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SAR Studies of 5-Aminopyrazole-4-carboxamide Analogues as Potent and Selective Inhibitors of *Toxoplasma gondii* CDPK1

Wenlin Huang,[†] Kayode K. Ojo,[‡] Zhongsheng Zhang,[†] Kasey Rivas,[‡] Rama Subba Rao Vidadala,[§] Suzanne Scheele,[∥] Amy E. DeRocher,[∥] Ryan Choi,[‡] Matthew A. Hulverson,[‡] Lynn K. Barrett,[‡] Igor Bruzual,[⊥] Latha Kallur Siddaramaiah,[†] Keshia M. Kerchner,[†] Matthew D. Kurnick,[#] Gail M. Freiberg,[#] Dale Kempf,[#] Wim G. J. Hol,[†] Ethan A. Merritt,[†] Georg Neckermann,[▽] Eugenio L. de Hostos,[▽] Nina Isoherranen,[○] Dustin J. Maly,[§] Marilyn Parsons,[∥,◆] J. Stone Doggett,[⊥] Wesley C. Van Voorhis,[*,‡,◆] and Erkang Fan[*,†]

[†]Department of Biochemistry, University of Washington, Seattle, Washington 98195, United States

[‡]Department of Medicine, Division of Allergy and Infectious Diseases, and the Center for Emerging and Re-emerging Infectious Diseases (CERID), University of Washington, Seattle, Washington 98109, United States

[§]Department of Chemistry, University of Washington, Seattle, Washington 98195, United States

[∥]Center for Infectious Disease Research (formerly Seattle Biomedical Research Institute), Seattle, Washington 98109, United States

[⊥]Portland VA Medical Center, Portland, Oregon 97239, United States

[#]AbbVie, N. Chicago, Illinois 60064, United States

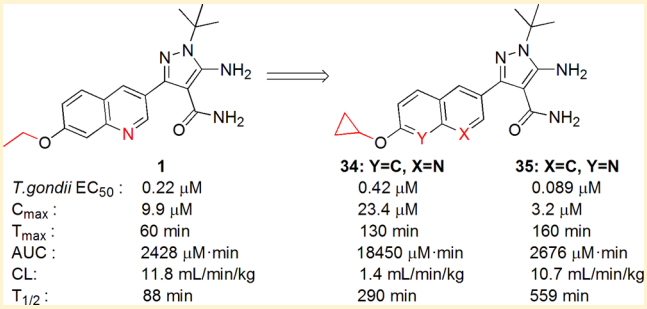
[▽]PATH, San Francisco, California 94102, United States

[○]Department of Pharmaceutics, University of Washington, Seattle, Washington 98195, United States

[◆]Department of Global Health, University of Washington, Seattle, Washington 98195, United States

Supporting Information

ABSTRACT: We previously discovered compounds based on a 5-aminopyrazole-4-carboxamide scaffold to be potent and selective inhibitors of CDPK1 from *T. gondii*. The current work, through structure–activity relationship studies, led to the discovery of compounds (**34** and **35**) with improved characteristics over the starting inhibitor **1** in terms of solubility, plasma exposure after oral administration in mice, or efficacy on parasite growth inhibition. Compounds **34** and **35** were further demonstrated to be more effective than **1** in a mouse infection model and markedly reduced the amount of *T. gondii* in the brain, spleen, and peritoneal fluid, and **35** given at 20 mg/kg eliminated *T. gondii* from the peritoneal fluid.



KEYWORDS: *Toxoplasma gondii, calcium-dependent protein kinase-1, enzyme inhibitor, structure–activity relationship studies*

Toxoplasma gondii is an apicomplexan protozoan that infects humans and domesticated animals. Infection by *T. gondii* presents major health concerns in immunocompromised patients.[1] Current therapies have some shortcomings,[2] thus new and effective therapy for treating *T. gondii* infection is needed.

The life cycle of *T. gondii* is regulated in part by a family of calcium-dependent protein kinases (CDPKs) that have no direct homologues in humans.[3,4] *T. gondii* CDPK1 (*Tg*CDPK1) has been shown both genetically and chemically to be critical for parasite reproduction, specifically in the process of cell invasion and egress.[3,5−7] Furthermore, *Tg*CDPK1 contains a glycine residue at the "gatekeeper" position, which is extremely rare for mammalian kinases.[8−10] The critical role of CDPK1 in combination with its unique active site gatekeeper makes this kinase an attractive target for antiparasitic drug discovery.[3,8,9,11−13] Three different chemical scaffolds of CDPK1 inhibitors have been reported previously: the pyrazolopyrimidine (PP) scaffold,[14−17] the acylbenzimidazole scaffold,[18,19] and the 5-aminopyrazole-4-carboxamide (AC) scaffold.[20] Overexpression of CDPK1 with a methioinine replacing the glycine mutant conveys resistance to certain inhibitors based on the PP scaffold.[8] However, it should be recalled that *T. gondii* is not transmitted from person to person (except in rare congenital acquisition), so that should resistant parasites arise, they would

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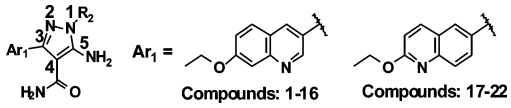
be confined to the treated individual. Preliminary structure−activity relationship (SAR) studies of the AC scaffold found a promising inhibitor (**1**, Table 1) which inhibited *Tg*CDPK1 with an IC_{50} value of 2.0 nM and exhibited 0.22 μM EC_{50} in the *T. gondii* cell assay.[20] However, its relatively low solubility, short half-life, and relatively high oral clearance could limit its further development. In this report, we describe further SAR studies of the AC scaffold based on compound **1**, which resulted in the discovery of compounds with improved aqueous solubility, potency, and/or pharmacokinetic (PK) properties.

Table 1. SAR Study of N1-Substitution

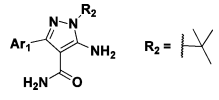


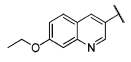
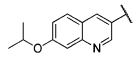
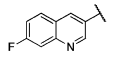
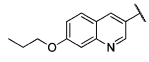
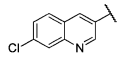
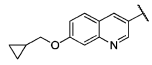
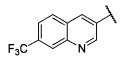
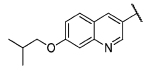
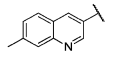
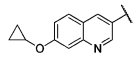
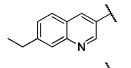
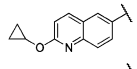
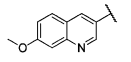
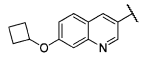
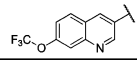
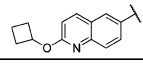
Compound Number	R_2	TgCDPK1 IC_{50}(nM)	Compound Number	R_2	TgCDPK1 IC_{50}(nM)
1		2.0±0.3	**12**		80±32
2		8.0±2.9	**13**		26±10
3		31±13	**14**		113±59
4		21±1	**15**		20±3
5		13±5	**16**		11±2
6		15±3	**17**		6.0±0.7
7		12±0	**18**		6.5±1.6
8		11±2	**19**		7.9±3.2
9		8.0±2.2	**20**		5.5±1.9
10		31±3	**21**		17±8
11		6.0±1.6	**22**		7.0±2.6

In the previous study most variations at N1 position were carried out with Ar_1 (on C3) being fixed as 2-naphthyl group, and for analogues containing other heteroaryl groups C3, only *t*-butyl analogues at N1 position were made.[20] Therefore, we reinvestigated a larger panel of N1 substitutions, while first fixing a 7-ethoxyquinolin-3-yl group at C3 (Table 1). Compounds with aliphatic chains (**2**−**5**) or rings (**6**−**11**) at the N1 position did not improve enzyme potency over **1**. Hydroxyl aliphatic chains were also introduced to decrease lipophilicity of compounds, resulting in compounds (**13**−**16**) with comparable activity relative to the hydroxyl-free compounds. Though the potency did not improve during the investigation of the N1 substitutions, some of the substituents, especially the hydroxyl aliphatic chains, improved the solubility of compounds and provided the opportunity to optimize the PK properties. Additional study of N1 substitutions was conducted with a 2-ethoxyquinolin-6-yl group at C3. All the compounds (**17**−**22**) exhibited low nanomolar potency against *Tg*CDPK1, indicating that the quinolin-6-yl moiety was as well accommodated in the hydrophobic pocket as the quinolin-3-yl. These two Ar_1 alternatives may lead to potent compounds with different pharmacological properties.

Because the *t*-butyl group seems to be the optimal N1 substitution, it was fixed at the N1 position, while we investigated the C3 substituent (Table 2) with the goal to optimize PK properties. Replacement of the ethoxy group on the 7-position of the quinolin-3-yl with fluorine, chlorine, trifluoromethyl, or alkane groups all produced decreased potency (**23**−**26**). Coming back to the alkoxy substitutions, small sized chain- or ring-alkoxy groups were potent inhibitors (**28**, **31**, **34**, and **36**), while the trifluoromethoxyl led to decreased activity. Similarly, the quinolin-7-yl analogues with 2-cyclopropoxy and 2-cyclobutoxy substitutions (**35** and **37**) were also potent for *Tg*CDPK1 inhibition.

Table 2. SAR Study of C3-Substitution



Compound Number	Ar_1	TgCDPK1 IC_{50}(nM)	Compound Number	Ar_1	TgCDPK1 IC_{50}(nM)
1		2.0±0.3	**30**		16±6
23		6.0±2.1	**31**		5.0±0.6
24		36±6	**32**		16±3
25		247±63	**33**		86±24
26		16±8	**34**		10±1
27		115±26	**35**		4.6±0.9
28		5.0±3.0	**36**		7.0±3.3
29		39±15	**37**		12±6

The consistent binding pose of the AC scaffold in the presence of chemical elaboration at Ar_1 and R_2 was confirmed by crystal structures of selected compounds bound to *Tg*CDPK1. In detail, the amine group and the oxygen atom of the amide group make hydrogen bonds to the hinge region of *Tg*CDPK1, projecting the Ar_1 group at the C3 position into the hydrophobic pocket adjacent to the gatekeeper residue and the R_2 group at the N1 position into the ribose-binding pocket. In particular the bound position of the Ar_1 quinoline is not significantly perturbed by the position of the ring N, but its precise orientation is constrained by the need to accommodate a distal cyclopropyl (compounds **34** and **35**) or cyclobutyl (compounds **36** and **37**) between *Tg*CDPK1 residues Leu 114 and Leu 198 (Figure 1). Co-crystallizations and structure determinations were performed as previously described.[14,20] Crystal structures showing the bound state of compounds **1**

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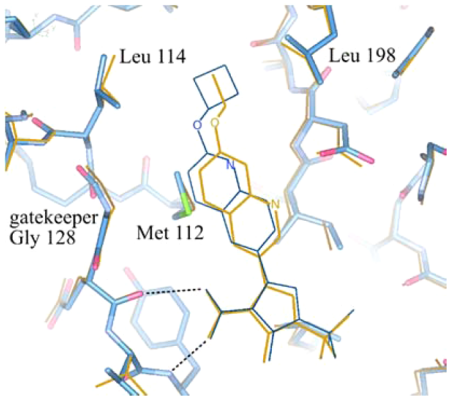

Figure 1. Superposition of crystal structures of *Tg*CDPK1 in complex with **1** (gold) and **37** (blue).

and **37** have been deposited in the PDB (entry codes 4ONA and 4YJN).

A select group of potent inhibitors were evaluated in a variety of assays to assess aqueous solubility (see Supporting Information), selectivity over mammalian kinases, potency against parasite cells, cytotoxicity to mammalian cells (Table 3), and, for select compounds, PK properties after oral (PO) dosing in mice (Table 4).

As a preliminary evaluation of the selectivity of the AC analogues, the top compounds were tested for inhibition of the human kinase SRC that contains a small gatekeeper residue using reported procedures.[16] As shown in Table 3, none of the top compounds except compounds **18** and **34** displayed significant inhibition of SRC activity at concentrations up to 10 μM. Though compound **34** showed an IC_{50} of SRC inhibition at 4.75 μM, the selectivity of *Tg*CDPK1 over SRC was more than 450-fold.

We investigated the efficacy of compounds against *T. gondii* cells (Table 3). With 7-ethoxyquinolin-3-yl at C3 position, the modification of the *t*-butyl group merely resulted in reduced potency compared to **1**. However, the C3 2-ethoxyquinolin-6-yl compounds were generally more potent than their 7-ethoxyquinolin-3-yl analogues, while compounds **18**, **17**, and **22** exhibited comparable potency to **1**. Modification of the 7-ethoxy group of quinolin-3-yl moiety resulted in compounds mostly with lower potency. Even the best compounds with this modification, the cyclopropoxy (**34**) and cyclobutoxy (**36**) substituted analogues, showed at least 2-fold less potency compared to **1**. Interestingly, for the last two quinoline pairs in Table 3, while the cyclobutoxy substituted pairs (**37** and **36**) showed similar potencies, the cyclopropoxy substituted pairs (**35** and **34**) were quite different, as **35** was ~5-fold more potent than **34**. Compound **35** was the most potent inhibitor found in this study, with increased activity against parasites compared to **1**. As an indicator of selectivity at cellular level, we measured the cytotoxicity of these inhibitors (Table 3) using human lymphocyte cell line (CRL-8155).[16] These compounds showed no significant inhibition of cell growth at concentrations up to 30 or 40 μM.

Select compounds were subjected to PK studies in mice following oral administration at 10 mg/kg (Table 4). Compounds **9** and **17** had a higher oral clearance than compound **1** resulting in lower maximum concentration (C_{max}) and total exposure (area under the curve, AUC) compared to **1**. This is likely due to lower bioavailability of compounds **9** and **17** as their half-lives were longer than that of compound **1**. Compound **22** had greatly improved solubility but had nearly 16-fold higher oral clearance than compound **1** resulting in significantly diminished C_{max} and AUC in comparison to **1**. Compound **32** exhibited improved PK profile though the potency is relatively poor. Other analogues with small alkyl rings on quinoline oxygen are **34** and **36** and their quinolin-6-yl counterparts **35** and **37**. While the cyclobutoxy compound **36** with poor solubility did not show improvement on PK properties, the cyclopropoxy compound **34** with improved solubility did show a significantly improved PK profile. The oral clearance of compound **34** (1.4 mL/min/kg) was the lowest of the tested compounds and about 8-fold lower than that of compound **1** (11.8 mL/min/kg). This decrease in CL was associated with a 3-fold longer half-life and 2.3-fold higher C_{max}. The exposure of compound **34** was 8-fold higher (Figure 2) in comparison to **1** likely due to both better bioavailability and lower systemic clearance (Figure 2). The PK profile of **35** was similar to **1** in terms of AUC and oral clearance (10.7 mL/min/kg). However, **35** is still promising considering its much longer $T_{1/2}$ (559 min) compared to **1** (88 min) and **34** (290 min) and its high potency against *T. gondii* (EC_{50} = 0.089 μM). The

Table 3. Comprehensive Evaluation of Top Inhibitors[a]

compd number	SRC IC_{50} (μM)	*T. gondii* EC_{50} (μM)	cytotoxicity (μM)	compd number	SRC IC_{50} (μM)	*T. gondii* EC_{50} (μM)	cytotoxicity (μM)
1	>30	0.22 ± 0.09	>30	**21**	ND	0.32 ± 0.02	ND
2	>30	0.72 ± 0.10	ND[b]	**22**	>10	0.21 ± 0.04	>40
6	>10	1.21 ± 0.14	>40	**23**	>30	2.25 ± 0.94	>40
7	>10	0.52 ± 0.003	>40	**28**	>30	0.40 ± 0.04	>40
8	>10	0.97 ± 0.40	>40	**30**	ND	1.21 ± 0.42	>40
9	>10	0.33 ± 0.07	ND	**31**	>10	0.69[c]	>40
11	>10	0.41 ± 0.0	>40	**32**	>10	1.47[c]	>40
16	>10	1.19 ± 0.14	>40	**34**	4.75	0.42 ± 0.10	>40
17	>10	0.20 ± 0.09	>40	**35**	>10	0.089 ± 0.044	>40
18	3.3−10	0.14 ± 0.003	>40	**36**	>10	0.48 ± 0.09	>40
19	>10	0.48 ± 0.07	>40	**37**	>10	0.48 ± 0.06	>40
20	>10	0.43 ± 0.01	>40				

[a]Selectivity at enzyme level was conducted using a human kinase (SRC) that has a small gatekeeper residue; efficacy against parasites was performed using a *T. gondii* growth assay,[15] values shown are the average ± standard deviation from two or more experiments; and cytotoxicity to mammalian cells was measured using the CRL-8155 cell line. Experimental details are provided in the Supporting Information. [b]ND: not determined. [c]Value from one experiment.

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Table 4. PK Profiles for Select Inhibitors[a]

compd	C_{max} (μM)	T_{max} (min)	AUC (μM·min)	CL (mL/min/kg)	$T_{1/2}$ (min)
1	9.9 ± 2.9	60 ± 52	2428 ± 277	11.8 ± 0.5	88 ± 19
9	4.6 ± 1.0	80 ± 35	1608 ± 182	16.4 ± 1.5	204 ± 34
17	5.3 ± 0.6	80 ± 35	1694 ± 379	16.7 ± 3.8	134 ± 28
22	2.4 ± 2.0	30 ± 17	140 ± 97	186 ± 75	<30
32	7.1 ± 0.6	140 ± 92	3713 ± 1073	7.1 ± 0.7	549 ± 131
34	23.4 ± 3.1	130 ± 17	18450 ± 470	1.4 ± 0.04	290 ± 48
35	3.2 ± 0.7	160 ± 69	2676 ± 784	10.7 ± 1.0	559 ± 209
36	4.1 ± 1.5	30 ± 0	458 ± 206	57 ± 4	42 ± 17

[a]Mice dosing at 10 mg/kg, po. Reported as average ± standard deviation of measurements from three mice.

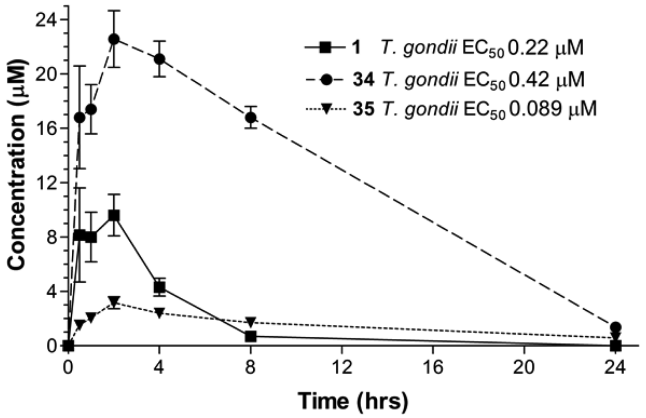

Figure 2. Comparison of oral PK curves of compounds **1**, **34**, and **35**.

human plasma protein binding properties of these three compounds were also measured, and all three compounds showed low protein binding with unbound fractions of 57% for **1**, 84% for **34**, and 88% for **35**. Based on the overall properties, **34** and **35** were selected for testing in a mouse model of *T. gondii* infection in comparison to **1**. All the three compounds get into the CNS, as estimated by a single time point, comparing plasma with brain tissue concentrations, where we found a brain/plasma ratio of 15.8% for **1**, 16% for **34**, and 43% for **35**.

In vivo efficacy of **34** and **35** was tested using a model of acute *T. gondii* infection with the Type I RH strain that is uniformly fatal in mice. Treatment was started 2 days after *T. gondii* inoculation to allow establishment of a robust systemic infection.[21] Compounds **1**, **34**, and **35** were administered via oral (po) dosing for 5 days. Compound **1** significantly reduced *T. gondii* infection (Figure 3A). However, **34** and **35** were much more efficacious than **1**, reducing *T. gondii* in the peritoneal fluid and spleen to near-undetectable levels at comparable or lower does than **1** (Figure 3B,C). Compound **34**, given at 50 mg/kg and 20 mg/kg, reduced the mean concentration of *T. gondii* in the peritoneal fluid by 99.6% and 93.4%, respectively. Compound **35**, given at 20 mg/kg, decreased *T. gondii* below the limits of detection (<100 parasites/mL). Similarly, **34** and **35** reduced the mean number of *T. gondii* in the spleen to less than 1% of controls. Both compounds also demonstrated more than 5-fold reduction in the mean concentration of detectable *T. gondii* DNA in the brain, suggesting that either both **34** and **35** achieve brain concentrations during active infection that are sufficient to inhibit *T. gondii* replication or **34** and **35** prevent brain infection from progressing by inhibiting systemic toxoplasmosis. None of the treated mice showed signs of infection or toxicity such as weight loss, altered grooming, or lethargy. However, one mouse in the **35**, 50 mg/kg group died on the fifth day of treatment, but the cause of death was not established. Based on the elimination of *T. gondii* in the peritoneal fluid by **35** and the trends of better efficacy in the brain and spleen, it appears that **35** is more potent than **34**. These experiments demonstrate that **34** and **35** are highly active against fulminant experimental toxoplasmosis.

Additional studies were performed using these two lead compounds **34** and **35** to address (1) the selectivity of AC series analogues beyond the single human kinase SRC in the preliminary study and (2) if the inhibitors acted on-target in parasite cells. For selectivity, we performed a fluorescence based binding inhibition assay as previously described[22] using a panel of ~80 diverse human kinases.[23] Compound **34** did not show any IC_{50} below 1 μM. Compound **35** only showed IC_{50}s below 1 μM for three of the 80 kinases (0.2−0.5 μM). This indicated that the top two compounds are quite selective against *Tg*CDPK1. A table of the binding inhibition results covering a selected group of 20 kinases is provided in the Supporting Information.

To study if the inhibitors acted on-target in parasite cells, we compared parasite growth inhibition against the parental cell line, a cell line transfected with a *Tg*CDPK1 expression plasmid, and a cell line transfected with a similar expression plasmid bearing CDPK1 with a G128M mutation at the gatekeeper position as described previously.[8] The results (see Supporting Information for additional figures) showed that neither **34** nor **35** produced significant shifts in parasite growth inhibition at low compound concentrations. However, these two compounds could not completely suppress parasite growth at high concentrations in the cell line containing the G128M mutant of *Tg*CDPK1. This suggests that the two leads only acted partially on-target, and therefore, testing AC series inhibitors with weaker IC_{50} against *Tg*CDPK1 may still lead to potent inhibitors at the parasite level. Additional study will be required to identify other potential cellular targets of the AC series inhibitors.

In summary, we described the structural optimization of 5-aminopyrazole-4-carboxamide derivatives as *Tg*CDPK1 inhibitors based on compound **1**. We evaluated over 30 analogues as potent and selective *Tg*CDPK1 inhibitors. Most of the compounds showed low nanomolar activity against the enzyme. These compounds also showed good efficacy against *T. gondii* cells, with the best compound **35** exhibiting an EC_{50} of 89 nM. Furthermore, no compound showed significant toxicity to mammalian cells at concentrations up to 30 μM. Compounds **34** and **35** also exhibited improved solubility and good pharmacokinetic properties. Compared to compound **1**, both **34** and **35** had improved efficacy in a mouse model of

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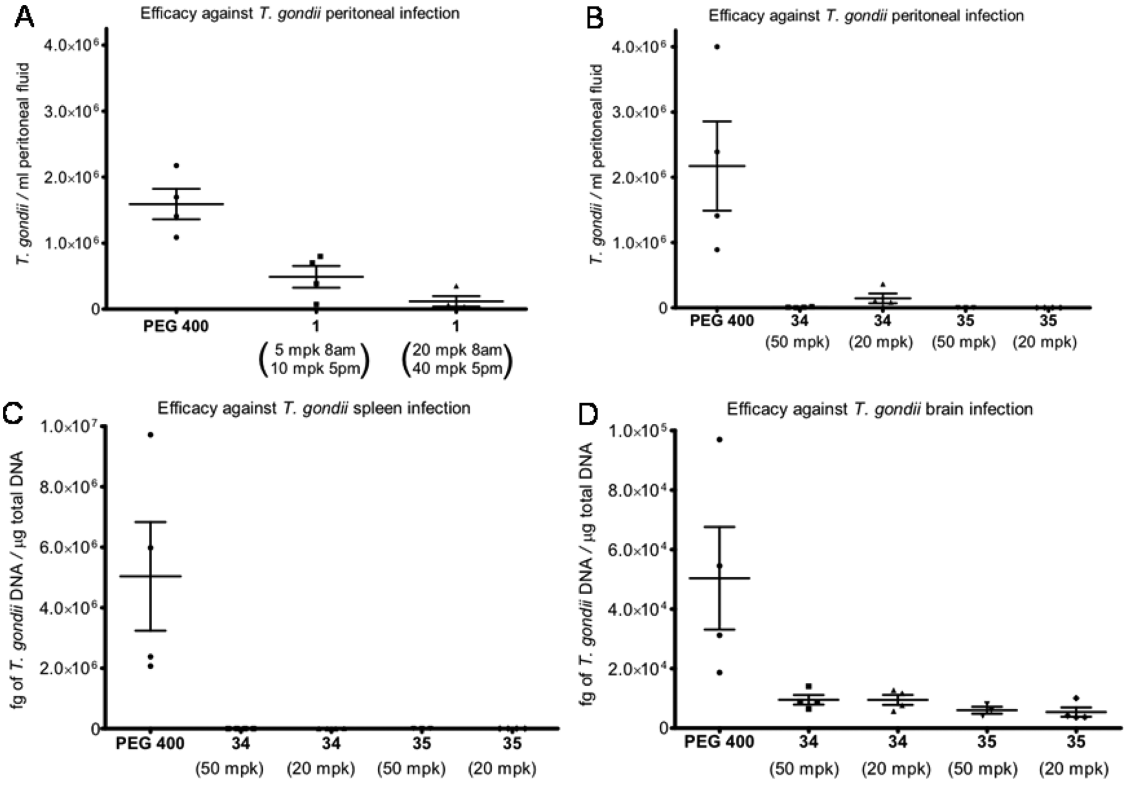

Figure 3. Efficacy of **1**, **34**, and **35** evaluated by measurement of *T. gondii* in peritoneal fluid, spleen, and brain. Mice were analyzed 1 day after the last dose. Groups consisted of 4 mice. (A) Efficacy of compound **1**, peritoneal parasite count. Due to its short half-life **1** was dosed twice daily. The higher dose reduced parasites by >10-fold compared to controls. Efficacy of compounds **34** and **35** (single daily dose): (B) peritoneal parasite count, (C) qRTPCR of spleen, and (D) qRTPCR of brain. The differences between all treatment groups and their respective controls and the difference between **34** and **35** on peritoneal *T. gondii* infection were statistically significant, while the differences between all other compared treatment groups were not statistically significant using a p-value of <0.05. Bars represent the mean and the standard error of the mean. PEG = polyethylene glycol; mpk = mg/kg; qRTPCR = quantitative real-time PCR. *Three mice in **35**, 50 mg/kg group were analyzed.

toxoplasmosis. These studies provide new lead compounds for further development of drugs for toxoplasmosis therapy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00319.

Synthesis and characterization data of compounds, biological assays, and structural studies (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: erkang@uw.edu.
*E-mail: wesley@uw.edu.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AC, 5-aminopyrazole-4-carboxamide; BKI, bumped kinase inhibitor; CDPK1, calcium-dependent protein kinase-1; PK, pharmacokinetic; PP, pyrazolopyrimidine; SAR, structure−activity relationship; *Tg*, *Toxoplasma gondii*

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