it a promising new antimalarial in the patient-exploratory stage of the MMV pipeline.

Tafenoquine

Although infection with *P. falciparum* represents the largest burden of malaria deaths, there is also a need to develop medicines that prevent the relapse of *P. vivax* and *P. ovale*. Unlike *P. falciparum*, both *P. vivax* and *P. ovale* have a dormant liver-stage form called a hypnozoite. Hypnozoites can reactivate without warning, leading to the onset of malarial symptoms. This dormant stage remains both a challenge to treat and a potent barrier to malaria elimination. Tafenoquine, an 8-aminoquinoline, is currently under development for the prevention of *P. vivax* relapse. Tafenoquine has high activity as a single-dose treatment and has promising anti-hypnozoite activity in humans[60]. However, tafenoquine has therapeutic restrictions similar to those of the current radical cure standard for *P. vivax* and *P. ovale*, primaquine, which might limit its therapeutic impact. Both primaquine and tafenoquine cause dose-dependent acute hemolytic anemia in individuals with glucose-6-phosphate dehydrogenase deficiency[61,62]. Because of its longer half-life, tafenoquine requires a higher glucose-6-phosphate dehydrogenase activity threshold than primaquine; thus, a greater proportion of individuals will be ineligible for tafenoquine treatment and primaquine will still be needed[63]. In July 2018, the US Food and Drug Administration approved tafenoquine under the trade name Krintafel. Tafenoquine is the first new antimalarial in 60 years to prevent relapse of *P. vivax*. However, its limitations highlight the need for development of additional compounds that target relapsing malaria.

Emerging new antimalarial targets

Continued efforts to dissect the basic biology of the complex malarial organism have yielded new therapeutic targets for the development of antimalarials. The *Plasmodium* Genetic Modification Project (*Plasmo*GEM) (http://plasmogem.sanger.ac.uk), a not-for-profit, open-access research resource, has advanced our understanding of *Plasmodium* by providing vectors for genome-wide manipulation. *Plasmo*GEM contains over 2,000 plasmids designed to tag or delete genes in *P. berghei*[64]. Provided without cost, these tools have been used in a recent large-scale knockout screen to identify essential genes. The essentiality of over 50% of the genome was tested in an *in vivo* mouse model of *P. berghei* infection[65]. Surprisingly, 44.9% of genes were found to be essential and an additional 18% showed reduced parasite blood-stage growth; therefore, 62.9% of genes are required in *P. berghei* for normal asexual growth[65]. The high percentage of essential genes and low functional redundancy suggest that *Plasmodium* may have considerably more drug targets than do bacteria, for example[65]. This genetic screen resulted in the identification of essential cellular processes in the parasite. Specific examples of pathways enriched with essential genes include glycosylphosphatidylinositol anchor biosynthesis, the mitochondrial tricarboxylic acid cycle, ubiquinone biosynthesis, and isoprenoid biosynthesis[65]. From this genetic screen, a searchable phenotype database was built (http://plasmogem.sanger.ac.uk/phenotypes).

A large forward genetic screen in *P. falciparum* parasites recently identified more than 2,680 genes that are likely essential for asexual blood-stage growth[66]. When high-throughput *piggyBac* transposon insertional mutagenesis was used in combination with quantitative insertion site sequencing[67,68], the mutability and fitness cost of 5,399 genes were evaluated[66]. The AT-richness of the *P. falciparum* genome (>81%) is well suited for *piggyBac* transposon-based mutagenesis because of the high density of the tetranucleotide insertion target sequence TTAA[66]. To quantify gene essentiality, a mutagenesis index score and mutagenesis fitness score were calculated for each locus. Together, these two independent measures were used to classify a gene as likely essential or dispensable, and this methodology may be expanded to identify essential genes for other life-cycle stages[66]. The essential genes and pathways discovered in both the *Plasmo*GEM *P. berghei* screen[65] and *piggyBac* transposon *P. falciparum* screen[66] will supplement ongoing studies and likely initiate investigation into novel putative antimalarial targets.

A final strategy to identify possible new antimalarial targets is the resistance screen. Resistance screens challenge the malaria parasite with low levels of an antimalarial compound to hinder development. This can lead to *in vitro* evolution and selection for resistance mutations that relieve growth suppression. Therefore, resistance screens can be used to discover both mediators of drug resistance and novel antimalarial drug targets[22,69]. Winzeler and colleagues recently performed a large resistance screen with 37 distinct compounds[70]. Whole genome sequencing of 262 compound-resistant parasite lines identified several candidate resistance mutations. Although the screen confirmed previously identified multi-drug resistance mechanisms and illuminated new drug target–inhibitor pairs, only two novel drug-resistance genes—*pfabcI3* and *pfaat1*[70]—were identified. Below, we highlight a few emerging antimalarial targets of particular promise (Figure 1).

PMIX and PMX

Two recent studies highlighted the importance of plasmepsins IX (PMIX) and X (PMX) for parasite development[71,72]. Plasmepsins comprise a family of 10 aspartic proteases in the *P. falciparum* genome. A number of studies have focused on the digestive vacuole plasmepsins I to IV (PMI to PMIV), including development of chemical inhibitors[73–76]. Subsequent functional genetic studies of PMI–PMIV revealed that they are not essential for parasite survival[77]. PMIX and PMX are expressed in asexual blood-stage parasites[78]. Conditional knockdown of PMIX in *P. falciparum* revealed that it is essential for red blood cell invasion[71,72]. PMX knockdown similarly interrupted red blood cell invasion but also revealed an additional requirement of PMX in red blood cell egress[72]. A recent study employed a combination of *in vitro* and *in vivo* rodent experiments to find that hydroxyl-ethyl-amine-based scaffold compound 49c (referred

to as 49c) inhibits both PMIX and PMX[71]. 49c is an effective inhibitor against *P. falciparum in vitro* and the rodent parasite *Plasmodium berghei in vivo*[79,80]. Treatment with 49c inhibits asexual, sexual, and liver-stage development, indicating that 49c or other PMIX or PMX inhibitors may have value to both treat symptomatic malaria and block transmission[71]. Together, these observations provide compelling evidence that PMIX and PMX are promising targets for antimalarial development.

Rab11a

In the recent *P. berghei* functional genetic screen, one of the cellular pathways most enriched with essential genes is that of isoprenoid biosynthesis[65]. A number of studies have previously highlighted the requirement of isoprenoid biosynthesis for *P. falciparum* asexual replication[81–83]. Isoprenoids are necessary for protein prenylation, the post-translational lipid modification of proteins. Because chemical inhibition of protein prenylation in the malaria parasite disrupts asexual parasite growth[84–90], prenylated malarial proteins are potential antimalarial targets. Recent chemical labeling approaches have revealed that only 15 to 19 proteins are prenylated in blood-stage malaria and a majority of these proteins are Rab GTPases[91,92]. Rab GTPases function in docking vesicles to membranes and their prenylation aids in association with target membranes[93]. Rab11a, a Rab GTPase, is expressed and prenylated in asexual blood-stage malaria parasites[91,92,94] and is essential for asexual parasite replication[95]. In the parasite, Rab11a functions as a mediator of PI4K signaling and is a binding partner of PI4K, the target of imidazopyrazines and MMV048[27,59,96]. Mutation of Rab11a confers resistance to the imidazopyrazine, KAI715[59]. Interestingly, Rab11a has very low genetic diversity when sequenced in 2,000 *Plasmodium* clinical isolates, and only one non-synonymous mutation has been identified[97]. These features suggest that Rab11a may represent a promising target because of both its prenylation and interactions with essential signaling pathways within the parasite. Additional studies are needed to evaluate the biological roles of the remaining prenylated proteins in blood-stage malaria.

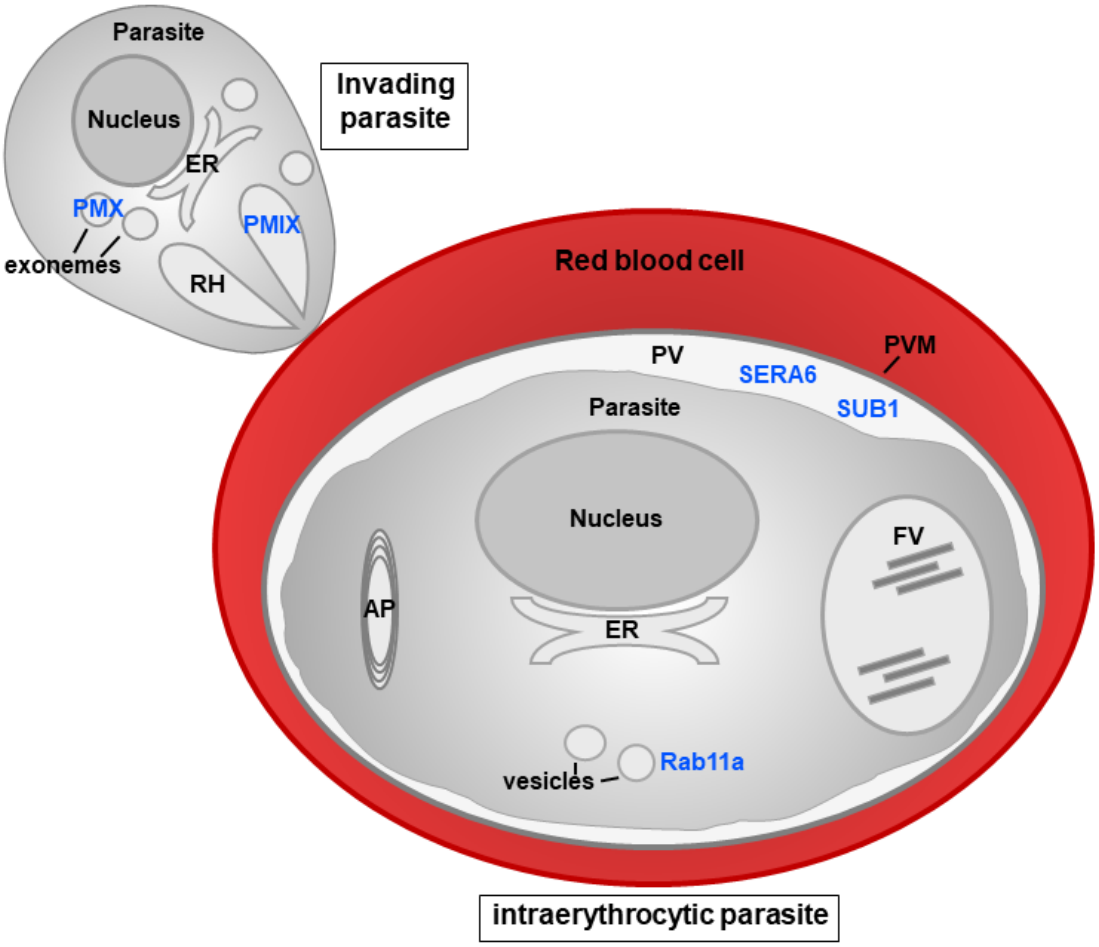

Figure 1. Localization of antimalarial targets in the asexual parasite. Shown are parasite organelles, including the nucleus, apicoplast (AP), endoplasmic reticulum (ER), food vacuole (FV), rhoptries (RH), exonemes, and vesicles. The intraerythrocytic parasite is located within the parasitophorous vacuole (PV), which is delineated by the PV membrane (PVM), where both SUB1 and SERA6 are found during egress[98]. Rab11a likely localizes to vesicles, which it guides to target membranes. In the invading parasite, plasmepsin IX (PMIX) is found in the bulbs of RH and plasmepsin X (PMX) localizes to exonemes[72].

SUB1 and SERA6

During asexual replication, the malaria parasite must exit the red blood cell before invading a new cell. This process, called egress, requires the rupture of both the parasitophorous vacuole membrane (PVM), which surrounds the parasite, and the red blood cell membrane (RBCM). Egress is protease dependent[99], and recently two proteases—SUB1 and SERA6—were identified as mediators of PVM and RBCM rupture[98]. SUB1, a serine protease, moves to the parasitophorous vacuole before egress[100–104] and cleaves multiple substrates[100,103,105–107]. One substrate cleaved by SUB1 is SERA6[108–110], a putative cysteine protease, which requires proteolytic processing by SUB1 to function[98]. *P. falciparum* parasites that lack SUB1 fail to rupture the PVM, thus stalling parasite development[98]. Interestingly, parasites that lack SERA6 can rupture the PVM, but RBCM rupture does not occur[98]. Therefore, SUB1 and SERA6 have distinct roles in parasite egress. Because SUB1 and SERA6 are essential for asexual blood-stage growth and orthologues are found in other *Plasmodium* species[98], compounds that inhibit these proteins may be useful in treating multiple types of malarial disease.

Discussion

Malaria continues to be a major global health concern. *Plasmodium* elimination will not be possible without substantial ongoing efforts, including diagnostic testing and treatment of confirmed and asymptomatic infections, mosquito vector control, preventative therapies, and surveillance systems. Although all of these areas of malaria control are crucial, antimalarial drug discovery is among the most pressing because of the continued spread of antimalarial resistance. The MMV has focused its efforts on developing drugs that treat disease, prevent transmission, and provide chemoprotection. This multifaceted approach to the antimalarial development pipeline provides assurance that new antimalarials will contribute to broad approaches of malaria control.

Studies in basic parasite biology remain extremely important to elimination efforts. Although the current antimalarial compounds under development have great potential, malaria control efforts will benefit from continued dissection of parasite biology. Ideally, new compounds will target proteins and pathways that are essential for parasite growth and transmission with diverse mechanisms of action. New antimalarials will almost certainly be employed in combination therapeutics, combining molecules of different chemical classes and with diverse mechanisms of action to slow the development of multidrug resistance. Novel drug targets will be uncovered through multiple approaches, such as large-scale genetic and resistance screens. A deeper understanding of essential parasite biology will also aid in other aspects of malaria control, including mosquito vector control, advancement of diagnostic tests, and development of preventative therapies. To date, *Plasmodium* has successfully adapted to sequential drug selective pressure in the field. However, the remarkable successes of recent efforts to develop new antimalarials and identify drug targets suggest an optimistic future in treatment of disease, prevention of transmission, and protection against infection.

Abbreviations

49c, hydroxyl-ethyl-amine-based scaffold compound 49c; DHODH, dihydroorotate dehydrogenase; MMV, Medicines for Malaria Venture; PI4K, phosphatidylinositol 4-kinase; *Plasmo*GEM, *Plasmodium* Genetic Modification Project; PMI, plasmepsin I; PMIV, plasmepsin IV; PMIX, plasmepsin IX; PMX, plasmepsin X; PVM, parasitophorous vacuole membrane; RBCM, red blood cell membrane

Competing interests

The authors declare that they have no competing interests.

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Version 1

1 **Emily Derbyshire** Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina, USA
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2 **Kirsten Hanson** Department of Biology and the South Texas Center for Emerging Infectious Diseases, University of Texas at San Antonio, San Antonio, Texas, USA
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