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The calcium-dependent protein kinase 1 from *Toxoplasma gondii* as target for structure-based drug design

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Abstract

The apicomplexan protozoan parasites include the causative agents of animal and human diseases ranging from malaria (*Plasmodium* spp.) to toxoplasmosis (*Toxoplasma gondii*). The complex life cycle of *T. gondii* is regulated by a unique family of calcium-dependent protein kinases (CDPKs) that have become the target of intensive efforts to develop new therapeutics. In this review, we will summarize structure-based strategies, recent successes and future directions in the pursuit of specific and selective inhibitors of *T. gondii* CDPK1.

Introduction

The phylum of Apicomplexa contains approximately 6000 unicellular, eukaryotic parasites including *Plasmodium* spp., the causative agent of Malaria, and *Toxoplasma gondii*, responsible for toxoplasmosis in many important farm animals and humans (Sato, 2011). Morphologically, all members of the apicomplexan family share a distinctive apical complex, together with species dependent apical-localized organelles. (McFadden and Yeh, 2017). These parasites employ complex life cycles including both sexual and asexual reproduction. Furthermore, in many cases their life cycles involve multiple hosts. *T. gondii*, first described in 1908 and often regarded as one of the most successful apicomplexan parasites, represents the key model organism of the phylum (Dubey, 2008; Weiss and Dubey, 2009; Szabo and Finney, 2017). Its primary hosts are members of the Felidae (cats) family while all other warm-blooded animals, including humans, are intermediate hosts. It is estimated that up to one third of the human population is infected with *T. gondii* and thus are potential carriers. Although the infection is usually asymptomatic in healthy individuals it can cause severe congenital disease during pregnancy (Kaye, 2011), and lead to life-threatening infections in immunocompromised patients including those suffering from HIV, receiving an organ transplant or undergoing cancer chemotherapy treatment (Flegr *et al.* 2014). Current toxoplasmosis treatment options are limited to a handful of antimicrobials such as sulphonamides, folic acid derivatives and certain macrolide antibiotics. However, these drugs often show limited efficacy and are associated with significant side effects (Alday and Doggett, 2017). Furthermore, there are no treatments available to target tissue cysts, the persistent form in which the parasite evades the host immune system, and to eradicate persistent *T. gondii* infections (Opsteegh *et al.* 2015). Therefore, new drug targets and novel therapies are urgently needed. In addition to high-throughput screening approaches (Norcliffe *et al.* 2014), structure-based methods in close combination with medicinal chemistry and biophysical and biological validation have become powerful tools in the search of new drugs and treatments (Hunter, 2009; Verlinde *et al.* 2009; Groftehaug *et al.* 2015; Hol, 2015; Muller, 2017).

The role of calcium-dependent protein kinases (CDPKs)

In *T. gondii* Ca²⁺-ions play key roles in cell signalling and in pathogen–host interactions including cell invasion, motility of the parasite within the host and differentiation during the parasites complex life cycle (Irvine, 1986; Nagamune *et al.* 2008; Lourido and Moreno, 2015). CDPKs are a family of serine/threonine kinases that are only found in plants and protists including ciliates and apicomplexan parasites. Importantly, these kinases provide the mechanistic link between calcium signalling and motility, differentiation and invasion (Tzen *et al.* 2007; Billker *et al.* 2009). These crucial roles of CDPKs have been proven through a range of knock-out studies in various species and underline the potential of CDPKs as targets for novel therapeutics (Long *et al.* 2016). CDPKs are members of the Calmodulin/Calcium kinase (CaM) family. They all share an N-terminal kinase domain (KD) linked *via* a junctional domain to a series of C-terminal Calcium-binding motifs. In *T. gondii* at least 12 different CDPKs have been putatively identified ranging in size from 507 (CDPK1) to more than 2000 amino acids (CDPK7, CDPK80) (Morlon-Guyot *et al.* 2014). Although there is probably overlap in functionalities, different sub-cellular locations and varying expression levels during the parasites' life cycle is likely to lead to different biological functions within the CDPK family

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(Hui *et al.* 2015). The shared sequence identities range from 51% (CDPK1 and CDPK3) (Treeck *et al.* 2014) to lower than 10% (Table 1). These variations in length and sequence support the notion that members of the CDPK family act upon a range of substrates and fulfil different functions in *T. gondii* biology. Recent knock-out studies using CRISPR-Cas9 indicate that CDPK4, CDPK5, CDPK6, CDPK8 and CDPK9, respectively, have no effect on virulence and on normal growth (Wang *et al.* 2016), however, knock-down studies have shown that CDPK7 is crucial for survival due to a critical role in parasite division (Morlon-Guyot *et al.* 2014). More detailed studies have been performed on the smaller family members. CDPK3 with 537 amino acids has been implicated in motility and host cell egress (McCoy *et al.* 2017). CDPK2 (711 amino acids) has been shown to act as key regulator of amylopectin metabolism (Uboldi *et al.* 2015). The loss of CDPK2 results in the build-up of amylopectin with fatal consequences for *T. gondii* in its chronic stage. Importantly, this family member contains an N-terminal carbohydrate-binding domain that may offer new opportunities for drug design (Uboldi *et al.* 2015). CDPK1, which is mainly located in the cytosol, has been shown to be required for the microneme secretion at the apical complex and parasite proliferation. The molecular mechanism, however, remains elusive (Lourido *et al.* 2010; Child *et al.* 2017). Here we will review strategies and recent results in the discovery, design and potency of inhibitors targeting the KD of CDPK1 from *T. gondii* (TgCDPK1).

Activation of TgCDPK1 by calcium

The mechanism of activation and inhibition was unravelled in 2010 when the crystal structures of both the auto-inhibited and the Ca²⁺-activated forms of TgCDPK1 were published (Ojo *et al.* 2010; Wernimont *et al.* 2010). These structures revealed the expected KD in similar overall conformations, however, the Ca²⁺-binding domain (also designated CPDK activating domain or CAD) adopted two vastly different conformations and orientations (Fig. 1). In its inactive state the CAD (shown in raspberry red) adopts an elongated form reminiscent of apo-calmodulin starting with a long helix followed by the first Ca²⁺-binding motifs (EF-hands), which is connected *via* another long helix to the second pair of C-terminal EF-hands (Fig. 1a). The first long helix has been suggested to be responsible for the auto-inhibitory effect by blocking the substrate binding site and providing a basic lysine residue to bind a cluster of conserved acidic residues. However, this may not be the only mechanism of deactivation as it has more recently been shown that removal of the regulatory domain alone does not lead to an active KD (Ingram *et al.* 2015). The CAD domain activated by Ca²⁺-binding appears to be required to maintain the KD in its active conformation. Calcium binding leads to a dramatic rearrangement and refolding of the protein chain (Fig. 1b) (Wernimont *et al.* 2010). The entire regulatory domain is shifted to the other side of the protein hence liberating the active site of the KD as shown in Fig. 1c. In addition, the regulatory calcium-binding domain is collapsed so that the two long helices are no longer arranged in an anti-parallel fashion but are partially unwound and interwoven to form a more globular overall shape. These structural changes are reminiscent to the calcium-bound structure of calmodulin (Kursula, 2014).

Comparison with human kinases

Historically, characterising (protozoan) kinases as potential drug targets and developing selective inhibitors has been considered challenging due to the fact that the overall protein fold and the active sites of all kinases are structurally well conserved (Scapin, 2002). The structural similarities of the KD are obvious when

Table 1. The protein sequence identities between the 12 putative calcium-dependent protein kinases (CDPKs) of *T. gondii*, rounded to the nearest whole number, derived from a multiple sequence alignment (MSA) generated using Clustal Omega (Sievers *et al.* 2011)

	CDPK1	CDPK2	CDPK2A	CDPK2B	CDPK3	CDPK4	CDPK4A	CDPK5	CDPK6	CDPK7	CDPK8	CDPK9
CDPK1		22%	23%	30%	51%	14%	24%	25%	9%	7%	6%	22%
CDPK2	22%		34%	34%	25%	14%	21%	32%	14%	7%	9%	26%
CDPK2A	23%	34%		37%	27%	20%	20%	32%	15%	9%	9%	24%
CDPK2B	30%	34%	37%		33%	16%	25%	36%	13%	8%	8%	26%
CDPK3	51%	25%	27%	33%		14%	25%	30%	12%	7%	7%	25%
CDPK4	14%	14%	20%	16%	14%		14%	15%	17%	11%	9%	14%
CDPK4A	24%	21%	20%	25%	25%	14%		21%	8%	6%	6%	18%
CDPK5	25%	32%	32%	36%	30%	15%	21%		13%	7%	8%	26%
CDPK6	9%	14%	15%	13%	12%	17%	8%	13%		16%	8%	12%
CDPK7	7%	7%	9%	8%	7%	11%	6%	7%	16%		8%	7%
CDPK8	6%	9%	9%	8%	7%	9%	8%	8%	8%	8%		7%
CDPK9	22%	26%	24%	26%	25%	14%	18%	26%	12%	7%	7%	

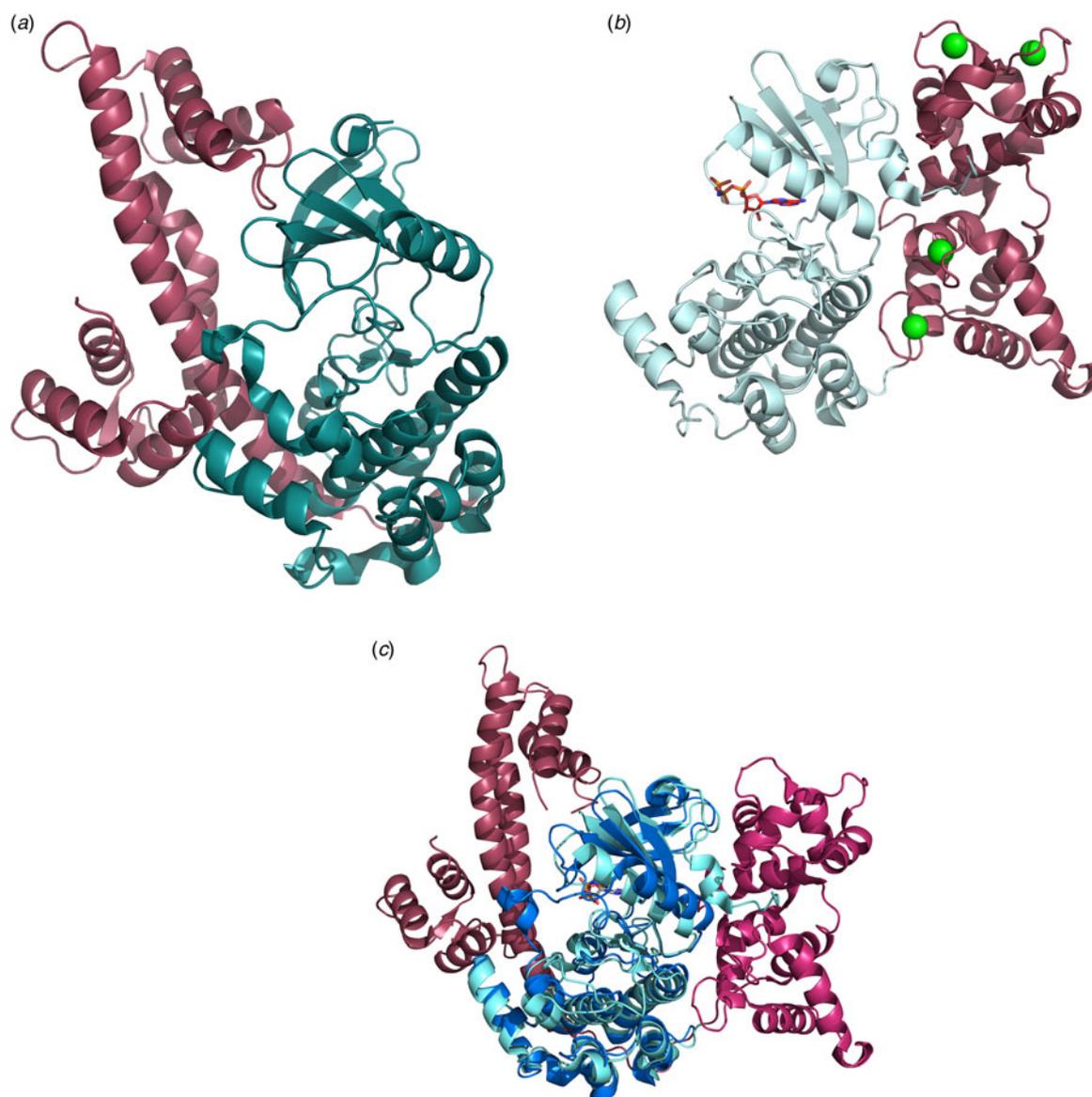


Fig. 1. Ribbon representation of the crystal structure of CDPK1 from *T. gondii* with the kinase domain depicted in cyan, the regulatory domain in raspberry red. (a) CDPK1 in its inactive auto-inhibited state (PDB code: 3KU2) (Wernimont *et al.* 2010). (b) CDPK1 in its calcium-bound, activated state with the Ca^{2+} -ions shown as green spheres and the non-hydrolysable ligand ANP in stick representation (PDB code: 3HX4) (Wernimont *et al.* 2010). (c) Ribbon diagram of the least-squares superposition of the inactive and active forms of *TgCDPK1* with the kinase domains shown in cyan (active) and blue (inactive), the regulatory domain in shades of red, respectively. Only the kinase domain was used to calculate the transformation matrix, which was then applied to the entire protein chain. CDPKs, calcium-dependent protein kinases.

comparing the crystal structures of the KD of *TgCDPK1* with the Calcium/Calmodulin (CaM) dependent-kinase II from *H. sapiens* (*HsCaMKII*) (Fig. 2a) (Rellos *et al.* 2010). These two proteins, which share a sequence identity of approximately 42% over 264 residues of the KD, display the same canonical kinase fold and superimpose with an overall root mean square deviation (rmsd) of approximately 1.5 Å. Note that the loop over the adenosine triphosphate (ATP) binding site adopts a very different conformation presumably due to an induced fit of binding of two very different ligands. *TgCDPK1* is bound to the ATP analogue ANP (Fig. 2a) while *HsCaMKII* is bound to a comparatively small inhibitor. More importantly there are significant differences in the ATP binding site, specifically a residue with no side chain (glycine) close to the adenine binding position. This residue, Gly128 is also termed the *gatekeeper* residue. Almost all mammalian kinases possess a large residue, a phenylalanine in *HsCaMKII* for example, in this position. Hence, CDPK1 feature an enlarged ATP binding site with a hydrophobic pocket that can be exploited

for structure-based drug design. This key structural difference in the binding pocket is shown in the surface representation where the ATP-analogue is shown as stick representation (Fig. 2b). The additional space at the end of the pocket below the surface of the gatekeeper residue Gly 128 in magenta is clearly visible.

Development of specific *TgCDPK1* inhibitors

Soon after the structural differences between *TgCDPK1* and the mammalian homologues were identified, two groups started to develop selective *TgCDPK1* inhibitors (Ojo *et al.* 2010; Wernimont *et al.* 2010). Initial compounds were based on known inhibitors previously developed for yeast kinases featuring amino acids with small side chains at the *gatekeeper* position. Importantly, these known kinase inhibitors, termed bumped kinase inhibitors (BKI) have been shown to be inactive against mammalian kinases (Hanke *et al.* 1996). Generally, BKIs are based on the planar pyrazolo[3,4-*d*]pyrimidin-4-amine

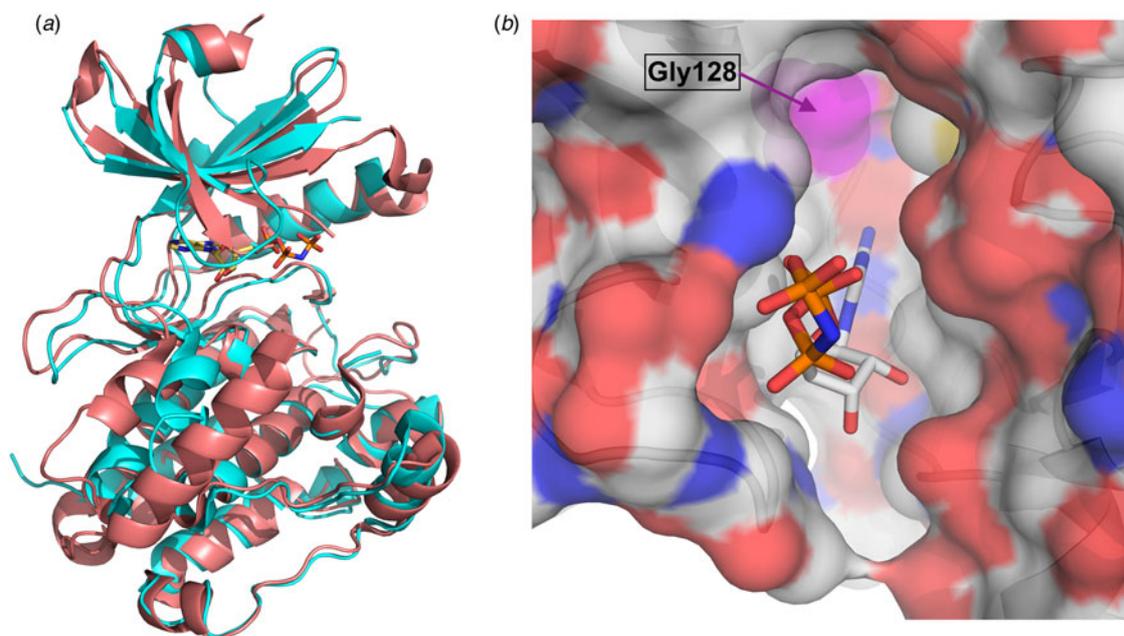


Fig. 2. (a) Least squares superposition of the kinase domain of *TgCDPK1* (depicted in cyan) in its active form with *HsCaMKII* bound to an inhibitor (PDB: 2VZ6) (shown in orange) (Rellos *et al.* 2010). The non-hydrolysable ATP analogue bound in *TgCDPK1* is presented as ball-and-stick representation to highlight the substrate binding site. (b) Surface representation of *TgCDPK1* viewing into the binding pocket with colour coding according to atom type (oxygen in red, nitrogen in blue, carbon in grey). The surface of Gly128 (gatekeeper residue) is shown in magenta at the top of the figure highlighting the additional space in the binding pocket. ATP, adenosine triphosphate; CDPKs, calcium-dependent protein kinases.

substituted with a bulky hydrophobic group on the C3 position (Bishop *et al.* 1998). The first example of a BKI with a sub-molar IC_{50} is 1-(1-methylethyl)-3-(naphthalen-1-ylmethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine. The co-crystal structure of *TgCDPK1* shows that the naphthalen-1-ylmethyl moiety fills the hydrophobic pocket created by the small gatekeeper residue Gly128 and lined by methionine and leucine residues, and one lysine residue (Fig. 3a and b). The chemically closely related 1-tert-butyl-3-naphthalen-2-yl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (Fig. 3c and d) adopts a similar conformation with the bulky aromatic substituent at the C3 position occupying the space next to the gatekeeper residue. Critically for the subsequent drug development was the fact that these and related BKIs reduced *T. gondii* proliferation significantly (Ojo *et al.* 2010; Sugi *et al.* 2010). These results sparked extensive medicinal chemistry efforts where a large number of compounds based on the BKI scaffold (4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidine) were synthesized and tested. A number of compounds exhibited sub- or low-nanomolar IC_{50} values and high activity in parasite growth models (EC_{50} in the low- and sub- μ molar range) while retaining specificity when compared with mammalian kinases (Lourido *et al.* 2013; Zhang *et al.* 2014; Moine *et al.* 2015). In addition to the pyrazolopyrimidine (PP) scaffolds, acylbenzimidazole and 5-aminopyrazole-4-carboxamide-based compounds have been shown to have similar properties (Fig. 4) (Zhang *et al.* 2012; Zhang *et al.* 2014; Huang *et al.* 2015). While the initial BKIs showed excellent potency *in vitro* and *in vivo* they also exhibited significant hERG (human Ether-Related Gene) inhibition thus posing potential cardiotoxicity (Doggett *et al.* 2014). Further extensive medicinal chemistry efforts finally led to the current lead *TgCDPK1* inhibitor, (1-{4-amino-3-[2-(cyclopropyloxy)quinolin-6-yl]-1*H*-pyrazolo[3,4-*d*]pyrimidin-1-yl}-2-methylpropan-2-ol) that combines high activity and selectivity with favourable pharmacokinetic properties and low hERG activity (Vidadala *et al.* 2016). Note that the compound is bound to the protein *via* H-bonds of the pyrimidin ring to the main chain, while the hydrophobic cyclopropyloxy-quinoline moiety forms a large

number of hydrophobic interactions (Fig. 5). Taken together, the structure-based approaches of drug development applied to *TgCDPK1* has led to three different series of compounds with high inhibitory activity, good pharmacokinetic parameters and promising efficacy in murine models.

CDPK1 inhibitors for related parasites

Based on the success of developing specific *TgCDPK1* inhibitors, recent work has branched out towards related apicomplexan parasites. For example, *Neospora caninum*, a cyst-forming parasite closely related to *T. gondii* represents the leading cause of abortion in cattle. This parasite expresses a CDPK1 with 96% sequence identity to *TgCDPK1* where all residues in the active site are conserved, bar one conservative variation from phenylalanine to tyrosine (Ojo *et al.* 2014). Consequently, the crystal structures of *TgCDPK1* and *NcCDPK1* show very similar overall structures (root mean square deviations (rmsd) on C-alpha atoms 0.5 Å) and the same binding mode of a BKI. Importantly, a number of BKIs display comparable *in-vivo* activities (Ojo *et al.* 2014; Winzer *et al.* 2015). Members of the *Cryptosporidium* genus are the causative agent of cryptosporidiosis in immune-compromised patients and malnourished children (Shultz *et al.* 2016). CDPK1 from *C. parvum* Iowa II (*CpCDPK1*) shares a sequence identity of approximately 41% with *TgCDPK1*, however, the active site residues including the gatekeeper residue are highly conserved. The screening of BKI libraries resulted in highly active *CpCDPK1* inhibitors based on the 5-aminopyrazole-4-carboxamide scaffold with clear potential for drug development (Castellanos-Gonzalez *et al.* 2016). High-throughput screening for *Plasmodium falciparum* CDPK1 (*PfCDPK1*) inhibitors resulted in five chemical series, including the PP scaffold (Fig. 4a). *PfCDPK1* shares a sequence identity of approximately 47% with *TgCDPK1* and the gatekeeper residue threonine harbours a slightly larger side chain compared to glycine. However, this side chain is still relatively small and mutational studies clearly indicated that these inhibitors bind at the same site (Ansell *et al.* 2014). More recent studies with

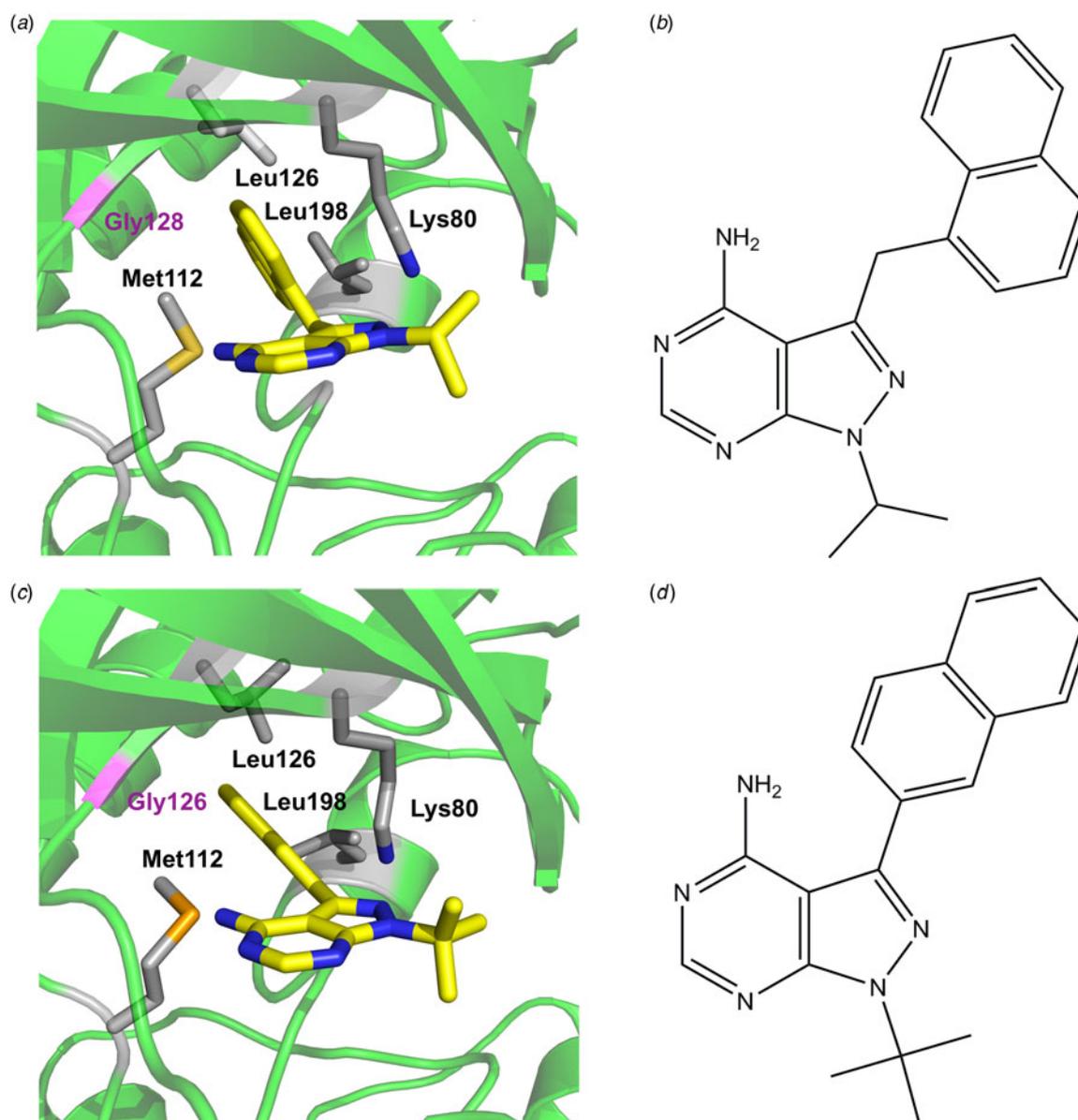


Fig. 3. Close-up of BKIs bound to *TgCDPK1* in the ATP binding site. The gatekeeper residue Gly128 is depicted in magenta, key hydrophobic residue of the binding site are labelled and shown in grey (a) 1-(1-methylethyl)-3-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine shown in ball-and-stick representation (PDB: 3i7b), (b) chemical structure of the ligand, (c) 1-tert-butyl-3-naphthalen-2-yl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PDB:3i7c) (Ojo *et al.* 2010) and (d) chemical structure of the ligand. ATP, adenosine triphosphate; CDPKs, calcium-dependent protein kinases.

PfCDPK1 inhibitors based on the chemically very similar Imidazopyridazine scaffold appear to show that these compounds also target cyclic GMP dependent kinases as well as Heat Shock Protein 90. These findings question the prospect of *PfCDPK1* inhibitors for further drug development (Green *et al.* 2015). Taken together, these recent results show the potential of BKIs for future drug development in *Toxoplasma* and related parasites but they also illustrate the limitations of transferring detailed structural data to more distantly related proteins.

Future challenges

Over the last 5 years there has been significant progress in the development of selective inhibitors of one of the key CDPKs from *T. gondii* achieved by taking advantage of a series of high-resolution crystal structures. While most of the previous research has focused on *T. gondii*, further efforts are currently underway to investigate inhibitors of CDPK1 from *Cryptosporidium* and *Plasmodium* spp. (Gaji *et al.* 2014; Green *et al.* 2015; Crowther

et al. 2016). However, further research is required to unravel the biological roles of *PfCDPKs* and their potential as future drug targets (Kumar *et al.* 2017).

Although the most promising *TgCDPK1* inhibitors show high efficacy in murine models, future research is required to increase solubility and bio-availability in order to proceed to clinical trials. Furthermore, current lead compounds only target the ATP binding site of *TgCDPK1*. However, allosteric kinase inhibitors and modulators have shown enormous potential to target specific kinases and could be further exploited (Fang *et al.* 2013). Additional binding sites in less conserved regions such as the carbohydrate binding site recently discovered in *TgCDPK2* can serve as starting points for the development of new inhibitors (Uboldi *et al.* 2015). Clearly, more work needs to be done to understand the role of the other members of the Apicomplexan CDPK family. In this regard, the recent development of CRISPR/Cas9 technology to modify the genes of members of the Apicomplexan family (Shen *et al.* 2014; Vinayak *et al.* 2015) will greatly facilitate the detailed analysis of the biological

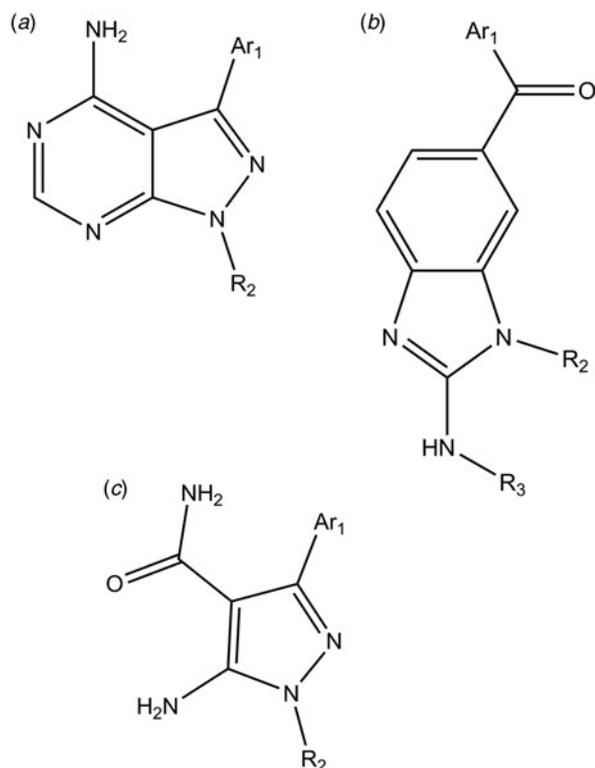


Fig. 4. The three different scaffolds for *Tg*CDPK1 inhibitors (a) pyrazolopyrimidines, (b) acylbenzimidazoles and (c) 5-aminopyrazole-4-carboxamide. CDPKs, calcium-dependent protein kinases.

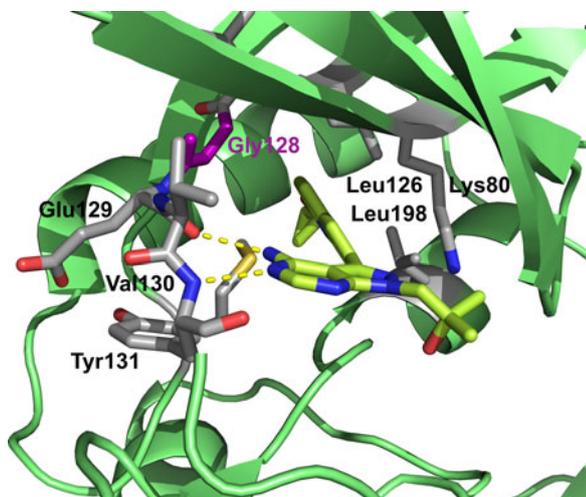


Fig. 5. Crystal structure of (1-[4-amino-3-[2-(cyclopropyloxy)quinolin-6-yl]-1*H*-pyrazolo[3,4-*d*]pyrimidin-1-yl]-2-methylpropan-2-ol) shown in stick representation bound to *Tg*CDPK1 shown in cartoon representation with selected residues depicted in sticks (Vidadala et al. 2016). CDPKs, calcium-dependent protein kinases.

function of CDPK family members (Long et al. 2016; Wang et al. 2016).

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