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Review

Roles of Apicomplexan protein kinases at each life cycle stage

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ABSTRACT

Inhibitors of cellular protein kinases have been reported to inhibit the development of Apicomplexan parasites, suggesting that the functions of protozoan protein kinases are critical for their life cycle. However, the specific roles of these protein kinases cannot be determined using only these inhibitors without molecular analysis, including gene disruption. In this report, we describe the functions of Apicomplexan protein kinases in each parasite life stage and the potential of pre-existing protein kinase inhibitors as Apicomplexan drugs against, mainly, *Plasmodium* and *Toxoplasma*.

Contents

1. Introduction

Some Apicomplexan parasites are known to be pathogens that cause lethal symptoms, including zoonoses, in humans, warm-blooded animals and insect vectors. Vaccines against the Apicomplexan parasites have not yet been developed. Although anti-protozoan drugs have been used in epidemic countries, drug-resistance frequently occurs. How is the damage caused by Apicomplexan parasites to be overcome?

Apicomplexan parasites, which have common characteristic organelles at the apex in the invasive forms, such as the dense granule, microneme, rhoptry, are the most-studied protozoans. Their life cycles are complex, compared to those of other biological species. Data described below suggest that protein kinases encoded by parasites are the main trigger molecules of life stage conversions. Addition of some protein kinase inhibitors causes specific inhibition of one parasite life cycle event. Phosphorylation by cellular protein kinases has been reported to regulate important cellular processes such as transcription, translation, protein synthesis, cell cycle, and apoptosis. Therefore, the protein kinases encoded by the parasite genomes may represent drug targets to block parasite-specific life events.

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However, the roles of each Apicomplexan protein kinase could not be determined using growth assays with protein kinase inhibitors. In this report, we reviewed the functions of Apicomplexan protein kinases by parasite life stage and the effects of pre-existing protein kinase inhibitors on, mainly, *Plasmodium* and *Toxoplasma*.

2. The functions of *Plasmodium* protein kinases in the life cycle and the effects of protein kinase inhibitors

The life cycle of *Plasmodium* (Fig. 1) is summarized below. Sporozoites are delivered into the bloodstream by the bite of an infected *Anopheles* mosquitoes. The sporozoites invade hepatocytes and produce several thousand merozoites. The merozoite invades erythrocytes and develops into the ring form, trophozoite, schizont. After rupture of the infected erythrocyte, the merozoites in mature schizonts burst out into the bloodstream and invade new erythrocytes, repeating the erythrocytic schizogony. During erythrocytic schizogony, a portion of parasites differentiate into micro- or macrogametocytes. Following the bite, gametogenesis occurs in the blood meal of the mosquito. After fertilization, the zygote is generated and develops into a motile ookinete. The ookinete establishes an oocyst at the basal lamina of the midgut. The oocyst generates sporozoites, which accumulate in the salivary glands.

In the life cycle, *Plasmodium* protein kinases regulate the life stage conversions (Fig. 1). The addition of protein kinase inhibitors causes specific inhibition of each life cycle event of *Plasmodium* although the targets of most of inhibitors are not specific. The method to knock out the specific gene of *Plasmodium* is useful for inhibit the specific protein kinase. However, the parasite in which the essential genes are knocked out for the life cycle event, especially blood stage, could not be produced. The conditional knockout system is not used as a large strategy and is still needed to be improved when used in *Plasmodium* species though one report showed successful knockdown using the destabilizing domain system [1]. We described the functions of *Plasmodium* protein kinases in the life cycle and the effects of protein kinase inhibitors below. The evaluation of effects of these pre-existing protein kinase inhibitors on *Plasmodium* is summarized in Table 1.

2.1. Invasion and egress

Protein kinases expressed in the invasive forms of parasites can play a role in the invasion step. *P. falciparum* protein kinase 2 (PfPK2), which is the only gene homologous to human calcium calmodulin-dependent protein kinase in the *P. falciparum* genome, is expressed in the merozoite [2]. W-7, a calmodulin antagonist,

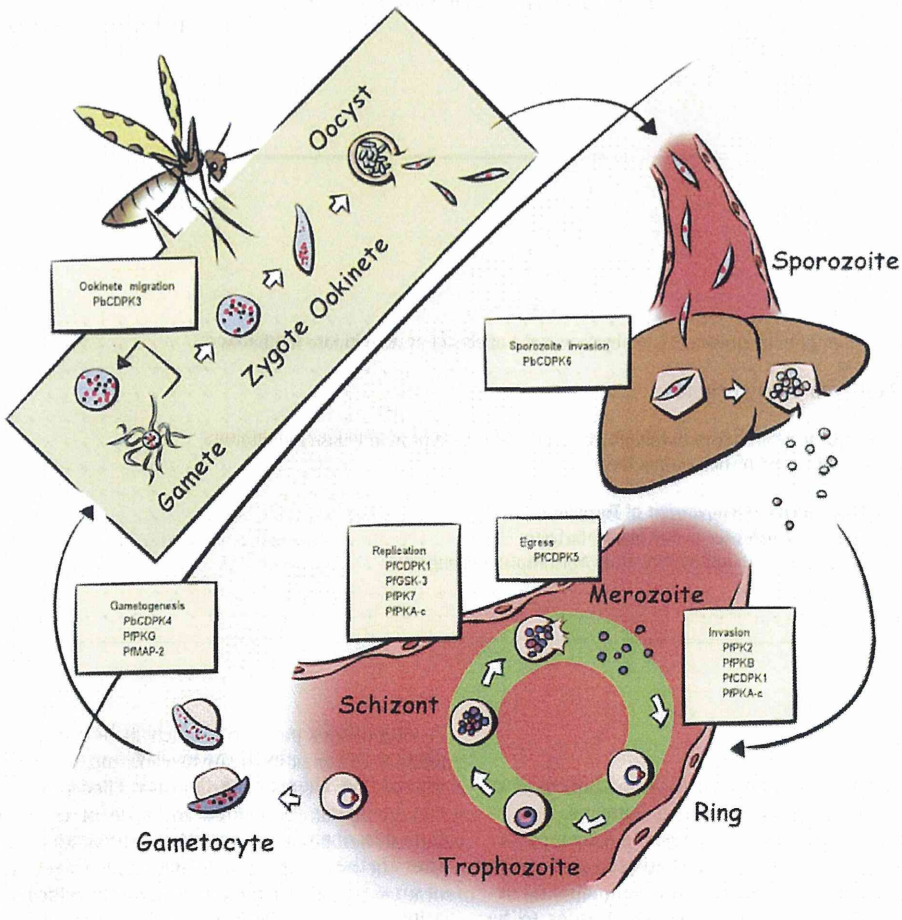

Fig. 1. Functions of *Plasmodium* protein kinases at each stage of the *Plasmodium* life cycle. *Plasmodium falciparum* protein kiase 2 (PfPK2) [2], protein kinase B (PfPKB) [3–6], calcium-dependent protein kinase 1 (PfCDPK1) [7] and PfPKA-C [8] are expressed in merozoites or associated with merozoite invasion. PfCDPK1 [17], glycogen synthase kinase-3 (PfGSK-3) [18] and PfPK7 [23] act on replication in erythrocytes. PfCDPK5 is critical for egress from the infected erythrocyte [1]. *Plasmodium berghei* calcium-dependent protein kinase 4 (PbCDPK4) and cGMP-dependent protein kinase (PfPKG) are essential for the exflagellation of male gametocytes induced by xanthurenic acid (XA) [36]. Mitogen-activated kinase-2 (PfMAP-2) is expressed in gametocytes [40]. PbCDPK3 regulates ookinete gliding mobility and penetration into the layer covering the midgut epithelium [14,15]. PbCDPK6 is critical for the switch to a hepatocyte-invasive phenotype [16].

Table 1
Evaluation of the effects of pre-existing protein kinase inhibitors on *Plasmodium*.

Inhibitor	Kinase family	Species	Strain	IC_{50} (nM)	Procedure						Reference
					Test	Parasitemia (%)	Hematocrit (%)	Starting stage	Incubation	Method	
Compound 1	PKG	Pf	3D7	<1000	Invasion	0.5	1	Schizont	24 h	Microscopy	11
				No effect in 1000	Grow (ring to troph)	0.5	1	Ring	24 h		
		Pf	3D7	2700	Growth	?	?	Early trophzoite	24 h	[^{3}H]-hypoxanthine uptake	25
				<300	Gametogenesis (rounding-up)	?	?	Gametocytes	10 min with XA and drug	Microscopy	
				<6000	Gametogenesis (exflagellation)						
		Pf	NF54	490	Growth	0.5	2.5	Early ring	48 h	[^{3}H]-hypoxanthine uptake	12
			Dd2	1300							
		Pb	?	Delayed mice mortality in 50 mg/kg	Growth	–	–	–	–	Virulence	
H89	PKA	Pf	D6	2900	Growth	0.1–0.5	1.5	?	42 h	[^{3}H]-hypoxanthine uptake	21
			W2	2500							
		Pb	234L clone	Reduced in 50,000	Gametologenesis (exflagellation)	?	10	Gametocytes	15 min	Microscopy	42
K252a	CDPK	Pf	3D7	348	Growth	0.5	2	Late stage schizont	48 h	Microscopy	7
KN-93	CaMK	Pg	8A	700	Zygotes to ookinetes	?	?	Zygotes	Overnight	Microscopy	41
W-7 (CAM antagonist)	CaMK	Pf	3D7	Reduced in 50,000	Invasion	?	?	Schizont	24 h	Microscopy	2
Go 6983	PKC	Pf	3D7	Reduced in 5000	Growth (schizont to ring)	1	?	Ring>80%	10–48 h	Microscopy	4
				Reduced in 2000, 5000	Invasion	?	?	Schizont	2–8 h	Microscopy	
U0126	MAPK	Pf	3D7	3000	Growth	0.5	5	Asynchronized	24 + 24 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	27
PD98059				30,000							
PD184352				7000							
Alsterpaullone	CDK	Pf	W2	4300	Growth	3	?	Ring>70%	24 + 18 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	29
			D6	4300							
		Pf	W2	4300							33
Butyrolactone 1		Pf	W2	11,700							29
			D6	11,700							
Indirubin-3′-monoxine			W2	1100							
			D6	600							
Indirubin		Pf	W2	1100							33
Isopentenyladenine		Pf	FCR-3	8340	Growth	0.5	1	Ring	24 h + 52 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	31
Kenpaullone		Pf	W2	3800	Growth	3	?	Ring>70%	24 h + 18 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	29
			D6	3800							
Olomoucine			W2	8000							
			D6	2700							
		Pf	K1	15,000	Growth	0.8	1.0	Ring	48 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	32
		Pf	FCR-3	8450	Growth	0.5	1	Ring	24 h + 52 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	31
Roscovitine		Pf	W2	28,000	Growth	3	?	Ring>70%	24 h + 18 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	29
			D6	33,000							
		Pf	FCR-3	5350	Growth	0.5	1	Ring	24 h + 52 h with [^{3}H]-hypoxanthine	[^{3}H]-hypothxanthine uptake	31
Purvalanol A		Pf	W2	9200	Growth	3	?	Ring>70%	24 h + 18 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	29
			D6	2600							
Purvalanol A (S-isomer)		Pf	FCR-3	420	Growth	0.5	1	Ring	24 h + 52 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	31
Purvalanol A (R-isomer)				550							
Purvalanol B				7070							

Table 1 (*continued*)

Inhibitor	Kinase family	Species	Strain	IC_{50} (nM)	Procedure						Reference
					Test	Parasitemia (%)	Hematocrit (%)	Starting stage	Incubation	Method	
Methyl-purvalanol B				47,450							
Aminopurvalanol				11,040							
Flavopiridol		Pf	K1	2000	Growth	0.8	1.0	Ring	48 h	[^{3}H]-hypoxanthine	32
Staurosporine	Most of kinase	Pk	?	250	Invasion into rhesus monkey RBC	?	?	Merozoite	Preincubation 5 min + invasion 2–3 h	Microscopy	9
		Pf	?	Reduced in 800	Invasion	?	?	Merozoite	20 h	Microscopy	10
		Pf	W2	150	Growth	3	?	Ring>70%	24 h + 18 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	29
			D6	190							
K510	Unclear	Pf	3D7	2500	Growth	?	?	?	48 h	[^{3}H]-hypoxanthine uptake	24
K109				1000							
K497				1500							
Xestoquione	Unclear	Pf	FCB1	3000	Growth	1	1.5	Mostly at ring	48 h	LDH activity	26
Oxindole-based inhibitor 14	CDK	Pf	W2	>20	Growth	3	?	Ring>70%	24 h + 18 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	29
			D6	>20							
Oxindole-based inhibitor 15			W2	>15.2							
			D6	>15.2							
Oxindole-based inhibitor 16			W2	>14.6							
			D6	>14.6							
Oxindole-based inhibitor 17			W2	>15.2							
			D6	>15.2							
Oxindole-based inhibitor 18			W2	>14.4							
			D6	>14.4							
Purine-derivative compound 99	CDK	Pf	FCR-3	830	Growth	0.5	1	Ring	24 h + 52 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	31
Purine-derivative compound 101				630							
Purine-derivative compound 40				540							
Purine-derivative compound 43				1120							
Purine-derivative compound 52				7100							
Purine-derivative compound 51				560							
Purine-derivative compound 52M				32,300							
Purine-derivative compound 59				530							
Purine-derivative compound 66				No inhibition at 10,000							
Sulfonamide-derivative compound 6	CDK	Pf	W2	17,000	Growth	3	?	Ring>70%	24 h + 18 h with [^{3}H]-hypoxanthine	[^{3}H]-hypoxanthine uptake	33
Sulfonamide-derivative compound 11				31,700							
Sulfonamide-derivative compound 12				24,700							
Sulfonamide-derivative compound 16				31,000							
Sulfonamide-derivative compound 20				21,200							
Sulfonamide-derivative compound 26				23,300							
Benzamides	Unclear	Pf	3D7, etc.	44–8000	Growth?	?	?	?	?	?	35
Imidazopyridazine compounds (Compound 2)	Unclear	Pf	?	5700	Growth?	?	?	?	?	?	34

–, needless to be described; ?, no description; Pf, *Plasmodium falciparum*; Pb, *Plasmodium berghei*; Pk, *Plasmodium knowlesi*; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; CDPK, calcium-dependent protein kinase; CaMK, calcium/calmodulin-dependent kinase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; CDK, cyclin-dependent protein kinase; XA, xanthurenic acid; LDH, lactate dehydrogenase.

inhibited phosphorylation of PfPK2 and reduced parasitemia of ring forms in an invasion assay.

P. falciparum protein kinase B (PfPKB) is expressed mainly in the schizonts and merozoites and phosphorylates *P. falciparum* glideosome-associated protein 45 (PfGAP45), an important component of the motor complex [3–6], which may help explain the role of PfGAP45 in erythrocyte invasion. *P. falciparum* calcium-dependent protein kinase 1 (PfCDPK1) is localized to the periphery of *P. falciparum* merozoites and acts on *P. falciparum* myosin A tail domain-interacting protein (PfMTIP) and PfGAP45 at the inner membrane complex [7]. Both PfMTIP and PfGAP45 can be efficiently phosphorylated by PfCDPK1 *in vitro*. Phosphorylation of *P. falciparum* AMA1 (PfAMA1) by the cAMP-dependent protein kinase catalytic subunit (PfPKA-C) affected invasion steps [8]. *P. falciparum* parasites expressing PfAMA1 (S610A) was impeded invasion. The enzyme responsible for the phosphorylation of PfAMA1 serine 610 was proved to be cAMP regulated PfPKA-C. However, cAMP does not have any effect on *T. gondii* invasion steps.

Pretreatment of *Plasmodium knowlesi* merozoites with staurosporine, a nonselective serine/threonine kinase inhibitor, inhibited invasion of rhesus monkey erythrocytes; this could be partially overcome with okadaic acid, a specific inhibitor of protein-serine/threonine phosphatases [9]. This effect is also found during invasion of human erythrocytes by *P. falciparum* [10].

Furthermore, trisubstituted pyrrole 4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1*H* pyrrol-3-yl] pyridine (Compound 1) is a more potent inhibitor of ring-stage formation than staurosporine in invasion assays [11]. Compound 1 is an ATP-competitive cGMP-dependent protein kinase (PKG) inhibitor. Treatment of schizonts for increasing lengths of time showed that they were unable to rupture and were irreversibly damaged after prolonged treatment. It was possible to reverse these effects using shorter treatment periods (1 to 3 h). This contrasted with staurosporine treatment, where even short periods of treatment led to irreversible inhibition of schizogony. Biochemical and molecular genetic analyses indicate that the chemotherapeutic target of Compound 1 in *P. falciparum* is PfPKG [12]. *P. falciparum* parasites expressing a Compound 1-insensitive PfPKG (T618Q) are insensitive to treatment with Compound 1 or a distinct anti-coccidian PKG inhibitor, Compound 2.

Go 6983 is an isoform-specific protein kinase C (PKC) inhibitor. The effect of this compound on recombinant PfPKB activity was tested, because *in silico* analysis suggested that the catalytic domain of PfPKB was most closely related to PKC, as compared to other AGC kinases, and because it appears that *P. falciparum* does not have a PKC homologue [4]. Go 6983 effectively inhibited both PfPKB activity and parasite growth. This growth inhibition effect was observed mainly during or after the schizont stages. Treatment with this inhibitor resulted in an almost 60% decrease in formation of new rings [4].

PfCDPK5, which is expressed in *P. falciparum* merozoites, is critical for egress from infected erythrocytes [1]. Parasites deficient PfCDPK5 were arrested as mature schizonts with intact membranes. The arrest was downstream of PfPKG function and independent of egress protease processing. Calcium signals are associated with micronemal secretion, which is related to egress and invasion.

Targeted disruption of the PbCDPK3 gene in *P. berghei* decreased ookinete ability to infect the mosquito midgut. PbCDPK3 regulates ookinete gliding motility and penetration into the layer covering the midgut epithelium [13–15].

PbCDPK6 is critical for the switch to a hepatocyte-invasive phenotype of sporozoite, a process accompanied by expression of the major sporozoite surface protein, circumsporozoite protein [16].

2.2. Plasmodium replication in the erythrocyte

Three N-terminal membrane anchor motifs of PfCDPK1 regulate the export of this protein to the parasitophorous vacuole (PV) of trophozoites and schizonts [17]. The Bisindolocarbazole inhibitor, K252a, is reported to inhibit PfCDPK1. Treatment of late schizont cultures with K252a inhibited growth of the parasites and invasion [7]. At high concentrations of K252a the egress of merozoites from schizonts was inhibited, whereas at intermediate concentrations egress was not affected, but merozoites failed to invade erythrocytes.

P. falciparum glycogen synthase kinase-3 (PfGSK-3) is predominantly expressed during the early erythrocyte stages. Once synthesized, PfGSK-3 is rapidly transported to the erythrocyte cytoplasm where it associates with vesicle-like structures, Maurer's clefts [18].

The host hormone melatonin increases cytoplasmic Ca^{2+} concentration, synchronizes the *P. falciparum* cell cycle [19] and induces an increase in cAMP levels and PfPKA-C activity [20]. H89, a specific PKA inhibitor, was equally effective against chloroquine-resistant and choloroquine-sensitive parasites (IC_{50}; 2.5 *vs.* 2.9 μM) [21]. The parasite overexpressing the PfPKA regulatory subunit (PfPKA-R) leads to down-regulation of host cell membrane anion conductance and a growth defect that can be restored by increasing the levels of intracellular cAMP [22].

The N-terminal region of PfPK7 is similar to fungal PKA [23]. PfPK7 is expressed in several *P. falciparum* developmental stages, both in the mosquito vector and in the host erythrocyte. In order to find PfPK7 inhibitors, a thermostability shift assay was performed to screen a kinase-targeted library of 568 compounds [24]. Some potential inhibitors are tested for their ability to inhibit asexual growth of *P. falciparum* in blood-stage cultures. Three K-series compounds (K510, K109, and K497) and PP2 were potent inhibitors. However, the two PP1 derivatives, 1NM-PP1 and 1NA-PP1, were less active.

Biochemical and molecular genetic analyses indicate that the chemotherapeutic target of Compound 1 in *P. falciparum* is PfPKG [12]. The *in vitro* growth of both chloroquine-sensitive and -resistant strains of *P. falciparum* in human erythrocytes is sensitive to treatment with Compound 1. Compound 1 extended survival of mice in a *P. berghei* acute infection model, but all mice eventually succumbed to the parasite. This compound also inhibited the parasite growth over 24 h started with the early trophozite stage [25].

Xestoquinone is a bioactive metabolite isolated from a new species of marine sponge, *Xestospongia*, collected in Vanuatu [26]. This molecule was isolated from a bioassay-guided fractionation based on a Pfnek-1 inhibition assay, and inhibited Pfnek-1 activity.

The highly selective MAPK/ERK kinase (MEK) 1/2 inhibitor, U0126, inhibited *P. falciparum* proliferation [27], despite the absence of typical MEK homologues in the *Plasmodium* kinome [28]. Furthermore, the structurally distinct allosteric MEK inhibitors, PD98059 and PD184352, also have parasiticidal activity. U0126 and PD184352 both blocked trophozoite development. These MEK inhibitors also have parasiticidal activity against *P. berghei*.

Staurosporine inhibits growth of *P. falciparum* at very low concentrations [29]. In addition, this compound inhibited infection of *Cryptosporidium parvum*, another Apicomplexan parasite [30].

Cyclin-dependent kinase (CDK) inhibitors have been used in many studies. The purvalanol series are 2,6,9-tri-substituted purine derivatives with an amino group at C2 and a benzylamino or anilino group at C6. This series of compounds showed a wide range of inhibitory activities against the growth of *P. falciparum* [31]. Both R and S isomers of purvalanol A displayed similarly high levels of inhibitory activity against *P. falciparum* proliferation [29,31]. Purvalanol B contains a carboxyl group on the 3-chloroanilino C6 substituent of purvalanol A. Although it is a potent CDK inhibitor, it has only a mediocre effect on *P. falciparum*. The methylated derivative of purvalanol B (methyl-purvalanol B) is less active against *P. falciparum*. An amino substituent on the chloroanilino group of purvalanol A (aminopurvalanol) does not significantly increase activity against *P. falciparum*, as compared to the carboxylated derivative (purvalanol B). Methylation of this compound (methyl-aminopurvalanol) decreases its antimalarial activity.

Olomoucine, roscovitine, and isopentenyladenine are purine derivatives. They are generated to inhibit CDKs. Several CDK inhibitors are based on purines, paullones and flavonoids, because they have been generated to target the ATP binding sites of CDKs. They displayed similar activity against *P. falciparum* [29,31,32]. On the other hand, iso-olomoucine shows minimal activity [31]. In the FCR-3 strain, which rarely produces gametocytes in culture, gametocyte formation was enhanced when it was exposed to the IC_{90} concentrations of olomoucine and roscovitine [31]. In other experiments, olomoucine and flavopiridol, semisynthetic flavonoid CDK inhibitors [32], significantly reduced incorporation of [^{3}H]hypoxanthine into malarial DNA. In addition, alsterpaullone [29], kenpaullone [29], indirubin [33], indirubin-3′-monoxime [29] and butyrolactone I [29] have been used as malarial CDK inhibitors in growth and *in vitro* kinase assays. Indirubin-3′-monoxime contains an oxindole moiety, and so other oxindole-based compounds were tested in the same assay [29]. However, none showed growth inhibitory activity. In contrast, sulfonamide compounds [33], purine derivatives [31], imidazopyridazine compounds [34] which are potent inhibitors of PfCDPK1, and benzamide compounds [35], which are candidate antimalarial kinase inhibitors, effectively inhibited live *P. falciparum*. The stage in which many CDK inhibitors affect remains to be investigated.

2.3. Gametogenesis

P. berghei CDPK4 (PbCDPK4) was essential for the exflagellation of male gametocytes induced by a mosquito molecule, xanthurenic acid (XA) [36]. PfCDPK4 is expressed in the gametocyte of *P. falciparum* and its phosphorylation is activated by an increase in Ca^{2+} concentration or pH and a decrease in temperature [37,38]. Compound 1 inhibited rounding-up of parasites, with XA-stimulated gametocytes clearly retaining their distinctive crescent shape [25]. By contrast, gametocytes treated with XA alone became spherical, indicating that gametogenesis had been initiated. Both rounding-up and exflagellation of *P. falciparum* gametocytes were inhibited by Compound 1 in a dose-dependent manner. However, in the same experiment, gametocytes from Compound 1-resistant PfPKG(T618Q) clones rounded up to almost normal levels in the presence of Compound 1 after stimulation with XA. Compound 2 showed similar results.

Pfnek-1, a *P. falciparum* gene homologous to never-in-mitosis A (NIMA)/NIMA-like kinase (Nek) family of protein kinase, is able to specifically phosphorylate PfMAP-2, a typical *P. falciparum* mitogen-activated protein kinase (MAPK) homologue, *in vitro* [39]. PfMAP-2 is expressed specifically in gametocytes, which is consistent with the fact that gametocyte extracts activated recombinant PfMAP-2 more efficiently than extracts from asexual parasites [40].

KN-93 is a specific inhibitor of Ca^{2+}/CaM-dependent protein kinase [41]. Differentiation of *P. gallinaceum* zygotes into motile ookinetes was inhibited by this compound, while this effect was not seen in control parasites or parasites treated with KN-92, an inactive analog of KN-93. Treatment with KN-93 did not interfere with expression of the ookinete-specific genes, Pgs28 and chitinase.

H89 also has an effect on gametogenesis. In a mini-scale exflagellation assay, this compound inhibited microgamete exflagellation of *P. berghei* [42]. Male gametocytes treated with H89 ceased development. The cAMP-/cGMP-dependent kinase inhibitors, H8 and H87, and staurosporine, also showed the same effects (staurosporine at lower concentrations).

3. The functions of *Toxoplasma* protein kinases in the life cycle and the effects of protein kinase inhibitors

T. gondii isolates from European and North American mainly belong to major three lines, referred to as types I, II and III. These three genetic types differ in bioactivity. Type I is most virulent, while type II and type III parasites have the moderate virulence. Among the protein kinases in *T. gondii* kinome, the ROP kinase family is diverged among the three genetic lines [43]. The life cycle of *Toxoplasma* (Fig. 2) is described below. In both the intermediate and definitive host feline species, *T. gondii* replicates quickly and causes acute infection as tachyzoites. Once the parasite is exposed to a stressful environment, which may be the host immune system or particular host cell types, the parasite replicates slowly, evades the immune system in the cyst wall and causes persistent infection as bradyzoites. *T. gondii* protein kinases work in each step of life cycle (Fig. 2).

The addition of protein kinase inhibitors causes specific inhibition of each life cycle event of *Toxoplasma*. The functions of *Toxoplasma* protein kinases in the life cycle and the effects of protein kinase inhibitors were described. The evaluation of effects of these pre-existing protein kinase inhibitors on *Toxoplasma* is summarized in Table 2.

3.1. Host cell invasion and egress of Toxoplasma tachyzoite

Toxoplasma can invade host cells in its tachyzoite, bradyzoite and sporozoite forms. However, the molecular mechanism of invasion of only the tachyzoite form has been well documented. Serine/Threonine protein kinases have been implicated in parasite invasion steps using simple inhibitor-based analyses with KT5926, staurosporine and Compounds 1 and 2. The general serine/threonine protein kinase inhibitor staurosporine was reported to inhibit micronemal secretion and host cell attachment [44] and also to inhibit calcium-induced egress [45]. KT5926, which is used for the inhibition of mammalian myosin light chain kinase family, was reported to inhibit invasion by affecting attachment [46] and gliding motility [47]. Compounds 1 & 2, which are the potent inhibitor of parasite PKG, inhibit invasion [48] by affecting motility and secretion [49]. In agreement with the inhibitor data, molecular genetics revealed the functions of several protein kinases in invasion and egress. TgCDPK1 is expressed in tachyzoites and localizes in the parasite cytosol [46,50]. Bumped kinase inhibitors (BKIs), which contain NA-PP1, NA-PP2, NM-PP1 and 3-MB-PP1, specifically inhibit TgCDPK1 and effectively inhibit tachyzoite invasion [51–53]. This effect was decreased in BKI-refractory mutated TgCDPK1-expressed parasites, suggesting that the main target of BKI is TgCDPK1. A conditional knockout study revealed the precise function of TgCDPK1 in calcium-dependent micronemal secretion steps, which are related to egress and invasion [53]. Considering that calcium signals are key in invasion steps [13], other CDPK must to be considered. *T. gondii* calmodulin-like domain protein kinase isoform 3 (TgCDPKif3: TgCDPK1_2 in ToxoDB (http://toxodb.org/) annotation) is expressed in tachyzoites and localized to the apical end under extracellular conditions. An *in vitro* kinase assay demonstrated that TgCDPKif3 can phosphorylate Aldolase 1 [54], a member of the glideosome. More than 20 CDPK-related kinases are encoded in the *T. gondii* genome [55]; thus, these may also contribute to invasion. TgPKG is also involved in invasion. The fact that mutant parasites that express PKG inhibitor-refractory TgPKG(T128Q) gained resistance to Compound 2 shows that the main target of Compound 2 is TgPKG [48]. MAPK and PKC inhibitors also inhibited invasion by *T. gondii* [56,57], though their target remains to be determined.

Most substrates of the protein kinases listed above have not been reported, and so how these protein kinases function in signal transduction and which proteins are phosphorylated remain unclear. However, similar with PfGAP45, TgGAP45, which is a member of the glideosome complex, is needed for active host cell penetration and is also phosphorylated in invasive parasites [58].

3.2. Parasite cell cycle regulation

H89, ATP competitive inhibitor of mammalian PKA-C, inhibited parasite growth effectively [59] and reduced the number of parasites per PV, while mammalian PKI, a PKA inhibitor peptide, did not reduce

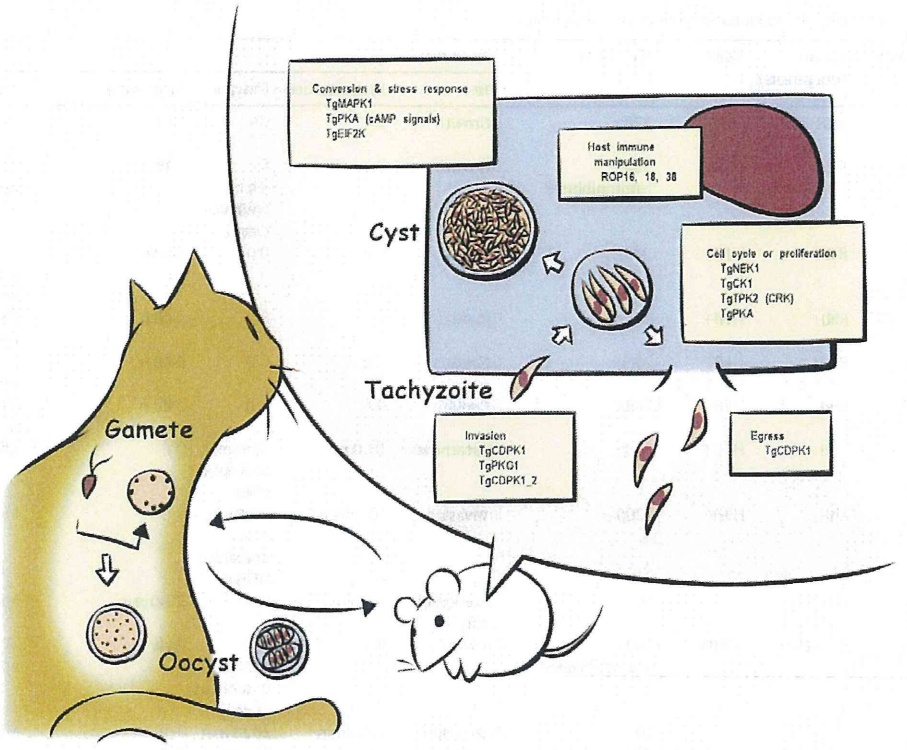

Fig. 2. Functions of *Toxoplasma* protein kinases in the parasite life cycle. No protein kinase function in the sexual stage of the definitive host feline species has been determined. In the tachyzoite growth cycle, *Toxoplasma gondii* calcium-dependent protein kinase 1 (TgCDPK1) [46,51–53], cGMP-dependent protein kinase (TgPKG1) [48] and TgCDPK1_2 [54] are reported to act in the invasion steps. TgCDPK1 is also reported to act during egress [53]. ROP16 [78,79], ROP18 [81,82] and ROP38 [43] are involved in host manipulation. cAMP-dependent protein kinase catalytic subunit (TgPKA-C) [60], NIMA-related kinase 1 (TgNEK1) [62], casein kinase 1 (TgCK1) [61] and cdc2 cyclin-dependent kinase (TgTPK2) [66] are involved in cell cycle regulation or functions required for growth. Mitogen-activated protein kinase 1 (TgMAPK1) [63], TgPKA-C [59] and eukaryotic initiation factor-2 kinase (TgEIF2K) [71,73] are involved in the stress response and conversion from tachyzoite to bradyzoite.

parasite numbers [60]. Of the CDK inhibitors, purvalanol A, aminopurvalanol, indirubin-3′-monoxime and alsterpaullone effectively inhibited parasite growth, but purvalanol B, hymenialdisine and kenpaullone did not [61].

The *T. gondii* temperature-sensitive cell cycle-deficient strain VA15 (cell cycle arrest by mitosis defect) can be rescued by complementing with the NIMA related protein kinase 1 (TgNEK1) gene, indicating that TgNEK1 is a regulator of the cell cycle [62]. TgMAPK1 is expressed in tachyzoites, the duplication time of which is slowed by addition of human p38α MAPK inhibitor [63–65]. TgTPK2 is a CDK homologue of *T. gondii*. Overexpression of TgTPK2 results in cell cycle arrest in S phase [66]. In agreement with the PKA inhibitor effect described above, cAMP or cGMP signal inducers or inhibitors have effects on *Toxoplasma* replication speed in the PV [67]. Inhibitor-based analysis demonstrated that cAMP signals are related to parasite cell cycle regulation, though cAMP signals of both host cells and parasites may have been affected. Which of these contributes to cell cycle arrest has not yet been determined. However, parasites overexpressing TgPKA-R replicate slowly [60], suggesting that only parasite cAMP signals can affect parasite cell cycle regulation.

3.3. Stress response and bradyzoite conversion of Toxoplasma

Some *T. gondii* tachyzoites in host cells may be converted into dormant bradyzoites. The pyridinylimidazole compounds (SB202190, SB203580 and RWJ67657), the imidazopyrimidine compound (RWJ68198) and the anthrapyrazolone compound (SP600125), are p38 MAPK inhibitors. These inhibited parasite growth, while PD98059, which is a MAPKK inhibitor, did not [68,69]. When RWJ67657 was administered to mice infected with *T. gondii* tachyzoites, survival was enhanced [69]. However, inhibitors may have affected both host and parasite MAPKs.

H89 and cAMP signal inhibitors can induce bradyzoite conversion [67]. Therefore, PKA might regulate switching between tachyzoite and bradyzoite forms. TgMAPK1 is upregulated in bradyzoites by stress [63]. Preincubation of parasites with a MAPK inhibitor before host cell infection leads to slow replication, suggesting that inhibition of parasite MAPK signals contributes to bradyzoite conversion [65]. However, host cell transcriptional changes induced by a protein kinase inhibitor also induce bradyzoite conversion [70].

T. gondii initiation factor-2 kinase (TgIF2K) phosphorylates *T. gondii* eukaryotic initiation factor-2 (TgIF2). TgIF2 phosphorylation status is altered upon induction of bradyzoite [71,72]. Two protein kinases possessing eIF2K activities have been reported. The first is localized to the nucleus, and the other to the endoplasmic reticulum [71]. The phosphorylation of TgIF2α also works in tachyzoite survival, especially in extracellular survival. Transgenic parasites which express the phosphorylation-negative mutant of TgIF2α showed lesser extracellular survival capacity and lower virulence in mice infection model than the wild type parasite [73]. The knockout of TgIF2α kinase reduces the extracellular survival of parasite [74].

3.4. Host cell manipulation by Toxoplasma and virulence factor

ROP kinase family is a secreted protein kinase family localized in the rhoptry body of *T. gondii*. ROP kinases have no homologue in mammalian genomes and are a promising drug target if these genes are virulence factors. Two ROP kinases, ROP16 of type I and III parasites and ROP18 highly expressed in type I and II parasites, function to regulate the immune system. ROP16 and ROP18 were found to be

Table 2
Evaluations of the effects of pre-existing protein kinase inhibitors on *Toxoplasma*.

Inhibitor	Kinase family	Strain[a] (organisms)	Cell	IC_{50} (nM)	Procedure					Reference
					Test	Preincubation	Invasion	Incubation	Method	
Compound 1	PKG	RH	HFF	230	Growth	0	0	48 h	[^{3}H]-uracil uptake	83
		RH	HFF	320 Not inhibited	Growth	0	0 4 h without drug	48 h	[^{3}H]-uracil uptake	49
		RH	HFF	210	Growth	0	0	5days	β-galactosidase activity	83
		RH	HFF	200	Growth	0	0	48 h	[^{3}H]-uracil uptake	61
		RH	HFF	235	Growth	0	0	48 h	[^{3}H]-uracil uptake	48
		RH	HFF	200	Growth	0	0	48 h	[^{3}H]-uracil uptake	84
		RH	HFF	370	Attachment	10 min	20 min to fixed HFFs	0	Microscopy	49
		Rh	HFF	1200	Invasion	10 min	20 min to unfixed HFFs	0	Microscopy	
		RH	–	60	Secretion of MIC2	10 min	0	30 min	Microscopy	
		E. tenelia	MDBK	160 Not inhibited	Growth	0	0 4 h without drug	48 h	[^{3}H]-uracil uptake	49
				40	Attachment	10 min	20 min	0	Microscopy	
				600	Invasion	10 min	20 min	0	Microscopy	
Compound 2	PKG (CDPK1, CK1α)	RH	HFF	24	Growth	0	0	48 h	[^{3}H]-uracil activity	48
H89	PKA	PLK (ME49)	HFF	Reduced in 3000	Growth	0	2 h without drug	24, 48, 72 h	CAT activity	59
KT5926	Myosin light chain kinase, CDPK	RH (EP)	HFF	90	Attachment	0	15 min	0	Microscopy	46
		β-Gal-expressing PLK strain	HFF	500–1000	Invasion	15 min	1 h	24 h after washed	β-galactosidase activity	47
		RH	HFF	500–1000	Motility	15 min	0	15 min at serum-coated	Trail distance	
		β-Gal-expressing RH strain	HFF	Significantly decreased	Attachment vs. invasion	15 min Rt	10 min	0	Microscopy	
NA-PP1	Analog sensitive kinase[b]	β-GAl-expressing RH strain	HFF	>10,000	Invasion	0	4 h without drug	20 h	β-galactosidase activity	51
				>10,000	Growth	0	0	20 h		
NA-PP2				>100	Invasion	0	4 h without drug	20 h		
				>10,000	Growth	0	0	20 h		
NM-PP1				<10,000	Invasion	0	4 h without drug	20 h		
				>10,000	Growth	0	0	20 h		
		RH	Vero	<50	Growth	0	0	4 days	Host lysis	52
				<500	Invasion	0	30 min	0	Microscopy	
3-MB-PP1	Analog sensitive kinase[b]	TATi-1	HFF	Reduced in 5000	Invasion	20 min RT	20 min	0	Microscopy	53
				Reduced in 5000	Secretion	20 min	0	15 min	Western blot	
3-BrB-PP1				<10,000	Growth	20 min RT	1 h	3 days after washed	Host lysis	
				<10,000						
Purvalanol A	CDKs	*RH*	*HFF*	1230	Growth	0	0	48 h	[^{3}H]-uracil uptake	61
Purvalanol B				>5000						
Aminopurvalanol				360						
Indirubin-3′-monoxime				500						
Hymenialdisine	CDKs (,GSK-3, CK1)			>5000						
Alsterpaullone	CDKs (,GSK-3)			1220						
Kenpaullone				>5000						

(continued on next page)

Table 2 (*continued*)

Inhibitor	Kinase family	Strain[a] (organisms)	Cell	IC_{50} (nM)	Procedure					Reference
					Test	Preincubation	Invasion	Incubation	Method	
PD98059	MAPK	RH	HeLa	Does not significantly inhibit	Growth	0	0	1, 5, 18 h→2 h with [^{3}H] uracil	[^{3}H]-uracil uptake	68
SP600125				Intracellular replication is decreased at 10,000						
SB203580	p38 MAPK	RH ME49	HFF	5000 1000	Growth	0	4 h without drug	48 h + 16 h with [^{3}H] uracil	[^{3}H]-uracil uptake	69
		RH ME49	HFF	5000 200	Growth	0	6 h without drug	18 h	[^{3}H]-methyluracil uptake	65
SB203580	p38 MAPK	RH ME49	HFF	8500 2500	Growth	0	4 h without drug	48 h + 16 h with [^{3}H] uracil	[^{3}H]-uracil uptake	69
		RH	HeLa	Intracellular replication is decreased at 10,000	Growth	0	0	1, 5, 18 h→2 h with [^{3}H] uracil	[^{3}H]-uracil upatke	68
		RH ME49	HFF	6000 500	Growth	0	6 h without drug	18 h	[^{3}H]-methyluracil uptake	65
RWJ67657	p38 MAPK	RH ME49	HFF	5000 800	Growth	0	4 h without drug	48 h + 16 h with [^{3}H] uracil	[^{3}H]-mtthyluracil uptake	69
RWJ68198		RH ME49		8600 2000						
Staurosporine	Most of kinases	RH 2F (β-Gal)	HFF Aldehyde-fied HFF	20 Reduced in 1000	Invasion Attachment	10 min 10 min	20 min 20 min	0 0	Microscopy β-galactosidase activity	44
		RH	HFF	Reduced in 10	Secretion of MIC2	?	?	?	Western blot	
		RH	LLC-MK2	<500	Calcium-ionophore-induced egress	0	40–50 min	2 h + 30 min with drug→wash and induced 15 min	Microscopy	45
		C. parvum	BFTE	<50,000	Growth	Cell + drug 60 min	0	24 h	Microscopy	30
Genistein	Tyrosine kinase	RH	LLC-MK2	<50,000	Calcium-ionophore-induced egress	0	40–50 min	2 h + 30 min with drug→wash and induced 15 min	Microscopy	45
		C. parvum	BFTE	25,000	Growth	Cell + drug 60 min	0	24 h	Microscopy	30

–, needless to be described; ?, no description; *E. tenella*, *Eimeria tenella*; *C. parvum*, *Cryptosporidium parvum*; PKG, cGMP-dependent protein kinase; CDPK, calcium-dependent protein kinase; CK, creatine kinase; PKA, cAMP-dependent protein kinase; CDK, cyclin-dependent protein kinase; GSK, glycogen synthase kinase; MAPK, mitogen-activated protein kinase; HFF, human foreskin fibroblast cell; MDBK, Madin–Darby bovine kidney cell; Vero, Vero cell; HeLa, HeLa cell; LLC-MK2, LLC-MK2 cell; BFTE, bovine fallopian tube epithelial cell; RT, room temperature; CAT, chloramphenicol acetyltransferase.

[a] RH, TATi-1 (RH clone expressing transactivator for the tet-off inducible gene knock system) and 2F (RH clone expressing beta-galactosidase) belong to type I parasites. ME49 and PLK belong to type II parasites.

[b] Analog sensitive kinase includes protein kinase whose gatekeeper amino acid is small and susceptible to inhibitor analog.

virulence factors by forward genetics using F1 progeny of virulent and avirulent strain parents [75,76]. ROP16 is related to suppression of host native immune systems. ROP16 from type I and III parasites phosphorylated STAT3 and STAT6, related to down-regulation of IL-12 [77]. Direct phosphorylation of STAT3 [78] and STAT6 [79] by ROP16 means that ROP16 regulated the host immune system by regulation of the phosphorylation state of STATs. Although *in silico* analysis failed to detect the eukaryotic typical tyrosine kinases in the *T. gondii* genome [43], the phosphorylation activity of tyrosine residue of STATs by these kinases exhibits atypical tyrosine kinase domain in this organism. ROP18 is related to regulation of the parasite cell cycle in HFF cells [80]. However, in mouse macrophage cells, ROP18 is related to the survival rate both *in vitro* and *in vivo* [81]. ROP18 on PV phosphorylates the mouse immunity-related GTPases (IRGs) family and saves the PV membrane from destruction by IRGs. The IRGs family does not exist in the human genome, but similar immune molecules in humans may inhibit another ROP kinase. ROP18 is also reported to phosphorylate activating transcription factor 6β (ATF6β), the host endoplasmic reticulum-bound transcription factor, and affect virulence in mice [82]. Comparison with genome sequences of type I, II and III *T. gondii* strains shows that ROP kinases are a divergent family [43]. ROP38 which is expressed in type III VEG strain and not expressed in type I RH strain manipulates host MAPK signals and regulates host cell apoptosis and the cell cycle [43].

4. Potentiality of the pre-existing protein kinase inhibitors as Apicomplexan drugs

Protein kinase inhibitors represent one of the best hopes for the development of drugs that block specific parasite life events without side effects, given that some parasite protein kinases have no homologous genes in the host genome. However, few *in vivo* parasite inhibition assays using animals infected with parasites have been reported. Therefore, animal model data are now needed if pre-existing protein kinase inhibitors, or those discovered by drug screening, are to be effective novel drugs against Apicomplexan parasites. Furthermore,

when effective drug candidates are found, improvement and modification of these compounds will probably be necessary.

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