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Review Article

Plasmodium falciparum protein kinase as a potential therapeutic target for antimalarial drugs development

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Abstract. Malaria is one of the most dangerous infectious diseases due to its high infection and mortality rates, especially in the tropical belt. *Plasmodium falciparum* (*P. falciparum*), the most virulent malaria parasite in humans, was recently reported to develop resistance against the final efficient antimalarial drug, artemisinin. Little is known about the resistance mechanisms, which further complicates the problem as a proper counteraction is unable to be taken. Hence, the understanding of drug mode of action and its molecular target is valuable knowledge that needs to be considered to develop the next generation of antimalarial drugs. *P. falciparum* protein kinase (*Pf* PK) is an attractive target for antimalarial chemotherapy due to its vital roles in all *P. falciparum* life stages. Moreover, overall structural differences and the presence of unique *Pf* PKs that are absent in human kinome, suggesting specific inhibition of *Pf* PK without affecting human cells is achievable. To date, at least 86 eukaryotic protein kinases have been identified in *P. falciparum* kinome, by which less than 40 were validated as potential targets at the erythrocytes stage. In this review, recent progress of the furthest validated *Pf* PKs; *Pf* Nek-1, *Pf* CDPK1, *Pf* CDPK4, *Pf* PKG, and *Pf* CLK-3 will be briefly discussed.

INTRODUCTION

Plasmodium falciparum (*P. falciparum*) is a protozoan parasite that causes the most virulent malaria cases in humans, followed by *Plasmodium vivax* (*P. vivax*) and *Plasmodium knowlesi*. Meanwhile, mild or moderate cases of malaria infections are inflicted by *Plasmodium malariae* and *Plasmodium ovale* (Rajahram *et al.*, 2012; Tougan *et al.*, 2020). Malaria is currently listed as one of the top 3 infectious diseases in the world due to its high infection and mortality rates (Osborn *et al.*, 2020). As reported by WHO in the latest Malaria report, 228 million malaria cases were recorded in 2018 (93% in Africa, 3.4% in Southeast Asia, and 2.1% in the Mediterranean), by which *P. falciparum* causes 71% of the total malaria infections. Malaria also accounted for almost half a million deaths annually since 2010, mostly children aged under 5 (67% in 2018) (WHO, 2019).

P. falciparum is an obligate parasite that requires two hosts, which are the invertebrate (female *Anopheles* sp. mosquito as a vector) and vertebrate, to complete its sexual and asexual lifecycle, respectively (Singleton, 2020). In humans, *Plasmodium* asexual life cycle is divided into exoerythrocytic (in the liver) and intraerythrocytic (in the red blood cell, RBC) stages. Saliva injected by an infected mosquito will transfer sporozoites into the bloodstream and enter liver cells. The

entry of sporozoites into the hepatocytes is highly precise due to the presence of the circumsporozoite protein that bears a ligand that only binds specifically to the liver basolateral domain (hepatocyte cell membrane). The entry of sporozoites will initiate the asexual reproduction, known as the pre-erythrocytic (PE) or primary exoerythrocytic schizogony (EE) cycles. Sporozoites then metamorphose into trophozoites and are further transformed into schizont and merozoites through schizogony (Miller *et al.*, 2013; Burda *et al.*, 2017).

The erythrocytic cycle starts once merozoites leave the liver cell to penetrate RBC. The merozoites then once again turn into trophozoites (early trophozoites or ring-stage and late trophozoites). Next, the late trophozoites developed into blood-stage schizont and released from ruptured RBC together with metabolic waste and residual bodies (responsible for the characteristic symptoms of malaria). After an indeterminate number of asexual cycles, some of the merozoites will enter erythrocytes and become macro- and microgamonts to be ingested by the mosquito for sexual reproduction and the cycle is repeated (Figure 1) (Cowman *et al.*, 2012; Abdel-Ghaffar *et al.*, 2019).

Most of the antimalarial class introduced so far are targeting *Plasmodium* at the RBC stage, such as 4-aminoquinolines (chloroquine, amodiaquine, piperaquine), amino alcohols (quinine, lumefantrine, mefloquine, halofantrine) and antibiotics (tetracycline, doxycycline, clindamycin) (Maerki *et al.*, 2006; Wilson *et al.*, 2013). The RBC stage is an attractive target for drug development owing to its apparent importance for *Plasmodium* survival. In addition, RBC stage screens also require a simple experimental setup for a vast compound library to be tested. Hence, asexual blood-stage will continue to be the main target in drug development (Baragaña *et al.*, 2015; Vos *et al.*, 2015; Swann *et al.*, 2016).

However, this approach causes most of the antimalarial compounds were developed without the knowledge of their mode of action as their activities were evaluated based on phenotypic assays. Their limited therapeutic window also causes quicker drug-resistance development. *Plasmodium* has been described as unique compared with other infectious diseases because they are capable

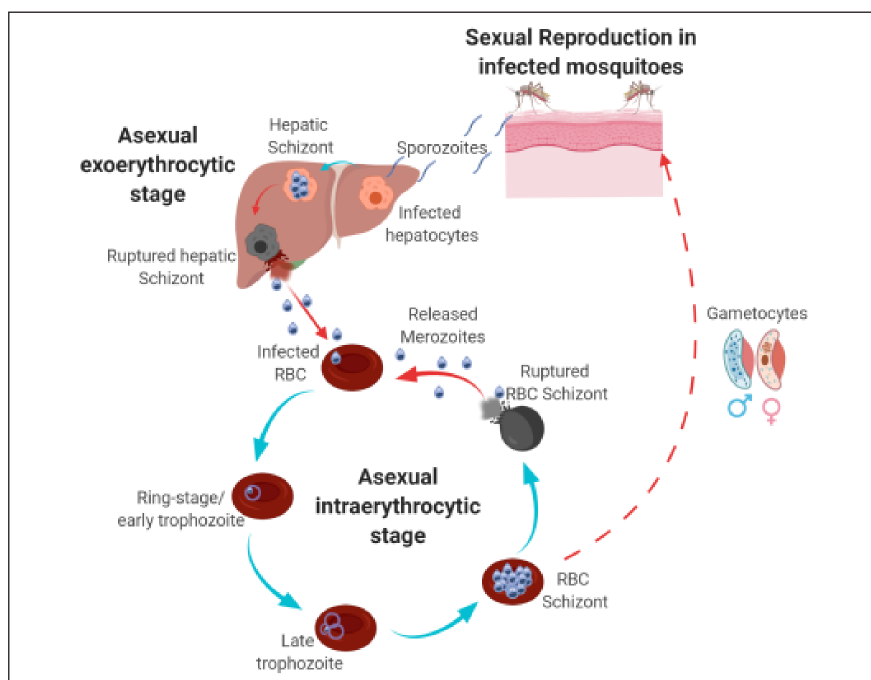

Figure 1. The life cycle of *Plasmodium falciparum*. (Created with BioRender.com)

of inducing specific resistance in the cellular target of the antimalarial drugs instead of random mutations (Goldberg *et al.*, 2012). For instance, the emergence of resistance against chloroquine (acting on trophozoite and schizont) and mefloquine (acting on ring-stage and merozoite) were reported in 12 and 5 years after their introduction, respectively (Maerki *et al.*, 2006; Wilson *et al.*, 2013; WHO, 2014). Mutation of *pfcrt* and *pfmdr1* has been consistently reported as the source of resistance against blood schizonticides in *P. falciparum* (Pulcini *et al.*, 2015; Agrawal *et al.*, 2017).

For this reason, artemisinin is widely accepted as the most effective antimalarial drugs ever introduced due to its ability to inhibit *P. falciparum* at all parasite life stages. Although artemisinin significantly delays the development of resistance strain, the artemisinin-resistance strain was finally reported in Southeast Asia, 40 years after its introduction (1972-2009) (Dondorp *et al.*, 2009; Su and Miller, 2015). Moreover, the actual target and mode of action of artemisinin are not well defined, which causes the identification of resistance mechanisms in *P. falciparum* is yet to be fully understood. So far, the mutation of 3D7K13 and VPS34myc are described as the possible sources of resistance in artemisinin-resistance strains (Bhattacharjee *et al.*, 2018; Rocamora *et al.*, 2018). Being the last efficient drug against *P. falciparum*, the recent issue associated with artemisinin is the most significant problem in malaria control (Cui and Su, 2009; Ross and Fidock, 2019).

Plasmodium falciparum protein kinase as a potential target

Based on the current problems, the next line of antimalarial agents should be identified based on an assay with a specific target, paired with the result of phenotypic or *in vivo* studies. *Plasmodium falciparum* protein kinase (*Pf* PK) is now gaining attention due to the accumulated knowledge of human kinase and successful chemotherapy application, targeting kinase protein (Dondorp *et al.*, 2010). In *P. falciparum*, *Pf* PK plays a fundamental role in numerous processes such as growth control, cell cycle regulation, signal transduction, and crucial for parasite invasion into human RBC (Soni *et al.*, 2017; Matthews *et al.*, 2018; Wilde *et al.*, 2019).

Moreover, recombinant *Pf* PKs can be easily adapted into a high throughput assay system against an unlimited number of compounds. It leads to the identification of numerous new scaffolds of *Pf* PKs inhibitor, which was never reported as antimalarial (Gamo *et al.*, 2010; Hallyburton *et al.*, 2017; Penzo *et al.*, 2019). With a proper target, *Pf* PKs may provide a new opportunity for antimalarial drug development. Bioinformatics analysis predicted that the *P. falciparum* genome is expressing at least 86 genes encoding for a eukaryotes protein kinases (ePKs), following Hanks kinase classification model, excluding atypical protein kinases (aPKs) such as phosphatidylinositol 3-kinase-related kinases (PIKK) and right open reading frame (RIO) kinase (Hanks and Hunter, 1995; Ward *et al.*, 2004; Anamika *et al.*, 2005; Miranda-Saavedra *et al.*, 2012).

Based on their structural features, these ePKs superfamilies can be further divided into those with and without human homologs. *Plasmodium* ePK with human homologs are CMGC (named after the initials of its members; CDK, MAPK, GSK3, CK2) (18 kinases/21%), PKs A, G and C (AGC/AGC-related) (8 kinases/9%), never in mitosis gene a (NIMA) (4 kinases/5%), casein kinase 1 (CK1/CK1-related) (2 kinases/2%), tyrosine kinase-like kinases (TKL/TKL-related) (5 kinases/6%) and "sterile-phenotype" kinases (STE) (2 kinases/2%). Meanwhile, more than 50% of *Plasmodium* ePKs are without human homologs which comprise of calmodulin-dependent protein kinase (CaMK) (17 kinase/ 20%), and orphan kinase (which include eukaryotic initiation factor-2 (eIF2), and a novel protein family named after a conserved Phe-Ile-Lys-Lys motif (FIKK)) (30 kinases/ 35%) (Sullivan *et al.*, 2004; Ward *et al.*, 2004; Doerig *et al.*, 2008; Abdi *et al.*, 2010; Solyakov *et al.*, 2011; Talevich *et al.*, 2012; Proellocks *et al.*, 2016; Murungi and Kariithi, 2017; Zhang *et al.*, 2018; Davies *et al.*, 2020) (Table 1).

Table 1. The list of eukaryotic protein kinases in *Plasmodium falciparum* and their current status as potential targets at the asexual red blood cell stage

No	Gene ID	Group	Family	Name	Genetic validation (RBC stage)
1	PF3D7_1431500 (PF14_0294)	CMGC	MAPK	*Pf* map-1	Dispensable
2	PF3D7_1113900 (PF11_0147)	CMGC	MAPK	*Pf* map-2	Essential
3	PF3D7_1014400 (PF10_0141)	CMGC	CDK	*Pf* mrk	Essential
4	PF3D7_0417800 (PFD0865c)	CMGC	CDK	*Pf* crk-1	Essential
5	PF3D7_0415300 (PFD0740w)	CMGC	CDK	*Pf* crk-3	Essential
6	PF3D7_0317200 (PFC0755c)	CMGC	CDK	*Pf* crk-4	Essential
7	PF3D7_1338900 (MAL13P1.196)	CMGC	CDK	*Pf* crk-4-related	Dispensable
8	PF3D7_0615500 (PFF0750w)	CMGC	CDK	*Pf* crk-5	Dispensable
9	PF3D7_1356900 (MAL13P1.279)	CMGC	CDK	*Pf* PK5	Essential
10	PF3D7_1337100 (PF13_0206)	CMGC	CDK	*Pf* PK6	Essential
11	PF3D7_1445400 (PF14_0431)	CMGC	CDK-like	*Pf* CLK-1/ *Pf* Lammer	Essential
12	PF3D7_144300 (PF14_0408)	CMGC	CDK-like	*Pf* CLK-2	Essential
13	PF3D7_1114700 (PF11_0156)	CMGC	CDK-like	*Pf* CLK-3	Essential
14	PF3D7_0302100 (PFC0105w)	CMGC	CDK-like	*Pf* CLK-4/ *Pf* SRPK1	Essential
15	PF3D7_1108400 (PF11_0096)	CMGC	CK2	*Pf* CK2	Essential
16	PF3D7_0312400 (PFC0525c)	CMGC	GSK3	*Pf* GSK3	Essential
17	PF3D7_0821100 (PF08_0044)	CMGC	GSK3	*Pf* PK1	Dispensable
18	PF3D7_1316000 (MAL13P1.84)	CMGC		*Pf* GSK3-related	Essential
19	PF3D7_0217500 (PFB0815w)	CaMK	CDPK	*Pf* CDPK1	Essential
20	PF3D7_0610600 (PFF0520w)	CaMK	CDPK	*Pf* CDPK2	Essential
21	PF3D7_0310100 (PFC0420w)	CaMK	CDPK	*Pf* CDPK3	Essential
22	PF3D7_0717500 (PF11_0060)	CaMK	CDPK	*Pf* CDPK4	Dispensable
23	PF3D7_1337800 (PF13_0211)	CaMK	CDPK	*Pf* CDPK5	Essential
24	PF3D7_1122800 (PF11_0239)	CaMK	CDPK	*Pf* CDPK6	Dispensable
25	PF3D7_1123100 (PF11_0242)	CaMK	CDPK	*Pf* CDPK7	Dispensable
26	PF3D7_1423600 (PF14_0227)	CaMK		*Pf* CDPK6-related	Dispensable
27	PF3D7_1450000 (PF14_0476)	CaMK		*Pf* CDPK6-related	Dispensable
28	PF3D7_0715300 (MAL7P1.73)	CaMK		*Pf* CDPK6-related	Dispensable

Table 1 continued...

29	PF3D7_0704500 (MAL7P1.18)	CaMK		*Pf* CDPK6-related	Dispensable
30	PF3D7_1104900 (PF11_0060)	CaMK		*Pf* CDPK6-related	Dispensable
31	PF3D7_0311400 (PFC0485w)	CaMK		*Pf* PKRP	Dispensable
32	PF3D7_0214600 (PFB0665w)	CaMK		*Pf* STK2	Dispensable
33	PF3D7_1238900 (PFL1885c)	CaMK		*Pf* PK2	Essential
34	PF3D7_131500 (PF13_0085)	CaMK		*Pf* PK9	Essential
35	PF3D7_1454300 (PF14_0516)	CaMK		*Pf* KIN	Essential
36	PF3D7_0203100 (PFB0150c)	STE		*Pf* PK8	Dispensable
37	PF3D7_0605300 (PFF0260w/MAL6P1.56)	STE-related	Aurora	*Pf* Ark-1	Essential
38	PF3D7_0309200 (PFC0385c)	AGC-related	Aurora	*Pf* Ark-2	Essential
39	PF3D7_1356800 (MAL13P1.278)	AGC-related	Aurora	*Pf* Ark-3	Essential
40	PF3D7_0934800 (PFI1685w)	AGC		*Pf* PKA	Essential
41	PF3D7_1246900 (PFL2250c)	AGC		*Pf* PKB	Essential
42	PF3D7_1436600 (PF14_0346)	AGC		*Pf* PKG	Essential
43	PF3D7_1145200 (PF11_0464)	AGC-related			Essential
44	PF3D7_1148000 (PF11_0488)	AGC-related			Essential
45	PF3D7_1441300 (PF14_0392)	AGC-related			Dispensable
46	PF3D7_0211700 (PFB0520w)	TKL		*Pf* TKL-1	Essential
47	PF3D7_1121300 (PF11_0220)	TKL		*Pf* TKL-2	Dispensable
48	PF3D7_1349300 (PF13_0258)	TKL		*Pf* TKL-3	Essential
49	PF3D7_0623800 (PFF1145c/MAL6P1.191)	TKL		*Pf* TKL-4	Dispensable
50	PF3D7_0926300 (PFI1290w)	TKL-related			Unknown
51	PF3D7_1228300 (PFL1370w)	NIMA		*Pf* nek-1	Essential
52	PF3D7_0525900 (PFE1290w)	NIMA		*Pf* nek-2	Dispensable
53	PF3D7_1201600 (PFL0080c)	NIMA		*Pf* nek-3	Dispensable
54	PF3D7_0719200 (MAL7P1.100)	NIMA		*Pf* nek-4	Dispensable
55	PF3D7_1136500 (PF11_0377)	CK1		*Pf* CK1	Essential
56	PF3D7_0926100 (PFI1280c)	CK1-related			Dispensable

Table 1 continued...

57	PF3D7_0107600 (PFA0380w)	Orphan	eIF2α kinase	*Pf* eiK1	Dispensable
58	PF3D7_1444500 (PF14_0423)	Orphan	eIF2α kinase	*Pf* eiK2	Dispensable
59	PF3D7_0628200 (PFF1370w)	Orphan	eIF2α kinase	*Pf* PK4	Essential
60	PF3D7_1428500 (PF14_0264)	Orphan			Unknown
61	PF3D7_0718100 (MAL7P1.91)	Orphan		*Pf* EST	Essential
62	PF3D7_0213400 (PFB0605w)	Orphan		*Pf* PK7	Dispensable
63	PF3D7_1247500 (PFL2280w)	Orphan			Dispensable
64	PF3D7_0102600 (PFA0130c)	Orphan	FIKK	*Pf* FIKK1	Dispensable
65	PF3D7_0301200 (PFC0060c)	Orphan	FIKK	*Pf* FIKK3	Dispensable
66	PF3D7_0424500 (PFD1165w)	Orphan	FIKK	*Pf* FIKK4.1	Dispensable
67	PF3D7_0424700 (PFD1175w)	Orphan	FIKK	*Pf* FIKK4.2	Dispensable
68	PF3D7_0500900 (PFE0045c)	Orphan	FIKK	*Pf* FIKK5	Dispensable
69	PF3D7_0726200 (MAL7P1.144)	Orphan	FIKK	*Pf* FIKK7.1	Dispensable
70	PF3D7_0731400 (MAL7P1.175)	Orphan	FIKK	*Pf* FIKK7.2	Dispensable
71	PF3D7_0805700 (MAL8P1.203)	Orphan	FIKK	*Pf* FIKK8	Essential
72	PF3D7_0902000 (PFI0095c)	Orphan	FIKK	*Pf* FIKK9.1	Dispensable
73	PF3D7_0902100 (PFI0100c)	Orphan	FIKK	*Pf* FIKK9.2	Dispensable
74	PF3D7_0902000 (PFI0105c)	Orphan	FIKK	*Pf* FIKK9.3	Dispensable
75	PF3D7_0902300 (PFI0110c)	Orphan	FIKK	*Pf* FIKK9.4	Dispensable
76	PF3D7_0902400 (PFI0115c)	Orphan	FIKK	*Pf* FIKK9.5	Dispensable
77	PF3D7_0902500 (PFI0120c)	Orphan	FIKK	*Pf* FIKK9.6	Dispensable
78	PF3D7_0902600 (PFI0125c)	Orphan	FIKK	*Pf* FIKK9.7	Dispensable
79	PF3D7_1016400 (PF10_0160)	Orphan	FIKK	*Pf* FIKK10.1	Dispensable
80	PF3D7_103900 (PF10_0380 R45)	Orphan	FIKK	*Pf* FIKK10.2	Dispensable
81	PF3D7_1149300 (PF11_0510)	Orphan	FIKK	*Pf* FIKK11	Dispensable
82	PF3D7_1200800 (PFL0040c)	Orphan	FIKK	*Pf* FIKK12	Dispensable
83	PF3D7_1371700 (MAL13P1.109)	Orphan	FIKK	*Pf* FIKK13	Dispensable
84	PF3D7_1476400 (PF14_0733/0734)	Orphan	FIKK	*Pf* FIKK14	Dispensable
85	PF3D7_0928800 (PFI1415w)	Orphan			Unknown
86	PF3D7_1433900 (PF14_0320)	Orphan			Unknown

Few notable differences exist between *P. falciparum* and human kinome. In general, most *Plasmodium* kinase shares around 30-60% of sequence identity to human kinases (Doerig, 2004; Ruiz-Carrillo *et al.*, 2018; Alam e*t al.*, 2019). Moreover, typical mammalian kinase group such as tyrosine kinase (TyrK) and (STE) are missing and exist in a low number in malaria kinome, respectively. Instead, most malaria kinome contains kinases such as FIKK (lacking the canonical glycine-rich motif in its ATP binding site, replaced with FIKK motif) and CDPKs that cannot be found in mammalian cells (Lin *et al.*, 2017; Ghartey-Kwansah *et al.*, 2020). Besides, the size of the ATP-gatekeeper in *Plasmodium* kinase is mostly guarded by small amino acid subunits, exposing its hydrophobic pocket in the ATP-binding site (Huang *et al.*, 2010; Tewari *et al.*, 2010; Bansal *et al.*, 2016). It can be exploited to identify a class of ATP-competitive inhibitor targeting this *Pf* PK hydrophobic pocket as most human kinases possess a bulky ATP-gatekeeper, preventing interaction (Van Voorhis *et al.*, 2017). Such differences indicate that species-selective inhibition is possible for antimalarial chemotherapy.

The most studied *P. falciparum* eukaryotic protein kinases

In general, the approach to target *Pf* PKs is still underexplored for antimalarial chemotherapy. However, continuous progress made so far has enriched our understanding of the physiological importance, regulation, and structural features of *Pf* PK, which will soon pave a pathway for *Pf* PK inhibitor to reach the clinical stage. This assumption is strengthened by the first *Plasmodium* lipid/protein kinase inhibitor reaching clinical test (MMV390048), targeting the ATP-binding site of *Pf* PI4K (aPK), a general feature shared by all kinases (Paquet *et al.*, 2017). So far, almost 40 *Pf* PKs have been genetically validated as a druggable target at the RBC stage (Abdi *et al.*, 2010; Solyakov *et al.*, 2011; Zhang *et al.*, 2018; Davies *et al.*, 2020) (Table 1). However, only a total of five *Pf* PKs have been validated as drug targets at genetic (knockout *Plasmodium* strain), phenotypic (*Pf* culture and enzymatic assay), and *in vivo* levels (*P. berghei* infected or humanized *P. falciparum*-infected mouse model).

a) *P. falciparum* NIMA related protein kinase 1 (*Pf* nek-1)

Pf nek-1 (along with *Pf* nek-2 to nek-4) is a member of *P. falciparum* kinome that shows the highest homology to the NIMA/NIMA-like kinase (Nek family). *Pf* nek-1 is the only member of *P. falciparum* NIMA kinase whose mRNA is detectable throughout the RBC stage (hence, the only essential member) and male gametocytes (Dorin *et al.*, 2001; Dorin-Semblat *et al.*, 2011). Structurally, *Pf* nek-1 possesses an extension on its C-terminal of the catalytic domain. In comparison to mammalian homolog (such as mouse nek-1 and human nek-2), the FXXT motif typically found in mammalian NIMA kinase is substituted by SMAHS motif in *Pf* nek-1 (Dorin *et al.*, 2001). This motif is unique against its mammalian homolog protein and other *Plasmodium* kinases, making it an attractive target (Kappes *et al.*, 1999; Tanaka and Nigg, 1999).

The importance of nek-1 protein on *Plasmodium* survival during the RBC stage was initially predicted from the failure to develop the nek-1 knockout (KO) in *P. berghei* strain (Tewari *et al.*, 2010). *Pf* nek-1 was then confirmed as an essential protein during the *P. falciparum* RBC stage based on a reverse genetic approach. Wild type (WT) and mutant *P. falciparum* 3D7 parasites bearing active and inactive *Pf* nek-1 (lacking in a subdomain for enzymatic activity), respectively, were subjected to blasticidin selection. PCR analysis was performed to detect the presence of WT and disrupted pfnek-1 locus. It was later confirmed that no integrated fragment was amplified (mutated *Pf* nek-1), implying the importance of *Pf* nek-1 for *P. falciparum* growth at the RBC stage (Dorin-Semblat *et al.*, 2011).

Pf nek-1 was identified as a druggable target for antimalarial chemotherapy, as indicated by the moderate antimalarial activity of xestoquinone (isolated from Vanuatu marine sponge *Xestospongia* sp.) on *P. falciparum* culture (*Pf* IC_{50}=3 μM) and

recombinant *Pf* nek-1 (*Pf* nek-1 IC_{50}=1 µM). This compound also displayed specific activity as it showed weak to no activity on other *P. falciparum* and human (*Homo sapiens*, *Hs*) kinases such *Pf* PK5, *Pf* PK7, *Pf* GSK-3, *Hs* GSK-3α/β, *Hs* CDK1 *Hs* CDK5 and *Hs* PKA. Although xestoquinone was shown to inhibit *P. berghei in vivo*, its relation with nek-1 inhibition was not confirmed. Besides, total parasitemia clearance was not achievable at 5 mg/kg (47% clearance), and the mice did not survive at 20 mg/kg dose (Laurent *et al.*, 2006).

Subsequently, alisiaquinones A (related to xestoquinone, also purified from marine sponges), was shown to inhibit both chloroquine-sensitive and -resistance *P. falciparum* strains with the IC_{50} of <10 µM. This compound was also shown to completely inhibited *Pf* nek-1 activity at 50 µM. Although alisiaquinones A showed slightly better parasitemia *in vivo* clearance (63% clearance at 5 mg/kg) than xestoquinone, its mortality rate at 20 mg/kg was also 100%. Due to its toxic activity, alisiaquinones A was not further pursued as an antimalarial agent (Desoubzdanne *et al.*, 2008).

Numerous studies reported other *Pf* nek-1 inhibitors such as roscovitine (*Pf* nek-1 IC_{50}=10 µM, *Pf* 3D7 IC_{50}=10.48 µM), compound 97 (purvalanol derivatives) (*Pf* nek-1 IC_{50}=0.2 µM), hymenialdisine 01 (*Pf* nek-1 IC_{50}=4 µM) (Dorin *et al.*, 2001; Houzé *et al.*, 2014), methyl (2,4-dibromo-3,6-dihydroxyphenyl) acetate (*Pf* nek-1 IC_{50}= 1.8 µM, *Pf* FcB1 IC_{50}=12 µM) (Lebouvier *et al.*, 2009) and recently compound 17/18 (both with *Pf* nek-1 IC_{50}=0.05 µM) (Penzo *et al.*, 2019). However, none of this study managed to established the connection between *Plasmodium* nek-1 inhibition and parasitemia clearance *in vivo*. More research is needed as this enzyme is an attractive target for antimalarial chemotherapy, given its unique structure and importance for *Plasmodium* survival.

b) *P. falciparum* calcium-dependent kinase 1 (*Pf* CDPK1)

Calcium-dependent kinases (CDPKs) represent a unique class of *Plasmodium* ePK for drug target since they have no homologs in humans but more related to the kinases of some algae, plant, and alveolates (Doerig *et al.*, 2005; Kadian *et al.*, 2017; Ghartey-Kwansah *et al.*, 2020). CDPKs are composed of four separate regions with distinct functions. The N-terminal plays two critical roles; variable regions (responsible for substrate recognition, localization, and interaction with target proteins) and the catalytic kinase domain (catalyzes the phosphorylation of serine/threonine residues in the substrate). Meanwhile, the C-terminal (a calmodulin-like domain (CLD)) is crucial for calcium-binding assisted by the EF-hand motifs and the J-domain (junction domain that is also referred as a calcium activation domain (CAD)) leading to the enzyme activation (Wernimont *et al.*, 2010). A total of seven members of CDPKs have been identified so far (CDPK1-7), by which *Pf* CDPK1 and *Pf* CDPK4 are the most progressed as both have been validated up to *in vivo* studies (Zhang and Choi, 2001; Harper and Harmon, 2005; Nagamune *et al.*, 2008).

Pf CDPK1 is mainly involved in the motility of parasite invasion of the host RBC by acting as a Ca^{2+}-dependent effector for microneme secretion and critical in the formation of both male and female gametes (Kumar *et al.*, 2017; Bansal *et al.*, 2018). *Pf* CDPK1 is validated as a druggable target based on failed initial attempts to produce a *Pf* CDPK1 KO strain, indicating its essential roles for parasite survival at the RBC stage (Kato *et al.*, 2008; Solyakov *et al.*, 2011; Zhang *et al.*, 2018). Numerous studies indicated successful *Plasmodium* clearance by *Pf* CDPK1 inhibitors based on *in vitro* phenotypic and enzymatic assays. For instance, 2,6,9-trisubstituted purines which inhibited the growth of *P. falciparum* 3D7 culture (*Pf* 3D7 IC_{50}=230 nm) and recombinant *Pf* CDPK1 (*Pf* CDPK1 IC_{50}= 17 nm) (Kato *et al.*, 2008). Besides, imidazopyridazine was also reported to show antimalarial activity (*Pf* 3D7 IC_{50}=0.034 µM) mediated by the inhibition of *Pf* CDPK1 (*Pf* IC_{50}=0.008 µM) (Green *et al.*, 2016).

Based on the phenotypic and recombinant assays, the inhibition of *Pf* CDPK1 causes accumulation of *P. falciparum* at late

schizogony. It was also suggested that *Pf* CDPK1 inhibition might affect *P. falciparum* during gametocytes that were later confirmed based on the gamete abnormality observed in the recent development of *P. falciparum* CDPK1 KO strains. This KO strain was generated using the CDPK1 T145M parasite, a mutant *P. falciparum* strain. This strain showed a slower growth rate than WT and the unaltered CDPK1 T145M strains. In addition, the CDPK1 KO parasite also produced an abnormal gamete and failed to infect the mosquitoes. This finding validated *Pf* CDPK1 as an essential enzyme in both the sexual and asexual stages (Bansal *et al.*, 2018).

A total of 47 imidazopyridazine series were synthesized from imidazopyridazine-compound 1 (as an early lead), to improve its potency against *Pf* 3D7 parasite culture, recombinant *Pf* CDPK1 and *P. berghei* (*in vivo*). Compound 10 and 41 were subsequently identified as the most potent compounds as both compounds showed *Pf* 3D7 EC_{50} of <0.1 µm and *Pf* CDPK1 IC_{50} of <15 nm, compared with compound 1 *Pf* 3D7 EC_{50} of <0.1 µm and *Pf* CDPK1 IC_{50} of <50 nm. However, a clear connection was not established between *in vitro* and *in vivo* studies since compound 41 showed moderate *P. berghei* clearance *in vivo* tested at 50 mg/kg (about 50% reduction, which is similar to compound 1). It might be contributed by the differences in CDPK1 importance and biology between *P. falciparum* and *P. berghei* (Chapman *et al.*, 2014).

c) *P. falciparum* calcium-dependent kinase 4 (*Pf* CDPK4)

Pf CDPK4 was shown to interact with *Pf* PKG at the asexual erythrocyte stage, especially at the final stage of the schizont or invasion of the new RBC. However, the knockdown of *Pf* CDPK4 alone does not affect parasite growth, which indicates its disposable role during the RBC stage (Fang *et al.*, 2018). Subsequently, the role of CDPK4 during the *Plasmodium* sexual phase was deduced based on the knockout of *P. berghei* CDPK4 (*Pb* CDPK4). Mice malaria is a suitable model organism as it encodes all members of *Pf* CDPKs except for *Pf* CDPK2. The *Pb* CDPK4 gene disruption was shown to cause sexual impair and altered the process of sporozoite invasion of hepatocyte cells (Billker *et al.*, 2004; Govindasamy *et al.*, 2016).

Hence, CDPK4 in *P. falciparum* is likely to be essential to maintain normal malaria life cycle, transmission, crucial for the transition of gametocytes into microgamete, male gametocyte exflagellation in the mosquito midgut and cell invasion (Billker *et al.*, 2004; Tewari *et al.*, 2010; Bansal *et al.*, 2018; Fang *et al.*, 2018). As most of the known antimalarial drugs are targeting *P. falciparum* during the RBC stage, *Pf* CDPK4 is an exciting target for drug development (Ahmed *et al.*, 2012). To identify *Pf* CDPK4 inhibitors, a series of kinase inhibitors that showed no activity on humans proteins known as "Bumped kinase inhibitor" (BKI) were tested (Ojo *et al.*, 2012).

BKIs are compounds with pyrazolo-pyrimidine backbone that was designed with a large R1 and R2 groups so that it will show no or little effect on human cells. The ATP binding pocket of human kinases is mostly guarded by bulky ATP-gatekeepers, thus preventing large compounds from binding (Uo *et al.*, 2018). Based on the prior screening of BKIs on CDPK1 of *Toxoplasma gondii* (*T. gondii*) and *Cryptosporidium parvum* (*C. parvum*), few selective inhibitors were identified that showed no activity on human kinases. This selectivity is due to a small ATP-gatekeeper (glycine) of these enzymes (Ojo *et al.*, 2010; Tewari *et al.*, 2010). Since *Pf* CDPK4 also possesses a small gatekeeper, many of these BKIs also inhibited *Pf* CDPK4 activity, by which BKI-1 being the most potent (IC_{50}=4 nM) (Ojo *et al.*, 2012).

As expected, BKI-1 did not inhibit the growth of parasite at the RBC stage, but showed potent *in vivo* inhibition of *P. falciparum* exflagellation (EC_{50}=35 nm), possibly mediated by *Pf* CDPK4 inhibition. BKI-1 was then tested on *P. berghei* infected mice at 10 mg/kg, and it exerted no effect on asexual parasitemia or gametocyte rates, but the dose adequate to block exflagellation.

Anopheles mosquitoes were then allowed to feed on the infected mice, 30 minutes after BKI-1 administration (10 mg/kg). Complete inhibition of oocyst production in the mosquito midgut was observed. At this point, *P. falciparum* exflagellation inhibition was strongly suggested due to the inhibition of *Pf* CDPK4 by BKI-1. However, it was not confirmed as the generation *Pf* CDPK4 mutant for further study was failed. In addition, BKI-1 bioavailability needs to be prolonged for better parasite gametocytes clearance (Ojo *et al.*, 2012).

To improve the activity of BKI-1, compound 1294 (BKI-1294) was synthesized that showed a reduced rate of microsomal metabolism while retaining comparable inhibitory to BKI-1. BKI-1294 blocked *P. falciparum* gametocyte exflagellation with the EC_{50} of 35 nm (BKI-1, EC_{50}=47 nm) and anti-*Pf* CDPK4 activity with the IC_{50} of 10 nm (BKI-1, IC_{50}=4 nm). BKI-1294 was also confirmed to exert transmission-blocking activity, evaluated with untransfected WT NF54 *P. falciparum* gametocytes grown in human blood (EC_{50}=23 nm). In this study, NF54 *P. falciparum* expressing mutant *Pf* CDPK4 (*Pf* CDPK4S147M), resulting in a bulky ATP-gatekeeper (Ser^{147}→Met). Hence, it restricted the space to accommodate bulky R1-group of BKI-1294 (ethoxynapthtyl), making it insensitive. BKI-1294 and BKI-1 were confirmed to inhibit *Pf* CDPK4 as these compounds showed low activity against *Pf* CDPK4 S147M recombinant enzyme, both with the IC_{50} of >2 µM. Moreover, BKI-1294 also exerted lower activity on the exflagellation of the NF54 S147M mutant (EC_{50}=0.292 µM). *In vivo* study later confirmed bioavailability of BKI-1294 was improved, and higher dosage to stop transmission exerted no adverse effect on mice (Ojo *et al.*, 2014).

d) *P. falciparum* cGMP-dependent protein kinase (*Pf* PKG)

In mammalian cells, PKG is known as an essential regulator of the cGMP signaling pathway that mediates numerous physiological processes such as apoptosis and cell differentiation (Haas *et al.*, 2009; Soh *et al.*, 2001). In humans, two isomers of PKG were identified (type I and type II) that exist as homodimers (Pearce *et al.*, 2010). PKG ortholog in apicomplexan parasites, including *P. falciparum*, was first identified in early 2000 (Gurnett *et al.*, 2002). In general, apicomplexan PKG exists as three cGMP-subunit. However, *Pf* PKG showed striking structural features as it has the fourth cGMP binding site known as the "degenerate fourth site" (Deng *et al.*, 2003). Besides, *Pf* PKG also equipped with a smaller ATP-gatekeeper subunit (threonine) than human PKGs (Huang *et al.*, 2010).

Pf PKG is an attractive drug target due to its role as a regulator of other *Pf* PKs such as *Pf* CDPK5, suggesting its function at various *P. falciparum* lifecycle phases such as asexual (RBC, liver) (Deng and Baker, 2002; Falae *et al.*, 2010) and sexual stages (gametogenesis and ookinete motility) (McRobert *et al.*, 2008; Brochet *et al.*, 2014; Alam *et al.*, 2015). The importance of *Pf* PKG during the asexual and sexual stage was first recognized based on its high level of mRNA and protein expression at these phases (Diaz *et al.*, 2006). Moreover, the *Pf* PKG knockout strain was unable to be generated, which further indicates its crucial role in *P. falciparum* survival during the erythrocytes cycle (Taylor *et al.*, 2010). *Pf* PKG was also indicated as indispensable during the RBC stage based on a mutagenesis index score that was calculated according to the number of piggyBac insertions relative to the number of TTAA target sites in *Pf* PKG gene. In general, the gene with low MIS is likely to be essential (Zhang *et al.*, 2018).

Besides, a chemical genetic approach exploiting the size of *Pf* PKG ATP-gatekeeper, generating an insensitive *P. falciparum* mutant (T618Q) strain, was constructed to confirm the importance of this enzyme for *P. falciparum* survival during RBC stage. This mutant strain is expressing *Pf* PKG with glutamine (bulky amino acid) as its ATP-gatekeeper instead of threonine (small). Potent anticoccidial agents, trisubstituted pyrrole (compound 1), and imidazopyridine (Compound 2 or ML1), targeting coccidian PKGs ATP-binding site were tested against the WT and T618Q mutant strains *in vitro* (Donald *et al.*, 2006). It was concluded that

the antimalarial activity of both compounds on WT *P. falciparum* growth is mediated by the inhibition of *Pf* PKG, as mutant T618Q *P. falciparum* strain was unaffected (Taylor *et al.*, 2010).

Recently, a total of 9 imidazopyridine derivatives (ML2-ML10) was synthesized from ML1. Each derivative was tested against WT *P. falciparum* culture/recombinant *Pf* PKG and mutant *P. falciparum* T618Q culture/recombinant *Pf* PKG T618Q. ML10 exerted the most potent activity against WT *P. falciparum in vitro* with the EC_{50} of 2.1 nm. This compound also showed a significant reduction (1000-fold) in inhibitory activity against *P. falciparum* mutant (T618Q) with the EC_{50} of 2430 nm. Besides, Enzymatic assay demonstrates the importance of the size of *Pf* PKG ATP-gatekeeper as the WT *Pf* PKG is 100 000-fold more sensitive towards ML10 with the IC_{50} of 0.16 nm, in comparison with mutant *Pf* PKG T618Q (IC_{50}=29 540 nm) (Baker *et al.*, 2017).

In a further study, the *in vivo* effect of ML10 was evaluated in an animal model using the *P. falciparum*-infected SCID mouse model engrafted with human RBC (infected with *P. falciparum* 3D70087/N9). ML10 was administered at two doses, 50 and 100 mg/kg daily for four days (oral administration). Both treatments significantly reduced the infection rate with >90% parasitemia clearance, by which a higher dosage completely eliminate the parasite. ML10 also tested against a panel of human kinases consisting of 80 kinase proteins representing all main families, including 14 human kinases with a small ATP-gatekeeper. The only significant inhibitory activity of ML10 was observed against human MLK3 (40% inhibition, tested at 100 nM). In addition, ML10 was also tested against four human cell lines to reveal a very high selectivity window (>4500 fold) (Baker *et al.*, 2017).

e) *P. falciparum* Cyclin-dependent-like kinase 3 (*Pf* CLK-3)

In *Plasmodium* kinome, the member of the CDK-like family consists of four members; *Pf* CLK1/*Pf* Lammer, *Pf* CLK-2, *Pf* CLK-3, and *Pf* CLK-4/*Pf* SRPK1. *Pf* CDK-like is closely related to the serine-arginine protein kinase (SPRK) family in mammalian cells. In general, SPRK mediates the phosphorylation of serine-arginine-rich (SR) proteins, for accurate assembly and catalytic activity of spliceosomes, hence pre-mRNA processing. Due to its possible role in the regulation of SR proteins, *Pf* CDK-like gain attention as a druggable target since mRNA expression is required at all *Plasmodium* life cycle (Ward *et al.*, 2004; Kern *et al.*, 2014).

Pf CLK-3 was recently validated up to the *in vivo* level as a druggable target for antimalarial drug development by which TCMDC-135051 was identified as its potent inhibitor. This compound was also previously reported to exert antimalarial activity against *P. falciparum* culture with the EC_{50} of 320 nM (Gamo *et al.*, 2010). In a more recent study, this compound was identified from a high-throughput screening program utilizing 13 533 compounds against recombinant *Pf* CLK-3. TCMDC-135051 showed a high degree of selectivity as it showed no activity on kinase *Pf* PKs such as *Pf* CLK-1 (closely related), *Pf* PKG, *Pf* CDPK1, and *Pf* CLK3. TCMDC-135051 also exerted no activity on closely related human kinases, pre-mRNA processing factor 4B (PRPF4B) (tested up to 50 µM), and CLK2 (29% sequence identity) (Alam *et al.*, 2019).

To confirm the molecular target of TCMDC_135051, strain with reduced sensitivity (TM051C strain) was produced by exposing *P. falciparum* D2d to an increasing concentration of TCMDC_135051. TM051C strain containing H529P ($His^{259}\rightarrow$Pro) mutation in its *Pf* CLK-3. The phenotypic assay revealed that the mutant strain is 12-fold less sensitive than the WT with the EC_{50} of 1.25 µM and 0.1 µM, respectively. Further genetic validation of *Pf* CLK-3 was performed using recombinant mutant *Pf* CLK-3, G449P ($Gly^{449}\rightarrow$Pro, at the C-terminus). This mutant enzyme was generated by swapping the residues between *Pf* CLK-1 (not inhibited by TCMDC_135051) and *Pf* CLK-3. Recombinant *Pf* CLK-3 G449P is almost 550-fold less sensitive towards TCMDC_135051 (IC_{50}=21.57 µM) than the WT (IC_{50}=0.04 µM) (Alam *et al.*, 2019).

In addition, changes in gene transcription in response to TCMDC_135051 was also evaluated. The genome-wide transcriptional pattern showed that the inhibition of *Pf* CLK-3 leads to the down-regulation/up-regulation of 799/155 genes in WT strain in comparison to 88/6 genes in mutant strains, respectively. These genes are involved in cell egress and invasion, cytoadherence, sexual stages, metabolism, lipid modification, and mitochondrial function (Alam *et al.*, 2019). Hence, proven the assumption of *Pf* CLK-3 as the regulator of mRNA processing (Kern *et al.*, 2014).

Given the proven roles of *Pf* CLK-3 in the mRNA processing, the effect of TCMDC_135051 at multiple stages of the *Plasmodium* life cycle was characterized. In the asexual RBC stage, TCMDC_135051 causes rapid *Plasmodium* clearance, which prevented the transition of parasite from ring-stage to trophozoites and trophozoites to schizont. In the sexual stage, TCMDC_135051 was first tested on the *P. falciparum* 2004 strain that produces a high-level of gametocytes and was shown to exert potent parasiticidal activity with the EC_{50} of 0.26 µM (Brancucci *et al.*, 2015). Subsequently, the mosquito membrane feeding assay was performed to study the effect of TCMDC_135051 on the transmission of *P. falciparum* to the mosquito vector. Stage II gametocytes were treated with the compound, and the parasites were allowed to develop to stage V, revealing a significant reduction of gametocyte number (EC_{50}=0.8 µM) (Alam *et al.*, 2019).

Finally, TCMDC_135051 was also shown to inhibit recombinant CLK-3 enzymes from different malaria parasites, especially *P. berghei* CLK-3 (IC_{50}=0.013 µM), which warranted *in vivo* study. The *P. berghei* infected mice were treated with daily intraperitoneal dosing of TCMDC_135051 for five days (maximal dose of 50 mg/kg) resulted in almost total parasite clearance. Inspection on *P. berghei* sporozoites in the liver showed potent activity with the EC_{50} of 0.40 µM (Alam *et al.*, 2019). Overall, *Pf* CLK-3 is currently one of the most convincing *Pf* PK to be targeted for antimalaria chemotherapy.

CONCLUSION

P. falciparum protein kinase represents an excellent opportunity for the development of a new line of antimalarial drugs with a well-defined mode of action and cellular target. Such information may prevent late-stage failure in the drug approval process since compound targeting *Pf* PKs will have little or no toxic effect on human kinases and cells. Besides, this approach may lead to the identification of antimalarial drugs with a longer clinical span as some inhibitors mimicking the interaction of ATP with ATP-binding sites. For example, imidazopyridine derivatives were shown to inhibit multiple *Pf* PKs at the same time, contributed by small ATP-gatekeeper subunits of most *Pf* PKs. Besides, the size of ATP-gatekeeper is generally conserved among the other human malaria and apicomplexan parasites, including *P. vivax*, *C. parvum*, and *T. gondii*. It suggests the possible application of antimalarial agents targeting *Pf* PKs to cure the other infectious diseases as well.

However, this approach is not without its challenges. As discussed in this review, some *Pf* PKs were unable to be studied due to the failure to produce knockout strains. Moreover, structural differences do exist between protein kinases across *Plasmodium* parasites, which hinder a proper evaluation of the tested drugs at an *in vivo* level that prevents their progression. Recent updates on malaria research approaches such as the combination of *P. falciparum* metabolomic profiling and phenotypic study may have widened the identification of *Pf* PKs (or the other cell components) targeted by the tested compounds, which was previously unidentifiable via the conventional methods (Muruthi *et al.*, 2020). It is no doubt that such techniques may eventually introduce *Pf* PKs inhibitor into the clinical trials.

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