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***Toxoplasma gondii* calcium-dependent protein kinase 1 is a target for selective kinase inhibitors**

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Abstract

New drugs are needed to treat toxoplasmosis. *Toxoplasma gondii* calcium-dependent protein kinases (TgCDPKs) are attractive targets because they are absent in mammals. We show that TgCDPK1 is inhibited by low nanomolar levels of bumped kinase inhibitors (BKIs), compounds designed to be inactive against mammalian kinases. Cocrystal structures of TgCDPK1 with BKIs confirm that the structural basis for selectivity is due to the unique glycine gatekeeper residue in the ATP-binding site at residue 128. We show that BKIs interfere with an early step in *T. gondii* infection of human cells in culture. Furthermore, we show that TgCDPK1 is the *in vivo* target of BKIs because *T. gondii* cells expressing a glycine to methionine gatekeeper mutant enzyme show significantly decreased sensitivity to this class of selective kinase inhibitors. Thus, design of selective TgCDPK1 inhibitors with low host toxicity may be achievable.

The food-borne apicomplexan protozoan *Toxoplasma gondii* is the causative agent of toxoplasmosis and may be the most common infectious eukaryotic parasite of humans, based

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Accession codes. Protein Data Bank: Atomic coordinates and structure factors have been deposited with accession numbers 3I79 (apo), 3I7c (NA-PP2 complex) and 3I7b (NM-PP1 complex).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

AUTHOR CONTRIBUTIONS

K.K.O., K.R.K., K.K.I. and W.C.V.V. were involved in the biochemical characterization and testing of inhibitors of TgCDPK1; L.J.C., K.K.O., K.R.K., A.J.N., C.L.M.J.V., F.S.B. and W.C.V.V. selected, cloned and purified the recombinant wild-type and mutant TgCDPK1 protein; E.T.L., J.E.K., T.L.A., L.Z., W.G.J.H. and E.A.M. crystallized and solved the structure of TgCDPK1; R.M. and D.J.M. synthesized the inhibitors; A.E.D. and M.P. performed the cellular *T. gondii* experiments; K.K.O., E.T.L., A.E.D., D.J.M., M.P., E.A.M. and W.C.V.V. wrote the paper; all authors reviewed and edited the paper.

COMPETING FINANCIAL INTERESTS

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on sero-surveys¹. Most infections are probably transmitted by undercooked meats, but accidental ingestion of cat feces can also transmit toxoplasmosis to humans². *T. gondii* is an obligate intracellular pathogen that can invade and replicate in most nucleated mammalian cells. Toxoplasmosis remains a major health concern in pregnancy, where it causes severe birth defects or miscarriage, and in immunocompromised hosts, where it causes toxoplasmic encephalitis^{3–5}. Infection is often asymptomatic in immunocompetent hosts, but prolonged disease does occasionally occur⁶. Attack rates in immunocompetent individuals can be high, as evidenced by laboratory-acquired infections⁷, and acute symptoms can be debilitating⁸, warranting *T. gondii*'s classification as a class B agent of concern for biodefense⁹. Toxoplasmosis is extremely difficult to treat, and only limited therapeutics are currently available in the United States (sulfadiazine and pyrimethamine or clindamycin)¹⁰. These drugs are problematic in that they can cause rash, leucopenia and nephrotoxicity¹¹, and sulfadiazine and pyrimethamine can result in complications during pregnancy. New therapeutics against *T. gondii* are needed.

Calcium levels have long been associated with *T. gondii*'s interrelated processes of invasion, gliding motility and secretion¹². The intracellular Ca^{2+} level oscillates during gliding motility and is promptly dampened upon cell invasion, preventing *T. gondii* from immediately gliding out of cells¹³. Calcium oscillations control many targets in the cell, and the mediation of invasion, micronemal secretion and gliding motility is thought to be largely due to *T. gondii* calcium-dependent protein kinases (*Tg*CDPKs)^{12,14}.

Protein kinases are generally attractive targets for new drugs against eukaryotic pathogens¹⁵ and *Tg*CDPKs are no exception, in part because they are more closely related to CDPKs found in plants and algae than any protein kinases found in mammalian cells^{16,17}. *Tg*CDPK1 is especially attractive for treatment of toxoplasmosis because, in addition to its key role in parasite invasion¹⁴, it contains a unique sequence variation in the ATP binding pocket: namely, a glycine at the so-called 'gatekeeper position'. This variation distinguishes *Tg*CDPK1 from all known mammalian kinases. Thus, design of selective inhibitors may be possible. To this end, we show here that *Tg*CDPK1 is inhibited by low nanomolar levels of bumped kinase inhibitors (BKIs), a class of compounds designed to be inactive against mammalian kinases. Crystal structures of *Tg*CDPK1 in complex with BKIs confirm the structural basis for selectivity and provide a structural look at the Ca^{2+} -binding regulatory domain in this class of kinases. Furthermore, we find that BKIs interfere with host-cell invasion in culture, and reverse genetic experiments with live parasites expressing a gatekeeper-residue mutant confirm that *Tg*CDPK1 is indeed the specific target of BKIs *in vivo*. These studies pave the way for the design of selective drugs against toxoplasmosis.

RESULTS

Enzymology of *Tg*CDPK1

Two types of enzyme assays were developed to follow *Tg*CDPK1 activity: a radiometric scintillation proximity assay measured the labeled γ -phosphate of ATP added to a biotinylated peptide substrate, and an ATP consumption assay monitored ATP consumption by luciferase and light production (KinaseGlo). Both assays gave similar results for the K_m of the substrates (less than two-fold differences in K_m values, Supplementary Table 1 and Supplementary Fig. 1a–d) and inhibitor concentrations for 50% enzyme inhibition (IC_{50} values) (Supplementary Tables 1 and 2). As expected, activity of *Tg*CDPK1 on the peptide substrates required addition of exogenous calcium (Supplementary Fig. 2). Indeed, calcium titration revealed that a concentration of 4.7 μM gave 50% of *Tg*CDPK1 kinase activity.

Structure of *Tg*CDPK1

We have determined X-ray crystal structures of Ca^{2+} -free *Tg*CDPK1 in the apo form and in complex with two potent inhibitors (Fig. 1 and Table 1). The structure of the catalytic domain is typical of serine/threonine-type protein kinases. The two Ca^{2+} -binding EF hand lobes and the connecting extended helical stem of the calmodulin-like regulatory domain lie along one face of the kinase domain, adjacent to the active site (Fig. 1a). The kinase and regulatory domains are connected by an intervening helical junction domain characteristic of this class of kinases¹⁸. The conformation represented by these structures is likely an inactive form of the enzyme, as the calmodulin-like domain occludes the surface required for recognition of target proteins and peptides. Notably, the ATP binding site remains accessible to small-molecule substrates and inhibitors. Comparison of the calcium-free *Tg*CDPK1 structure (Fig. 1a) with the calcium-activated structure¹⁹ (PDB 3HX4) shows that the regulatory domain undergoes major structural rearrangement and is repositioned to lie against the opposite surface of the kinase domain upon calcium binding. This dramatic structural change allows access of the protein substrates to the active site.

Bumped kinase inhibitors bind and inhibit *Tg*CDPK1

Most known kinase inhibitors bind in the ATP binding pocket of the active site^{20,21}. These inhibitors exploit many of the same hydrophobic contacts as the purine ring of ATP and make at least one conserved hydrogen bond to the hinge region. Potent inhibitors also occupy at least one hydrophobic pocket adjacent to the ATP binding site. These additional hydrophobic interactions increase both binding affinity and target selectivity of the inhibitor because sequence heterogeneity is greater among different kinases in these regions. Examination of the *Tg*CDPK1 sequence in the vicinity of the ATP binding pocket (Fig. 1b) shows that it contains a glycine residue at a position that has been termed the gatekeeper residue because it constrains access to the ATP binding site^{22–24}. The glycine at this position in *Tg*CDPK1 (Gly128) is expected to create a much larger pocket off the ATP binding site than is typically observed in protein kinases. Comparison of the *Tg*CDPK1 structure with other kinases shows that this is indeed the case. This difference in the active-site architectures may be exploited for design of selective inhibitors against *Tg*CDPK1.

Previous work²⁵ has shown that mutation of bulky gatekeeper residues to glycine renders mutant kinases uniquely susceptible to inhibition by BKIs. BKIs are analogs of 4-amino-1-*tert*-butyl-3-phenylpyrazolo[3,4-*b*]pyrimidine (Table 2) that are derivatized at the C3 position with bulky aromatic groups²⁶. The large side chain of the gatekeeper residue in most kinases prevents bulky aromatic substituents at the C3 position from accessing a hydrophobic pocket at the back of the catalytic cleft, rendering them insensitive to BKI inhibition^{22,23}. Large gatekeeper residues, like methionine or phenylalanine, severely restrict access by the BKIs to a large hydrophobic pocket, whereas small gatekeeper residues, such as the glycine present in *Tg*CDPK1, allow access of BKIs to this hydrophobic pocket^{23,24,27,28}. Studies with genetically engineered mice that express mutant kinases with small gatekeeper residues (glycine or alanine), have shown that BKIs preferentially target the mutant kinases^{29–31}. The well-documented lack of BKI inhibition of mammalian kinases suggests that this class of compounds may be very selective for *Tg*CDPK1 during *T. gondii* infection^{29–31}.

Based on structural and sequence analysis of the differences in ATP binding sites of typical mammalian kinases and *Tg*CDPK1 with a small gatekeeper (Fig. 1a,b), we synthesized three BKIs (NA-PP1, NA-PP2 and NM-PP1) and showed that two of the three potentially inhibit *Tg*CDPK1, with IC_{50} values in the low double-digit nanomolar range (Table 2). To show that the glycine gatekeeper is the primary determinant of BKI inhibition, we engineered a glycine-to-methionine gatekeeper mutant of *Tg*CDPK1 (G128M). Indeed, the wild-type enzyme was inhibited much more effectively by BKIs compared to the G128M mutant enzyme (Table 2).

For NA-PP1, there was a 175-fold difference between IC₅₀ values for wild type versus the G128M mutant; for NA-PP2, a 1,200-fold difference; and for NM-PP1, a 550-fold difference (Table 2).

We determined the crystal structures of *Tg*CDPK1 in complex with two potent BKIs (NA-PP2, Fig. 1c and Supplementary Fig. 3a; NM-PP1, Fig. 1d and Supplementary Fig. 3b) and, as predicted, these inhibitors bound in the ATP binding site, with the C3 bulky aromatic substituent occupying the pocket adjacent to the glycine gatekeeper. Superposition of an orthologous CDPK containing a methionine gatekeeper onto our BKI complexes showed impairment of the favorable binding mode observed for the BKIs in *Tg*CDPK1 (Fig. 1e). The bulky gatekeeper side chain clashes with the C3 bulky aromatic substituent, providing a structural basis for the insensitivity of typical kinases toward BKI inhibition, as verified experimentally with the G128M mutant of *Tg*CDPK1 (Table 2). These results implicate *Tg*CDPK1 with its unique glycine gatekeeper as a promising drug target for selective treatment of toxoplasmosis.

***Tg*CDPK1 appears in the cytoplasm and nucleus of *T. gondii* cells**

The mode of action of *Tg*CDPK1 is unknown, but its localization in the cell might provide clues to its cellular function. To this end, we fused *Tg*CDPK1 with green fluorescent protein (GFP) to facilitate its localization within live intracellular *T. gondii*. We found GFP-labeled *Tg*CDPK1 in both the cytosol and the nucleus (Fig. 2a). We obtained similar findings when the GFP tag was replaced with a C-terminal hemagglutinin (HA) tag (data not shown). Thus, *Tg*CDPK1 may phosphorylate target proteins in both the cytosol and the nucleus.

Effects of BKIs on *T. gondii* cell entry and growth

We tested three BKIs for effects on host-cell invasion and parasite growth, as earlier work on *Tg*CDPK1 (ref. 14) suggested a key role for this enzyme in *T. gondii* invasion. BKIs profoundly reduced *T. gondii* proliferation if added simultaneously with cellular infection (Fig. 2b) and modestly reduced proliferation when added 4 h after the start of invasion (Fig. 2c). Thus, inhibition of *Tg*CDPK1 activity by BKIs has a stronger effect on invasion than on intracellular growth. This was confirmed by microscopic examination of invasion (data not shown) and is consistent with previous observations on the role of *Tg*CDPK1 (refs. 12-14). We expect that longer exposure to BKIs would increase the impact of the drug on *T. gondii* growth as egressed parasites are prevented from invading new cells. We tested each BKI for its effects on human fibroblast replication; the EC₅₀ values were found to be ~1,000-fold higher (not shown) than those seen in the *T. gondii* 'invasion' assay.

Tg*CDPK1 is the dominant *in vivo* target of BKIs in *T. gondii

We transfected the *T. gondii* cell line with expression plasmids encoding an HA tag fused to the C terminus of either wild-type *Tg*CDPK1 or the G128M gatekeeper mutant. Immunoblot analysis showed that the wild-type and G128M mutant *Tg*CDPK1s were expressed to similar levels (Fig. 3a). Compared to the parental cell line, parasites expressing the G128M mutant were relatively resistant to BKIs NA-PP2 and NM-PP1 when they were added before invasion (Fig. 3b,c). In contrast, parasites overexpressing the wild-type *Tg*CDPK1 protein showed only a small shift in resistance. Although NA-PP2 was somewhat less effective against wild-type parasites in this experiment, the results were identical in the relative potency of the inhibitors, in that NA-PP2 was more potent than NM-PP1 in blocking *T. gondii* invasion, following the potency observed against the *Tg*CDPK1 enzyme. Furthermore, in both a microscopic assay of invasion³² (Supplementary Fig. 4) and a mixed cellular infection using wild-type and G128M mutant *Tg*CDPK1 (Supplementary Fig. 5), parasites expressing the gatekeeper mutant were markedly resistant to NA-PP2, whereas cells expressing either wild-type *Tg*CDPK1 or GFP

controls were not. These findings show that BKIs work primarily through the *Tg*CDPK1 target to prevent *T. gondii* mammalian-cell entry.

DISCUSSION

We have shown that *Tg*CDPK1 is a promising drug target for the therapy of toxoplasmosis. Its kinase activity is uniquely sensitive to inhibition by BKIs, and BKI treatment in turn blocks entry of the parasite into mammalian cells. Blocking cell entry is important because *T. gondii* is an obligate intracellular parasite and cannot replicate without invasion. Clinical toxoplasmosis is caused by the actively dividing tachyzoite form of the parasite, which exits its host cell and invades a new cell every few days. *Tg*CDPK1 has a unique ATP binding site with a small gatekeeper residue, as opposed to the larger gatekeeper residues present in mammalian protein kinases. This key difference in the ATP binding pockets of mammalian kinases and *Tg*CDPK1 allowed us to use BKIs to selectively inhibit *Tg*CDPK1 without untoward effects on the mammalian host cell. Crystal structures revealing the binding mode of BKIs in *Tg*CDPK1 (Fig. 1c,d and Supplementary Fig. 3a,b) support the selectivity we observed. Consistent with the proposal that *Tg*CDPK1 is the target of BKIs, the order of increasing potency (NA-PP2 > NM-PP1 > NA-PP1) was identical in both the enzymatic and cellular assays. *Tg*CDPK1 sensitivity to BKIs was altered by deliberate mutation of the gatekeeper glycine residue to a methionine (G128M) that is more typical of mammalian kinases. Expression of this G128M mutant led to resistance in three different types of assays when assessed using model compound NA-PP2 (Fig. 3b and Supplementary Figs. 4 and 5). These data support the contention that *Tg*CDPK1 is the major target for BKIs in *T. gondii* cells. These experimental findings, therefore, address any concerns about quantitative differences between enzyme inhibition and cellular effects, thereby validating the utility of *Tg*CDPK1 structures in complex with BKIs to drive drug development for toxoplasmosis therapy. The concept of using BKIs for the therapy of toxoplasmosis is bolstered by the fact that BKIs have been used in mouse studies with no demonstration of toxicity or troublesome effects to the animals^{29–31}. Therefore, BKIs have promise as a selective drug for toxoplasmosis therapy because they prevent cell entry, and thus the replication, of *T. gondii* and are likely to be nontoxic to the mammalian host.

Because we determined the structure for the inactive, Ca²⁺-free form of *Tg*CDPK1, there may be some concern that this form would not be optimal to guide the design of small-molecule inhibitors with improved potency and selectivity. It is apparent from structural and biochemical studies presented here, however, that small-molecule inhibitors can still access the ATP binding site of the Ca²⁺-free conformation of *Tg*CDPK1. Notably, the structure of the active site near the ATP binding pocket, particularly in the vicinity of the gatekeeper residue, the hinge region and the activation loop, is not substantially [AU: Correct?] altered between the structures shown here and that of the Ca²⁺-bound enzyme in complex with adenylyl-imidodiphosphate (AMP-PNP)¹⁹ (PDB 3HX4). Thus, structure-guided optimization of small-molecule inhibitors that target this region is possible using the inactive, Ca²⁺-free form of *Tg*CDPK1.

*Tg*CDPK1 was localized in the cytoplasm but also found in the nucleus. Some plant CDPKs are also partially localized to the nucleus. In those CDPKs, nuclear localization is mediated by a signal in the junction domain¹⁸, but the *T. gondii* protein is not homologous in this region, and no nuclear localization signal is predicted by standard programs. Because the size of the protein is above the threshold for free diffusion through the nuclear pore, we propose that the protein could bear a noncanonical nuclear-localization sequence or could piggyback into the nucleus on another protein. These results raise the possibility that *Tg*CDPK1 phosphorylates specific nuclear proteins in addition to its presumably cytosolic targets involved in gliding motility. Furthermore, as previously noted, CDPK1 undergoes a large Ca²⁺-dependent structural rearrangement that repositions the regulatory domain to the opposite side of the

catalytic domain.¹⁹ This raises the intriguing, but speculative, possibility that Ca^{2+} could modulate CDPK1 localization by revealing or occluding the region of the kinase required to mediate nuclear import.

Drug-resistant mutations of the *Tg*CDPK1 gatekeeper to a bulky residue could eventually emerge under selective pressure of BKi therapy. A primary strategy to suppress the emergence of resistance is the coadministration of two drugs targeting different proteins. As *Tg*CDPK1 is not the target of any existing drug, the development of an anti-*Tg*CDPK1 compound could provide a partner drug for coadministration with an existing drug. Most transmission of *T. gondii* is not from person to person (although this can occur in pregnancy, transplantation or transfusion) but rather through zoonotic cycles where drug pressure is not exerted¹. This suggests that, if drug resistance emerges, it will largely be confined to the individual, posing little threat to the utility of the drug in other infected persons.

As other apicomplexan pathogens use CDPK enzymes with a small gatekeeper residue, this work may have broader applicability. For instance, the *Tg*CDPK1 ortholog in *Cryptosporidium parvum*, also an apicomplexan parasite, likewise has a glycine residue at the gatekeeper position (Fig. 1b), suggesting that BKIs targeting *T. gondii* could also be effective for the therapy of cryptosporidiosis, another potentially life-threatening infection with poor therapeutics. CDPKs from other apicomplexan parasites have smaller gatekeeper residues, such as serine and threonine (Fig. 1b). An extended search of all reported human kinase ATP binding pockets found none with a glycine or alanine gatekeeper residue, though ~20% do contain threonine²². Thus, the results presented here may have implications in the rational design of anti-apicomplexan CDPK agents devoid of toxic side effects to the host cells.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structure of calcium-free *TgCDPK1*. (a) Association of the kinase domain (green) and the calmodulin-like calcium regulatory domain (orange) buries approximately 1,400 Å² of accessible surface area per domain. The junction domain, connecting the kinase and regulatory domains, is shown in brown. This interaction surface between kinase and regulatory domains is entirely lost during the transition to the Ca²⁺-bound form in which the regulatory domain relocates to lie along the opposite surface of the kinase domain¹⁹ (PDB 3HX4), the left side in the orientation shown. The active site is shown with bound inhibitor NA-PP2 (magenta). (b) Comparative analysis of the ATP binding pocket of kinases reveals the presence of a glycine in the position of the gatekeeper residue (bold) in *TgCDPK1* and a *CpCDPK1* ortholog. Medium-sized side chains, threonine and serine, are found at this position in other apicomplexan CDPKs, *Eimeria tenella* (*EtCDPK1*) and *Plasmodium falciparum* (*PfCDPK1* and *PfCDPK4*). Animal CDPKs *Xenopus tropicalis* (*XtCaMK*), *Macaca mulatta* (*MmCaMK*), including the closest orthologs in humans (*HsCaMK*), tend to have large gatekeeper residues such as methionine or phenylalanine. (c,d) View of the active site with the bumped kinase inhibitors NA-PP2 (c) or NM-PP1 (d) bound. Residues within 6 Å of the inhibitors are shown as sticks. The unusual glycine gatekeeper residue that permits inhibition by bumped kinase inhibitors is highlighted in cyan. Difference electron density, contoured at +3.5σ (green mesh) and −3.5σ (pink mesh), was calculated after omission of the inhibitor. (e) *TgCDPK1* active site in the vicinity of the gatekeeper residue. The surface of *TgCDPK1* is shown in white and is slabbed through to show the adenine pocket of the ATP binding site. The glycine at the

gatekeeper position of *Tg*CDPK1 enlarges the active-site pocket and facilitates binding of bumped kinase inhibitors (NA-PP2, magenta sticks; NM-PP1, violet sticks). Larger residues, such as methionine shown here (brown sticks and protruding surface) from superposition of a *C. parvum* ortholog¹⁹ (PDB 3HKO; bound adenylyl-imidodiphosphate, tetralithium salt shown as brown sticks) are more typical at this gatekeeper position and prevent binding of compounds with large, hydrophobic ‘bumps’ on the adenine ring. This is made apparent by the brown surface protrusion of the methionine clearly clashing with the bump of each of the BKIs in our crystal structures.

Fig. 2a

<i>T. gondii</i> (GI: 12484153)	<i>TgCDPK1</i>	122	gyFYLV G EVYtGGELFDEI	140
<i>C. parvum</i> (GI:126644493)	<i>CpCDPK1</i>	146	ssFYIV G ELYtGGELFDEI	164
<i>T. gondii</i> (GI:77022110)	<i>TgCDPK3</i>	147	rnYYLV M EVYrGGELFDEI	165
<i>E. tenella</i> (GI:77022114)	<i>EtCDPK1</i>	117	gyFYLV T EVYtGGELFDEI	135
<i>P. falciparum</i> (GI: 50401836)	<i>PfCDPK1</i>	139	kyFYLV T EFYeGGELFEQI	157
<i>P. falciparum</i> (GI: 23498846)	<i>PfCDPK4</i>	141	nyYYLV S DVYtGGELFDEI	159
Human (GI:4502553)	<i>HsCDPKI</i>	89	ghLYLI M QLVSGGELFDRI	107
<i>X. tropicalis</i> (GI:213625749)	<i>XtCDPK</i>	83	gfHYLV F DLVtGGELFEDI	101
<i>M. mulatta</i> (GI:109075456)	<i>MmCDPK</i>	84	gfHYLV F DLVtGGELFEDI	102

Fig. 2b

Fig. 2c

Fig. 2d

Fig. 2e

Figure 2.

Cellular localization of TgCDPK1 and effects of bumped kinase inhibitors on *T. gondii* binding to and invasion of mammalian cells. **(a)** TgCDPK1 is localized to the nucleus and cytosol of *T. gondii*. Live *T. gondii* cells transiently transfected with wild-type GFP-TgCDPK1 were fixed in 4% (w/v) paraformaldehyde. Samples were probed with anti-GFP antibody followed by anti-rabbit Ig antibody coupled to Texas red, stained with 4',6-diamidino-2-phenylindole (DAPI) and viewed. GFP, green; DAPI, blue. For the binding and invasion assays, *T. gondii* cells expressing β -galactosidase (10^3 per well) were allowed to infect fibroblasts in a 96-well plate. **(b)** For invasion assays compounds were added to the wells before infection with *T. gondii*. **(c)** For growth assays, compounds were added to the indicated final concentration 4 h after infection with *T. gondii* cells. After 20 h, the cells were lysed and β -galactosidase activity was assessed by adding 0.5 mM chlorophenol red- β -D-galactoside, incubating at 37 °C, and then measuring absorbance at 595 nm. Data were collected from at least three independent

experiments, the mean and s.d. normalized to the no compound (vehicle alone) control are presented.

Localization of CDPK1 to the nucleus and cytosol of *T. gondii*

Figure 3.

TgCDPK1 gatekeeper mutant reduces sensitivity to BKIs. *T. gondii* clonal lines expressing hemagglutinin (HA)-tagged (HA-CDPK1) versions of *TgCDPK1* (WT) or its G128M mutant along with green fluorescence protein (GFP) and β -galactosidase were generated. (a) Western blot of protein separated on a 10% acrylamide gel and probed with anti-HA antibody. Anti-GFP antibody served as a loading control. (b,c) Invasion assays were performed with increasing concentrations of BKIs NA-PP2 (b) and NM-PP1 (c) using the parental cell line and the transfectants additionally expressing wild-type *TgCDPK1* and *TgCDPK1* G128M. Assays were done in triplicate, and mean and s.d. normalized to the 'no compound' (vehicle alone) control are shown.

Fig. 4a

Fig. 4b

Figure 4.

Fig 5a

Fig 5b

Fig 5c**Figure 5.**

Table 1

Data collection and refinement statistics

	Peak SAD ^a (SeMet)	Apo (native)	NA-PP2 (SeMet)	NM-PP1 (native)
Data collection				
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁
Cell dimensions				
<i>a</i> , <i>b</i> , <i>c</i> (Å)	48.2, 72.0, 67.5	48.1, 71.9, 67.3	48.1, 72.5, 67.1	47.2, 72.9, 65.7
α , β , γ (°)	90.0, 102.9, 90.0	90.0, 103.0, 90.0	90.0, 103.8, 90.0	90.0, 98.8, 90.0
Wavelength	0.9792	0.9792	0.9791	0.9795
Resolution (Å)	50–2.30 (2.38–2.30)*	50–2.04 (2.12–2.04)	50–1.98 (2.06–1.98)	50–1.99 (2.06–1.99)
Unique reflections	20,099 (1,986)	28,324 (2,799)	30,080 (2,996)	30,350 (2,975)
<i>R</i> _{merge}	0.11 (0.95)	0.05 (0.67)	0.09 (0.70)	0.06 (0.74)
<i>I</i> / σ <i>I</i>	13.1 (2.4)	17.0 (2.4)	8.4 (2.1)	14.2 (1.9)
Completeness (%)	100 (100)	99.9 (100)	98.1 (96.6)	99.8 (98.1)
Redundancy	7.7 (7.7)	4.2 (4.2)	3.7 (3.6)	4.2 (4.1)
Wilson <i>B</i> factor (Å ²)	43.7	34.8	26.6	29.7
Refinement				
Resolution (Å)		36.89–2.04	29.72–1.98	35.72–1.99
No. reflections		26,871	29,224	28,797
No. reflections test set		1,431	1,556	1,536
<i>R</i> _{work} / <i>R</i> _{free}		0.213 / 0.258	0.193 / 0.245	0.192 / 0.228
No. atoms				
Protein		3,674	3,776	3,743
Ligand/ion		4	24	40
Water		75	170	127
<i>B</i> -factors (Å ²)				
Protein		60.2	42.5	48.7
Ligand/ion		56.0	30.7	42.1
Water		26.1	26.5	18.3
R.m.s. deviations				
Bond lengths (Å)		0.006	0.007	0.006
Bond angles (°)		0.866	0.978	0.878

Each dataset was collected from a single crystal. Values in parentheses are for highest-resolution shell.

^aSingle-wavelength anomalous dispersion.

Table 2Bumped kinase inhibitor inhibition of wild type and *Tg*CDPK1 G128M

Inhibitor	NA-PP1	NA-PP2
Wild-type <i>Tg</i> CDPK1	0.989	0.018
<i>Tg</i> CDPK1 G128M	173	21.44
Chemical structure

The IC₅₀ (μM) values were calculated by nonlinear regression using Prism (GraphPad Software). The assay was performed three times, and the mean IC₅₀ is shown. The assay was performed using coupling with KinaseGlo at 20 nM *Tg*CDPK1, 10 μM ATP, 40 μM peptide and an incubation time of 30 min.