An Overview of *Plasmodium* Protein Kinases

B. Kappes, C.D. Doerig and R. Graeser

Protein kinases are key regulators of many biochemical processes in eukaryotic cells. Malaria parasites, in spite of all their peculiarities, are not likely to represent an exception in this respect. Over the past few years, several genes encoding Plasmodium *protein kinases have been cloned and characterized; these molecular studies extend previous data on kinase activities in parasite extracts. Here, Barbara Kappes, Christian Doerig and Ralph Graeser present available data on this topic, with an emphasis on cloned protein kinase genes, and discuss the potential outcome of such research in the context of drug development.*

One of the main biochemical processes used by eukaryotic cells to transmit signals is protein phosphorylation. The addition of phosphate groups brings about striking changes in enzymatic activity, stability, binding properties or subcellular localization of protein substrates. Furthermore, the process is readily reversed by protein phosphatases, making the modulation of the phosphorylation state of proteins a very flexible and finely tunable control system. Based on their sequence similarity and their enzymatic specificity, protein kinases of eukaryotes can be classified into the superfamily of Ser, Thr and Tyr kinases, whose members catalyse the transfer of phosphate from ATP to Ser, Thr or Tyr, respectively, and the His kinase family, whose members autophosphorylate on a conserved His residue[1,2]. No sequence homology exists between these two types of protein kinases, although extensive sequence conservation is found between members of either type. Using phylogenetic trees, the superfamily of Ser, Thr and Tyr kinases was subdivided into five major groups, which contain closely related protein kinase families[1].

In recent years, several protein kinases belonging to the superfamily of Ser, Thr and Tyr kinases, as well as phosphatases (outlined in Ref. 3) have been isolated from the malaria parasite. Table 1 summarizes most of the data gained from the individual protein kinase genes and their gene products; major results are discussed in the text. We classified the malaria protein kinases according to the above-mentioned scheme. Starting from the general characteristics of the respective group or family the individual kinases belong to, we speculate on their possible functions in the malaria parasite. These functional predictions might not prove true; *Plasmodium* is evolutionarily divergent from yeast and higher eukaryotes, on which most of the comparative analysis is based. However, they might provide a guide for the functional analysis of the kinases, which is urgently required for a better understanding of basic regulatory processes in the malaria parasite.

Barbara Kappes is at the Center of Biochemistry, Heidelberg University, Im Neuenheimer Feld 328, D-69120 Heidelberg. Christian Doerig is at Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 511, Centre Hospitalier Universitaire Pitié-Salpêtrière, 91 Boulevard de l'Hôpital, 75013 Paris, France. Ralph Graeser is at the Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire, UK EN6 3LD. **Tel: +49 6221 544171, Fax: +49 6221 545586, e-mail: barbara.kappes@urz.uni-heidelberg.de**

CMGC group

The CMGC group includes the family of cyclin-dependent protein kinases (CDKs), the mitogen-activated protein kinase (MAPK) family, the glycogen-synthase kinase 3 (GSK-3) family, the CDK-like kinase (CLK) family and other close relatives. Most of these enzymes are proline-directed kinases, phosphorylating substrates at sites close to proline residues[1].

CDK family. CDKs have been found in all eukaryotes investigated so far, and most are involved in cell cycle regulation. Apart from their role in the cell cycle, CDKs are involved in brain development and in the regulation of phosphate and glycogen metabolism in yeast[4–6]. The active forms are composed of a catalytic subunit (a CDK) and a regulatory subunit (a cyclin). The temporary association of both subunits, together with the phosphorylation state of the CDK subunit, results in well-defined intervals during which a CDK is active. Several CDKs and cyclins coexist in eukaryotic cells, with given combinations being responsible for progression of the cell cycle through particular phases[7]. In higher eukaryotes, CDK7 or MO15 is the kinase that activates CDK1, -2, -4 and -6 by phosphorylating a Thr residue in the conserved T-loop[8]. Full catalytic activity requires binding of cyclin H and phosphorylation on both Ser146 and Thr169 (human CDK7)[9].

CDK-related kinases show significant homologies to CDKs, but a binding partner is absent, or has not yet been isolated. They include the human PITSLRE protein kinases, which are present as 58–130 kDa products. Whereas the smaller isoforms are activated by caspases during apoptosis, the larger ones seem to play a role during cell proliferation in transcriptional and/or splicesosome regulation[10,11].

Three CDK-related kinases have been described in *Plasmodium falciparum*: PfPK5 (Ref. 12), Pfmrk (Ref. 13) and Pfcrk-1 (Ref. 14).

PfPK5 was the first malaria CDK-related kinase to be cloned[12]. Its closest relatives are the mammalian CDK1 and CDK5. No cyclin partner has yet been identified, although the existence of such a component is suggested by the low kinase activity of the bacterially expressed enzyme when compared with immunoprecipitated kinase of schizont extract[15,16]. As in mammalian CDK5, the PSTAIRE region, which is involved in cyclin binding, is not fully conserved in PfPK5. The activating partner of CDK5, p35, shows only very limited similarity to other cyclins[17], which might explain why no partner for PfPK5 has been discovered on the basis of sequence homology.

The protein level and activity of PfPK5 both peak at the time of the parasite's nuclear division cycles. A function of PfPK5 in the regulation of the S-phase of the nuclear division cycles is suggested by two findings: (1) the kinase activity of immunoprecipitated PfPK5 is raised in schizont-stage parasites blocked with aphidicolin, a DNA polymerase α inhibitor; and (2) olomoucine and

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Table 1. Summary of the fully cloned and described kinase genes from *Plasmodium falciparum*[a]

	CMGC					CAMK				AGC	Others		
P. falciparum gene name	*PfPK5*	*Pfmrk*	*Pfcrk 1*	*PfMAP 1*	*PfPK1*	*PfCDPK 1*	*PfCDPK 2*	*PfKIN*	*PfPK2*	*PfPKA*	*PfCK1*	*PfPK4*	*PfFEST*
Closest relatives	*cdk1, cdk5*	*MO15/cdk7*	*PITSLRE*	*ERK1, ERK2*	*GSK-3*	*CDPK, CAMK*	*CDPK, CAMK*	*SNF1*	*PKA/C, CAMK*	*PKA*	*CK1*	*HRI* kinase	NA
Identity (%)[b]	60	46	49	60	39	39	39	40	30	47	59	37	NA
Ref.	11, 14, 15	12	13	19–21	27	34–36 BK unp.[c]	37	39	41	CD unp.[c]	48	53	55
Accession no.	Q07785	U73195	X80759	U36377	X83707	X67288	X99763	Z22868	Q02595	AJ224444	AF017139	X94118	U40232
Chromosome	13	10	4	14	ND	ND	ND	ND	ND	ND	ND	ND	7
Size(s) of transcripts (kb)	1.8	2.5	2.5/3.5	3.7–4.2	ND	3.2, 3.3	2.5	ND	3.8, 4, 5	ND	2.4, 3.2	ND	ND
Protein (kDa) (pre/app)[d]	30/30	38/ND	86/ND	100/40, 80 100, 150	100/70	58/60	56/60	91/ND	56/72	38/ND	38/37	124/80, 90	276/210, 220
Extensions/insertions[e]													
Size (aa)	NA	NA	370	503	178, 330	NA	NA	400	108, 143	NA	NA	265, 559	1334, 128, 86, 150, 514
Location	NA	NA	N	C	5, 6	NA	NA	N	N, C	NA	NA	N, 4	N, 1, 4, 8, C
Composition	NA	NA	K, N	K, I/E	N, D, K	NA	NA	K, N, I	N, K	NA	NA	K, N	N, K
Repeats	NA	NA	+	+	+	NA	NA	−	−	NA	NA	+	+
Stage-specific presence[f]													
Transcript													
R	−							−			++		
T	+			+				−			−		
Sch	+	+	−	−	ND	+	+		+	ND	−	ND	ND
Seg	+							−			+		
Sx	ND	++	++	++		ND	ND	+	ND		ND		
Protein													
R	−			−	+	++			−			+	+
T	+			+	+	+			+			+	++
Sch	++	ND	ND	++	+	+	ND	ND	++	ND	ND	++	++
Seg	+			++	+	++			+			++	++
Sx	ND			+	ND	ND			ND			ND	ND
Activity													
R	−			−		−					+		
T	−			+		+					++		
Sch	++	ND	ND	+	ND	++	ND	ND	ND	ND	−	ND	ND
Seg	+			++		++					−		
Sx	ND			ND		ND					ND		
Kinase[g]													
Activity													
In vitro	+	ND	ND	+	ND	+	+	ND	ND	ND	+	+	ND
In vivo	+	ND	ND	+	+	+	ND	ND	ND	ND	+	ND	ND
Substrates													
In vitro	Casein histone H1	ND	ND	MBP	Casein	Casein histone H1, MBP	MBP	ND	ND	ND	Casein, peptide	Peptide	ND
In vivo	ND	ND	ND	ND	ND	β spectrin, band 4.1, band 4.9	ND	ND	ND	ND	Cytosolic proteins	ND	ND
Inhibitors (IC_{50}, μM)	Olo (15), flavo (0.06)	ND	ND	ND	ND	Stauro (0.05) TFP (40)	ND	ND	ND	ND	CK1–7 (<10)	Hemin	ND
Localization	Cyto/nuc (IF)	ND	ND	Cyto/nuc (IF)	Cyto/M/O (WB)	M/O (WB, IF); mem (EM)	ND	ND	M/O (WB)	ND	ND	M/O, RBC mem (WB); rhoptries (IF)	Mem, RBC mem (EM)

[a] Abbreviations: for CMGC, CAMK and AGC group definitions, see text; NA, not applicable; ND, not determined.
[b] The % amino acid identity with their catalytic domain.
[c] BK, CD unp. are unpublished data from B. Kappes and C. Doerig, respectively.
[d] Predicted (pre) and observed apparent (app; SDS-PAGE) molecular mass of the gene product(s).
[e] The size and position of sequences larger than 50 amino acids (aa) added either N- or C-terminally to the catalytic kinase domain (extensions) or inserted within it (insertions) when compared with the closest relative. The amino acid composition (ie. amino acids that appear at a frequency of more than 10% in the sum of all of the additional sequences of the given protein), and the presence of repeat structures within these sequences are listed. Amino acids are given in the single letter code.
[f] Stage-specific production of the transcript and the protein as well as the level of kinase activity (R, ring; T, trophozoite; Sch, schizont; Seg, segmenter; Sx, sexual stages). A '−' does not necessarily mean absent, it might be undetectable by the methods applied. +, present; ++, present at high level or high activity.
[g] Kinase activity and substrates determined as well as tested inhibitors (MBP, myelin basic protein; olo, olomoucine; flavo, flavopiridol; stauro, staurosporine; TFP, triflouroperazine). Localization of the protein (if known) (cyto, cytoplasmic; EM, electron microscopy; IF, immunofluorescence; mem, membrane; M/O, membrane and organelle fraction; nuc, nuclear; RBC, red blood cell; WB, western blot).

flavopiridol, two specific CDK inhibitors, significantly reduce the rate of parasite DNA synthesis[16].

Pfmrk shows high similarity (46%) with the CDK-activating kinase MO15/CDK7 (Ref. 13). The pattern of synthesis of Pfmrk indicates a role during the differentiation of the parasite to sexual stages[11]. If active recombinant Pfmrk could be generated, PfPK5, Pfcrk-1 or other CDK homologs might be tested for their ability to serve as substrates. This could be achieved by mimicking activatory phosphorylation by replacement of the appropriate residues by negatively charged amino acids. In this way, one third of the normal activity of *Xenopus* MO15 could be obtained in the absence of cyclin H[9].

The closest relatives of Pfcrk-1 are the PITSLRE kinases[14]. However, the 370 amino acid sequence located N-terminally of the catalytic domain of Pfcrk-1 shows only weak homologies to any of the PITSLRE isoforms, and the caspase recognition motif is missing in Pfcrk-1 (Refs 11,14). A role for Pfcrk-1 in sexual development is suggested by the gametocyte-specific accumulation of Pfcrk-1 transcripts[14].

Additional kinases related to CDKs have been identified in the context of the Malaria Genome Project or by other means (C.D. Doerig *et al*., unpublished; D. Chakrabarti *et al*., unpublished). Hence, it appears that *P. falciparum* uses a large number of CDK-related enzymes.

MAPK family. MAPKs play a role in a wide array of signal transduction processes, inducing cell proliferation, growth arrest or commitment to apoptosis. They are activated by phosphorylation brought about by dual specificity MAPK kinases (MAPKKs) on a Tyr and a Thr residue in the TXY motif located between subdomains VII and VIII[18]. In *Saccharomyces cerevisiae*, six genes encoding MAP kinases have been found, each used in a distinct signaling pathway, but some of them sharing the same MAPKKs[19].

A MAPK termed PfMAP, Pfmap-1 or PfMRP has been isolated independently in three laboratories[20–22]. The recombinant PfMAP gene product autophosphorylates at the Tyr and the Thr residues in the TXY motif, which is a typical feature of MAPKs, and results in a slight increase in kinase activity[22,23]. In the parasite, the protein is present as four distinct forms with apparent molecular masses of 40, 80, 100 and ~150 kDa, as shown by western blotting and immunoprecipitation[21,22]. As suggested by their tyrosine phosphorylation, a fraction of the 40 and/or 80 kDa forms of the PfMAP kinase is active during the asexual stages. Total MAPK activity, immunoprecipitated from the parasites, increases with parasite maturation[22]. The 100 kDa form is found in gametes and zygotes[21]. Whether PfMAP is involved in growth control, as suggested by the heterologous expression results[22], or in sexual differentiation, as might be hypothesized from the high abundance of the malaria MAP kinase transcript in sexual stages[21], and whether the various isoforms fulfil a variety of functions, awaits further elucidation. A second member of the MAPK family has been identified in *P. falciparum* (C.D. Doerig *et al*., unpublished).

GSK-3 family. Two subfamilies have been grouped to the GSK-3 family: the GSK-3 kinases themselves, and the casein kinase 2 (CK2)-type enzymes.

GSK-3 activity is usually high in cells. Many of its substrates are kept in a repressed state by GSK-3 phosphorylation, and become activated by dephosphorylation upon inactivation of GSK-3 mediated by an external stimulus[24]. Two genes, GSK-3α and -β, exist in mammals, their gene products phosphorylating proteins with functions ranging from metabolic pathways to transcription factors[24,25].

CK2 is a ubiquitous protein kinase known to phosphorylate >100 substrates, many of which are involved in the control of cell division and in signal transduction. It forms a heterotetrameric complex generally composed of two catalytic (α and/or α′) and two non-catalytic β subunits, which might cooperate in the modulation of CK2 targeting/activity[26]. CK2 enzymes, like CK1s, have acidic recognition sequences, and thus often modify sites close to previously phosphorylated amino acids[27].

Besides PfPK1 (Ref. 28), which shares sequence similarities with GSK-3 also outside of the catalytic domain, the malaria parasite might have (an)other potential GSK-3 homologue(s) – two expressed sequence tag (EST) fragments (0113c3/T02508 and 2118c3/N98054) show between 40% and 50% identity with the human GSK-3 genes. There is strong evidence for the existence of CK2-related enzymes in the parasite – an EST tag with high homology to CK2 (0111c3/T02506) and a PCR fragment encompassing subdomains II–VI (R. Graeser *et al*., unpublished).

CaMK group

The Ca^{2+}–calmodulin kinase (CaMK) group includes the family of protein kinases regulated by Ca^{2+}–calmodulin, the calcium-dependent protein kinases (CDPKs), the SNF1 (sucrose non-fermenting)/AMP-activated kinase (AMPK) family and other close relatives. In general, the CaMK group of protein kinases are basic amino acid-directed enzymes, phosphorylating substrates at Ser/Thr sites close to Arg and Lys residues[1].

CDPK family. CDPKs are characterized by the presence of two functional domains within their polypeptide sequence: a protein kinase catalytic domain and a Ca^{2+}-binding regulatory domain similar to calmodulin. These kinases are activated directly by Ca^{2+} and do not depend on calmodulin as a mediator protein. CDPKs have been found only in plants and some protozoan species, where they usually appear as multigene families[29–32]. They are involved in stress and hormone response, germination and possibly membrane biogenesis[33,34]. The CDPKs might substitute for the function of the CaMKs and protein kinase C (PKC) isoenzymes in some unicellular eukaryotes, as is likely to be the case in plants[34].

PfCDPK1 was the first member of this kinase family isolated from *P. falciparum*, and has been studied in detail[35]. The activity of the recombinant protein is strongly Ca^{2+} dependent. Maximal kinase activity is achieved if two Ca^{2+} ions are bound per PfCDPK1 molecule[36]. Phosphatidylserine also has a stimulatory effect on PfCDPK1 kinase activity[37]. Calmodulin antagonists inhibit PfCDPK1 kinase activity, but at much higher concentrations than those required to inhibit calmodulin-mediated effects[37]. PfCDPK1 localizes to areas of the parasite with active membrane biogenesis, such as the growing parasitophorous vacuole membrane in trophozoites, the tubular–vacuolar system (eg. Maurers clefts) and the intraparasitic vacuoles of

trophozoites and schizonts, as well as the surface of immature merozoites in segmenters. PfCDPK1 colocalizes with MSP-1 in segmenters and on isolated merozoites, on which it is accessible to *in vitro* iodination, suggesting a surface localization of the kinase (B. Kappes *et al*., unpublished). All of these results point to a role for PfCDPK1 in erythrocyte invasion and/or membrane biogenesis. The presence of PfCDPK2, which has been partially characterized[38], and of additional plasmodial CDPK genes and gene fragments (B. Kappes *et al*., unpublished; G. Jaureguiberry *et al*., unpublished) indicates that CDPKs form a multigene family in the malaria parasite.

SNF1/AMPK family. The SNF1/AMPK family is involved in stress response. Mammalian AMPK is activated by high levels of AMP and in yeast the SNF1 complex is activated in response to glucose deprivation. Although in this latter case the signal transduction elements have not been identified, SNF1 activation is associated with the depletion of ATP and elevation of AMP. Both kinases provide an adaptive mechanism to control cellular metabolism of glucose and fatty acids and to limit ATP utilization[39].

PfKIN has been described as a malarial homologue of SNF1 (Ref. 40). However, its C-terminus, which by analogy with the mammalian AMPKs would be involved in the binding of the regulatory β- and γ-subunits[41], does not show any significant homology with that of its potential relatives. Thus, it is difficult to speculate on a similar role for PfKIN in the malarial parasite.

PfPK2 shows homology with the CaMKs, PKAs and PKCs[42]. Thus, it seems to belong to both the CaMK and the AGC group, reflecting the fact that the AGC and CaMK groups fall close to one another in the phylogenetic tree[1]. PfPK2 has been characterized to some extent, but its function is still unclear.

AGC group

The AGC group includes the cyclic nucleotide-dependent families of PKA and PKG, the PKC family, the β-adrenergic receptor kinase family, the ribosomal S6 kinase family and other close relatives. Like the kinases of the CaMK group, AGC protein kinases tend to be basic amino acid-directed[1].

PKA family. cAMP-dependent protein kinases play an important role in various signal transduction processes[43]. In some unicellular organisms, like the slime mold *Dictyostelium*, cAMP levels regulate the differentiation process leading to stalk formation[44]. Several experiments have been aimed at the question of whether cAMP plays a role in the induction of gametocytogenesis in blood-stage parasites, but the results have been controversial[45,46]. In *P. falciparum*, cAMP-dependent kinase activity has been observed in extracts, partially purified and characterized as a type I cAMP-dependent kinase[47]. A *P. yoelii* cAMP-dependent protein kinase, PycAPK[48], and a *P. falciparum* PKA homologue (C.D. Doerig, C. Syin and G. Langsley, unpublished) have been isolated (Table 1).

Other protein kinase families

Casein kinase 1 (CK1) family. Like CK2, CK1 has acidic recognition sequences, and is often involved in hierarchical protein phosphorylation reactions, acting on a wide range of substrates[27].

A malaria CK1 homologue has been isolated and characterized (Ref. 49). PfCK1 is very similar to the human α-isoform in that it lacks a C-terminal extension sequence, which in some CK1 isoforms mediates subcellular localization or is the target of inhibitory autophosphorylation[50,51]. The kinase activity of recombinant PfCK1 can be stimulated by heparin, and abolished by the CK1 inhibitor CK1-7 at concentrations similar to those required for the bovine isoform. CK1 activity, partially purified from soluble extracts of *P. falciparum*, displays identical properties, indicating that PfCK1 is the major CK1 enzyme in the parasite. The overall characteristics of this enzyme make it very likely that this is the CK1 kinase described by Wiser and co-workers[52]. Although still unknown, its high level of conservation suggests that it might play a similar role in the parasite to that played by CK1s in other eukaryotes.

Kinases with possible homologues in other species. PfPK4 has highest homologies to eIF-2α kinases, which are involved in the regulation of protein synthesis in eukaryotic cells by phosphorylating the α-subunit of eukaryotic translation initiation factor 2 (eIF-2α)[53]. PfPK4 phosphorylates a synthetic peptide, P45–56, which is an *in vitro* substrate of eIF-2α kinases[54]. The kinase is present as two major polypeptides of 80 and 90 kDa in asexual blood stages and shows the typical spotted pattern of a rhoptry-associated protein in segmenters and merozoites, where it colocalizes with the *P. falciparum* antigen, apical membrane antigen 1 (Ref. 54).

Erythrocytes contain small amounts of free hemin, which has dissociated from hemoglobin into the cytosol and into the erythrocytic membrane; the level of free hemin is increased in aged erythrocytes or pathological cells of sickle cell anemia and β-thalassemia[55]. Because hemin inhibits PfPK4, the kinase might help the parasite to sense its environment during the invasion process. Whether PfPK4 functions as a sensor, and whether it regulates initiation of translation like its mammalian homologues, remains to be shown.

Kinases without known homologues. FEST, *P. falciparum* exported Ser/Thr protein kinase is present in all asexual stages, being more abundant in late schizonts and segmenters. It is associated with the parasite plasma membrane, Golgi-like structures (Maurers clefts), vesicles and other membranous structures in the cytoplasm of the infected erythrocyte, and knobs in the parasitized erythrocyte plasma membrane[56]. Because homology in the catalytic kinase domain to other protein kinases is minimal, and there are no homologies in the other domains, it is very hard to speculate on a function for this enzyme.

Conventional protein tyrosine kinase group

To date, no convincing evidence has emerged for the existence of tyrosine kinases in *P. falciparum*. This apparent lack of tyrosine kinases, and the relatedness of the apicomplexans to the plant kingdom suggests that if a receptor protein kinase system is present in the parasite it might be of the type found in plants. In contrast to the animal kingdom, with receptor kinases autophosphorylating at tyrosine residues, the plant receptor kinases belong to the Ser/Thr kinase family[57]. However, none of the conserved motifs present in the extracellular domain of plant receptor protein kinases could be identified in any of the currently known malaria protein kinases.

How far have we come?

Validation of a given kinase as a drug target requires the proof that it is essential for parasite growth and/or differentiation. Gene knockout might present an attractive means of demonstrating the function of a protein kinase in sexual differentiation; the mutant parasites would be unaffected in their ability to proliferate, but could no longer form sexual stages. A similar experiment for a kinase with an essential function during the asexual multiplication cycle is hampered by the fact that it would be expected to have a lethal phenotype (in *Leishmania*, an essential role for a CDK-related kinase has indeed been suggested by the repeated lack of success of attempts at generating null mutants[58]). A system for establishing conditional knockouts (eg. using the cre–lox system)[59] needs to be developed in *Plasmodium* for this purpose.

A second essential feature for a molecule to be considered as a potential drug target is that it must be distinguishable from its counterpart in the human host. The CDPKs might prove very valuable in this respect – mammals do not have this type of protein kinase. An interesting feature of many *Plasmodium* enzymes (including some protein kinases) is the presence of insertion or extension sequences, which are absent from their relatives in other eukaryotes. Sequences added N- or C-terminally of the catalytic kinase domain might function as individual domains; the unusually large insertion sequences within the catalytic kinase domain itself found in some malaria kinases (PfPK1, PfPK4 and FEST) are surprising. However, as shown for PfPK1 and -4, they are compatible with enzymatic activity[27,53]. The position and the hydrophilicity of the inserts suggest that they form surface loops that might be accommodated into the three-dimensional structure of the kinases. In terms of drug development, it remains to be shown whether the insertions/extensions alter the characteristics of a given kinase enough to distinguish it chemically from its mammalian counterpart. In most cases, and without considering the insertions discussed above, the homology of malarial kinases to their mammalian counterparts falls within the range of 40–60% at the amino acid level. This suggests that structural differences might be significant, therefore allowing specific inhibition – an avenue well worth exploring, considering the urgency for novel antimalarials.

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