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The Gatekeeper Residue and Beyond:

Homologous Calcium Dependent Protein Kinases as drug development targets for
veterinarian Apicomplexa parasites

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SUMMARY

Specific roles of individual CDPKs vary, but in general, they mediate essential biological functions necessary for parasite's survival. A comparative analysis of the structural-activity relationships (SAR) of *Neospora caninum*, *Eimeria tenella* and *Babesia bovis* Calcium-dependent Protein kinases (CDPKs) together with those of *Plasmodium falciparum*, *Cryptosporidium parvum*, and *Toxoplasma gondii* was performed by screening against 333 Bumped kinase inhibitors (BKIs). Structural modeling and experimental data revealed that residues other than the gatekeeper influence compound-protein interactions resulting in distinct sensitivity profiles. We subsequently defined potential amino-acid structural influences within the ATP binding cavity for each orthologue necessary for consideration in the development of broad-spectrum apicomplexan CDPK inhibitors. Although the BKI library was developed for specific inhibition of glycine gatekeeper CDPKs combined with low inhibition of threonine gatekeeper human SRC kinase; some library compounds exhibit activity against serine or threonine containing CDPKs. Divergent BKI sensitivity of CDPK homologs could be explained on the basis of differences in the size and orientation of the hydrophobic pocket and specific variation at other amino-acid positions within the ATP binding cavity. In particular, *Bb*CDPK4 and *Pf*CDPK1 are sensitive to a larger fraction of compounds than *Et*CDPK1 despite the presence of threonine gatekeeper in all the three CDPKs.

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Keywords

Apicomplexa; Calcium-dependent Protein kinases; bumped kinase inhibitors

INTRODUCTION

The apicomplexa are a diverse group of unicellular parasites that infect humans and animals. *Plasmodium falciparum* and *Toxoplasma gondii* are among the best-known apicomplexa being among the leading cause of morbidity and mortality worldwide (Tenter *et al.* 2000; Guinovart *et al.* 2006). Malaria is endemic in 104 countries putting an estimated 3.4 billion people at risk with 207 million reported cases and 627,000 deaths globally in 2012 (WHO 2013). Other pathogens of importance include *Cryptosporidium*, *Babesia*, *Eimeria*, and *Neospora caninum* which collectively can cause devastating and debilitating encephalitis, diarrhea, and fetal abortion in the host. Treatment options, which rely heavily on chemotherapeutics, are limited and an issue of immediate importance due to increasing resistance of many apicomplexa to available therapeutic agents (Nair *et al.* 2011). Currently available therapies include antifolate and macrolide antimicrobials such as sulfadiazine and pyrimethamine or clindamycin, spiramycin, and imidocarb. All of these treatments present obstacles by exhibiting poor pharmacokinetic properties, toxicity, teratogenicity, tolerability and allergenicity (Katlama *et al.* 1996; Luft *et al.* 1993; Norrby 1978; Reeves and Wilkinson 1979).

Most apicomplexan parasites are obligate intracellular pathogens that must invade a host cell to grow and replicate (Morrisette and Sibley 2002), they have distinctive structural and phenotypic characteristics that can be targeted for the development of safe and effective antimicrobial therapies. Recent studies of apicomplexa kinase biology have described the transmission of calcium signals under the control of calcium-dependent protein kinases (CDPKs) to inter-related pathways as essential in facilitating host cell invasion, namely gliding motility and microneme secretion (Lovett *et al.* 2002). Each CDPK has defined regulatory functions at distinct stages of the apicomplexan life cycle, which are integrated into various key phosphorylation associated signaling events (Ojo *et al.* 2013). Hence, expression levels and specific functions in each organism may vary based on the specific life cycle requirements which are not completely understood at present. However, we have previously shown that interruption of these enzymes' ability to transmit signals by the application of specific inhibitors resulted in inhibition of parasites' cell replication and growth in *in vitro* and *in vivo* models for *T. gondii* (Ojo *et al.* 2010; Johnson *et al.* 2012; Doggett *et al.* 2014), *C. parvum* (Murphy *et al.* 2010; Castellanos-Gonzalez *et al.* 2013) and *P. falciparum* (Ojo *et al.* 2012; Ojo *et al.* 2014; Vidadala *et al.* 2014) without harmful effects to the hosts. The inhibitors library structure-activity relationships (SARs) for these parasites are similar. A structure-activity relationship is an iterative study that defines the contribution of each molecular group within an inhibitor's chemical structure to the overall biological activity associated with it (inhibitor). Specific inhibition of CDPKs was achieved using an adenosine-like pyrazolopyrimidine scaffold which binds to the hinge region of the active site, similar to the binding motif of ATP, but with an added bulky aromatic R1-substituent at the C3-position - the so called "bump" of bumped kinase inhibitors (BKIs)

(Ojo *et al.* 2010). Topologically, the aromatic R1-group projects into a hydrophobic pocket adjacent to the gatekeeper position of the ATP-binding site (the gatekeeper pocket), a feature exclusive to protein kinases with small gatekeepers like many apicomplexan CDPKs. Most mammalian kinases typically have larger gatekeeper residues that exclude the “bump” of BKIs, although few mammalian protein kinases like SRC do contain relatively smaller gatekeeper residues (threonine). Therefore, great care must be taken to maintain selectivity towards apicomplexan CDPKs over such human kinases. Fortunately, preliminary results also suggest that slight topographical deviations of the ATP binding region of apicomplexan CDPKs from SRC namely, the ribose binding pocket, also confer alternate selectivity to BKIs. Larson *et al.* 2012 previously demonstrated that mammalian kinases such as SRC have shallow ribose pockets as compared to apicomplexan CDPKs. Co-crystal structures of BKIs in complex with *Tg*CDPK1 have revealed a hydrogen bond between residue E135, a conserved glutamic acid found within the ribose binding pocket and shared among other apicomplexan CDPKs, and inhibitors with a 4-piperidinylmethylene R2 group, an interaction which further enhances selectivity. Together, the gatekeeper residue and the differing topology of the ribose binding pocket allow selectivity for broad spectrum anti-apicomplexan inhibitors with specificity for CDPKs over human kinases.

Furthering our work with apicomplexan CDPKs, this study reports the analysis of sensitivity of additional apicomplexan CDPK orthologues to a compound library of 333 BKIs. Using purified CDPK orthologues of veterinary pathogens *E. tenella*, *N. caninum*, and *B. bovis*, we investigate the impact of BKIs on several pathogens of great relevance to food security and veterinary medicine and further define potential structural constraints for consideration in the development of broad spectrum apicomplexan CDPK inhibitors.

MATERIALS AND METHODS

Compound library

Chemical synthesis of BKIs used in this study was previously described (Murphy *et al.* 2010; Johnson *et al.* 2012; Zhang *et al.* 2012). The library is comprised of fifty-three benzoylbenzimidazole; fourteen imidazo [1, 5-a] pyrazine analogues, two hundred and fifty-nine pyrazolo [3,4-d] pyrimidine analogues, with seven other bumped inhibitors. The purity of all compounds (>98%) was confirmed by reverse-phase HPLC and ¹H-NMR. The <2% impurity should have insignificant effect on inhibitory activity at the final reaction concentration of 3 μM.

Molecular cloning, protein expression and purification of parasite CDPK enzymes

The complete coding region of wild type apicomplexan calcium dependent protein kinase enzymes *Tg*CDPK1 (*T. gondii* ME49), *Cp*CDPK1 (*C. parvum* Iowa II), *Nc*CDPK1 (*N. caninum* Liverpool), *Pf*CDPK1 (*P. falciparum* 3d7), *Pf*CDPK4 (*P. falciparum* 3d7), *Et*CDPK1 (*E. tenella*) and *Bb*CDPK4 (*B. bovis* T2Bo) were PCR amplified from cDNA library of the respective parasite strain in parenthesis. Since the ATP binding sites of apicomplexan CDPKs seem to be highly conserved even between genera, we do not anticipate any structural differences or response to inhibitors between strains of the same species as shown by previous studies in *T. gondii* (Ojo *et al.* 2010; Doggett *et al.* 2014). The

amplicons were cloned into the ligation independent cloning (LIC) site of expression vector AVA0421 or the Maltose binding protein variant (AVA0421_MBP) (Mehlin *et al.* 2006; Alexandrov *et al.* 2004). The inserts were sequenced and confirmed to correspond with GenBank entries for their respective gene. Site-directed mutants of *Tg*CDPK1, in which the gatekeeper amino acid was substituted for a serine or threonine residue (*Tg*G128SCDPK1 and *Tg*G128TCDPK1 respectively), were also included to further our understanding of the gatekeeper residue influence. Recombinant expression was in Rosetta® 2(DE3) competent *E. coli* cells (Novagen EMD, Billerica, MA) using Studier auto-induction protocols at 20°C (Studier 2005). Soluble enzymes were purified by immobilized metal-affinity chromatography (IMAC) in a Ni²⁺-NTA (Qiagen, Valencia, CA) column as earlier described (Ojo *et al.* 2011). The binding buffer is composed of 20 mM HEPES pH 7.25, 500 mM NaCl, 5% glycerol, 30 mM imidazole, 0.5% CHAPS, and 1 mM TCEP. Purified proteins were eluted with the same buffer supplemented with imidazole up to 250 mM. Human kinase SRC was included as a control to examine off-target liabilities of threonine gatekeeper kinases. SRC was expressed and purified according to an established protocol (Seeliger *et al.* 2005).

Apicomplexan CDPK enzyme activity assay

Compound screening included 333 compounds at 3 µM final concentration in the experimental reaction. Each screen for a given enzyme was performed in three replicates. Inhibition concentration to give 50% reduction in enzyme activity (IC₅₀) was determined in 8 serial dilutions of compounds from 2 µM to 0.0001 µM using the non-radioactive Kinaseglo® luciferase assay (Promega, Madison, WI). Kinaseglo® is a reagent containing the enzyme luciferase, which utilizes ATP to give off light. Phosphorylation of peptide substrate (PLARTLSVAGLPGKK) (American Peptide Company, Inc. Sunnyvale, CA) was indirectly assessed as previously described (Ojo *et al.* 2010). Enzyme activity assays in the presence of 20 µM peptide substrate; 2.11 nM *Tg*CDPK1; 0.80 nM *Nc*CDPK1; 2.0 nM *Cp*CDPK1; 4.06 nM *Et*CDPK1; 1.84 nM *Tg*G128SCDPK1; 146.5 nM *Pf*CDPK4; 8.80 nM *Pf*CDPK1; 18.72 nM *Bb*CDPK4; or 7.32 nM *Tg*G128TCDPK1 were performed in a buffered solution containing 1 mM EGTA (pH 7.2), 10 mM MgCl₂, 20 mM HEPES pH 7.5 (KOH), 0.1% BSA and enzyme activation reagent containing 2 mM CaCl₂. The *Pf*CDPK4 recombinant enzyme is marginally soluble even as a maltose binding protein fusion, hence the necessity for a higher concentration per reaction. The reaction was stopped after 90 minutes incubation at 30° C with the addition of 5 mM EGTA. Each experiment included reactions without peptide substrate as controls and no ATP consumption was noted without substrate.

SRC enzyme activity assay

Similarly, for the human SRC enzyme inhibition assay, Kinaseglo® luciferase assay was used to generate inhibition data with the compound library. Final concentration of components of assay buffer included 40 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 1 mM MnCl₂, 1 mM DTT, and 0.1% BSA. SRC enzyme was used at a final concentration of 1.97 nM (25 µL final reaction volume), while SRC substrate sequence Ac-EIYGEFKKK (GenScript, Piscataway, NJ) was used at a final concentration of 61 µM. SRC substrate was purified on a C-18 reverse phase prep column on a Varian Prostar® HPLC with an

acetonitrile/water gradient containing 0.05% TFA. A final concentration of 10 μ M ATP was added per well to initiate the reaction. The assay was allowed to proceed for 90 minutes at 30°C. Enzyme inhibition was obtained indirectly by assessing the percentage of unused ATP in the reaction via luminescence with Kinaseglo® and comparing it to SRC wells with no inhibitor (in the presence of 1% DMSO to account for the DMSO in experimental wells). This assay also included reactions without peptide substrate as controls. Luminescence was read on a MicroBeta2® plate reader (PerkinElmer, Waltham, MA) after addition of Kinaseglo®.

Human cell toxicity assay

Cell viability assays were performed to determine potential toxicity of compounds against mammalian cells. CRL-8155 human lymphocytic cells (ATCC, WIL2-NS) cultured in RPMI-1640 growth medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, and 1 mM L-glutamine, were incubated in the presence of compounds. The Alamar Blue® assay (Life Technologies, Grand Island, NY), which measures general cellular metabolism, was used to quantify cell growth. Mid-log cells were seeded in 96-well flat-bottom plates at a density of 3×10^5 cells/mL containing compounds (20 mM stock solutions dissolved in DMSO) at six concentrations (40 μ M, 20 μ M, 10 μ M, 5 μ M, 2.5 μ M, and 1.25 μ M) in quadruplicate and grown for 48 hours at 37°C in a 5% CO₂ humidified incubator. 1/10th volume of Alamar Blue® developing reagent was added to each well and incubated for an additional 3 hours and fluorescence was measured at the respective excitation and emission wavelengths of 560 nm and 590 nm in a FLx800 microplate reader (Biotek, Winooski, VT). Percent growth inhibitions by test compounds were calculated based on DMSO vehicle and negative (50 μ M Quinacrine) controls, which represent 0% and 100% growth inhibition, respectively. Additionally, Quinacrine was used as a positive control in each assay for quality assurance.

Data analysis

Luminescence data were analyzed and equated to percent ATP usage. The results were then converted to percent inhibition produced by each compound, subsequent IC₅₀ analysis was performed using Graphpad® Prism software (GraphPad Software, San Diego, CA). Statistical analysis of data using Pearson's Correlation test was performed using Microsoft® Excel analysis package. For each enzyme's reaction plate, the Z'-factor, a coefficient that defines assay signal dynamic range and data variation associated with signal measurements were determined as earlier described (Zhang *et al.* 1999). The Z'-factor was calculated as an intra-assay assessment of the quality of the assay using the equation below (Zhang *et al.* 1999).

$$Z' - \text{Factor} = 1 - \frac{3 \times (\text{Standard Deviation of negative control}) + 3 \times (\text{Standard deviation of positive control})}{|\text{Mean of negative control} - \text{Mean of positive control}|}$$

RESULTS

Overall inhibition of CDPKs and SRC from the BKI library

In order to establish the structure activity relationships (SAR) of BKIs against the CDPKs, we assayed the entire BKI library for inhibition of the recombinant apicomplexan CDPKs at a concentration of 3 μ M. Sensitivity to a compound at this final reaction concentration was defined as the inhibition of enzyme kinase activity by greater than 70% relative to the no inhibitor full reaction (positive control). Resistance was defined as the inhibition of an enzyme kinase activity by less than 30% relative to the positive control. The average Z' -factor of the compound reaction plate for enzymes screened ranged from 0.7112 for *Bb*CDPK4 to 0.8949 for *Cp*CDPK1 (Table 1). A high Z' -factor reflects two characteristics of the assay. It reflects a wide separation between the maximum signals of the assay (in this case, highest amount of luminescence in zero ATP consumption found in negative controls) and the lowest signal (lowest amount of luminescence found in positive enzyme reactions with no inhibitors causing a reduction of the initial ATP concentration). It also shows that standard deviations are low when assessing positive and negative controls. A Z' -factor value of greater than 0.5 implies a quality assay, and a perfect assay will approach 1.0 (Zhang *et al.* 1999). The most sensitive CDPK orthologues to our BKI compound library were wild type *Nc*CDPK1, *Tg*CDPK1 and *Cp*CDPK1, where 90% (301 compounds), 89% (296) and 86% (287) of inhibitors exhibited over 70% to absolute inhibition of kinase activity, respectively (Table 1). These three enzymes share a glycine gatekeeper residue. These results were followed by *Pf*CDPK4 with 55% (183 compounds), *Pf*CDPK1 with 44% (145), *Bb*CDPK4 with 39% (129), *Et*CDPK1 with 21% (71) and SRC with 10% (33) effective inhibitors. *Pf*CDPK4 has a serine gatekeeper, while *Pf*CDPK1, *Bb*CDPK4, *Et*CDPK1, and the human enzyme SRC all share a threonine gatekeeper. The level of resistance to BKIs was much higher for *Et*CDPK1 and SRC compared to other kinases (Table 1).

The selectivity of the BKIs' inhibition towards the different protein kinases tested was variable. Fourteen of the 333 compounds have greater than 90% inhibition of kinase activity in all of the wild type apicomplexan CDPK enzymes tested. Of these 14 compounds, 5 were completely non-selective as they also inhibited the human kinase SRC activity by greater than 90%. Forty compounds have >80% inhibition of all CDPKs with 11 of these 40 compounds also inhibiting SRC by greater than 80%. Sixty of the 333 compounds caused >70% inhibition of CDPKs where 17 also inhibited SRC by greater than 70%. Of these 60 compounds, none are benzoylbenzimidazole analogues, 4 are imidazo[1,5-a]pyrazine analogues, and 56 are pyrazolo [3,4-d]pyrimidine analogues. Thus, when BKIs inhibit all of the CDPK orthologues, similar inhibition of SRC occurs at a ratio of 1 compound in approximately 4 (17 out of 60 compounds). Human SRC was chosen as a control because it contains a threonine gatekeeper. When the 14 compounds with >90% inhibition of all apicomplexan CDPK enzymes tested were assayed for cytotoxicity in human cells, 4 compounds achieved significant inhibition in the <10 μ M range *in vitro* against the human lymphotic cell line CRL8155 (Table 2). Hence, the data suggest that the anti-proliferative effects of off-target BKIs on mammalian cells are likely not mediated by the inhibition of SRC.

Correlation relative to identity of the protein kinase domain

The apicomplexan CDPKs tested shared highly conserved ATP binding domains. Multiple alignments of the amino acid residues proposed to interact with BKIs, as well as a matrix portraying the identity of the protein kinase domain between orthologues as a percentage are shown in Fig. 1 and Table 3. *Tg*CDPK1 and *Et*CDPK1 share 94% amino acid identity within their ATP binding sites as defined by *Tg*CDPK1 residues K52 to L198 (accession number XP_002371666). This is an important pair to compare because *Et*CDPK1 is the least sensitive CDPK homologue to the BKI library, despite its close identity to *Tg*CDPK1, the enzyme against which the focused BKI library was modeled. For comparison, *Tg*CDPK1 and *Et*CDPK1 also share identities to the ATP binding sites of *Pf*CDPK4 (77%), *Cp*CDPK1 (74% and 73% respectively), *Bb*CDPK4 (71% and 72%, respectively) and *Pf*CDPK1 (54% and 55%, respectively). Amino acid identity information can sometimes be correlated to drug screening results. In theory, the greater the identities of the orthologous CDPKs, the more similar their drug inhibition profiles. A good example is illustrated by the comparison between *Tg*CDPK1 and *Nc*CDPK1 where in addition to 99% shared residues within the ATP binding site, their activities with BKIs are similar. On the contrary, *Cp* and *Tg*CDPK1 are very similar with respect to inhibitor binding, and yet are only 74% identical at the ATP binding site where as *Tg* and *Et*CDPK1 are more similar at the ATP binding site (94%) but are the most different in terms of inhibitor binding.

As orthologues, the apicomplexan CDPKs collectively share a positive correlation amongst one another against the BKI library. However, when assessing an individual compound amongst the enzymes, the level of sensitivity to the library varies (Table 2). This may be reflective of each enzyme's active site variations for BKI inhibition (Table 2). The shared concordance could be correlated by Pearson's R value (Table 4). Relative to other enzyme pairings, *Tg*CDPK1, with glycine gatekeeper residue, and *Et*CDPK1, with threonine as the gatekeeper residue, have only fair correlation (Table 4), despite high protein kinase active site identity (Fig. 1 and Table 3). *Cp*CDPK1's inhibition spectrum toward BKIs is very similar to *Tg*CDPK1 and *Nc*CDPK1 (Table 4), and distinct from the BKIs that inhibited *Et*CDPK1 (Pearson R value = 0.4252). *Pf*CDPK4, the only CDPK studied with a serine gatekeeper, had fair to poor correlation when compared with other enzymes, achieving the most correlation with *Tg*CDPK1 (Pearson R = 0.6028), *Cp*CDPK1 (Pearson R = 0.6921), and *Nc*CDPK1 (Pearson R = 0.5851 (Table 4).

Effects of a gatekeeper residue mutation on sensitivity and characteristics of inhibitory compounds

Mutants were generated to explore the effect of the gatekeeper residue on the inhibition pattern. A *Tg*CDPK1 threonine mutant, *Tg*G128TCDPK1, was shown to have an almost perfect correlation and BKI compound sensitivity compared with *Et*CDPK1 (Tables 1 and 4). The compelling similarity between *Et*CDPK1 and the mutant *Tg*G128TCDPK1 under the same conditions, as well as their relative resistance to compounds compared to the other orthologues with smaller gatekeeper residues further supports the hypothesis that gatekeeper residues are key drivers of compound binding. However, when comparing *Tg*G128TCDPK1's SAR to *Bb*CDPK4 and *Pf*CDPK1, sensitivities to the compound library showed discordance (37% for *Bb*CDPK4 and 44% for *Pf*CDPK1 versus 21% for

TgG128TCDPK1 or *EtCDPK1*, respectively). Thus, *EtCDPK1* and *TgG128TCDPK1* are more resistant to the library than *BbCDPK4* and *PfCDPK1*.

Weighted identity scores ([WS] in Fig. 1) of conserved differences in amino acids proposed to directly interact with BKIs were derived by assigning a unitless value of 1 for every property difference, based on well-established properties of charge, polarity, and size as previously described (Livingstone and Barton, CABIOS 1993). The gatekeepers were excluded from this analysis since the influence of these residues on BKI inhibition of CDPKs is well defined. By this measurement, *PfCDPK1* and *CpCDPK1* have the highest divergence from the *TgCDPK1* ATP binding site with a score of 15 for *PfCDPK1* and 9 for *CpCDPK1* (Fig. 1). However, *CpCDPK1* showed identical SAR profile and with high sensitivity toward the library, much like *TgCDPK1*, probably due to the higher influence of the shared glycine gatekeeper residue. Conversely, the SAR profiles of *Nc/TgCDPK1* versus those of *PfCDPK1*, *BbCDPK4*, *EtCDPK1* make it challenging to attribute all the inhibitor library observed characteristics to the gatekeeper size alone. In the latter case, we can partially attribute higher sensitivity to increased divergence from the model of *TgCDPK1* on which compound design was based. However, it is likely that a combinatory effect resulting from differences in types and chemical properties of amino acids within the active sites of apicomplexa CDPKs can produce a variety of BKI inhibition SARs. Analysis of amino acid differences between *PfCDPK1* and *TgCDPK1* demonstrated deviations capable of promoting the differential sensitivity observed. Through modeling based on previous crystal structures of *TgCDPK1* and *CpCDPK1* co-crystallized with BKI inhibitors, probable structural models were created to simulate binding motifs of other CDPK enzymes. *PfCDPK1* was compared to this effect with *TgCDPK1* (Fig. 2). Three possible differences from the sequences of *PfCDPK1* and *TgCDPK1* were noted that may influence the binding of BKIs to *PfCDPK1*. These include *TgCDPK1* F134V (Residue 117 in *PfCDPK1*), M112I (Residue 129 in *PfCDPK1*), and I194V (Residue 221 in *PfCDPK1*). Perhaps the most influential variation is at the position of methionine 112 in *TgCDPK1* (isoleucine 129 in *PfCDPK1*). The larger (glycine->threonine) gatekeeper residue on one side of the binding pocket is paired with a complementary smaller residue (methionine->isoleucine) on the opposite side of the binding pocket (Fig. 2). This allows the BKI binding pose to shift slightly away from the gatekeeper, something that is otherwise sterically restricted in *TgCDPK1* and *EtCDPK1*. Thus the effect of the larger threonine gatekeeper in *PfCDPK1* is partially offset by the presence of a complementary amino acid variation elsewhere in the active site, which may explain its increased BKI sensitivity relative to *EtCDPK1* (Fig. 2).

Within the *TgG128TCDPK1* inhibitor SAR, patterns of compound characteristics were identified. In general, inhibition of *TgG128TCDPK1* and its highly similar threonine partner, *EtCDPK1*, requires relatively small substituents at the C3 position (R1 group), such as an alkoxybenzyl group, 4-chlorophenyl, or naphthalen-2-yl (Fig. 3). Presumably, larger substituents exhibit steric clash with the threonine gatekeeper, disfavoring binding. However, a few compounds with larger R1 groups, such as 23j, 24j, 31j, 32j, 33j, 26a, 24a, 25j, 30a, 39a, 27a, 34a, 6j, did cause >70% inhibition of *EtCDPK1* kinase activity, irrespective of the R2 groups size. Such constraints on inhibitors were not as obvious with the other threonine enzymes *BbCDPK4* and *PfCDPK1*. A wider range of compounds were

able to achieve inhibition for these orthologues (*Bb*CDPK4 and *Pf*CDPK1) relative to *Et*CDPK1, including compounds with larger R1 moieties (6-alkoxy- and 6-benzyloxy-naphthol) without the need of a smaller R2 group that may be associated with the spatial constraints of enzymes with larger gatekeeper residues.

Contrary to the near identical sensitivity and correlation seen with *Et*CDPK1 and *Tg*G128TCDPK1, the serine mutant, *Tg*G128SCDPK1 was sensitive to 241 (72%) of compounds while its serine gatekeeper partner *Pf*CDPK4 was sensitive to 183 (55%). Although this enzyme pair shared the same gatekeeper residue, there was a difference in sensitivity patterns to compounds and change in correlation (Table 4: Pearson R value 0.7714) that was not dependent on the gatekeeper. A series of compounds was isolated which differentially inhibited *Tg*G128SCDPK1 over *Pf*CDPK4. This series shifted inhibition towards *Tg*G128SCDPK1 by greater than 30% over *Pf*CDPK4. Compounds with this pattern include 15k, 4a, 13a, 31a, 32a, 36a, 35a, 37a, 30a, 41b, 43b, 40b, (45j), 25b, 6b, 12b, 10b, 1h.

*Pf*CDPK4 seems to accommodate R1 groups such as a naphthyl ring connected in an ortho configuration, while the larger threonine gatekeeper residue enzyme, *Pf*CDPK1, was able to accommodate quite large and extended groups at this position, such as 6-alkoxy- and 6-benzyloxynaphthol substituents. In fact, a group of compounds with extended moieties inhibited *Pf*CDPK1, yet against *Pf*CDPK4 with a serine gatekeeper residue, did not achieve the same level of inhibition. These compounds include 35a, 7a, 8a, 9a, 13a, 36a, 35a, 11j, 10b (Fig. 3).

DISCUSSION

A comparative analysis of the structural activity relationship of veterinary pathogens *N. caninum*, *E. tenella* and *B. bovis*; zoonotic pathogens *C. parvum* and *T. gondii* CDPKs and those of human pathogen *P. falciparum* CDPKs was determined by screening against 333 BKI analogues. The degree of sensitivity or resistance of CDPK enzymes to BKIs was earlier postulated to be due to the size and characteristic of the gatekeeper residue and the adjacent pocket. Indeed, correlation of screening data was best among enzymes with the same gatekeeper residue, which corroborates the gatekeeper hypothesis (Table 4). However, sensitivity to compounds among enzymes with the same gatekeeper residue does not correlate perfectly, suggesting alternate modes of CDPK-drug interaction. We explored this by constructing *Tg*CDPK1 mutants that substituted serine or threonine for the wild type glycine at the gatekeeper position, and comparing their BKI sensitivity profile to that of the homologues with the corresponding wild type gatekeeper residue. i.e. *Tg*CDPK1G128S can be compared to *Pf*CDPK4, and *Tg*CDPK1G128T can be compared to *Pf*CDPK1, *Et*CDPK1, and *Bb*CDPK4 (Tables 3 and 4). Although *Et*CDPK1 has the closest sequence identity to wild type *Tg*/*Nc*CDPK1 in the ATP binding pocket (Table 3: 94%), it nonetheless proved to be the least sensitive enzyme to BKIs, with poor correlation to the sensitivity profile of wild type *Tg*CDPK1. However the sensitivity of *Et*CDPK1 correlates very well with that of *Tg*CDPK1G128T. The BKI sensitivity of two other enzymes with a threonine gatekeeper, *Bb*CDPK4 and *Pf*CDPK1, is also more similar to that of the *T. gondii* G128T mutant than to that of the wild type *Tg*CDPK1. However, these orthologues share less sequence identity

with the *T. gondii* enzyme and have weaker correlation with the sensitivity of the *Tg*CDPK1G128T mutant. *Tg*CDPK1 and *Nc*CDPK1 are also similar in their ATP binding domain identity (>99%) and predictably share similar sensitivity to compounds. Yet, *Cp*CDPK1 is not as similar in identity to *Tg*CDPK1 as *Et*CDPK1 (94% identity versus 74%) or *Nc*CDPK1 (>99% versus 74%) (Table 3). However, the fact that *Tg/Nc*CDPK1 and *Cp*CDPK1 are the most sensitive enzymes to compounds, yet share less sequence similarity (74% identity) than that of *Et*CDPK1, returns us to the enormous role played by the size of the gatekeeper residue in determining the level of sensitivity to BKIs. This assertion was supported with experimental evidence showing excellent correlation and characteristics of structure activity relationship between *Et*CDPK1 and *Tg*G128TCDPK1, completely reversing susceptibility of *Tg*CDPK1.

Although the similarity of a homologue to *Tg*CDPK1's ATP binding site sequence is an attractive marker for determining sensitivity to BKIs for enzymes whose full binding pocket is uncharacterized, the binding pocket size, charge, and hydrophobicity are all factors that ultimately determine sensitivity to BKIs. A combination of these factors contributes directly or indirectly to the sensitivity of CDPKs to BKIs. Further research is necessary to elucidate the relative impact of each factor to CDPK sensitivity to BKIs. Crystal structures of these new homologues may help understand the complex nature of the binding pocket. With this new information, we conclude that a large gatekeeper residue does not necessarily mean complete resistance to BKIs. In the example of our threonine gatekeeper enzymes, low nanomolar inhibition was achieved, even by BKIs designed to target small gatekeepers.

It is important that future drug development focus on exploiting other characteristics of the drug binding pocket in addition to the gatekeeper. In this respect, compounds could be optimized to target the conserved apicomplexan ribose binding pocket. Similarly, targeting the calcium binding regulatory domain could prevent activation of CDPK enzymes with a resultant disruption of downstream process. Compounds that inhibit *Et*CDPK1 but not SRC, are important as lead compounds for the development of broad spectrum antimicrobials. Because of its threonine gatekeeper and unaccommodating binding pocket towards BKIs, if potent compounds to target *Et*CDPK1 are synthesized, then clinically, these compounds can be useful as monotherapy with low likelihood of easily conferring resistance across CDPKs of the apicomplexa. Within our BKI library, 34 compounds effectively inhibited *Et*CDPK1 by >70% and inhibit SRC kinase activity by <30%. The structure and characteristics of these compounds will be the focus of future studies. These compounds include 19a, 19j, 22j, 23j, 26j, 24j, 21j, 31j, 33j, 25j, 3j, 38a, 30a, 42b, 19b, 18b, 10j, 17j, 6j, 25L, 19e, 16j, 19q, 19r, 19t, 19v, 19w, 20j, 5j, 5L, 19s, 20a, 28j, 28j.

CONCLUSION

It is apparent that the highly conserved nature of the ATP binding domain shared by apicomplexan CDPK homologues could be exploited to some extent in development of potential broad spectrum inhibitors if potential structural constraints are well defined and understood. A combination of these constraints concerning the gatekeeper residue, the structure and characteristic of the binding pocket, and differences among compounds, all contribute subtle distinctions in sensitivity to compounds among these highly conserved

CDPKs. Further research into the structural constraints for the development of broad spectrum, potent inhibitors of apicomplexan CDPKs is warranted.

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	57	58	59	60	61	62	63	64	65	66	67	77	78	79	80	81	112	113	114	115	116	117	125	126	127	128	129	130	131	132	179	180	181	182	183	192	193	194	195	196	WS
<i>Cp</i> CDPK1	L	G	K	G	S	F	G	E	V	L	K	Y	A	V	K	V	M	K	L	F	E	I	Y	I	V	G	E	L	Y	T	N	I	L	L	E	K	I	I	D	F	9
<i>Nc/Tg</i> CDPK1	L	G	K	G	S	F	G	E	V	I	L	C	A	V	K	V	M	K	L	Y	E	F	Y	L	V	G	E	V	Y	T	N	L	L	L	E	R	I	I	D	F	
<i>Pf</i> CDPK4	L	G	K	G	S	F	G	E	V	I	L	Y	A	I	K	V	M	K	L	Y	E	F	Y	L	V	S	D	V	Y	T	N	L	L	L	E	K	I	I	D	F	3
<i>Pf</i> CDPK1	L	G	S	G	A	Y	G	E	V	L	L	K	A	I	K	V	I	K	L	F	D	V	Y	L	V	T	E	F	Y	E	N	I	L	L	E	K	I	V	D	F	15
<i>Et</i> CDPK1	L	G	K	G	S	F	G	E	V	I	L	Y	A	V	K	V	M	K	L	Y	E	F	Y	L	V	T	E	V	Y	T	N	L	L	L	E	R	I	I	D	F	
<i>Bb</i> CDPK4	L	G	K	G	S	F	G	E	V	L	L	Y	A	V	K	V	M	K	I	F	E	F	Y	F	V	T	E	L	Y	S	N	L	L	L	E	R	I	I	D	F	4

Fig. 1. Multiple alignments of the amino acid residues proposed to interact with BKIs

Regions contributing to the active site as seen in crystal structures of several *Tg*CDPK1 orthologs are shown. Numbering were based on *Tg*CDPK1 protein database accession number XP_002371666. Specific residues that may confer differential sensitivity to some BKI are shaded yellow. Residues that are shaded grey are conserved between all the enzymes. The gatekeeper residues are shown in red. Weighted identity score (WS) of differences in amino acids, compared with *Tg/Nc*CDPK1 (note, *Tg* identical to *Nc* in the active site) proposed to directly interact with BKIs in each enzyme is shown on the far right. The gatekeepers were excluded from this analysis since the influence of these residues on BKI inhibition of CDPKs is well defined.

Fig. 2. BKI interactions in the ATP binding domains of PfCDPK1 and TgCDPK1

Model of *Pf*CDPK1 based on previous crystal structure of *Tg*CDPK1 in complex with inhibitor 19j (Larson *et al.*, 2012; PDB 3sx9). Gatekeeper residues and others residues postulated to affect inhibition are shown as red (*Tg*CDPK1) and yellow (*Pf*CDPK1) sticks. The larger G128M (Residue 145 in *Pf*CDPK1) gatekeeper residue is paired with a complementary smaller residue M112I (Residue 129 in *Pf*CDPK1), and I194V (Residue 221 in *Pf*CDPK1) in *Pf*CDPK1. This partially offset the effect of the larger gatekeeper in *Pf*CDPK1 by the presence of a complementary amino acid variation elsewhere in the active site, which may explain its increased BKI sensitivity relative to *Et*CDPK1.

Fig. 3.
R1 and R2 substructures of compounds

Table 1

Recombinant enzymes tested, gatekeeper residues, evaluation of reproducibility of assays (Z'-factor) and % BKIs that effectively inhibited the various enzymes

Enzyme	Gatekeeper Residue	Average Z'-factor	Sensitive (>70%)		Resistant (<30%)	
			Count	%	Count	%
<i>Tg</i> CDPK1	glycine	0.880	296	89	15	5
<i>Nc</i> CDPK1	glycine	0.869	301	90	15	5
<i>Cp</i> CDPK1	glycine	0.895	287	86	22	7
<i>Pf</i> CDPK4	serine	0.844	183	55	59	18
<i>Tg</i> G128SCDPK1	serine	0.809	241	72	36	11
<i>Pf</i> CDPK1	threonine	0.716	145	44	104	31
<i>Bb</i> CDPK4	threonine	0.711	129	39	142	43
<i>Et</i> CDPK1	threonine	0.898	71	21	192	58
<i>Tg</i> G128TCDPK1	threonine	0.854	73	22	187	56
SRC	threonine	0.808	33	10	241	72

Table 2

Experimental enzymes IC₅₀ and human cells CRL 8155 EC₅₀ values of compounds with >90% inhibition of kinase activity at single dose of 3 μ M

Compound	TgCDPK1 (μ M)	CpCDPK1 (μ M)	PfCDPK1 (μ M)	PfCDPK4 (μ M)	NcCDPK1 (μ M)	EtCDPK1 (μ M)	BbCDPK4 (μ M)	Src (μ M)	CRL8155 (μ M)
26j	0.002	0.002	0.027	0.030	0.004	0.176	0.102	3.063	>10
31j	0.008	0.009	0.167	0.161	0.010	0.511	0.514	1.759	>10
32j	0.007	0.008	0.110	0.133	0.009	0.308	0.359	0.375	>10
33j	0.005	0.004	0.067	0.036	0.005	0.168	0.303	1.551	6.844
44b	0.046	0.417	0.012	0.136	0.035	0.583	0.026	0.261	>10
16b *	0.004	0.002	0.061	0.018	0.003	0.492	0.547	1.287	>10
17j	0.003	0.002	0.077	0.007	0.003	0.300	0.355	>10	>10
2b	0.005	0.036	0.338	0.399	0.019	0.638	0.350	0.129	>10
3b	0.005	0.022	0.074	0.133	0.003	0.093	0.091	0.037	>10
19w	0.004	0.001	0.110	0.004	0.013	0.217	0.046	8.228	>10
19u	0.004	0.001	0.083	0.008	0.004	0.190	0.017	0.729	>10
14d	0.005	0.001	0.012	0.010	0.003	0.081	0.242	0.073	<10
45j	0.001	0.001	0.002	0.003	0.001	0.012	0.012	0.020	<10
28j	0.004	0.004	0.005	0.012	0.003	0.099	0.671	>7	<10

* = imidazole aniazad [a-5',1] oxazimid

Table 3

Comparison of amino acid identities (%) of CDPK orthologues' ATP binding sites as defined by *Tg*CDPK1 residues K52 to L198 (accession number XP_002371666). The smallest sum probability by BLASTP search is shown in parenthesis.

Enzyme	Similarity (identity)					
	<i>Pf</i> CDPK1	<i>Et</i> CDPK1	<i>Bb</i> CDPK4	<i>Tg</i> CDPK1	<i>Cp</i> CDPK1	<i>Nc</i> CDPK1
<i>Pf</i> CDPK4	53 (5.0 e ⁻⁴²)	77 (5.0 e ⁻⁷²)	66 (4.0 e ⁻⁶⁰)	77 (8.0 e ⁻⁷²)	68 (4.0 e ⁻⁵⁷)	78 (2.0 e ⁻⁷²)
<i>Pf</i> CDPK1		55 (3.0 e ⁻⁴⁹)	53 (2.0 e ⁻⁴⁵)	54 (2.0 e ⁻⁴⁷)	53 (4.0 e ⁻⁴⁶)	54 (2.0 e ⁻⁴⁶)
<i>Et</i> CDPK1			71 (4.0 e ⁻⁶⁶)	94 (5.0 e ⁻⁸⁹)	73 (6.0 e ⁻⁷⁵)	93 (1.0 e ⁻⁸⁸)
<i>Bb</i> CDPK4				72 (2.0 e ⁻⁶⁵)	68 (3.0 e ⁻⁵⁹)	72 (5.0 e ⁻⁶⁶)
<i>Tg</i> CDPK1					74 (6.0 e ⁻⁶⁵)	99 (5.0 e ⁻⁹⁵)
<i>Cp</i> CDPK1						73 (4.0 e ⁻⁶⁴)
<i>Nc</i> CDPK1						

Table 4

Pearson correlation R values: Statistical comparative analysis of inhibition data for each enzyme relative to the other using Pearson's Correlation test. Higher correlations among CDPKs with similar gatekeeper residues support their influence on BKI inhibition. The data showed relatively lower inhibition correlation of most CDPK enzymes with human SRC Kinases.

Enzyme	<i>Pf</i> CDPK1	<i>Et</i> CDPK1	<i>Bb</i> CDPK4	<i>Tg</i> CDPK1	<i>Tg</i> G128SCDPK1	<i>Tg</i> G128TCDPK1	<i>Cp</i> CDPK1	<i>Nc</i> CDPK1	SRC
<i>Pf</i> CDPK4	0.607	0.597	0.544	0.603	0.771	0.593	0.692	0.585	0.261
<i>Pf</i> CDPK1		0.854	0.865	0.578	0.719	0.846	0.534	0.563	0.483
<i>Et</i> CDPK1			0.866	0.447	0.639	0.962	0.425	0.433	0.456
<i>Bb</i> CDPK4				0.454	0.632	0.858	0.401	0.438	0.369
<i>Tg</i> CDPK1					0.855	0.449	0.911	0.989	0.310
<i>Tg</i> G128SCDPK1						0.658	0.794	0.847	0.348
<i>Tg</i> G128TCDPK1							0.421	0.431	0.495
<i>Cp</i> CDPK1								0.901	0.294
<i>Nc</i> CDPK1									0.303