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Optimizing small molecule inhibitors of calcium-dependent protein kinase 1 to prevent infection by *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is sensitive to bulky pyrazolo [3,4-*d*] pyrimidine (PP) inhibitors due to the presence of a Gly gatekeeper in the essential calcium dependent protein kinase 1 (CDPK1). Here we synthesized a number of new derivatives of 3-methyl-benzyl-PP (3-MB-PP, or **1**). The potency of PP analogs in inhibiting CDPK1 enzyme activity *in vitro* (low nM IC_{50} values) and blocking parasite growth in host cell monolayers *in vitro* (low μM EC_{50} values) were highly correlated and occurred in a CDPK1-specific manner. Chemical modification of the PP scaffold to increase half-life in the presence of microsomes *in vitro* led to identification of compounds with enhanced stability while retaining activity. Several of these more potent compounds were able to prevent lethal infection with *T. gondii* in the mouse model. Collectively the strategies outlined here provide a route for development of more effective compounds for treatment of toxoplasmosis, and perhaps related parasitic diseases.

Keywords

Serine/threonine protein kinase; gatekeeper; calcium signaling; toxoplasmosis; chemotherapy

INTRODUCTION

Toxoplasma gondii is a widespread protozoan parasite of animals that frequently causes zoonotic infections in humans [1]. Humans are infected by accidental ingestion or inhalation of oocysts, spore-like stages shed in the feces of cats, or via ingestion of tissue cysts found

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Supporting Information Available
A summary of the synthesis schemes, chemical purity and identity is provided. This material is available free of charge via the Internet at http://pubs.acs.org

in undercooked meat from infected animals. During acute infection, the tachyzoite stage of the parasite disseminates widely, infecting a wide range of nucleated cell types, before being controlled by innate and adaptive immune responses [2]. Despite this vigorous immune response, the infection is not eradicated, but rather the parasite differentiates into a slow growing cyst stage that is found within long-lived cells such as neurons in the central nervous system (CNS) [3]. Although most infections resolve without complications, they can lead to serious outcomes following congenital infection or from chronic infections that present a risk of reactivation in immunocompromised patients [4]. Additionally, toxoplasmosis has been associated with serious ocular disease in otherwise healthy adults from some regions of Brazil, where it is widespread [5]. Effective antibiotic therapy is available, most commonly in the form of combination therapy of pyrimethamine and sulfa drugs, yet it suffers from problems of intolerance, allergic reactions, and an inability to cure the chronic infection [6].

Toxoplasma gondii is an obligate intracellular parasite, capable of actively penetrating and developing in virtually any nucleated cell from a wide range of vertebrate hosts. Host cell invasion is driven by parasite motility that depends on an actin-myosin motor that is anchored beneath the parasite plasma membrane [7]. Motility is also dependent on secretion of adhesive proteins from apically located organelles called micronemes. Once discharged onto the parasite cell surface, these transmembrane adhesins serve to link extracellular adhesion with the actin myosin motor and translocation of these complexes drives forward motility [7]. Microneme secretion is controlled by elevated calcium in the parasite cytosol and blocking this signal disrupts both motility and cell invasion [8, 9]. Using a conditionally regulated genetic system it was shown that elevated calcium controls microneme secretion through the action of calcium dependent protein kinase 1 (CDPK1), which is an essential gene in *T. gondii* [10].

CPDKs are uniquely found in plants and protozoa, and they consist of an N-terminal serine/threonine protein kinase domain followed by a series of 4 EF hands that form a calmodulin-like regulatory domain (CRD) [11]. However, unlike calmodulin-dependent kinases (CamK) in animal cells, which are regulated by a C-terminal region that interacts with calmodulin, the CRD of CDPKs is directly fused to the C terminus of the kinase domain. Recent structural studies reveal that in the absence of calcium, the CRD binds to the kinase domain and prevents substrate access, while addition of calcium causes a massive reorganization of the CRD to unmask the kinase domain for activation [12, 13].

The structure of TgCDPK1 also revealed that it has a small gatekeeper, a feature shared by its orthologue in *C. parvum* [12, 13]. Small gatekeeper residues are rare in nature and this feature has previously been utilized to engineer mammalian and yeast kinases to become selectively sensitive to pyrazolo-pyrimidine (PP) inhibitors that contain bulky groups that can occupy the so-called “gatekeeper pocket” [14-17]. Taking advantage of the naturally small gatekeeper residue, it was shown that PP analogs are potent inhibitors of TgCDPK1 *in vitro* and that this property depends on the small gatekeeper [10, 12, 18]. Moreover, PP analogs were shown to block parasite invasion into host cells, an effect that was reversed in the lines expressing mutants of TgCDPK1 where the small gatekeeper was altered to Met [10, 12, 18], thus validating TgCDPK1 as the primary target of these inhibitors in *T. gondii*. More complete analysis of the effects of PP inhibitors revealed that they specifically block microneme secretion, thus inhibiting parasite motility, cell invasion, and egress, and effectively blocking growth *in vitro* [10].

The potent activity of PP analogs and selectivity for kinases with small gatekeeper residues, suggests that these compounds would be promising leads for development of potent inhibitors for *T. gondii* and possible related parasites like *Cryptosporidium parvum*, which shares the small gatekeeper feature of CDPK1 [13, 19]. Indeed several previous reports have identified a number of substitutions of this scaffold that result in excellent selectivity *in vitro* [20-22]. Here we exploited the versatile nature of the PP scaffold to develop unique potent and stable inhibitors of TgCDPK1 and assess their ability to control infection *in vivo* in the mouse, a natural host for infection and transmission.

RESULTS AND DICSUSSION

Chemical Synthesis

To identify PP analogs with increased potency against TgCDPK1 and improved bioavailability for efficacy *in vivo*, we synthesized compounds with different functional groups (R1) at the C3 position, in combination with either an isopropyl or *tertiary* butyl group (R2) at the N1 position (Figure 1). The parent compound, 3-methyl-benzyl pyrazolo [3,4-*d*] pyrimidine (3-MB-PP, or **1**) inhibits kinases with small (Gly) or medium (i.e. Ala, Ser, Thr) gatekeeper residues at low and high nM, respectively [2316]. We designed and synthesized a series of PP analogs with various functional groups at the C3 position of the PP scaffold. Most of these compounds are close structural analogs of **1**: they contain either a single halogen substituting for the methyl group (i.e. **2**, **3**, and **4**) or a di-halogen substitution pattern on the benzyl moiety (i.e. **9-12** & **17-16**) to confer additional stability against metabolism *in vivo* [24, 25]. The remaining ones contain either a naphthyl group (i.e. **5**, **6**, and **7**) or a substituted phenyl groups (i.e. **8**, **13-16**) [14]. All the PP analogs except **25** and **26** were synthesized using an established route with slightly altered reaction conditions (Scheme 1) [14]. Compounds **25** and **26** were synthesized using a varied route to furnish modification to the benzylic methylene group (Supplementary Scheme 1).

PP analogs act rapidly to prevent parasite infection of host cells

Previous studies have shown that PP analogs block *T. gondii* growth in host cells cultured *in vivo* [10, 12, 22]; however, these studies did not address whether these compounds were toxoplasmacidal or simply static. To assess how rapidly PP analogs work against live parasites, we treated tachyzoites of *T. gondii* with compounds in the presence of host cells for 4 h *vs.* 24 h, followed by washing to remove extracellular parasites and compounds. Monolayers were then returned to culture and parasites allowed to grow for a total of 72 h, prior to harvest and determination of parasite growth based on β-galactoside (β-gal) activity. The parent compound **1** proved to be extremely potent in preventing parasite invasion during the treatment period and hence blocking growth, with an EC_{50} of ~ 0.1 μM following exposure for either 4 or 24 h in culture (Figure 2A). In contrast, treatment with pyrimethamine, which acts against the bifunctional dihydrofolate reductase-thymidylate synthase enzyme to block DNA synthesis, required at least 24 h to demonstrate potent inhibition (Figure 2A). These findings indicate that PP analogs act rapidly, likely as a consequence of their potent inhibition of parasite invasion into host cell, as described previously [10, 12].

Potency of PP analogs for inhibition of CDPK1 *in vitro* and parasite growth in host cells

To develop a SAR profile, we compared the potency of the series of PP analogs (Table 1) at inhibiting recombinant TgCDPK1 enzyme *in vitro* to that at inhibiting parasite growth in host cells (Table 1). The *in vitro* activity against TgCDPK1 was based on an ELISA that detects phosphorylation of the syntide-2 peptide, while measurement of parasite growth was based on β-gal activity, as described above. The inhibitors showed a range of potency in blocking enzyme activity *in vitro vs.* parasite growth in host cells (Table 1), and there was an excellent correlation between the results of these two assays (Figure 2B). The strong correlation of enzyme inhibition with growth inhibition suggests that the major target of these compounds in the parasite is CDPK1, although the specificity of these compounds is further addressed below. The most potent analogs in both assays were **3** the parent compound **1**, as well as several new analogs that contain two halogen atoms on the phenyl ring (i.e. **11**, **21**) (Figure 2B). A number of chemical modifications resulted in much lower potency in both assays (i.e. **13**, **15**, and **16**), suggesting they disrupt the binding of the compound in the nucleotide-binding pocket of TgCDPK1 without providing additional favorable interactions in the hydrophobic pocket to compensate.

Consistent with prior work on mammalian kinases [14-17], co-crystal structures of TgCDPK1 with 2-napthyl-PP (**5**) or 1-napthylmethyl-PP (**6**) have shown that the glycine gatekeeper allows binding of bulky substitutions at the 3 position of the PP scaffold, and that extensions at this position protrude into a deep hydrophobic pocket that is inaccessible in kinases with larger gatekeeper residues [12]. Based on this unique relationship, a variety of PP analogs have previously been synthesized and tested for activity against TgCDPK1 and the ortholog in *C. parvum* called CpCDPK1 *in vitro* and in cell culture-based assays [20, 22]. Efforts to extend the PP scaffold initially focused on changing R1 at the C3 position to various aryl groups. A variety of such PP derivatives are effective including addition of methyl or Cl at the 3 position of the phenyl ring (**7b** and **7g** respectively in [22]); however, these compounds were 5-fold less potent than **1** and 30-fold less than **3** tested here *in vitro*.

Prior studies have also indicated that bulkier substitutions at the C3 position, including 2-napthyl or quinoline and napthyl-methylene such as **6**, results in compounds that are more selective for kinases with a small Gly gatekeeper *vs.* Src and ABL kinases that have Thr gatekeepers [22]. For these substitutions, direct aryl linkages were again favored due to improved ability to selectively target the Gly residue in CDPK1 *vs.* Thr in mammalian kinases [20, 22]. However, inclusion of these bulkier ring structures results in a trade off, as they are less potent than **1** or **3** tested here against TgCDPK1 *in vitro* or against parasite growth *in vivo* (Table 2).

The observed potency of the substituted benzyl modifications in our studies suggests that the additional methylene linkage in the benzyl group is important for conferring flexibility of substitutions at the C3 position to fit optimally in the hydrophobic pocket, while still allowing the PP base to make two H-bond contacts to the hinge region. When the benzyl compounds are overlaid with the PP in the published crystal structures, our substitutions at the C3 position are consistent with the activity of C6 substitutions of the napthyl group in **5**, reported previously [21].

Additionally, prior studies revealed that modifications to the R2 group by addition of a piperidine ring via a methylene linkage to C1 of the PP scaffold increased potency against both parasite CDPK enzymes, while reducing potency for Thr containing kinases such as Src [22]. The improved specificity of this inhibition was attributed to a favorable salt-bridge formed between the N of the ring and a glutamate residue in the pocket of TgCDPK1 and CpCDPK1 [21]. We have not tested this modification in combination with the best derivatives found here, but future studies should reveal whether this combination further enhances the potency and specificity of PP for CDPK1.

Selectivity for PP analogs against CDPK1

The potency of PP analogs has been attributed to the natural occurrence of a small gatekeeper residue in TgCDPK1, where glycine occupies a position that is normally a bulky hydrophobic residue in most kinases [16, 17]. Previous studies have shown that conversion of this Gly residue to Met in TgCDPK1 renders the enzyme insensitive to **1** [10] as well as other PP analogs [12, 18]. To further explore the influence of amino acid side chains at this position of TgCDPK1 on the sensitivity to PP analogs, we generated a number of point mutants in the gatekeeper position and purified TgCDPK1 from *E. coli* for testing *in vitro*. All of these mutant alleles retained strong activity in phosphorylating the syntide-2 peptide *in vitro* and showed roughly similar Km values for ATP and overall catalytic efficiencies (Table 2). To compare their sensitivities to **1** they were each tested at their respective Km values for ATP and using the amount of enzyme that led to the half-maximal activity. Consistent with previous reports, conversion of the Gly residue to Met rendered TgCDPK1 completely insensitive to **1**, while alteration to Ala or Ser shifted potency by only ~ 3-5 fold, and substitution to Thr resulted in an additional 10-fold decrease in inhibition (Table 2). These findings reveal that while **1** has strong potency for the Gly containing gatekeepers it also has some activity against CDPK1 containing Ala or Ser at this position. Although we have not tested other amino acid substitutions, it is likely based on previous work [16, 17], that other charged or bulky hydrophobic residues would be highly similar to the Met substitution in terms of resistance to **1**. Consistent with this, human CamKIIα, which has a Phe residue at the gatekeeper position, was completely resistant to **1** (Table 2).

To further evaluate the specificity of PP analogs, we made use of a conditional knockout of CDPK1 (CDPK1-cKO) that constitutively expresses either naturally sensitive (Gly gatekeeper) or resistant alleles (Met substitution) of TgCDPK1, as described previously [10]. Treatment with anhydrotetracycline (Atc) results in shut down of the endogenous *CDPK1* gene, without disrupting expression of the Gly or Met alleles, which are comparably active and support normal parasite growth [10]. Following shutdown of TgCDPK1 with Atc treatment, treated parasites were evaluated using a host cell lysis assay to monitor parasite invasion and subsequent growth. We analyzed the four most potent PP analogs as defined above (Figure 2B). All four compounds showed strong inhibition of the parasites expressing the wild-type Gly gatekeeper allele of TgCDPK1, while parasites expressing the Met allele were almost 100-fold more resistant to PP analogs (Figure 3). These findings support the hypothesis that the major target of PP analogs in *T. gondi* is the naturally sensitive Gly gatekeeper TgCDPK1, although they do not rule out the possibility of off-target effects either at higher concentrations or with prolonged treatment.

Stability of PP analogs in the presence of rat liver microsomes

Although PP analogs are chemically stable *in vitro*, their ability to withstand metabolism by enzymes involved in detoxification *in vivo* remains untested. As a preliminary screen, we subjected a series of PP analogs to treatment with rat liver microsomes *in vitro*, and then assessed compound stability by mass spectrometry. For this analysis, we included one member of each of the compound pairs that showed high or medium activity *in vitro* (Figure 2B), plus several analogs that had been specially modified to potentially improve stability (Table 3). The parent compound **1** was extremely unstable, being converted to metabolites so rapidly that the clearance rate could not be estimated accurately (Table 3). The instability of this compound is likely due to the susceptibility of the benzylic methylene to cytochrome P450 metabolism (unpublished data). By contrast, analogs bearing halogen substitutions were more stable, such as **3** and 3-BrB (**4**) (Table 3). Addition of a second halogen group on the benzyl ring resulted in a further decrease in metabolic conversion as seen in **11**, which contains a second Cl group at position 2, or **10**, which contains an extra F atom (Table 3). However, not all doubly modified scaffolds showed this reduced metabolism in the presence of microsomes (Table 3). Modification of the methylene linkage between the benzyl group and the core PP moiety by addition of an OH or F side group (i.e. **25**, **26**) also led to enhanced stability (Table 3). However, these later two compounds lost potency against the enzyme and in blocking parasite growth (Table 3), potentially due to restricted rotation around the substituted methylene linker and resulting steric interference.

Potency of PP analogs in preventing toxoplasmosis in the murine model

Having established that modification of PP analogs can dramatically affect their potency in inhibiting TgCDPK1 enzyme *in vitro* as well as parasite growth and metabolism, we sought to test some of the best analogs in a murine model for toxoplasmosis. We utilized a well-established model for monitoring the efficacy of compounds against the acute infection based on challenge with high doses of a type II strain that has intermediate virulence in laboratory mice [26]. The mouse is a natural host for *T. gondii*, and following infection the parasite initially expands by replication within host cells and disseminates throughout the body, eventually resulting in death of the animal or immunological control that leads to chronicity [1]. For comparison, we chose the parent compound **1** along with **3** and two additional compounds that showed enhanced resistance to microsome degradation (i.e. **11**, **24**). Given the relatively rapid metabolism of these compounds *in vitro*, we reasoned that it would likely be necessary to provide repeated doses of the compounds, hence they were injected daily from day 1-10 at 5 mg/kg body weight. Although infection with *T. gondii* led to death in 60% of animals in the presence of vehicle (DMSO), treatment with PP analogs provided significant protection against infection (Figure 4A).

One of the key goals in developing treatments for toxoplasmosis is the ability to prevent chronic infection, or reactivation of parasites within tissue cysts. Therefore, we tested the surviving mice from the above treatment groups to ascertain their burden of chronic infection by examining brain homogenates for the presence of tissue cysts. All four PP analogs significantly reduced the number of tissue cysts found in the brain of chronically infected mice (Figure 4B). However, there was considerable variation in the reduction of cyst numbers seen between mice, especially in animals treated with **3** or **11**. In contrast, **24** provided much more potent and uniform protection against chronic cysts burden (Figure 4B). It is unlikely that the differences in potency or the failure to cure infection in the CNS is due to altered expression of he CDPK1 target as previous microarray studies have shown that tachyoites and bradyzoites express comparable levels of CDPK1 (http://ToxoDB.org).

At present it is uncertain if potency differences *in vivo* result from differences in the bioavailability of the compounds in various tissues including the CNS, enhanced stability against metabolism *in vivo*, or a combination of the above factors. However, one interesting feature that may underlie this increased potency is the relationship between hydrophilicity (LogP) and the stability of compounds in the microsome clearance assay (Figure 4C). Most compounds exhibited a trend of increased microsome stability with increasing LogP, which might result in nonspecific protein and/or membrane binding, leading to slower clearance (Figure 4C). However, molecules with LogP values above 3 (and below 1) are less likely to penetrate the blood brain barrier and are therefore less likely to have adequate CNS distribution [27, 28]. In contrast to this trend, **24** and **25** show increased microsome stability without a concomitant increase in LogP. Although **25** is inactive against *T. gondii* for reasons that are discussed above, **24** was the most effective in reducing the chronic burden of CNS infection. Despite having poor microsome stability and LogP > 3, compounds **1** and **3** were extremely potent *in vitro* (Table 1) and both were effective in preventing toxoplasmosis in mice (Figure 4A). Although **24** was much less potent *in vitro* (Table 1), its LogP value is closer to the optimal range for blood brain barrier penetration and it was better able reduce tissue cysts in the brain of chronically infected mice (Figure 4C). Taken together these observations highlight the importance of a combined approach in drug development whereby potency and favorable physical properties of drug molecules are simultaneously optimized.

A previous study reported that oral administration of 1-NM-PP (**6**) failed to protect mice against lethal challenge with the highly virulent RH strain, although 10-fold higher doses resulted in modest reduction in parasite burdens when administered by i.p. inoculation [29]. The higher efficacy in preventing lethal infection observed in the present study may be due to the use of a less virulent challenge with a type II strain, which cause the majority of human infections in North American and Europe [30] and thus may represent a more appropriate model for monitoring protection. Alternatively, the different efficacy may stem from different potency and bioavailability of the PP analogs. Compound **6** was less potent in blocking parasite growth *in vitro* than any of the inhibitors tested *in vivo* in the present study (Table 2). It is also possible that **6** does not have favorable *in vivo* properties since it contains the susceptible methylene linker between the PP scaffold and the unsubstituted naphthyl substituent. Despite this, previous studies have shown that **6** is capable of entering the CNS and targeting an inhibitor-sensitive variant of CamKIIα engineered to contain a small gatekeeper [31].

Overall, the best of the PP derivatives reported previously [20, 22] or described here, show potent inhibition of CDPK1 activity *in vitro* (IC_{50} values ranging from sub-nanomolar to low nanomolar), high activity in blocking parasite growth *in vitro* (EC_{50} ~ 1 μM) and minimal activity against other host enzymes (μM - high μM). A combination of features will need to be balanced with improved properties for *in vivo* activity. In particular, the susceptibility of PP analogs to metabolism highlights the importance of *in vitro* assays for metabolism, such as the microsome stability assay used here, as a means of profiling compounds for stability prior to *in vivo* testing. The compounds examined here have some suitable properties for *in vivo* administration, while others are not considered optimal, in particular for CNS penetration [28]. Positive attributes include satisfaction of Lipinski's rule of five - the relative small size (i.e. < 350 daltons), small number of heteroatoms (≤5), and LogP < 5. We are currently pursing further refinement in the chemical synthesis of PP derivatives to reduce LogP values to < 3.0, improve solubility, and increase half-life in the microsome clearance assay, thereby improving efficacy *in vivo*.

CONCLUSIONS

1. *Toxoplasma gondii* and *C. parvum* are unique in sharing a Gly gatekeeper in the essential kinase CDPK1, which controls microneme secretion and hence cell invasion and egress. By targeting this small gatekeeper residue with a series of PP analogs we have demonstrated excellent potency against the enzyme *in vitro* and in blocking growth of *T. gondii in vitro*.

2. Chemical genetic approaches validate that TgCDPK1 is the primary target of PP analogs in *T. gondii* and demonstrate that these agents act rapidly to prevent parasite infection *in vitro*.

3. Structural modification of PP analogs reveals the importance of a methylene linkage of the benzyl group in **1**, which is markedly improved in potency by substitution of Cl at the 3 position. Addition of halogens at other positions on the benzyl ring was largely tolerated, while bulkier substitutions often led to decreased activity.

4. Modification of the PP scaffold to enhance stability led to improved resistance to clearance in the presence of rat liver microsomes, and was associated with enhanced efficacy *in vivo*.

5. Several PP analogs show excellent promise for preventing acute and chronic infection, in particular **24**, which also has LogP < 3 and hence may favor CNS distribution. However, further optimization is will likely be required to optimize stability and *in vivo* distribution for controlling toxoplasmosis.

EXPERIMENTAL PROCEDURES

Chemical synthesis

Compounds **1** – **24** were synthesized using previously described procedures with slight modifications [14, 32], Scheme 1. Compounds **25** and **26** were synthesized using a varied route to furnish modification to the benzylic methylene group (Supplementary Scheme 1). Details on chemical synthesis, purity and spectral analysis for all compounds are provided in the Supporting Information.

Expression and purification of active kinases

Full length *CDPK1* was PCR amplified from a *T. gondii* RH cDNA library generated using the SMART cDNA synthesis kit (Clontech). The primers used (5′-GCGCATATGATGGGGCAGCAGGAAAGCAC and 5′-GCGCTCGAGGTTTCCGCAGAGCTTCAAGAGC) contained restriction sites that were used to directionally clone the PCR product, NdeI to XhoI, into the pET-22b(+) vector, in frame with a C-terminal hexahistidine tag. Single mutation of the codon corresponding to glycine 128 was achieved using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies), with specific primers designed according to manufacturer instructions. Plasmids were transformed into BL21(DE3)V2RpAcYc-LIC+LamP *E. coli*, which express the LamP phosphatase, as described previously [33]. Following overnight growth in Terrific Broth at 37°C, cells were diluted 1:50 in fresh medium and cultured for 3 h at 37°C, then cooled to 15°C, induced by addition of 1 mM IPTG, and cultured overnight. Cells were lysed in CelLyticB solution (Sigma Aldrich), and proteins purified using HIS-select Nickel Affinity Gel following manufacturers instructions (Sigma-Aldrich). Purified proteins were dialyzed (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.125% Chelex 100) and stored in 20% glycerol at -80°C. Protein purity and concentration were determined by SDS-PAGE followed by staining with SYPRO Ruby (Invitrogen).

Enzyme assays—Kinase assays were conducted using a peptide-based ELISA based on the syntide-2 peptide (Calbiochem). Syntide-2 peptide (10 mg/ml) was used to coat 96-well plates by overnight incubation in carbonate coating buffer (pH 9.6) at 4°C. Following washing in Tris tween (50 mM Tris-HCl, pH 7.5, 0.2% Tween20), plates were blocked with 3% BSA in Tris-tween for 2 h at room temperature, and further washing steps were conducted with Tris-tween. Kinase reactions were conducted at 30°C for 20 min in kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM DTT, 2.5 mM CaCl2, 0.1 mM EGTA, 0.005% Tween20) containing appropriate amounts of ATP (Km for each enzyme) and enzyme dilutions (see below). Phosphorylated syntide peptides were detected with mAb MS-6E6 (MBL Intl. Corp.,), followed by peroxidase-conjugated goat-anti-mouse IgG, developed with the substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) and detected by absorbance at 450 nm. The activity of human calmodulin dependent kinase II alpha (αCaMKII) was tested using the CaM Kinase II Assay CycLex kit (MBL Intl. Corp.,).

For testing different point mutants of CDPK1, purified enzymes were tested with increasing amounts of purified enzyme added to establish their half-maximal activity from a dose-response curve. The Km for ATP was determined for each enzyme tested at its half-maximum and by serial dilution of ATP. The sensitivity of wild type and mutant forms of CDPK1 were tested at their individual half-maximal activities and Km values for ATP. Samples were conducted in triplicate for all assays. For screening different PP analogs, serial dilutions of compounds were under the optimized conditions for CDPK1. The human calmodulin dependent kinase II (αCamKII) (Cyclex) was tested using the manufacturers recommendations. Data were analyzed using Prism (GraphPad) to determine IC_{50} values by plotting normalized, log-transformed data (X axis), using non-linear regression analysis as a sigmoidal dose response curve with variable slope.

Inhibition of parasite growth *in vitro*—Inhibition of parasite growth was determined using the 2F clone of the type I RH strain that expresses the *E. coli* β-galactoside enzyme (β-Gal), as described previously [34]. Compounds were dissolved in DMSO at 10 mM stock and diluted in medium containing 1% DMSO, which also served as a no compound control. Freshly harvested parasites were mixed with compounds and preincubated for 20 min at room temperature before being used to challenge confluent monolayers of human foreskin fibroblasts (HFF) grown in 96-well plates containing DMEM supplemented with 10% FBS. All samples were tested in triplicate. Following addition of 5×10^2 parasites / well containing dilutions of compounds and/or 1% DMSO, plates were centrifuged at ~ 300 *g* for 5 min and returned to culture at 37°C, 5% CO_2. To compare the efficacy of pyrimethamine *vs.* compound **1**, HFF cultures were challenged with parasites and compound dilutions, washed in warm PBS after 2, 4, 24 and 48 hours, and returned to culture in DMEM supplemented with 10% FBS at 37°C, 5% CO_2. To compare the effects of different PP analogs, HFF cultures were challenged with parasites and compound dilutions, washed in warm PBS after 4 h, and returned to culture in DMEM supplemented with 10% FBS at 37°C, 5% CO_2. Replication was stopped at 72 h by addition of 1% Triton X-100 and β-gal activity was determined following addition of 1 mM chlorophenol red-β-D-galactopyranoside and monitoring of absorbtion at 570 nm, as described previously [34]. Data were analyzed using Prism (GraphPad) to determine EC_{50} values by plotting normalized, log-transformed data (X axis), using non-linear regression analysis as a sigmoidal dose response curve with variable slope.

Monolayer lysis assay—A conditional knockout strain of CDPK1 (CDPK1-cKO), which expresses CDP1 under control of a tet-off promoter, complemented with either wild type or $G^{128}M$ CDPK1 expressed under its endogenous promoter [10], was used to assess the specificity of PP inhibitors. Parasite strains were grown for 72 h in the presence of 1 μg/ml anhydrotetracycline (ATc; Clontech), and kept in the presence of ATc for the course of the assay. Parasites were harvested and incubated 20 min at 37°C in DMEM containing 10% FBS and different compound concentrations or vehicle control (1 % DMSO). Confluent monolayers of HFF cells in 96-well plates were infected with the pre-treated parasites at a concentration of 10^5 per well, and sedimented onto the host cells by centrifugation at 300*g* for 2 minutes. Infection was allowed to proceed at 37°C, 5% CO_2, for 1 hour, prior to rinsing the monolayers 5 times with 37°C PBS to remove extracellular parasites. DMEM containing 10% FBS and 1 μg/ml ATc was added to all wells, and parasites were allowed to replicate for 72 hours. Host cell lysis was quantified by staining monolayers with crystal violet and measuring the absorbance at 570 nm.

***In vitro* microsome stability assays—**The stability of PP analogs *in vitro* was tested in using rat liver microsomes as performed by Absorption Systems Inc (Exton, PA). In brief, compounds were mixed with rat liver microsomes activated with 1 mM NADPH and loss of compound was followed at 10, 20, 30 and 60 min. The percentages of remaining compounds were calculated from the peak area ratio vs. standard for each starting compound by LC-MS. The half-life was estimated as $t_{1/2} = 0.693 / K$, where K is the slope of a plot of the natural log of percent of remaining compound *vs.* time. The intrinsic clearance (Cl_{int}) was calculated as k/D, where K is defined above and D is the protein concentration in the microsome preparation. LogP was estimated using ChemDraw Ultra.

Murine infection model of toxoplasmosis—To assess the ability of CDPK1 inhibitors to protect against lethal infection *in vivo*, we used a murine model and challenge with lethal doses of a type II strain of *T. gondii*, similar to previously described protocols [26]. Balb/C female mice at 8 to 10 weeks of age were injected i.p. with 10^4 PRU-Luc-GFP parasites (kindly provided by J. Boothroyd, Stanford University School of Medicine, CA) per animal. Compounds were reconstituted in dimethyl sulfoxide (DMSO) and diluted in PBS prior to injection into mice. Mice were treated beginning on the day of infection and continuing for ten days with daily i.p. injections of the specified compound at 1-5 mg/kg containing 5% DMSO, or DMSO control. Survival was monitored for 30 days following infection. After 30 days, animals were sacrificed, brains removed and homogenized, and tissue cysts were enumerated by microscopic examination after staining with fluorescently labeled lectin (*Dolichos biflorus*) as described previously [35]. Animals were maintained in an AAALAC-approved facility overseen by the Institutional Animal Care Committee at Washington University.

Statistics—Kaplan-Meier survival plots were generated in Prism (Graph Pad) and differences on survival compared using a Log rank (Mantel-Cox) test. Differences in chronic infection burdens were compared using a non-parametric Mann Whitney test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Atc	anhydrotetracycline
3-BrB-PP	3-bromo-benzyl pyrazolo [3,4-*d*] pyrimidine
3-ClB-PP	3-chloro-benzyl pyrazolo [3,4-*d*] pyrimidine
CDPK1	calcium-dependent protein kinase 1
EC_{50}	half-maximum effective concentration
IC_{50}	half-maximum inhibitory concentration
3-MB-PP	3-methyl-benzyl pyrazolo [3,4-*d*] pyrimidine
PP	pyrazolo [3,4-*d*] pyrimidine

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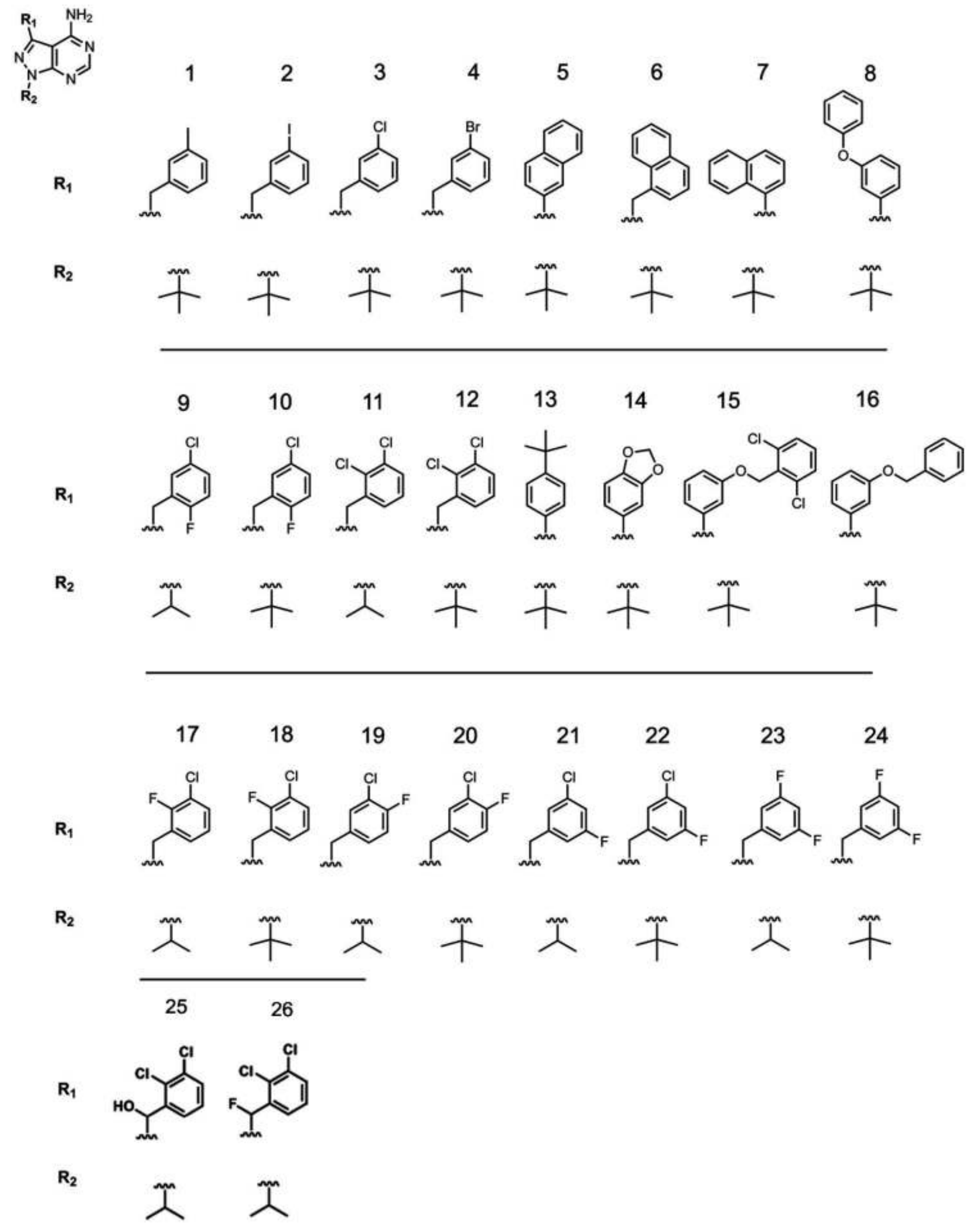

Figure 1.

Structures of PP analogs used in this study. The parent scaffold of pyrazolo [3,4-*d*] pyrimidine (PP) is shown at the top with side groups R1 and R2 indicated. The various analogs are listed based on their substitutions at R1 and R2.

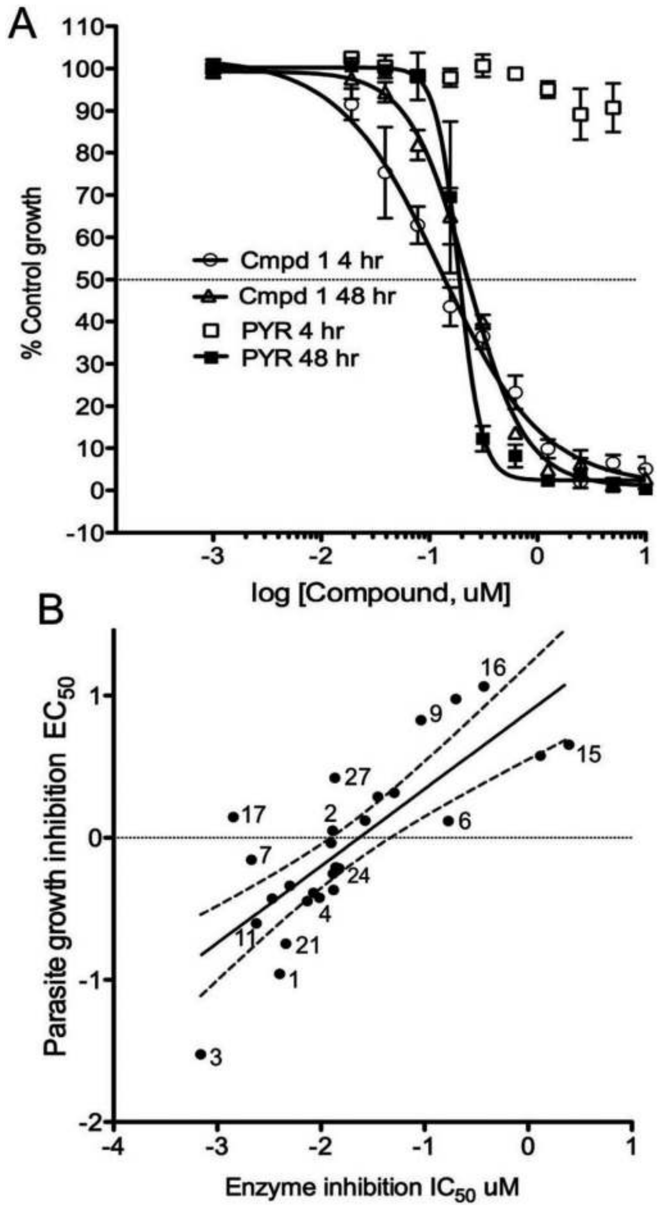

Figure 2.
Comparison of the potency and selectivity of compounds for inhibiting *T. gondii* growth *in vitro*. (A) Comparison of the potency of pyrimethamine (PYR) *vs.* compound **1** inhibiting *T. gondii* growth *in vitro* following a 4 h *vs.* 24 h treatment. Following addition of parasites to host cells and treatment with compounds for defined time intervals, monolayers were washed and returned to culture for 72 h when parasite growth was measured by β-gal activity. Activity at each compound dose is expressed as a % of growth in the absence of inhibition. Curves were fit using non-linear regression as sigmoidal dose responses. Representative of 4 or more similar assays, n = 4 replicates per data point. (B) Comparison of the relative potency of PP analogs based on inhibition of wild type TgCDPK1 enzyme activity *in vitro* (IC_{50}) *vs.* inhibition of parasite growth *in vitro* (EC_{50}). Values derived from Table 1. Plot represents linear regression with 95% confidence interval. The identities of selected inhibitors are shown.

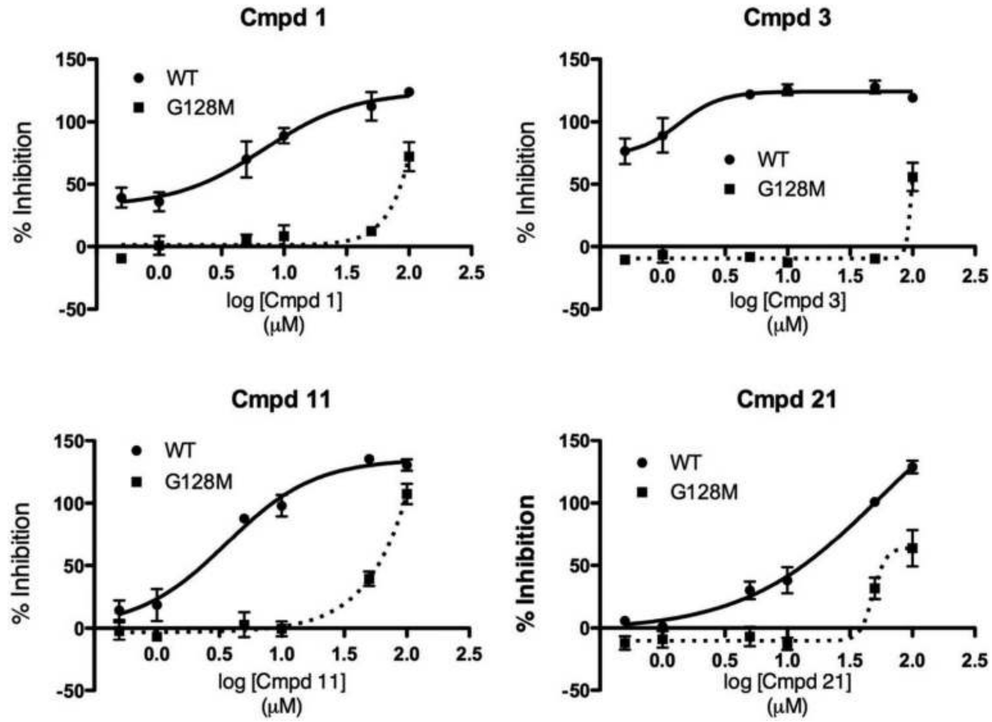

Figure 3.

Selectivity of PP analogs as shown by inhibition of *T. gondii* growth *in vitro*. Compound sensitivity of the conditional knockout of TgCDPK1 treated with Atc and expressing either wild type (WT, solid lines, circles) or PP-resistant mutant alleles of TgCDPK1 ($G^{128}M$, dashed lines, squares), as described previously [10]. Following addition of parasites to host cells and treatment with compounds for 1 h, monolayers were washed and returned to culture. Inhibition was monitored by lysis of host cell monolayers after 72 h of growth as detected by staining with crystal violet and monitoring absorbance at 570 nm. Inhibition is reported as % of control cultures treated with DMSO. Representative of 3 experiments, means ± s.d., n = 3 replicates.

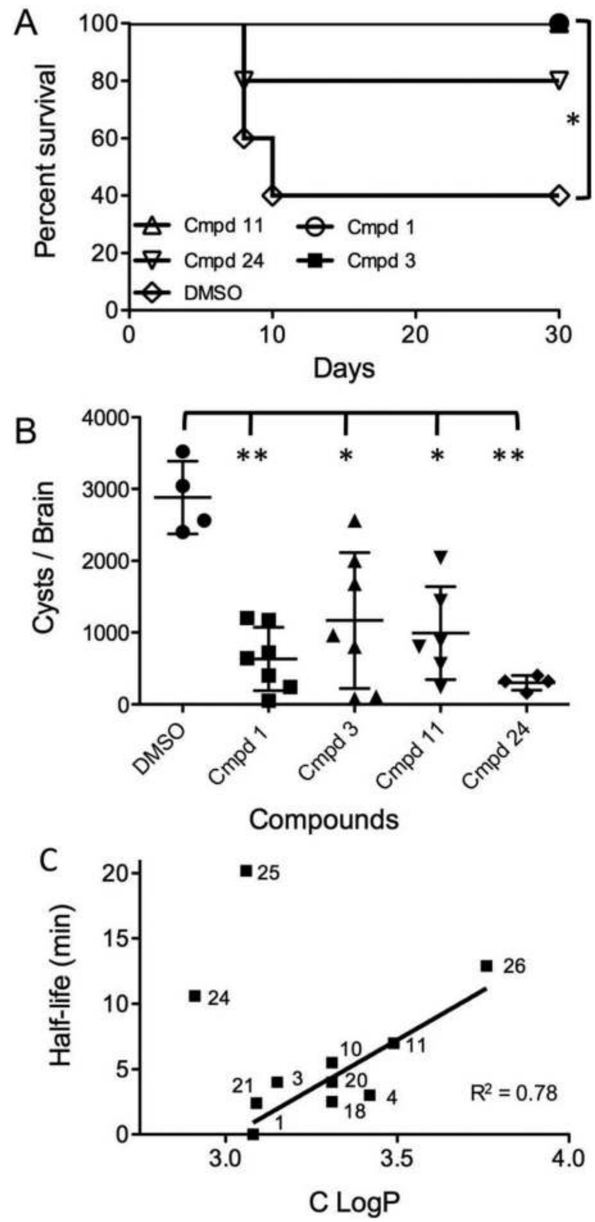

Figure 4.

Ability of PP analogs to prevent acute and chronic toxoplasmosis in the murine model. (A) Mice were infected with the type II Pru-LUC strain by i.p. injection with 10^4 tachyoites on day 1. Compounds were given by daily i.p. injection from days 0-10 (5 mg/ kg in 5% DMSO). Survival was followed for 30 days. n = 5 animal per group. Representative of three or more similar experiments. * $P \leq 0.05$, Log rank (Mantel-Cox) test. (B) Cyst burdens from surviving mice were determined at 30 days post-infection by microscopic examination of homogenates stained with fluorescently labeled lectin (*Dolichos biflorus*). * $P \leq 0.05$, ** $P \leq 0.01$, Mann Whitney test. (C) Relationship between lipophilicity and microsome clearance for a subset of compounds. Linear correlation for the majority of compounds shows increasing CLogP values are correlated with increasing resistance to clearance (Half-life (min)). Compounds **24**, **25** show an exception to this pattern. Linear regression line excludes the data points for **24**, **25**.

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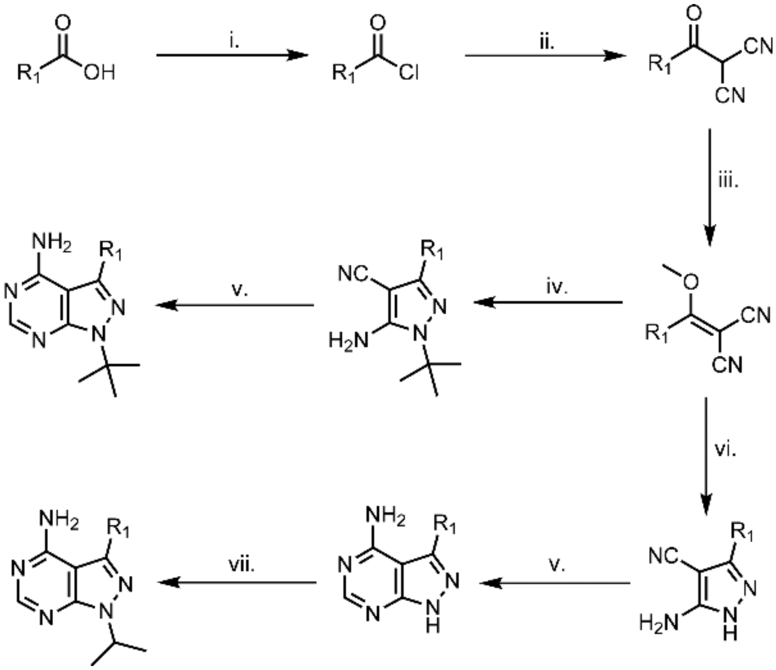

Scheme 1.

- **i.** oxalyl chloride, DMF, hexane, RT, 1 h;
- **ii.** NaH, malononitrile, THF, RT, 1 h;
- **iii.** dimethyl sulfate, $NaHCO_3$, dioxane/H_2O, reflux, 1 h;
- **iv.** NH_2NH_2 hydrate, EtOH, RT, 1 h;
- **v.** formamide, 160-180°C, 8 h;
- **vi.** t-$BuNHNH_2$, EtOH, reflux, 1 h;
- **vii.** 2-iodopropane, Na_2CO_3, DMF, RT, 8 h.

Table 1

Inhibition of enzyme activity and parasite growth by PP derivatives

Cmpd #	IC_{50} [μM][a]	EC_{50} [μM][b]
1	0.00399	0.11
2	0.01295	1.12
3	0.00069	0.03
4	0.00964	0.38
5	0.03541	1.94
6	0.16950	1.31
7	0.00213	0.70
8	0.02665	1.32
9	0.09236	6.73
10	0.01251	0.92
11	0.00238	0.25
12	0.00338	0.38
13	1.31000	3.77
14	0.01385	0.62
15	2.47100	4.53
16	0.37400	11.59
17	0.00143	1.40
18	0.00737	0.36
19	0.01311	0.56
20	0.00847	0.41
21	0.00459	0.18
22	0.01323	0.43
23	0.05085	2.07
24	0.01496	0.61
25	0.20020	9.46
26	0.01363	2.63

[a] *In vitro* inhibition of CDPK1 enzyme activity

[b] *In vitro* infection of HFF cells by wild type *T. gondii*

Table 2

In vitro enzyme activity and susceptibility to 3-MB-PP

Enzyme	Form	Enzyme Conc[a]	ATP Conc[b]	IC_{50}[c]
CDPK1	Wild type	19.0	11.0	4.1 ± 0.6
	$G^{128}M$	48.0	6.0	> 10,000
	$G^{128}A$	60.0	4.0	13.7 ±7.1
	$G^{128}S$	72.0	7.0	22.9 ± 7.5
	$G^{128}T$	72.0	30.0	221.8 ± 43.7
αCamK II	alpha	25.0	50.0	> 10,000

Activities determined using syntide-peptide ELISA as described in methods

[a] nM; determined as the half-maximal activity concentration

[b] μM; Km at half-maximal activity

[c] nM; mean ± S.D.

Table 3

In vitro stability in microsomes

Compound	CLogP[d]	*In vitro* microsome clearance assay[a]	
		$T_{1/2}$ (min)	Intrinsic clearance[b]
1	3.08	~	~
3	3.15	4.0	> 0.35
4	3.42	3.0	> 0.35
10	3.31	5.5	0.25
11	3.49	7.0	0.19
18	3.31	2.5	> 0.35
20	3.31	4.0	> 0.35
21	3.09	2.4	> 0.35
24	2.91	10.6	0.13
25	3.06	20.2	0.068
26	3.76	12.9	0.107

[c] Rat, following 5 mg/kg i.v. dose

[a] Rat liver microsome clearance *in vitro*

[b] mL / min / mg protein

~clearance was too rapid to estimate the rate

[d] LogP values were calculated using the LogP function in ChemDraw Ultra