Plasmodium Kinases as Potential Drug Targets for Malaria: Challenges and Opportunities

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ABSTRACT: Protein and phosphoinositide kinases have been successfully exploited as drug targets in various disease areas, principally in oncology. In malaria, several protein kinases are under investigation as potential drug targets, and an inhibitor of Plasmodium phosphatidylinositol 4-kinase type III beta (PI4KIIIβ) is currently in phase 2 clinical studies. In this Perspective, we review the potential of kinases as drug targets for the treatment of malaria. Kinases are known to be readily druggable, and many are essential for parasite survival. A key challenge in the design of Plasmodium kinase inhibitors is obtaining selectivity over the corresponding human orthologue(s) and other human kinases due to the highly conserved nature of the shared ATP binding site. Notwithstanding this, there are some notable differences between the Plasmodium and human kinome that may be exploitable. There is also the potential for designed polypharmacology, where several Plasmodium kinases are inhibited by the same drug. Prior to starting the drug discovery process, it is important to carefully assess potential kinase targets to ensure that the inhibition of the desired kinase will kill the parasites in the required life-cycle stages with a sufficiently fast rate of kill. Here, we highlight key target attributes and experimental approaches to consider and summarize the progress that has been made targeting Plasmodium PI4KIIIβ, cGMP-dependent protein kinase, and cyclin-dependent-like kinase 3.

KEYWORDS: Plasmodium, protein kinase, lipid kinase, target validation, malaria, drug discovery

Malariain humans, caused by five species of Plasmodium, is still a massive problem in many parts of the world, causing approximately 228 million clinical cases and 405,000 deaths in 2018, mainly among children under 5 and pregnant women in sub-Saharan Africa.4 In addition to active cases, there are likely to be significant numbers of asymptomatic carriers. The most severe form of malaria results from infection with P. falciparum. The other predominant strain causing human disease is P. vivax, which causes a relapsing form of the disease. Human disease is also caused by P. ovale, P. malariae, and P. knowlesi. Control and ultimate elimination of malaria is likely to require the application of multiple different strategies, including the use of insecticide-impregnated bed nets, appropriate drug therapies, and vector control through the use of insecticides and vaccines. Despite significant progress in reducing the incidence of the disease and associated deaths, this seems to have plateaued in recent years.5 Undoubtedly, there are multiple reasons for this, including emerging resistance to current drugs within the parasite population as well as resistance to commonly used insecticides within the mosquito vector population. Of particular concern is the reported increase in resistance to artemisinin, a core component of current front-line artemisinin combination therapies (ACTs), in South East Asia. There is growing fear that resistance to artemisinin-based therapies will spread to other areas, thus threatening the effectiveness of this mainstay therapy. Indeed, a recent study from Uwimana and colleagues demonstrated that P. falciparum samples isolated from patients in Rwanda were resistant to artemisinin in vitro, the first report of this type from Africa. In addition to new drugs to tackle the problem of drug resistance, there is an urgent need for new drug classes with novel mechanisms of action to aid the elimination and/or eradication agenda: chemoprotection, especially important for vulnerable groups in areas of low transmission and where malaria is seasonal; transmission-blocking agents to remove gametocytes from the blood, thus blocking transmission; a radical cure for P. vivax malaria.

New medicines for malaria are developed as combination therapies in order to slow the emergence of resistance and to improve clinical efficacy. Medicines for Malaria Venture

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One class of molecular drug targets that has been extensively investigated in multiple disease areas, in particular oncology, is protein kinases, both protein and lipid. Currently, an inhibitor of the human malaria parasite *Plasmodium falciparum* phosphatidylinositol 4-kinase type III beta (PfPI4KIIIβ) is in the clinical development for malaria, identifying PfPI4KIIIβ as an exciting, exploitable molecular target. In this Perspective, we assess kinases as potential antimalarial drug targets and discuss the challenges associated with developing drugs that specifically inhibit these enzymes. Here, we focus on protein and phosphoinositide kinases (PIKs). For information related to other kinases, such as choline kinase and hexokinase (glucokinase), a reference is made to the specific literature. In addition, a recent review from Cabrera and colleagues deals with *Plasmodium* kinases as drug targets and specific classes of inhibitors.

Protein and phosphoinositide kinases represent attractive drug targets for multiple reasons. In general, kinases are readily druggable; there is a huge knowledge base to help guide the development of specific kinase inhibitors, and multiple kinase-focused compound libraries are available for screening to provide starting points for drug discovery. There is also significant structural information available to support the design and optimization of inhibitors.

Protein kinases have a plethora of physiological roles within cells ranging from signal transduction to cell fate control. At the most basic level, these enzymes catalyze the transfer of γ-phosphate from ATP (or GTP) to protein substrates, most commonly at the site of serine, threonine, or tyrosine residues. In comparison to mammalian kinases, the understanding of the physiological roles of *Plasmodium* kinases and their substrates is in its infancy. The improvement of the understanding of the specific and possibly unique functions of *Plasmodium* kinases will be crucial in facilitating the full exploitation of this enzyme class for antimalarial drug discovery.

**Figure 1.** Overview of the *P. falciparum* and human kinomes. Eukaryotic protein kinase (ePK) groups include AGC (named after protein kinase A, G, and C families, containing cyclic nucleotide and calcium/phospholipid-dependent kinases), CAMK (calmodulin/calcium-dependent kinase), CK1 (casein or cell kinase 1), CMGC (named after CDK, MAPK, GSK3, and CLK families), STE (homologues of yeast STE7, STE11, and STE20 genes, including kinases in MAPK pathways but not MAPKs), TK (tyrosine kinase), TRL (tyrosine kinase-like serine/threonine kinase), RGC (receptor guanylate cyclase), and other (other protein kinases including the NEK (never in mitosis A (NIMA)-related kinase) family). The aPK-related kinase family FIKK (Phe (F)–Ile (I)–Lys (K)) consists of 19 members, of which 18 are specific to *P. falciparum* and closely related species. Atypical protein kinases (aPKs) include PIKKs (phosphatidylinositol 3-kinase-related kinases, all three of which are classified as PIKs in the section below) and RIO (right open reading frame) families in *Plasmodium*.

(MMV) has developed the following *Target Product Profiles* (TPPs) for malaria.

- **TPP-1 case management:** Treatment of acute uncomplicated malaria, relapsing malaria, and severe malaria, including population-based strategies such as the treatment of asymptomatic infections and transmission blocking

- **TPP-2 chemoprotection:** For outbreak prevention and use in subjects migrating to endemic areas

These are used to derive *Target Compound Profiles* (TCPs), outlining the properties required in an individual molecule. These include *in vitro* and *in vivo* activity, physicochemical, pharmacokinetic, and safety pharmacology properties. The TCPs include the following:

- **TCP1:** Molecules that clear asexual blood-stage parasites to cure blood-stage malaria

- **TCP3:** Molecules active against hypnozoites to provide a radical cure of *P. vivax* malaria

- **TCP4:** Molecules active against liver schizonts to provide chemoprotection

- **TCP5:** Molecules active against gametocytes to block transmission

- **TCP6:** Molecules that target the mosquito to block transmission

Up until now, most compounds developed for malaria have been identified phenotypically. However, there is increasing interest in target-based drug discovery for malaria. It is important that when selecting a drug target the inhibitors of this molecular target are capable of satisfying at least one of the TCPs.

One class of molecular drug targets that has been extensively investigated in multiple disease areas, in particular oncology, is kinases, both protein and lipid. Currently, an inhibitor of the human malaria parasite *Plasmodium falciparum* phosphatidylinositol 4-kinase type III beta (PfPI4KIIIβ) is in the clinical development for malaria, identifying PfPI4KIIIβ as an exciting, exploitable molecular target. In this Perspective, we assess kinases as potential antimalarial drug targets and discuss the challenges associated with developing drugs that specifically inhibit these enzymes. Here, we focus on protein and phosphoinositide kinases (PIKs). For information related to other kinases, such as choline kinase and hexokinase (glucokinase), a reference is made to the specific literature. In addition, a recent review from Cabrera and colleagues deals with *Plasmodium* kinases as drug targets and specific classes of inhibitors.

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### THE PLASMODIUM KINOME

The *Plasmodium* kinome consists of between 60 and 90 protein kinases, depending on the species and the stringency of the classification method, with kinases accounting for ~1.7% of coding genes within *P. falciparum*. While many of these kinases can be classified into one of the established eukaryotic protein kinase (ePK) groups (AGC, CAMK, CK1, CMGC, STE, TKL, or other; see the Figure 1 legend for definitions of the different kinase groups in this paragraph) or atypical kinase (aPK) groups (PIKK and RIO), the *Plasmodium* kinome displays significant genetic divergence from the kinomes of other eukaryotes and importantly from its human host (Figure 1). Many *Plasmodium* kinases have no clear human orthologue, and in cases where orthologues exist, atypical features and significant structural differences are often apparent. These typically include large insertions within kinase domains as well as pronounced differences in regulatory regions, suggesting that the regulatory mechanisms and functions of these kinases may differ substantially from their human orthologs. Unique features that distinguish the *Plasmodium* kinome from the human kinome include: (1) the absence of tyrosine kinases (TK), the largest kinase group in humans, and the structurally related receptor guanylate cyclases (RGCs); (2) no clearly recognizable MAPKK homologue, despite the presence of two related receptor guanylate cyclases (RGCs); (3) the CAMK group, which includes a 7-member family of calcium-dependent protein kinases (CDPKs) also found in plants and other protists; (4) the presence of FIKKs, an ePK-related family unique to apicomplexan parasites. The kinome is largely conserved between *Plasmodium* species, although the exact numbers of kinases within a given group can vary, suggesting possibly divergent roles and/or redundancy. On the basis of the analysis by Miranda-Saavedra et al., the kinome of the most virulent species, *P. falciparum*, consists of 65 ePKs, 5 aPKs, and 19 FIKKs (Figure 1). This is substantially larger than the kinomes of other *Plasmodium* species, principally due to an expansion of the FIKK family in *P. falciparum* to 19 members while other *Plasmodium* species maintain only one. All 18 of these unique FIKKs have export signal sequences and are thought to play specific roles in virulence through the modulation of host factors. A recent quantitative phosphoproteomics study by Davies et al. showed unique phosphorylation patterns for *P. falciparum* FIKK kinases, including FIKK-dependent phosphorylation of parasite virulence factors and host erythrocyte proteins.

### PLASMODIUM PHOSPHOINOSITIDE KINASES

Lipid phosphoinositides are universal signaling molecules that play fundamental roles in almost all aspects of cellular function including growth, cell division, organelle identity, and membrane trafficking. Different phosphoinositide species are generated by the highly regulated activity of lipid PIKs and phosphatases. PIKs, particularly phosphoinositide 3-kinases (PI3Ks), have been associated with a variety of human diseases including cancer and immunodeficiency disorders (review by Burke). Dysregulation of human PIKs, particularly phosphoinositide 3-kinases (PI3Ks), has been associated with a variety of human diseases including cancer and immunodeficiency disorders (review by Burke). In addition, human PIKs are important host enzymes that are hijacked by pathogens in order to mediate viral replication and bacterial infections. Consequently, concerted efforts have been made to exploit PIKs for drug discovery. These efforts have predominantly focused on PI3K and PI4K enzymes; in comparison, less is known about phosphatidylinositol phosphate kinases (PIPKs).

The *P. falciparum* genome encodes 7 putative PIKs (Figure 2B). Even though *P. falciparum* PI4KIIId (PF3D7_0509800) is a clinically validated drug target for malaria, the role of the other PIKs in *Plasmodium* and their potential as drug targets are largely unexplored. Although interest in targeting *Pf*PI3K (PF3D7_0515300, also referred to as *Pf*Vps34) has been expressed, genetic essentiality demonstrated, and human PI3K inhibitors with potent in vitro *Pf*PI3K and antiplasmo-

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**Figure 2.** Overview of human and *Plasmodium* phosphoinositide kinases (PIKs) and their putative substrates. (A) Human PIKs and substrates clustered into 3 groups based on structure similarity. (B) *Plasmodium* PIKs/PIK-related proteins and substrates. Proteins are labeled using PlasmoDB (https://plasmodb.org/) gene identifiers for the *P. falciparum* 3D7 strain. Putative PIKs that have not yet been studied are enclosed by dotted lines (PF3D7_0419900, PF3D7_0311300, PF3D7_1129600, PF3D7_1412400). Proteins are colored to match the human PIK(s) that they interact with. Essential: cross (X), nonessential; question mark (?), conflicting data between studies.

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**A. Human PIKs**

| Type III | Type II | Type I |
| --- | --- | --- |
| PI4KIIIa | PI4KIIIb | PI3PK |
| PISK Class I | P110c, P110d | PI100c, PI100y |
| PISK Class II | PI4KIIc, PI4KIIa | PI10b |
| PISK Class III | PI4KIVc, PI4KIVb, PI4KIVa | PI10a, PI10f |

**B. Plasmodium PIKs**

| Type III | Type II | Type I |
| --- | --- | --- |
| PF3D7_0419900 | PF3D7_0509800 | PF3D7_0515300 |
| PF3D7_0515300 | PF3D7_0509800 | PF3D7_0515300 |
| PF3D7_0515300 | PF3D7_0515300 | PF3D7_0515300 |

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to our knowledge, no major medicinal chemistry programs targeting this *Plasmodium* enzyme have been reported to date. As is the case for PI3K class III enzymes in other organisms, PfPI3K is thought to play a role in the regulation of autophagy. In addition, PfPI3K has been shown to play a role in hemoglobin endocytosis and trafficking and has been controversially implicated in *PfKelch*-13-mediated artemisinin resistance.  

![Figure 3. Key conserved features of the kinase domain and ATP binding site.](image)

**KEY STRUCTURAL FEATURES OF KINASES**

Over five thousand unique high-resolution structures of kinase domains, including protein and related kinases that act on nonprotein substrates such as PIKs, have been deposited in the Protein Data Base (PDB). The three-dimensional structure of the kinase domains is highly conserved, and key features of ePKs have been well described. Despite low sequence conservation, ePKs, aPKs, and PIKs share a similar three-dimensional kinase fold and many other of the key structural features.  

High-resolution structures of at least 11 different *Plasmodium* protein kinases have been solved (*Pb*CDPK1 (3Q5I), *Pf*CDPK2 (4MVF), *Pf*CDPK4 (4QOX, 4RGJ), *Pf*CK2 (5XVU), *Pf*CLK1 (3LLT), *Pf*MAPK2 (3NIE), *Pb*MAPK2 (3N9X), *Pf*PK5 (e.g., 1OB3), *Pf*PK7 (e.g., 2PMN), *Pf*PKG (5DYK), and *Pf*PKG (e.g., 5DZC). Recently, structures of *P. falciparum* choline kinase (*Pf*ChoK) and hexokinase have also been reported. To our knowledge, no *Plasmodium* PIK structures have been elucidated to date.

The key features of the conserved ePK domain are illustrated in Figure 3 using a high-resolution structure of the kinase domain of *Plasmodium vivax* cGMP-dependent protein kinase (*Pf*PKG) as an example. The ATP binding site is situated between the small N-terminal lobe, made up of a five-stranded β-sheet (β1–β5) and the catalytic αC-helix, and the larger α-helical-rich C-terminal lobe. The hinge region connects the two lobes and contains a conserved valine that interacts with adenine of ATP as well as the so-called hinge-binding motif of small molecule ATP-competitive kinase inhibitors via "donor−acceptor−donor" interactions. The gatekeeper residue (*Pf*PKG Thr611) is situated at the end of β5 before the hinge region. The size of the gatekeeper residue affects the accessibility to a small pocket adjoining the ATP binding site, typically referred to as the back pocket. Small molecule kinase inhibitors, referred to as bumped-kinase inhibitors, that selectively target enzymes with small gatekeeper residues (Thr/Ser) can extend into this back pocket. A glycine-rich loop in ePKs situated between β1 and β2, commonly referred to as the phosphate binding loop (P-loop), acts as a lid over the phosphate channel, securing the ATP in position. The P-loop is also present in the aPKs and PIKs, serving an analogous function; however, the glycine-rich sequence motif (GxGxxG) is not conserved. The P-loop is followed by a conserved valine residue (*Pf*PKG Val548) that...
forms hydrophobic interactions with the adenine of ATP and many ATP-competitive inhibitors. Kinase domains exist in different conformations depending upon their activation state. A number of key features have been identified that distinguish active and inactive kinase conformations. The conserved catalytic lysine residue (PvPKG Lys563), within the conserved AxK signature sequence in β3, forms a salt bridge with the catalytic glutamate (PvPKG Glu582) in the αC-helix in the active “αCin” conformation and interacts with the α and β phosphates of the bound ATP. In PIKs, a larger hydrophobic residue is sometimes observed in the alanine position of the AxK motif.

The C-terminal lobe forms the base of the ATP binding pocket and contains the activation segment and the catalytic loop, which play key roles in substrate binding and catalysis. The side chain of the catalytic aspartate (PvPKG Asp675), at the start of the activation segment within the conserved DFG motif, points toward the ATP binding site in the active “DFGin” conformation. There are generally one or more phosphorylation sites within the activation segment (Thr688 in PvPKG), and phosphorylation is required for activation in some kinases. The APE motif (697APE699 in PvPKG), found at the end of the activation segment, is highly conserved in ePKs. This motif is not conserved in PIKs, which have a conserved PFxLT motif at the end of the activation segment. The catalytic loop contains the conserved HRD signature (655YRD657 in PvPKG) with the catalytic aspartate thought to assist in the transfer of the phosphoryl group from ATP to the substrate. In the aPKs and PIKs, the arginine and histidine are typically mirrored on the other side of the aspartate residue (atypical DRH motif).

**SELECTING AN APPROPRIATE PLASMODIUM KINASE FOR DRUG DISCOVERY**

The ATP binding site, present in all kinases, is highly druggable, and the structural requirements for designing potent ATP-competitive kinase inhibitors are well understood (Figure 3). This, together with the large number of *Plasmodium* kinases that have been identified as genetically essential for parasite survival, results in a long list of potential kinase targets that could form the basis of target-focused antimalarial drug discovery programs. However, not all
of these kinases are likely to be good targets. In this section, we discuss the different features that may determine whether or not a particular kinase will be a good drug target. This is based on criteria used to evaluate drug targets in infectious diseases\textsuperscript{53–55} with a focus on kinases. This includes a discussion of: (1) the essentiality and vulnerability across different stages of the parasite life cycle; (2) druggability and mode of inhibition; (3) conservation across \textit{Plasmodium} species and potential for pan-species activity; (4) homology with human kinases and the potential for selectivity; (5) stage specificity, speed of parasite killing, and potential to fulfill TCP requirements; (6) a propensity for resistance; (7) the availability of target-based assays; (8) the availability of structural information. Target-based drug discovery programs are most productive when they are closely integrated with phenotypic approaches, allowing for phenotypic and \textit{in vivo} validation of the putative target as early as possible (Figure 4).

Fortunately, in the case of malaria, there are good cellular models (subject to pharmacokinetic factors) and animal models of infection. However, the ultimate validation of any drug target is through the clinic, providing definitive evidence that a compound inhibiting a specific molecular target is capable of curing disease.

**Essentiality and Vulnerability Across Different Stages of the Parasite Life Cycle.** Cabrera and colleagues have previously discussed the four levels of target validation within the context of \textit{Plasmodium} kinases\textsuperscript{14}. For a kinase to be exploitable for drug discovery, it must first be confirmed as essential for parasite survival: i.e., the inhibition of the kinase results in parasite death. This can be achieved through genetic validation, chemical validation, or a combination of both. Genetic validation\textsuperscript{51,52} can be achieved through a number of approaches including: classical gene knockout by homologous recombination or more recently by CRISPR-cas9 gene editing (reviewed by Lee et al.\textsuperscript{56}). The inability to knockout a specific gene can provide indirect evidence that the gene is essential. A variety of conditional knockdown (cKD) systems are now available to interrogate target essentiality, vulnerability, and function in \textit{Plasmodium}. Target vulnerability refers to the duration and extent to which the levels or activity of the target need to be reduced to achieve parasite death. cKD approaches allow the endogenous levels of specific proteins within parasites to be depleted without being entirely ablated. cKD lines are also increasingly used to confirm that compounds remain on-target in the whole cell environment and thus provide an important route for chemical validation. Cell lines where targets of interest have been depleted can also be used in high-throughput screening to rapidly identify chemical matter capable of inhibiting the target within parasites. However, the gold standard for genetic validation of drug targets in \textit{Plasmodium} remains the generation of conditional knockout (cKO) lines using DiCre recombinase,\textsuperscript{57} FKBP-destabilization

**Figure 5.** Examples of \textit{Plasmodium} kinase inhibitors. (A) PI4K inhibitors, (B) PKG inhibitors, and (C) CLK3 inhibitor.
domains,58 glnS ribozyme,59 or other similar systems. Here, endogenous genes can be removed and replaced by versions of the target that can be switched on or off, allowing their role in parasite survival to be definitively established. These approaches can also provide an early and important indication of the speed of cell killing associated with a specific target.

There are many examples where gene knockout/disruption studies have yielded conflicting results (http://phenoplasm.org/) even when carried out in the same Plasmodium species. For example, conflicting results for tyrosine kinase-like 3 (TKL3) have been reported in both P. falciparum (PF3D7_1349300) and P. berghii (PBANKA_1362100). Reverse genetics studies in P. falciparum demonstrated that TKL3 is likely essential for blood-stage schizogony.51,60 In contrast, subsequent data from a piggyBac insertion mutagenesis screen in P. falciparum suggests that TKL3 is dispensable.30 Similarly, conflicting results have been reported in P. berghii.52,61 While the reasons for these discrepancies remain unclear, in a more recent study, a P. falciparum TKL3 cKD line showed only minor growth defects, corroborating the finding that TKL3 is nonessential for asexual blood-stage development in P. falciparum.62 It is important to note that compensatory mechanisms activated due to reduced levels of the target or reduced activity of a target may not be evident in gene knockout studies and may also differ from compensatory mechanisms activated during chemical inhibition of the target. Many proteins have more than one function, including important structural functions. Knocking out a gene entirely will remove all of these functions of the corresponding protein, and it will not be immediately clear which function is responsible for killing the parasite. It should be noted, however, that gene knockout strategies correspond to 100% inhibition of a specific target. It is extremely challenging to achieve this level of inhibition pharmacologically, particularly for any extended period of time. Indeed, the lower the percentage inhibition required to elicit a pharmacological response, the better it is. Collectively, these issues illustrate the difficulty in validating drug targets and highlight the need for multiple complementary approaches to confirm the essentiality of any given target.

Chemical validation is an alternate approach to validate molecular targets. This requires development of a compound that is highly selective for the target of interest;63 if this is achieved, then it should be possible to obtain a direct correlation between inhibition of the enzyme and parasite death. However, it is challenging to develop a completely selective inhibitor of a kinase, owing to the highly conserved structural features of many kinases. Indeed, many human kinase inhibitors are clinically efficacious precisely because they inhibit more than one kinase.40 Target deconvolution studies for compounds developed phenotypically can also lead to the validation of novel molecular targets; for instance, PI4KIIIβ was shown to be the primary target of KDU69164 and MMV390048, now in clinical trials (Figure 5).8,9

Chemogenetics approaches provide an alternative method for the chemical validation of kinases, whereby key active site residues are mutated, altering the sensitivity of the mutated kinase to a specific inhibitor. For example, in the “bump and hole” approach developed by Shokat and co-workers,47,65 the ATP binding site is made larger by genetically engineering in a small gatekeeper residue, thus opening up the binding pocket and allowing very selective inhibitors to be designed. Again, this approach can be utilized to probe the physiological function of an individual protein kinase.

**Druggability and Mode of Inhibition.** A key advantage of targeting kinases for malaria drug discovery is that they are a known druggable target class. There are a number of different types of inhibitors of protein kinases with different ways of binding:40,60,65

- **Type I:** ATP site targeting active form (DFG-in)
- **Type I1/2:** ATP site targeting inactive form (DFG-in)
- **Type II:** ATP site targeting inactive form (DFG-out)
- **Type III:** allosteric (binding to a cleft between the large and small lobes but not the ATP binding site)
- **Type IV:** allosteric
- **Type V:** binds in at least two different parts of the active site
- **Type VI:** covalent

The multiple different binding modes of inhibitors may offer alternative drug discovery strategies to inhibit protein kinases. This may be important in deriving selectivity or appropriate physicochemical properties of compounds. Similarly, different inhibitor binding modes also apply to PIKs, although the range of binding modes observed in high-resolution structures is more limited.68 Most published inhibitors of human kinases bind to the highly druggable ATP binding site and have the typical donor–acceptor– (donor) motif for binding to the hinge. Binding interactions are well understood for compounds that bind in this site. While the most common scaffolds bind to multiple different kinase targets, many are more selective, preferentially binding to specific kinase families.69 Despite overall structural similarity, the shape of the ATP binding site in aPKs and PIKs differs significantly from ePKs. PIK inhibitors typically exploit unique interactions in the so-called affinity and/or selectivity pockets.68,70 Interactions with the affinity pocket are available due to the unique side chain orientation of a residue at the start of β-sheet 4, toward the binding site, as well as the orientation of the gatekeeper residue, which typically blocks interactions with this back pocket in ePKs. Furthermore, the unique composition of residues in the P-loop of the PIKs results in the specificity pocket being occupied by propeller-shaped inhibitors, which are less flat than typical kinase inhibitors. Hinge-binding scaffolds are typically very flat, and many have low solubility, providing an additional challenge to compound development.

Ideally, an antimalarial will be in BCS (Biopharmaceutics Classification System) Class I (high solubility and high permeability).

A key issue with developing compounds for the treatment of malaria is that TCFs dictate antimalarial must have a sufficiently long half-life to facilitate single dose treatment regimens. This usually requires compounds to have a suitable balance of low clearance and high volume of distribution. It is important that these features are built into a molecule during the optimization process.

**Conservation across Plasmodium Species: Pan-Species Activity.** Essentiality and structural conservation across different human pathogenic Plasmodium species are important to ensure that compounds will have pan-species activity. This is an important consideration for clinical use in the field. Generally, activity is only tested against one or two Plasmodium kinase orthologues in vitro. In addition, many experimental models rely on Plasmodium species that cause infection in animals with the assumption that the targets are conserved.
While ATP binding sites are typically highly conserved between species, small structural differences in the kinase domain may affect the potency in a compound specific manner. Furthermore, allosteric sites may be more poorly conserved. For example, the PfPI4KIIIβ and PvPI4KIIIβ have an overall sequence identity of only 58%; however, their kinase domains are highly homologous sharing 97% sequence identity, and the residues making up the ATP binding site are predicted to be identical.

**Plasmodium and Human Kinases: Selectivity.** A key issue in developing kinase inhibitors for malaria is selectivity over human kinases to prevent toxicity. There are relatively small differences in the ATP binding sites of different kinases, both Plasmodium and human. Therefore, it is always important to, where possible, identify the closest human homologues for counterscreening. Screening key compounds against a panel of human kinases is important to highlight any significant inhibition likely to cause toxicity to the host. This is a costly screen, so only a few compounds can be screened against a whole panel. However, if several human kinases are identified as potential “anti-targets”, it is much more feasible to counterscreen against those on a regular basis.

It is often very challenging to design compounds that are selective for a *Plasmodium* kinase target relative to human kinases at the outset of a project. However, if one or two human kinases are specifically inhibited, then designing in selectivity for the *Plasmodium* kinase is much more feasible. The best starting point is one with no significant activity against human kinases. It must also be noted that, even where there is a direct human homologue to a pathogen target, it is still possible to obtain compounds with a high degree of selectivity; an example inhibitor developed against *Leishmania CRK3.* Development of selective inhibitors capable of targeting the parasite enzyme and not its human homologue can be greatly facilitated by structure-based approaches. Furthermore, there are examples of human ATP-competitive kinase inhibitors that have been developed to specifically inhibit only one isoform of several very closely related human kinases. For example, both pan-P13K class I inhibitors and inhibitors that display selectivity for each of the individual class I isoforms have been developed. This demonstrates that selectivity can be obtained even in the case of highly homologous ATP binding pockets. Selective kinase inhibitors typically exploit nonconserved regions on the periphery of the ATP binding site or subtle differences in the accessibility, flexibility, and/or conformation of subpockets adjoining the ATP site. While high resolution inhibitor-bound structures can often be used to rationalize selectivity, many of these isoform specific binding features may only become apparent after a selective inhibitor is identified and an inhibitor-bound structure is elucidated (e.g., the so-called “specificity pocket” in P13K isoforms that opens upon binding of propeller-shaped inhibitors). In many cases, even where structural information is available, key aspects of selectivity remain unclear.

The determination of the selectivity window necessary to mitigate the toxicity risk due to human kinase off-target activity can be challenging. In *in vitro* kinase inhibition data in the form of IC50 values can provide a good indication of selectivity, but it is important to note that these values are ATP concentration dependent. The determination of the inhibitory constant (Ki) more accurately predicts relative binding affinity. However, differences in the Ki_{ATP} / ATP concentrations, and drug exposure at target sites (inside different parasite compartments at different life-cycle stages) and off-target sites (different human cells/cellular compartments) may further confound off-target activity predictions. In addition to selectivity based on inhibition of the enzyme (K, *Plasmodium* kinase/K, human kinase), there is also selectivity based on biological function. In this case, inhibition of the enzyme in one species can have a much more profound effect on the cellular growth than inhibition of the same enzyme in another species. Furthermore, the relatively short duration of malaria treatment may mitigate toxicity that would otherwise be observed in the case of a high dose, long-term treatment for chronic diseases such as cancer.

An attractive strategy to find very selective kinase inhibitors is to target an allosteric pocket remote from the ATP binding pocket. Allosteric pockets are usually specific to a particular enzyme. Thus, an allosteric pocket in a malaria kinase is unlikely to be found in any human kinases. A caveat is that allosteric pockets may have a greater propensity to mutate than highly conserved active site residues, and this may lead to an increased resistance potential for drugs targeting these pockets. Allosteric inhibitors do not need to compete with high cellular levels of ATP. Another approach to develop selective inhibitors would be to use a covalent inhibitor that targets a nucleophilic residue in the active site/allosteric pocket of the *Plasmodium* enzyme that is absent in the human orthologue(s).

**Stage Specificity, Rate of Action, and Potential to Fulfill TCP.** Different kinases are expressed at different life-cycle stages. It is important to understand at what stage the kinase of interest is expressed and its vulnerability to inhibition at each stage. The ideal scenario is that a kinase is essential across multiple life-cycle stages. Targeting a kinase essential to the liver, asexual blood, and the gametocyte or mosquito stages of the life cycle has the potential to deliver an antimalarial with prophylactic, curative, and transmission-blocking activity. Ideally, the inhibition of a kinase leads to a fast rate of kill of the blood stage form of *Plasmodium*, or if it has a moderate/slow rate of kill, this occurs on multiple life-cycle stages. The rate of kill can be measured in *in vitro* using the parasite reduction ratio (PRR) assay, in *vivo* using the SCID mouse model, and ultimately in clinical studies in humans.

**Propensity for Resistance.** Resistance is a key challenge for anti-infectives in general but particularly for antimalarials. There are a number of different mechanisms by which resistance can emerge in pathogens. These can include the following: mutations in the target enzyme; amplification of the target enzyme; increased influx of the compound from the parasite due to amplification of the parasites’ efflux transporters; reduced uptake of the compound due to down-regulation of parasite importers; upregulation of bypass pathways; increased uptake of key intermediates from the human host; direct metabolism of the inhibitor. In *Plasmodium*, drug resistance is most commonly driven by mutation rather than amplification, and *in vitro* drug resistance selections followed by whole genome sequencing are regularly used to assist in target identification and to estimate the resistance potential of development compounds. It is unclear if resistance is an intrinsic feature of specific targets, inhibitors, or both. However, some indication that resistance is difficult to obtain is valuable in validating a given target. First, there should be no significant cross-resistance to existing antimalarials, which can be examined by screening against parasite strains resistant to existing antimalarial. Second, it is possible to measure the minimum inoculum for resistance (MIR).
Compounds with a MIR of $<10^5$ are considered high risk, while those with a MIR of $>10^7$ are much lower risk. Some examples of clinically used compounds are reported by Ding et al.\textsuperscript{24} The MIR depends on the \textit{Plasmodium} cell line used. Fold increases in EC\textsubscript{50} values of resistant clones relative to sensitive parasites and the associated fitness cost to the parasite are also considered when assessing resistance potential.\textsuperscript{34} Another important feature is the level of conservation of the active site across different \textit{Plasmodium} species and clinical isolates. This information can be used to assess the risk of pre-existing, naturally occurring resistance once the drug is used widely in the field.

\textbf{Availability of Target-Based Assays.} A variety of different assay formats have been developed for the study of kinases with many amenable to high-throughput screening.\textsuperscript{75,76} Radiometric incorporation assays utilizing radioisotope labeled γ-ATP are considered the gold standard. Nonradiometric assay formats include universal ADP-detection assays typically utilizing either coupled enzyme reactions (e.g., Promega ADP-Glo Kinase Assay, DiscoverX ADP Hunter, and ADP Quest Assays) or antibody-based methods (e.g., Bellbrook Laboratories Transcreener ADP\textsuperscript{5} Fluorescent Polarization, and Fluorescent Intensity Kinase Assays) to quantify ADP formation. Other methods detect the phosphorylation of labeled substrates and include FRET-based methods (e.g., Cisbio KinEASE TR-FRET kits) and mobility shift assays (e.g., Caliper Life Sciences microfluidic chip-based assays). High-throughput mass spectrometry detection methods (e.g., Agilent RapidFire Mass spectrometry platform) allow for sensitive and direct quantitative detection of native substrates and products. Despite this, there are still a number of challenges in developing a robust assay. First, soluble, enzymatically active protein must be readily generated by recombinant expression. The genome of \textit{Plasmodium} is extremely AT rich, particularly in \textit{P. falciparum}, making recombinant expression in bacterial and eukaryotic expression systems particularly challenging. This issue can be circumvented if \textit{Plasmodium} expression constructs are codon-optimized for the desired expression system prior to expression. Some \textit{Plasmodium} proteins have large inserts, consisting of low complexity regions of amino acid homorepeats, which are particularly prevalent in \textit{P. falciparum}. These repeated sequences again make recombinant expression difficult, and in some cases, it is necessary to use a closely related orthologue from another \textit{Plasmodium} species more conducive to expression and subsequent purification. Second, many kinases are activated by phosphorylation or other post-translational modifications and may not be isolated in their active form. Third, it is necessary to find a substrate protein or peptide for the kinase to support assay development. This can often prove challenging where there is no knowledge of the physiological function of the kinase or it is natural substrate.

\textbf{Structural Biology.} Structural information surrounding targets of interest can be extremely valuable in compound optimization to drive both potency improvement and selectivity against a human orthologue. This again is dependent upon recombinant expression of soluble and stable protein. Advances in X-ray crystallography and cryo-electron microscopy now enable high-resolution structures to be generated for challenging proteins. High-throughput cloning and protein production strategies enable the rapid assessment of multiple engineered protein constructs for structural biology.\textsuperscript{27,77} In addition, antibodies/nanobodies can be used as chaperones for structure determination to improve sample homogeneity/aid crystallization and/or stabilize protein targets in a desired conformation.

\textbf{CDPK1: An Example.} Many of the challenges associated with target selection and validation have been highlighted by \textit{Plasmodium} CDPK1, one of the first \textit{Plasmodium} kinases explored extensively as a drug target. CDPK1 initially appeared to fulfill many of the key requirements of a promising target including (1) genetic essentiality for asexual blood-stage development and transmission based on unsuccessful attempts to knock out \textit{cdpk1}, (2) druggability, (3) conservation across \textit{Plasmodium} species, (4) unique structural features with no orthologue in humans, and (5) a small gatekeeper residue that could be exploited for selectivity.\textsuperscript{52,79,80} Recombinant expression of \textit{Plasmodium} CDPK1, development of a biochemical assay suitable for high-throughput screening, and a high-resolution crystal structure of PfCDPK1 (PDB 3Q5I) provided the key tools to drive target-based drug discovery efforts.\textsuperscript{81}

Despite these promising features and the identification of potent CDPK1 inhibitors with good antiplasmodium activity, the validity of CDPK1 as a drug target for TCP1 has been brought into question. Follow up medicinal chemistry efforts showed a poor correlation between CDPK1 enzyme inhibition data and antiplasmodium activity, suggesting that other targets might be involved and hindering phenotypic target validation.\textsuperscript{82} Despite multiple, unsuccessful attempts to knock out or disrupt \textit{cdpk1} in \textit{P. falciparum}, Jebiwo et al. successfully disrupted \textit{cdpk1} in \textit{P. berghei}.\textsuperscript{83} Subsequent studies, utilizing engineered parasites with gatekeeper mutations, demonstrated that PKG-mediated pathways can compensate for reduced CDPK1 activity,\textsuperscript{84,85} consistent with the observation that the conditional knockdown of CDPK1 in \textit{P. falciparum} shows no significant phenotype.\textsuperscript{62} In an elegant study, Green et al. showed that the imidazopyridazine series that potently inhibits PfCDPK1 could be split into two classes on the basis of the mode of action in asexual blood-stage parasites with the primary target being either PKG or heat shock protein 90 (Hsp90) rather than CDPK1.\textsuperscript{86} CDPK1 has been shown to be essential for transmission, so the inhibition of CDPK1 may still be a good strategy for TCP\textsubscript{S}.\textsuperscript{55,87} This example highlights the importance of phenotypic validation, the complexity of compensatory mechanisms, and the potential role of multiple targets in antiplasmodium activity. This also serves as a reminder that small structural modifications within the same chemical series can lead to a change in mode of action and/or implicate additional targets, particularly when the target is part of a large multigene family.

\section*{opportunities for polypharmacology}

Many drugs are known to inhibit more than one molecular target at pharmacological doses.\textsuperscript{88} This could be perceived as an advantage for an anti-infective agent, for several reasons, if it is active against more than one of the pathogen’s enzymes. First, if both enzymes are essential, then this can limit the potential for resistance to emerge, since the likelihood of a resistance-generating mutation spontaneously occurring in two independent targets at the same time in one parasite is extremely low.\textsuperscript{89} This is illustrated in bacteria, where compounds such as the fluoroquinolones inhibit both gyrase and topoisomerase II.\textsuperscript{90} Second, compounds demonstrating polypharmacology have the potential to increase the efficacy. Targeting multiple enzymes within the same or related
pathways may result in synergistic effects, as seen with dihydropyrimidine synthase and dihydrofolate reductase inhibitors with both acting on the folate metabolism. Synergism potentially allows improved efficacy at a given dose. However, one potential risk is if resistance occurs to one of the agents and the other is dosed sub-therapeutically, then treatment with the combination could fail. Third, polypharmacology will have the added advantage of beneﬁtting patients by minimizing the pill burden and cost associated with combination drug regimens, which currently requires two or more small molecules that inhibit diﬀerent single targets to be combined. It is noteworthy that building the appropriate combination, can provide a more complete picture of the eﬀect of compounds on multiple pathways within the parasite. Coupled with genetic approaches, comparative omics studies can be used to distinguish “on-target” eﬀects of inhibitors from those resulting from additional/secondary targets.

There are numerous examples of Plasmodium kinase inhibitors reported to inhibit more than one kinase from different kinase families as well as closely related analogues shown to primarily act through essential kinases. These compounds could serve as starting points for the design of dual or multikinase inhibitors. We anticipate that, as more data sets are generated showing the beneﬁts of targeting multiple Plasmodium kinases, a so-called “magic shotgun” approach rather than a “magic bullet” approach, in which drugs are purposefully designed to interact selectively with multiple molecular targets, may gain traction.

### EXAMPLES OF KINASES BEING TARGETED IN MALARIA

Several Plasmodium kinases are being advanced and show promise. Our previous review covered some of this work. Here, we give an update on 3 chemically validated kinases that have been demonstrated to be essential to multiple stages of the Plasmodium life cycle and look exciting from a malaria drug discovery perspective (Table 1). **PI4K**. PI4KIIIβ was first reported as a promising antimalarial target in 2013 after it was identiﬁed as the primary target of novel imidazopyrazine/pyridine compounds (e.g., KDU691, Figure 5A) displaying potent prophylactic liver-stage, asexual blood-stage, and transmission-blocking antiplasmodium activity in mouse models of malaria. Target identiﬁcation involved in vitro resistant selections that resulted

| TARGET  | PI4K | PKG | CLK3 |
|---------|------|-----|------|
| PLASMO DB ID | PF3D7_0509800 | PF3D7_1436600 | PF3D7_1114700 |
| VALIDATION LEVEL | Clinically (Phase II) | In vivo | In vivo |
| TOOL COMPOUNDS | KDU691, MMV390048 | MI10, MMV30084 | TCMDC-135051 |
| LIFE-CYCLE STAGE ACTIVITY | ASB, T, L | ASB, T, L | ASB, T, L |
| HUMAN ORTHOLOGUE | PI4KIββ (~43%) | PRKGI1/2 (~30%) | PRP4 (~50%) |
| IN VITRO RATE OF ACTION * | moderately slow (fast acting in clinic) | slow | fast |
| RESISTANCE PROPENSITY | moderate (MIR ~10^6) | low | low (MIR ~10^7) |
| CELL-BASED TOOLS | resistant lines | cKD, gatekeeper mutant | resistant lines, G449P mutant |
| BIOCHEMICAL ASSAY | ✓ | ✓ | ✓ |
| STRUCTURE | Homology model | Homology model |

| PI4K ^a, ^b, ^c, ^d | Phosphatidylinositol 4-kinase type III beta; PKG, ^e, ^f, ^g | cGMP-dependent protein kinase; CLK3, ^h, ^i | cyclin-dependent-like kinase 3. ^j, ^k |
|---------------------|---------------------------------|---------------------------------|--------------------------|
| ^a^PI4K, ^b^9, ^c^, ^d^64 | ^e^Phosphatidylinositol 4-kinase type III beta; PKG, ^f^62, ^g^99 | cGMP-dependent protein kinase; CLK3, ^h^97 | cyclin-dependent-like kinase 3. ^i^6, ^k^ |
| ^j^See Figure 5 for chemical structures of tool compounds. ^k^Tool compound/s showed activity in assays testing for asexual blood-stage (ASB), transmission-blocking (T), and prophylactic liver-stage (L) antiplasmodium activity. ^l^Closest human orthologue (percentage shared sequence identity); ^m^PI4KIIIβ, phosphatidylinositol 4-kinase type III beta (UniProt Q9UBF8); PRKGI1/2, protein kinase cGMP-dependent 1 and 2 (UniProt Q13976 and Q13237); PRP4, pre-mRNA-processing factor 4 (UniProt Q13523). ^n^Based on data from the in vitro parasite reduction ration assay. ^o^Based on the minimum inoculum of resistance (MIR) determined for tool compounds in in vitro drug-resistance selections and EC50 fold change associated with resistance. ^p^ML10 or MMV30084 resistance selections did not identify PKG mutations. The continuous exposure of DD2-B2 parasites to 3 × EC50 of MMV30084 yielded resistant parasites with a T1268R mutation in tyrosine kinase-like protein 3 (TKL3; PF3D7_1349300) that showed a 2.9-fold EC50 shift compared with the parental line. Continuous exposure of 10^6 DD2-B2 TKL3 knockout parasites did not result in parasite recrudescence. ^q^cKD, conditional knockdown. ^r^Engineered inhibitor-insensitive P. falciparum PKG T618Q parasite line. ^s^Engineered inhibitor-insensitive P. falciparum CLK3 G449P parasite line. ^t^High-resolution structures of PfPKG (PDB ID: 5DYK) and PvPKG (PDB IDxs: 4RZ7, 5F0A, SDYL, SDZC, 5FET, 5EZR). |
in drug resistant *P. falciparum* lines with mutations in the kinase domain of PI4KIIIβ and Rab11a, a small GTPase known to interact with PI4KIIIβ in humans. To confirm the role of these mutations in resistance, they were then engineered into *P. falciparum* Dd2.64 Subsequent biochemical assays using recombinantly expressed *P. vivax* PI4KIIIβ confirmed that these inhibitors are potent ATP-competitive inhibitors of *Plasmodium* PI4KIIIβ. These compounds were not progressed, but PI4KIIIβ was subsequently identified as the primary target for a very promising series of amino-pyridines/pyrazines.8,100 The most advanced *Plasmodium* kinase inhibitor, the 2-aminopyridine MMV390048 (Figure 5A), is currently in Phase II clinical trials for the treatment of malaria targets PI4KIIIβ.8,9

Using a phenotypic-based approach, MMV390048 and other related compounds were developed from whole cell screening hits identified from a Soft Focus Kinase Library without prior knowledge of their targets. Target identification studies, including resistance selections in *P. falciparum*, chemoproteomic studies, and biochemical assays, all pointed to PI4KIIIβ as the primary target. In addition, a good correlation between *in vitro* antiplasmodium activity and enzyme inhibitory potencies was observed for this series.8 More recently, MMV390048 was reported to potently inhibit *P*/*I13K in addition to *P*/*I13Kβ in *in vitro*, but the contribution of *P*/*I13K inhibition to compound efficacy is not yet known.52 MMV390048 displayed good *in vitro* selectivity for *Plasmodium* PI4K with limited off-target activity against human kinases and the potential to fulfill TCP1, TCP4, and TCP5, consistent with the essential role of PI4KIIIβ in multiple stages of the life cycle.53 MMV390048 showed a moderately slow rate of kill in the *in vitro* PRR assay and a moderate rate of kill *in vivo* but a fast killing profile in the clinic.

*P*/I4KIIIβ competitive inhibitors show a moderate propensity for resistance *in vitro* with a MIR of 10<sup>5</sup>–10<sup>7</sup>.8,100 Nine different PI4KIIIβ single point mutations within the kinase domain as well as PI4K copy number variations and a single point mutation in Rab11a have been reported for laboratory resistant lines generated against a range of PI4KIIIβ inhibitors.8,64,100,101 These resistant lines displayed low to moderate EC<sub>50</sub> shifts (3- to 22-fold) with some PI4K inhibitors.8,64,100,101 Additional kinase targets were implicated for a subset of thiazoles that were shown to be fast acting *in vitro*, deviating from the slow rate of kill typically observed for PKG inhibitors.8 More recently, PKG was identified as the primary target for the trisubstituted imidazole MMV300884 (Figure 5B) that shows potent antiplasmodium activity across the parasite life cycle and structural similarity with PKG inhibitors identified from the GSK Full Diversity Collection.62 In line with the described roles of PKG, MMV300884 inhibited hepatocyte invasion by sporozoites; merozoite egress from asexual blood-stage schizonts, and male gamete exflagellation. Resistance selections carried out with both MMV300884 and the imidazopyridine MLI0 did not lead to mutations in PKG, suggesting that PKG is a target with a low propensity for resistance.62,99 In the case of MMV300884, a continuous exposure of the drug resulted in resistant parasites with a T1268R mutation in tyrosine kinase-like protein 3 (TKL3, PF3D7_1349300) conferring a 2.9-fold EC<sub>50</sub> shift compared with the parental line.62 The molecular basis for TLK3-mediated resistance is not clear, and it is yet to be determined if this TKL3 mutation results in cross-resistance to other compounds targeting PKG. In addition to PKG cKD and cKO approaches, a chemogenetics approach making use of engineered inhibitor-insensitive parasite lines containing a gatekeeper mutation has served as a useful tool to demonstrate on-target whole cell activity and study PKG function.96,99,111–114 On the basis of the previously reported series targeting P/PKG, very significant PKG inhibition appears to be required for good whole cell activity with a difference of roughly 2 orders of magnitude typically observed between PKG IC<sub>50</sub> values (generated in the presence of ATP at a concentration equal to the K<sub>m</sub> of ~20 μM) and *P. falciparum* IC<sub>50</sub> values.

Unlike both human PKGs and many other human serine/threonine protein kinases, *Plasmodium* PKG has a small gatekeeper residue, allowing inhibitors access to the back pocket. Even though reported *Plasmodium* PKG inhibitors exploit this back pocket, providing a degree of selectivity, human kinase off-target selectivity remains a key concern for drug development. The mechanism of *in vitro* allosteric inhibition of *P*/PKG using cGMP analogues has recently been described.115 However, allosteric P/PKG inhibitors with potent antiplasmodium activity are yet to be reported.

While PKG inhibitors have been shown to be slow acting in the *in vitro* PRR assay,8 PKG as a target has other favorable properties including the potential to deliver antimalarials with multistage activity that have a low propensity to generate...
resistance. It is also still not yet clear how in vitro speed of kill data for PKG inhibitors will translate into the clinic. The availability of inhibitor-bound high-resolution structures, biochemical assays suitable for high-throughput screening, and chemogenetic tools to test for on-target whole cell activity makes Plasmodium PKG an attractive target for structure-based drug design.

**CLK3.** *P. falciparum* cyclin-dependent-like kinase 3 (CLK3, PF3D7_1114700) has emerged as a promising drug target with the potential to yield antimalarials with multistage activity.97 TCMDC-135051 (Figure 5C), a CLK3 inhibitor displaying liver stage prophylactic, asexual blood-stage antiparasitoid activity and transmission reducing potential, has recently been identified from a target-based screen.97 CLK3 is one of four CLKs in *Plasmodium*.15 CLKs 1–3 have been shown to be genetically essential for asexual blood-stage development.30,52,96,116 In humans and other eukaryotes, CLKs and chemogenetic tools to test for on-target whole cell activity for the parasite life cycle, making them attractive targets requiring chemical validation.94,117 On the basis of this premise, CLK1 and CLK3 were recombinantly expressed and biochemical assays suitable for high-throughput screening were established.97 A screen of nearly 30,000 compounds composed of several kinase-focused libraries identified CLK1 and CLK3 selective small molecule inhibitors in addition to inhibitors targeting both CLK1 and CLK3. In *in vitro* resistance selections, using CLK3 selective inhibitor TCMDC-135051, resulted in point mutations within CLK3 and putative RNA processing protein PjUSP39 (PF3D7_1317000) giving rise to a 4- to 11-fold shift in EC50. On target whole cell activity for TCMDC-135051 was confirmed using a chemogenetics approach, phenotypically validating CLK3 as a target. The chemogenetics approach relied on mutating a CLK3 specific active site residue to its CLK1 counterpart (G449P), resulting in a CLK3 variant and corresponding parasite line with reduced sensitivity to TCMDC-135051. Furthermore, TCMDC-135051 showed rapid killing in *in vitro* PRR assays and selectivity for *Plasmodium* CLK3 relative to its human ortholog USP39 and closely related human CLK1. Selectivity based on a panel of representative human kinases is yet to be reported. A transcriptome analysis study revealed that CLK3 inhibition resulted in the downregulation of more than 400 genes thought to be essential to parasite survival, most of which contain introns, consistent with the role of CLK3 in regulating RNA splicing. Initial medicinal chemistry optimization efforts focused on TCMDC-135051 have been reported,118 and we anticipate new target-based programs focused on CLK3 and other promising CLKs (e.g., CLK2), including structural biology efforts, to follow.

### COMMENT

In summary, kinases are promising drug targets that have been relatively poorly explored in malaria, compared to the effort on human kinases. There is a large amount of literature available to guide the design of inhibitors for this family of enzymes. Furthermore, many compounds have been developed in human kinase inhibitor programs, some of which could act as leads for antimalarial drug discovery. The obtaining of selectivity over human kinases can be very challenging and chemistry resource intensive, but it is worth noting that not all off-target activity will necessarily lead to toxicity.

Many factors, in addition to genetic essentiality, need to be considered when selecting a kinase as a target. Target-based drug discovery efforts need to be closely integrated with phenotypic approaches so that phenotypic validation can be achieved at an early stage. Phenotypic whole cell screening of kinase-focused libraries followed by target deconvolution studies is a good way to identify promising kinase— inhibitor pairs. Kinase-specific chemoproteomic approaches, coupled with advances in genome editing technologies for *Plasmodium*, have provided a powerful set of tools to assist in target identification and validation.

The development of a panel of *in vitro* *Plasmodium* kinase assays together with more structural information would greatly facilitate drug discovery. Target-based screens using purified kinases also have the potential to identify non-ATP competitive modes of action that may overcome some of the challenges related to selectivity. This may circumvent the problem that few ATP-competitive inhibitors are BCS Class I. The potential for resistance needs to be monitored, particularly due to the high rates of clinical resistance seen for kinase inhibitors in cancer. Given the structural similarity between kinases, specifically designing in polypharmacology is a very attractive proposition119 but is still very much in its infancy.

In this Perspective, we have highlighted the many factors that require consideration when prioritizing a kinase as a target. While the absence of a known human homologue may be an attractive feature, the conserved nature of the ATP site across this large superfamily of enzymes makes it difficult to predict potential kinase off-targets (or adverse effects caused by off-target activity), and the obtaining of selectivity may still be challenging. On the other hand, *Plasmodium* kinases with human orthologues may have specific features that allow for the selective inhibition of the *Plasmodium* enzyme. It is difficult to say if any specific class of kinases represents a superior target class for the development of antimalarials. However, *Plasmodium* kinases in general certainly merit further investigation.

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