Dual-Stage Picolinic Acid-Derived Inhibitors of *Toxoplasma gondii*

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T. gondii tachyzoites | *T. gondii* bradyzoites

Improved logD
Improved solubility
Growth inhibition:
tachyzoite EC_{50} = 0.32 μM
bradyzoite EC_{50} = 0.23 μM
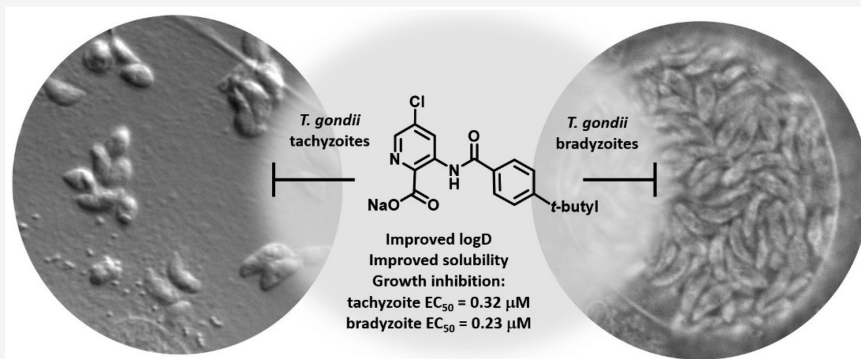
ABSTRACT: *Toxoplasma gondii* causes a prevalent human infection for which only the acute stage has an FDA-approved therapy. To find inhibitors of both the acute stage parasites and the persistent cyst stage that causes a chronic infection, we repurposed a compound library containing known inhibitors of parasitic hexokinase, the first step in the glycolysis pathway, along with a larger collection of new structural derivatives. The focused screen of 22 compounds showed a 77% hit rate (>50% multistage inhibition) and revealed a series of aminobenzamide-linked picolinic acids with submicromolar potency against both *T. gondii* parasite forms. Picolinic acid **23**, designed from an antiparasitic benzamidobenzoic acid class with challenging ADME properties, showed 60-fold-enhanced solubility, a moderate $LogD_{7.4}$, and a 30% improvement in microsomal stability. Furthermore, isotopically labeled glucose tracing revealed that picolinic acid **23** does not function by hexokinase inhibition. Thus, we report a new probe scaffold to interrogate dual-stage inhibition of *T. gondii*.

KEYWORDS: *Toxoplasma gondii*, picolinic acid, parasite hopping, inhibitor, multistage

Toxoplasma gondii is responsible for a commonly acquired parasitic infection that is estimated to affect 30−50% of the human population.[1] Mainly contracted through consumption of contaminated food or water,[2] *T. gondii* parasites rapidly proliferate in the tachyzoite stage, causing an acute infection that recruits a robust host immune response. Chemotherapeutic or immune pressure induces tachyzoite parasites to differentiate into metabolically quiescent bradyzoite cysts that evade the host immune system and are impenetrable to current first-line therapeutics.[2,3] Failure to clear parasites from the host results in a lifelong opportunistic chronic infection with significant risk of fatal complications by reactivation in immunocompromised patients.[4] While immunocompetent individuals who become infected are typically asymptomatic or may suffer self-limiting flulike illness, ocular lesions have been reported.[5] Maternal infection during pregnancy can lead to miscarriage or visual or motor disabilities in newborns.[6−8] The acute infection, caused by *T. gondii* tachyzoites, is frequently treated with a synergistic combination of the antifolate drugs pyrimethamine and sulfadiazine.[9,10] Unfortu-

nately, significant side effects[11−13] and resistance[14] have been reported with use of these drugs, and persistent bradyzoite parasites evade this therapy.[15,16] Despite new development[17−24] and drug repurposing efforts,[25−28] effective treatments for *T. gondii*, especially those capable of clearing the bradyzoite stage of the parasite, remain an important unmet medical need.[15,29−31] We previously described a series of benzamidobenzoic acids, exemplified by **1** (Figure 1), that inhibited the glycolytic hexokinase enzyme of the kinetoplastid parasites *Trypanosoma brucei* and *Leishmania major*.[32]

Through broader screening, we determined that unrelated parasites may be inhibited by these same compounds, though
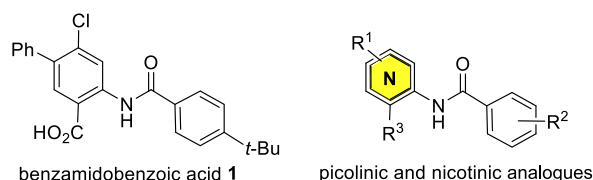
Figure 1. Probe **1** and next-generation aza analogues.

the mechanism may not be shared. For instance, when our compound library was assessed against the glucokinase of the pathogenic free-living protozoa *Naegleria fowleri*, a neuro-invasive amoeba that causes a rare but highly lethal brain infection, hits were identified, and distinct structure–activity relationships (SARs) emerged from testing these compounds against various parasites.[33,34] This parasite-hopping approach[26,35−37] reveals opportunities for therapeutic intervention and divergent scaffold optimization due to differences in SARs across a spectrum of familial and distantly related parasites. With this in mind, we curated a small library of 22 compounds from our collection that included compound **1**, several related analogues, and a collection of new picolinic and nicotinic acid derivatives that evolved from the benzamido-benzoic acids (Table 1S).

Compounds were evaluated against both tachyzoite[38] and bradyzoite[39] forms of the *T. gondii* parasite in separate in vitro screens. Briefly, human foreskin fibroblast (HFF) host cells harboring either *T. gondii* tachyzoites or in vitro-differentiated bradyzoites were treated with each of the compounds at a single compound concentration of 10 μM.[40] Pyrimethamine, which is known to be active against the tachyzoite stage but not the bradyzoite stage, was included as a control (Figure 2).[41] Compound **1** displayed >98% growth inhibition for both
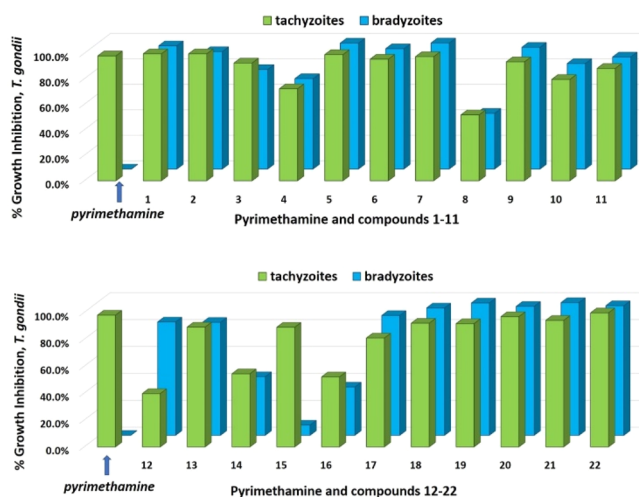

Figure 2. Percent growth inhibition of *T. gondii* tachyzoites and bradyzoites with compounds **1−22** at 10 μM.

T. gondii parasite stages at 10 μM, and several of the related 1-arylbenzamidobenzoic acids (compounds **18−22**) also resulted in high levels of inhibition (see Figure 2 and Table 1S). These compounds provided some initial SAR insights; however, their high lipophilicity (cLogD$_{7.4}$ = 5.0−7.4) limited their solubility and development potential. We were encouraged to see that many of the picolinic acid derivatives were also active, as these compounds comparatively had improved physiochemical properties.

The synthesis of several of the benzamidobenzoic acids[32] has been previously disclosed, and new analogues in that series followed similar protocols (see the Supporting Information). The sequence of steps was modified, but the picolinic acid derivatives were generally prepared by benzoylation of 3-amino-5-chloropicolinic acid esters followed by Suzuki coupling when a bromine atom was installed at C6 (Scheme 1). Hydrolysis of the ester functionality revealed the desired

Scheme 1. Synthesis of Picolinic Acid Derivatives[a]
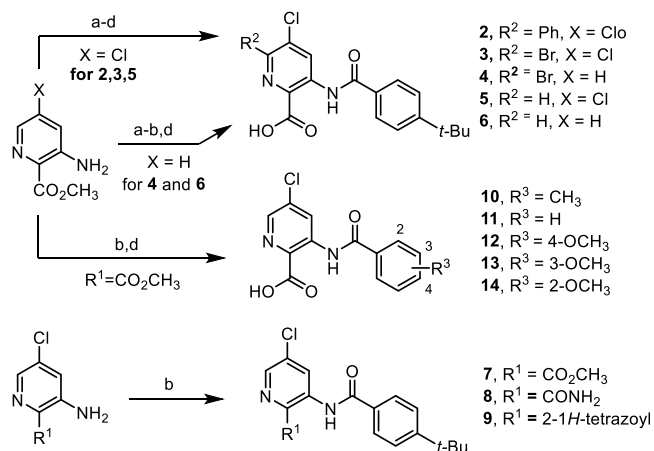
[a]Reagents and conditions: (a) 2.6 M Br$_2$ in AcOH, 2 M H$_2$SO$_4$, rt, 4−72 h, 51−55%; (b) ArCOCl, Et$_3$N or *i*-Pr$_2$EtN, THF or CH$_3$CN, rt to 150 °C, 30−89%; (c) PhB(OH)$_2$, Na$_2$CO$_3$, Pd(PPh$_3$)$_2$Cl$_2$, 10:1 CH$_3$CN/H$_2$O, 100 °C, 0.5 h, 67%; (d) 1.3 M aq LiOH, 3:2 THF/H$_2$O, rt, 12−48 h, 31−93%.

acids (compounds **2−6** and **10−14**). In some cases, replacement of the carboxylic acid with a bioisostere was surveyed by using the appropriately substituted picolinic derivative and employing the same benzoylation procedure (compounds **7−9**). Nicotinic acid analogues **15** and **16** were generated by similar methods (see the Supporting Information).

In parallel with compound **1**, these second-generation aza analogues **2−16** were subsequently evaluated in dose–response-formatted growth inhibition assays for each stage of the *T. gondii* parasite.[38,39] General cytotoxicity was assessed in uninfected HFF cells over 72 h for comparison with the parasitic assays performed in the same cell line (Table 1). Our benchmark, compound **1**, inhibited both tachyzoites and bradyzoites with similar submicromolar potency. Integration of the nitrogen atom into the core to generate the analogous picolinic acid **2** resulted in comparable tachyzoite and bradyzoite inhibition (EC$_{50}$ = 0.56 and 0.41 μM, respectively). Across the aza series, inhibition was generally congruent between tachyzoites and bradyzoites for a given compound, except in four cases in which inhibition for one stage was significantly favored. The effort revealed that the C6-phenyl ring appended to the picolinic acid core was not necessary to achieve submicromolar inhibition, and removing this moiety helped to reduce the lipophilicity. Conversely, the C5-chloro substituent of the aza analogue set was important for retaining activity (compounds **4−6**). Compound **5** showed the best tachyzoite inhibition (EC$_{50}$ = 0.24 μM) with commensurate bradyzoite activity; however, some apparent toxicity in HFF cells was also noted, affording selectivity indices of 10.4 to 7.1, respectively. The methyl ester derivative **7** showed activity

Table 1. *T. gondii* Tachyzoite and Bradyzoite Inhibition and Cytotoxicity Studies with Compounds 1−16

cmpd	structure	inhibition, EC$_{50}$ µM (SI)		CC$_{50}$[b]
		tachyzoite[a]	bradyzoite[a]	
1		0.46 ± 0.01[c] (> 108.7)	0.99 ± 0.09 (> 50.5)	> 50
2		0.56 ± 0.10 (86.1)	0.41 ± 0.11 (117.6)	48.2
3		3.85 ± 0.27 (> 13.0)	4.09 ± 0.33 (> 12.2)	> 50
4		5.14 ± 0.22 (> 9.7)	3.96 ± 3.22 (> 12.6)	> 50
5		0.24 ± 0.04 (10.4)	0.35 ± 0.01 (7.1)	2.5
6		1.11 ± 1.34 (8.1)	2.16 ± 0.59 (4.2)	9.0
7		0.43 ± 0.01 (> 116.3)	0.57 ± 0.09 (> 87.7)	> 50
8		> 10	> 10	> 50
9		0.80 ± 0.29 (1.5)	2.45 ± 1.32 (0.5)	1.2
10		1.48 ± 0.12 (12.3)	1.10 ± 0.54 (16.5)	18.2
11		2.42 ± 0.38 (> 20.7)	4.47 ± 0.48 (> 11.2)	> 50
12		7.10 ± 0.79 (> 7.0)	2.41 ± 2.07 (> 20.7)	> 50
13		1.82 ± 0.60 (> 27.5)	6.20 ± 1.86 (> 8.1)	> 50
14		3.50 ± 0.61 (> 14.3)	4.90[c] ± 3.32 (> 10.2)	> 50
15		1.69 ± 0.37 (> 29.6)	> 10	> 50
16		> 10	> 10	23.6

[a]Compounds were minimally tested in triplicate ($n \geq 3$) in HFF cells unless otherwise indicated. SI is the selectivity index, given by SI = CC$_{50}$/EC$_{50}$. [b]HFF cell line, 72 h exposure. [c]Tested in duplicate ($n = 2$).

against both parasite forms, though hydrolysis to reveal an active acid metabolite (compound 5) is likely, especially for the bradyzoite assay, which involves base-mediated maturation of parasites. Notably, though, this analogue did not manifest the same levels of toxicity toward HFF cells as observed with compound 5. Other isosteric replacements of the carboxylic acid were also surveyed, revealing that a primary amide isosteric replacement of the acid was inactive; the toxicity of tetrazole 9 confounded the interpretation of any inhibitory effect.

The necessity of the 4-*tert*-butyl appendage of the benzamide moiety was also evaluated. Exchange of the *tert*-butyl group of 5 with a methyl group (compound 10) or a hydrogen atom (compound 11) eroded the potency against both parasitic forms in a trend that suggests that hydrophobic interactions may be important in this region of the scaffold. This hypothesis was further supported by the more pronounced loss of potency when a more polar 4-methoxyphenyl group was installed in place of the 4-*tert*-butylphenyl moiety (compound 12). Migration of the methoxy group to other positions on the phenyl ring was not

advantageous for potency over other structural modifications already discussed (compounds 13 and 14). Nicotinic acid derivatives 15 and 16 were devoid of inhibitory activity against the bradyzoite form. The benzamidobenzoic acid series, represented by compound 1, possessed challenging physiochemical properties. For instance, lipophilic probe 1 had an experimental LogD$_{7.4}$ of >5, along with modest solubility and microsomal stability (Table 2). The aza analogues were designed to address these liabilities. Selected picolinic analogues and compound 1 were evaluated for LogD$_{7.4}$, solubility, microsomal and plasma stability, and brain permeability (PAMPA). Compound 5 had a desirable LogD$_{7.4}$ value of 2.7 and the most improved solubility and microsomal stability relative to compound 1. Despite the presence of the carboxylic acid, the BBB PAMPA assay predicted that the compound should achieve brain exposure. A pharmacokinetic mouse study was planned; however, we found that compound 5 was difficult to formulate into a solution for proper administration. To enhance the solubility of 5, the sodium salt of the carboxylic acid was generated to afford analogue 23 (Table 2). Parasite growth inhibition was verified,

Table 2. ADME Results for Selected Compounds

cmpd[1]	1	2	5	7	23
tachyzoite EC_{50} (μM)	0.46 ± 0.01	0.56 ± 0.10	0.24 ± 0.04	0.43 ± 0.01	0.32 ± 0.02
tachyzoite SI[2]	> 108.7	86.1	10.4	>116.3	6.6
bradyzoite EC_{50} (μM)	0.99 ± 0.09	0.41 ± 0.11	0.35 ± 0.01	0.57 ± 0.09	0.23 ± 0.01
bradyzoite SI[2]	> 50.5	117.6	7.1	> 87.7	9.1
CC_{50}, (μM)[3]	> 50	48.2	2.5	> 50	2.1
$LogD_{7.4}$[4]	5.4	3.9	2.7	5.3	2.6
solubility[5]	2.7 μM	6.4 μM	33 μM	< 1.6 μM	164 μM
microsomal stability[6]	69.2%	85.1%	75.4%	2.7%	100%
plasma stability[7]	100%	97.1%	98.4%	24.7%	88.1%
BBB PAMPA[8]	6.7	5.4	6.9	ND	ND

[1]R = NHCO-4-*tert*-butylphenyl. [2]Selectivity index, given by SI = CC_{50}/EC_{50}. [3]CC_{50} in HFF cells after 72 h = cytotoxic concentration at which cell viability is reduced by 50%. [4]Experimentally determined. [5]Kinetic aqueous solubility at pH 7.4. [6]Mouse microsomes; value reported as percent parent remaining after 1 h of exposure [7]Mouse plasma; percent parent remaining after 1 h of exposure. [8]BBB PAMPA, Pe (10^{-6} cm/s). ND = not determined.

revealing EC_{50} values for salt **23** comparable to those for the parent compound **5** against both tachyzoites and bradyzoites. Notably, the solubility of salt **23** was improved 5-fold over that of **5**, which enabled us to find a particle-free formulation for pharmacokinetic evaluation.

To assess the plasma and brain exposure of compound **23**, a snapshot pharmacokinetic experiment in mice was conducted. Compound **23** was administered to male BALB/c mice at 15 mg/kg via ip injection as a clear solution. Plasma concentrations were assessed 30 min and 2 h postadministration, and brain exposure was measured at the 2 h time point. Terminal mean plasma and brain exposure (AUC_{2h} = 7883 ng/mL and AUC_{2h} = 816 ng/g, respectively) were determined, affording a brain-to-plasma (B/P) ratio of 0.1. These values translate to 22 μM in plasma and 2.3 μM in brain 2 h postadministration, though because of high protein binding (99.5%), the free fraction is substantially reduced. Encouragingly, compound **23** demonstrated exposure at a low dose in both plasma and brain; however, additional work will be required to improve the selectivity index and increase the exposure to permit suitable dosing in mice for efficacy assessment. The ester of compound **7** may offer potential insight into addressing these characteristics.

Contrary to the tachyzoites, the in vitro-derived bradyzoites used in the primary screen and SAR assessment were not susceptible to pyrimethamine as a control, indicating that the bradyzoites were differentiated. Nonetheless, we evaluated whether the observed inhibition of in vitro-derived bradyzoites was recapitulated with ex vivo-derived bradyzoite cysts using compound **23**. Bradyzoites were harvested from the brains of four *T. gondii*-infected mice 30+ days postinfection, purified, and transferred to fibroblast cells (HFF). Compound **23** was independently added to the cells at three separate concentrations (1, 3, or 6 × EC_{50}) followed by incubation for 3 days. Monolayers were fixed and incubated with an anti-BAG1 antibody that recognizes a bradyzoite-specific antigen, BAG1, which is absent in tachyzoites.[42] Fluorescence imaging (Incucyte) of the infected cells showed that compound **23** reduced the fluorescence signal corresponding to BAG1 in a dose-dependent manner compared with the DMSO control, thus demonstrating that compound **23** inhibits ex vivo bradyzoite cysts (Figure 3).
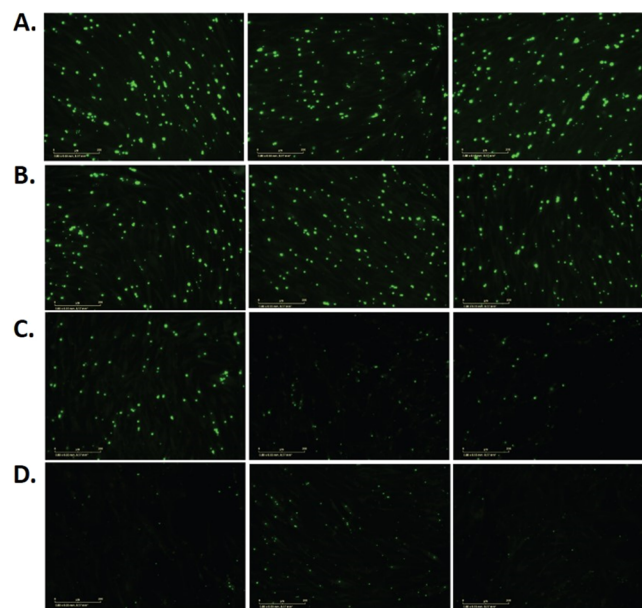

Figure 3. Ex vivo harvested *T. gondii* cysts from mouse brains in HFF cells incubated with BAG1 antibody and fluorescently imaged after 3 day treatment with (A) DMSO control or (B−D) compound **23** at (B) 1 × EC_{50}, (C) 3 × EC_{50}, or (D) 6 × EC_{50}. Antibody-labeled cysts appear in green. The experiment was performed twice, each time in triplicate, but only one experiment is shown for clarity (see Figure 1S and Table 2S).

A biological target of the picolinic acids is currently unknown. However, the benzamidobenzoic acids from which these compounds were designed, represented by **1**, were originally developed as inhibitors of the *T. brucei* parasite glycolytic enzyme hexokinase 1 (TbHK1).[32] *T. gondii* possesses a single copy of its hexokinase (*T. gondii* hexokinase, TgHK),[43,44] and like *T. brucei* parasites, *T. gondii* bradyzoites are glycolysis-dependent; however, the tachyzoite stage can use alternative metabolic pathways when necessary. To determine whether compound **23** inhibited TgHK, *T. gondii*-infected HFF cells were treated with DMSO control or with compound **23** at either 2 or 6 × EC_{50} 4 h postinfection. After 5 h, the medium was changed to glucose-free RPMI medium supplemented with isotopically labeled $^{13}C_6$-glucose.[45] After 10 min of labeling, infections were rapidly quenched, processed, and subjected to broad-spectrum mass spectrometry analysis. Data were evaluated by the Metabolomics Analysis and Visualization Engine (MAVEN)[46] software. Analysis of $^{13}C_6$-labeled metabolite production from the initial glycolysis reactions (Figure 4A) revealed two notable outcomes related to the absence or presence of compound **23**.
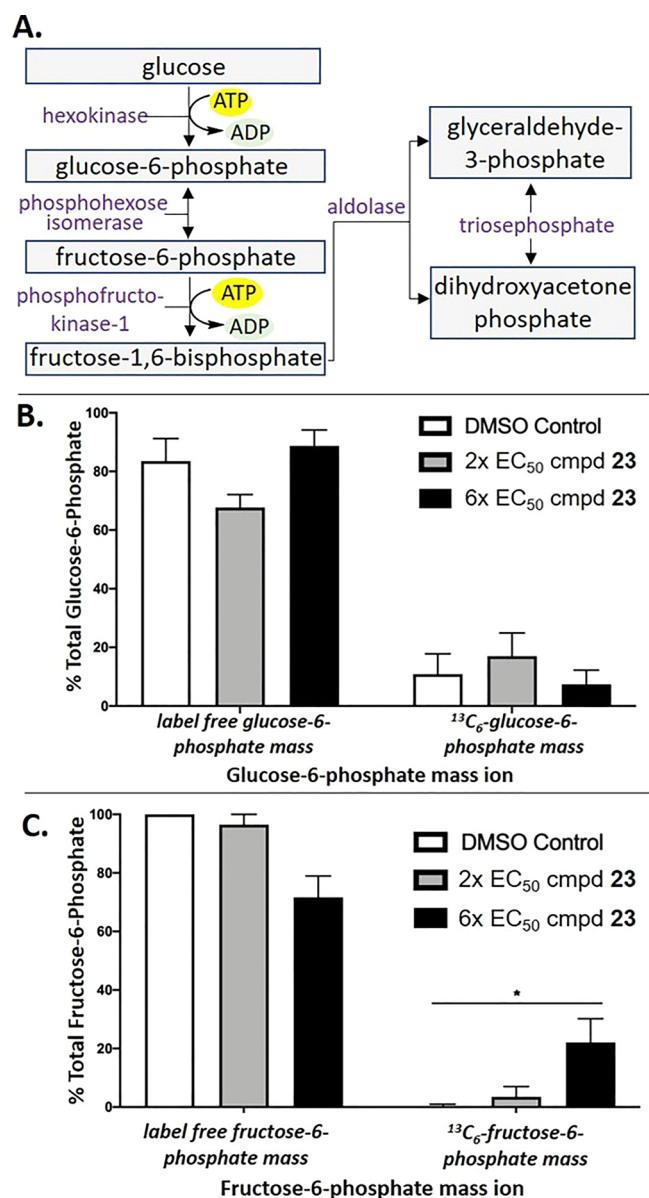
Figure 4. (A) Schematic of initial *T. gondii* glycolysis reaction pathways. (B, C) Mass spectrometry analyses showing the percentages of the total label-free or $^{13}C_6$-labeled (B) glucose-6-phosphate and (C) fructose-6-phosphate in *T. gondii*-infected HFF cells treated with DMSO (white bars) or with compound **23** at $2 \times$ EC$_{50}$ (gray bars) or $6 \times$ EC$_{50}$ (black bars). Error bars = 95% confidence interval. Asterisks indicate a significant difference in $^{13}C_6$-labeled fructose-6-phosphate mass ion ($p = 0.02$, t test corrected for multiple comparison using the Holm–Sidak method) between the DMSO and $6 \times$ EC$_{50}$ results. Shown is one representative experiment of two, each performed in triplicate.

In the presence of compound **23**, $^{13}C_6$-glucose-6-phosphate, the product of the hexokinase, did not change significantly in treated parasites compared with the DMSO control, suggesting that the hexokinase was not appreciably inhibited (Figure 4B). In contrast, $^{13}C_6$-fructose-6-phosphate accumulated in a statistically significant amount compared with DMSO-treated tachyzoites (Figure 4C). An impact of compound **23** on other pathway metabolites was not observed (data not shown). Elucidating the biological target of compound **23** that leads to the observed increase in $^{13}C_6$-fructose-6-phosphate will require

more study; however, these results show that **23** impacts the glycolytic pathway, although inhibition of the hexokinase is not the primary mechanism of action.

Herein we have described the results of a parasite-hopping approach revealing a novel picolinic acid template that inhibits both tachyzoite and bradyzoite *T. gondii* parasites. Generally, a good correlation between the in vitro potencies toward the two parasite forms was found, and structure–activity relationships were identified for a preliminary collection of 18 compounds, several of which showed submicromolar inhibition. Compared with the first-generation benzamidobenzoic acids, the second-generation picolinic acid derivative **23** was more balanced in terms of its lipophilic character and demonstrated a >30% improvement in mouse microsomal stability and a 60-fold improvement in solubility. Some cellular toxicity in HFF cells was observed, and additional work will be required to enhance the exposure, especially in brain tissue, where the bradyzoite burden is significant. As current FDA-approved therapies are ineffective against *T. gondii* bradyzoites, these compounds represent an opportunity to study the acute and chronic stages of *T. gondii* infection with a new probe scaffold.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00267.

Assay, experimental details, synthetic procedures, and NMR spectra for all new compounds reported in this work (**2**−**23**) (PDF)

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■ ABBREVIATIONS

AUC, area under the curve; BAG1, *T. gondii* bradyzoite specific heat shock protein; BBB, blood−brain barrier; CC_{50}, cytotoxic concentration that results in death of 50% of the cells; cLogD, calculated partition coefficient for an ionizable compound at a given pH; EC_{50}, effective concentration of compound necessary to inhibit 50% of the parasites; HFF, human foreskin fibroblasts; PAMPA, parallel artificial membrane permeability assay; SAR, structure−activity relationships; SI, selectivity index; TbHK1, *Trypanosoma brucei* hexokinase 1; TgHK, *T. gondii* hexokinase.

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