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Letter

Acridones Are Highly Potent Inhibitors of *Toxoplasma gondii* Tachyzoites

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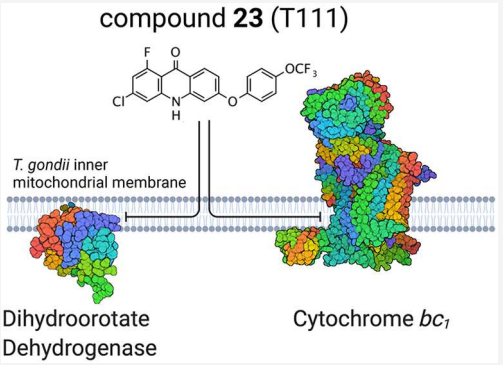
ABSTRACT: Acridone derivatives, which have been shown to have *in vitro* and *in vivo* activity against *Plasmodium* spp, inhibit *Toxoplasma gondii* proliferation at picomolar concentrations. Using enzymatic assays, we show that acridones inhibit both *T. gondii* cytochrome bc_1 and dihydroorotate dehydrogenase and identify acridones that bind preferentially to the Q_i site of cytochrome bc_1. We identify acridones that have efficacy in a murine model of systemic toxoplasmosis. Acridones have potent activity against *T. gondii* and represent a promising new class of preclinical compounds.



KEYWORDS: *toxoplasma, acridone, cytochrome bc_1, dihydroorotate dehydrogenase, drug discovery*

Toxoplasma gondii, a member of the phylum *Apicomplexa*, is a unicellular protozoal pathogen distributed throughout the world that can infect birds and mammals, including humans.[1] Although 80% of primary infections are asymptomatic,[2] severe and potentially fatal neurologic and ocular disease can occur in immunocompromised persons and developing fetuses. Following primary infection, *T. gondii* establishes latent infection as tissue cysts. Reactivation of latent infection can occur under conditions of immunosuppression, causing pneumonitis, encephalitis, or death.

Current first-line therapy for *T. gondii* infection is a combination of the antifolates pyrimethamine and sulfadiazine, plus leucovorin to minimize host bone marrow toxicity. This regimen has a number of shortcomings: it has a high rate of toxic side effects, does not eradicate latent infection, and requires courses of therapy that are weeks to months in duration.[3] There is an urgent need for new medications for toxoplasmosis that address these deficits.

Antimalarial acridones (hereafter referred to as "acridones") were developed to create a bifunctional molecule that reverses chloroquine resistance in *Plasmodium* spp. and binds heme, blocking the essential process of hemozoin formation.[4−6] Later development of a new acridone chemotype[5,6] provided evidence that these acridones inhibit cytochrome bc_1. On the basis of the high degree of homology between *Plasmodium falciparum* cytochrome bc_1 and that of *T. gondii*, we hypothesized that acridones would inhibit *T. gondii* proliferation by targeting cytochrome bc_1. Extending these findings to other ubiquinone-binding enzymes, we found that acridones inhibit *T. gondii* dihydroorotate dehydrogenase (*Tg*DHOD) with nanomolar enzyme−inhibitor dissociation constants (K_i). Finally, we tested acridones known to have *in vitro* activity against *P. falciparum* and *in vivo* efficacy against *P. yoelli* in a mouse model of acute toxoplasmosis and identified those with *in vivo* activity against *T. gondii*.

Acridones Have Picomolar Potency against *T. gondii* *in Vitro*. Acridones were tested against RH-strain *T. gondii in vitro* in a plate-based proliferation assay. The EC_{50}'s (50% inhibitory concentration) summarized in Tables 1 and 2 range from 0.030 nM to 485 nM. The synthesis and structural characterization of all final target compounds **1**−**57** (Tables 1 and 2) have been described in previous publications.[5,6] All compounds have no toxicity against cultured human HepG2 cells[5,6] at the concentrations studied.

Acridones Inhibit *T. gondii* Cytochrome bc_1. The ability of acridones to inhibit the enzymatic reduction of cytochrome *c* by cytochrome bc_1 was investigated using mitochondria isolated from RH-strain *T. gondii* parasites.

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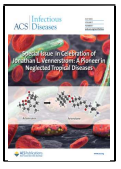

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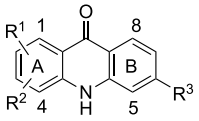
Table 1. *In Vitro* Activities of 6- and 7-Alkoxy Acridones against RH-Strain *T. gondii*



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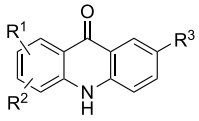
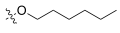
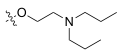
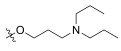
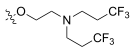
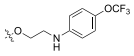
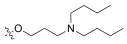
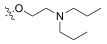
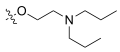
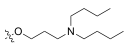
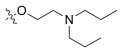
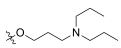
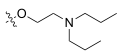
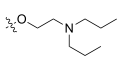
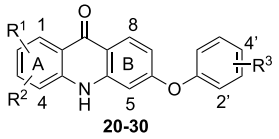


16-19

compd	R^1	R^2	R^3	EC_{50} (nM)[a] vs RH-strain *T. gondii*
1	H	H	H	93.3 (79.4–110)
2	1-F	3-OMe	OMe	11.5 (9.27–14.2)
3	1-F	3-F		0.900 (0.692–1.35)
4	1-F	3-F		0.168 (0.138–0.205)
5	1-F	3-F		3.64 (0.767–8.08)
6	1-F	3-F		57.7 (37.6–88.6)
7	1-F	3-F		0.135 (0.100–0.182)
8	1-F	3-F		14.3 (7.91–25.7)
9	1-Cl	2-Cl		1.35 (0.614–2.95)
10	1-Cl	2-Cl		176 (118–264)
11	2-Cl	3-OMe		104 (79.1–138)
12	2-Cl	3-OMe		423 (243–1620)
13	2-Cl	3-OMe		313 (261–466)
14	H	3-OMe		185 (164–211)
15	1-N(pr)$_2$	3-F		169 (110–263)
16	1-Cl	2-Cl	OMe	152 (130–161)
17	2-Cl	3-Cl	OMe	62.1 (48.1–68.4)
18	1-F	3-F		37.5 (29.7–47.4)
19	1-Cl	2-Cl		485 (259–910)
ATV	-	-	–	10.6 (4.45–25.3)

[a]95% confidence intervals are within parentheses; ATV, atovaquone.

Table 2. *In Vitro* Activities of 6- and 7-Aryloxy/Arylamino/-Aryl/Benzyloxy Acridones against RH-Strain *T. gondii*



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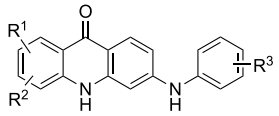

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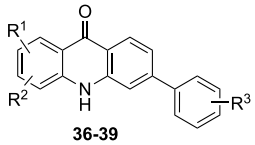

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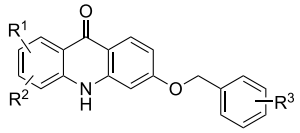

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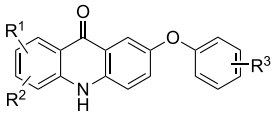

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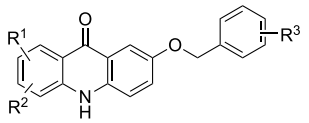

56 and 57

compd	R^1	R^2	R^3	EC_{50} (nM)[a] vs RH-strain *T. gondii*
20	H	3-F	4′-OCF_3	3.73 (3.31–4.20)
21	1-F	H	4′-OCF_3	0.628 (0.447–0.871)
22	1-F	3-F	4′-OCF_3	0.053 (0.041–0.068)
23	1-F	3-Cl	4′-OCF_3	0.030 (0.013–0.040)
24	1-Cl	3-F	4′-OCF_3	1.01 (0.070–1.45)
25	H	3-OMe	4′-OCF_3	0.379 (0.223–0.644)
26	1-F	3-OMe	4′-OCF_3	0.170 (0.121–0.238)
27	2-F	3-OMe	4′-OCF_3	0.630 (0.476–0.851)
28	2-Cl	3-OMe	4′-OCF_3	3.99 (3.47–4.60)
29	2-Cl	3-OMe	3′-OCF_3	9.09 (7.63–10.8)
30	2-F	3-OMe	2′-OCF_3	8.93 (7.28–10.9)
31	1-F	3-F	4′-OCF_3	5.38 (2.48–11.7)
32	1-F	3-OMe	4′-Cl	30.9 (27.7–34.5)
33	2-F	3-OMe	4′-OCF_3	5.67 (4.5–7.16)
34	2-F	3-OMe	3′-OCF_3	7.63 (6.10–9.56)
35	2-F	3-OMe	2′-OCF_3	1.75 (1.33–2.30)
36	1-F	3-F	H	11.1 (9.73–12.6)
37	1-F	3-F	4′-OCF_3	5.23 (4.56–6.04)
38	1-F	3-OMe	4′-OCF_3	0.310 (0.260–0.370)
39	1-F	3-OMe	4′-Cl	2.24 (1.93–2.60)
40	1-F	3-F	H	8.45 (7.16–10.0)
41	1-F	3-F	4′-CF_3	0.180 (0.121–0.180)
42	1-F	3-F	4′-OCF_3	1.75 (1.55–1.97)
43	1-F	3-F	4′-SCF_3	0.350 (0.299–0.420)
44	1-F	3-F	3′-OCF_3	0.100 (0.063–0.141)
45	1-F	3-F	2′-CF_3	0.260 (0.204–0.320)
46	1-Cl	2-Cl	4′-Cl	0.320 (0.213–0.464)
47	2-Cl	3-OMe	4′-CF_3	4.70 (3.57–6.20)
48	2-F	3-OMe	4′-OCF_3	16.9 (9.29–32.1)
49	2-F	3-OMe	4′-CF_3	1.70 (1.34–2.16)
50	H	3-Cl	4′-OCF_3	1.52 (1.26–1.85)
51	H	3-Cl	4′-CF_3	1.45 (1.14–1.83)
52	1-F	3-F	4′-OCF_3	2.50 (2.02–3.08)
53	1-F	3-OMe	4′-OCF_3	22.1 (19.5–25.4)
54	1-OMe	3-F	4′-OCF_3	32.8 (28.3–37.8)
55	H	3-OMe	4′-Cl	5.22 (3.74–7.47)
56	1-F	3-F	4′-CF_3	2.43 (2.04–2.87)
57	1-F	3-F	4′-Cl	2.23 (1.49–3.23)
ATV	–	–	–	10.6 (4.45–25.3)

[a]95% confidence intervals are within parentheses; ATV, atovaquone.

Estimates of K_i obtained from global fits to rate data are presented in Table 3. All of these values are well under 1 μM, demonstrating that the acridones are potent inhibitors of *T. gondii* cytochrome bc_1.

Acridones Show Q_i-Site Specificity. Acridones were tested against an RH-derived line of *T. gondii* which harbors a threonine to proline substitution at position 222 in the Q_i site of its cytochrome *b*. This strain was previously created with chemical mutagenesis and selection with the endochin-like quinolone (ELQ) derivative, ELQ-316[7] (Figure 1). We hypothesized that substitutions on ring A of the acridone skeleton are a major driver of site specificity. Accordingly, we tested acridones with a variety of substitutions at positions 1, 2, and 3, including compound **27** (T65) (Table 2), which is most analogous to ELQ-316 (2-F, 3-OMe, see Figure 1). To investigate the role that substitutions on ring B of the acridone skeleton play in site specificity, we also tested 1,3-difluoro acridones with chemically diverse position 6 substituents. A comparison of the EC_{50}'s obtained against the T222P strain

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Table 3. K_i of Acridones against *Tg* Cytochrome bc_1

compd	code name	K_i vs *Tg* bc_1 (nM)[a]
3	T45	42.2 (10.9−201)
7	T41	205 (78.0−516)
21	T68	26.8 (12.5−55.7)
27	T65	1.70 (0.714−3.96)

[a]95% confidence intervals are within parentheses.

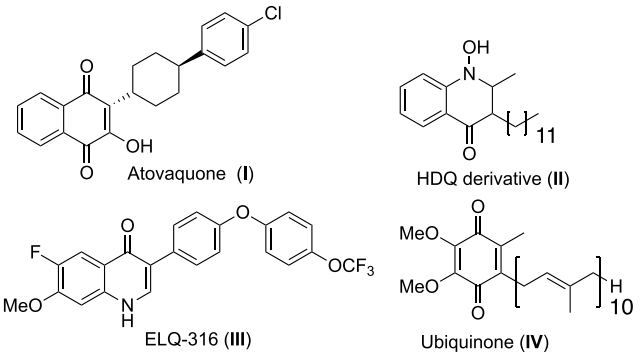

Figure 1. Structures of representative cytochrome bc_1 inhibitors atovaquone (**I**), HDQ derivative (**II**), ELQ-316 (**III**), and ubiquinone (**IV**).

versus those obtained from RH-strain parasites is presented in Table 4. Although the T222P mutation confers resistance to

Table 4. EC_{50}s of Acridones against the T222P Strain of *T. gondii* with RH Strain Values Provided for Reference

compd	EC_{50} vs RH (nM)[a]	EC_{50} vs T222P (nM)[a]	fold Δ[b]
3	0.900 (0.692−1.35)	2.36 (1.54−3.60)	2.62
4	0.168 (0.138−0.205)	0.232 (0.044−0.274)	1.38
7	0.135 (0.100−0.182)	0.002 (0.004−0.001)	0.01
21	0.628 (0.447−0.871)	0.054 (0.022−133)	0.09
22	0.053 (0.041−0.068)	0.080 (0.051−0.126)	1.51
23	0.030 (0.018−0.039)	0.096 (0.061−0.152)	3.20
24	1.01 (0.700−1.45)	0.704 (0.538−0.921)	0.70
25	0.379 (0.223−0.644)	3.45 (2.83−4.22)	9.10
26	0.170 (0.121−0.238)	0.028 (0.018−0.042)	0.16
27	0.630 (0.476−0.851)	5.61 (4.23−7.45)	8.90

[a]95% confidence intervals are within parentheses. [b]Fold change is calculated as T222P EC_{50} divided by RH EC_{50}.

compounds **3** (T45), **23** (T111), **25** (T113), and **27** (T65) it has no significant effect against compounds **4** (T42), **22** (T44), and **24** (T110).

The K_i of compound **27** (T65) was measured against T222P-derived mitochondria and found to be significantly elevated compared to that of the parental RH-strain mitochondria: 41.5 nM [17.6−96.3] for T222P versus 1.70 nM [0.714−3.96] for RH.

Acridones Inhibit *T. gondii* DHOD. As acridones displayed competitive inhibition kinetics with respect to ubiquinone in the cytochrome bc_1 activity assays, we hypothesized that acridones may inhibit other ubiquinone- or ubiquinol-binding proteins.

We tested the ability of acridones to inhibit *Tg*DHOD-VSSM, a mutated *Tg*DHOD in which the N-terminal membrane anchor has been removed to enhance solubility in aqueous solution.[8] A variety of acridones with substitutions at positions 1, 2, 3, 6, and 7 (Tables 1 and 2) were tested in order to fully explore the structure−activity relationships (SAR) of the inhibition of *Tg*DHOD-VSSM by acridones.

The K_i's of acridones against *Tg*DHOD-VSSM are shown in Table 5, and demonstrate that acridones are effective inhibitors

Table 5. K_i of Acridones against *Tg*DHOD-VSSM

compd	code name	K_i vs *Tg*DHOD (nM)[a]
3	T45	1484 (924−2820)
4	T42	348 (283−570)
7	T41	313 (125−883)
15	T179	154 (86−283)
20	T67	1175 (771−1074)
21	T68	98 (54−183)
22	T44	2029 (993−8091)
23	T111	273 (151−540)
24	T110	59 (21−148)
25	T113	62 (21−163)
26	T96	462 (283−808)
27	T65	2251 (1639−3365)
28	T63	717 (567−932)
29	T98	632 (396−1073)
30	T86	199 (129−312)
31	T60	127 (83−195)
33	T70	1524 (827−3948)
34	T74	488 (296−865)
35	T73	189 (120−303)
ATV	–	2715 (1547−6586)

[a]95% confidence intervals are within parentheses; ATV, atovaquone.

at low nanomolar to low micromolar concentrations. The cytochrome bc_1 inhibitor atovaquone was also evaluated and has a K_i of 2715 nM.

Acridones Are Efficacious against *T. gondii in Vivo*. We hypothesized that acridones which had previously been shown to be efficacious in a murine model of *Plasmodium yoelli* infection would be most likely to be efficacious against *T. gondii* infection.[6] Using a murine model of systemic *T. gondii* infection,[9] we tested compounds **18**, **23**, **42**, and **44** at doses of 5, 20, and 40 mg/kg as well as the drugs pyrimethamine (at 20 mg/kg) and pyrimethamine/sulfadiazine (at 5/60 mg/kg). The doses of pyrimethamine and sulfadiazine were chosen to approximate those used in clinical practice. The survival curves are graphed in Figure 2 (E). Compound **23** at 20 mg/kg and 40 mg/kg and compound **42** at 20 mg/kg and 40 mg/kg conferred a survival benefit that was statistically significant compared to control. Pyrimethamine alone at 20 mg/kg provided no survival benefit, but the combination of pyrimethamine and sulfadiazine at 5/60 mg/kg did. No overt clinical toxicity or behavior changes were observed in any mice.

Acridones show exceptional *in vitro* potency against *T. gondii* and are appreciably more potent *in vitro* than drugs that are currently approved for use against *T. gondii*, such as pyrimethamine and sulfadiazine (402 and 26 000 nM, respectively, against RH strain parasites[10]). The acridone EC_{50}'s compare favorably with other cytochrome bc_1 inhibitors such as atovaquone (**I**, 10.6 nM), a 1-hydroxy-2-dodecyl-4(1*H*)quinolone (HDQ, **II**) derivative (0.8 nM in a 24 h assay[11,12]) and the endochin-like quinolones[13] (ELQs **III**, 0.003 nM−210 nM). Representative structures of these molecules and ubiquinone (**IV**) are shown in Figure 1. Taken as a class, the cytochrome bc_1 inhibitors are remarkably

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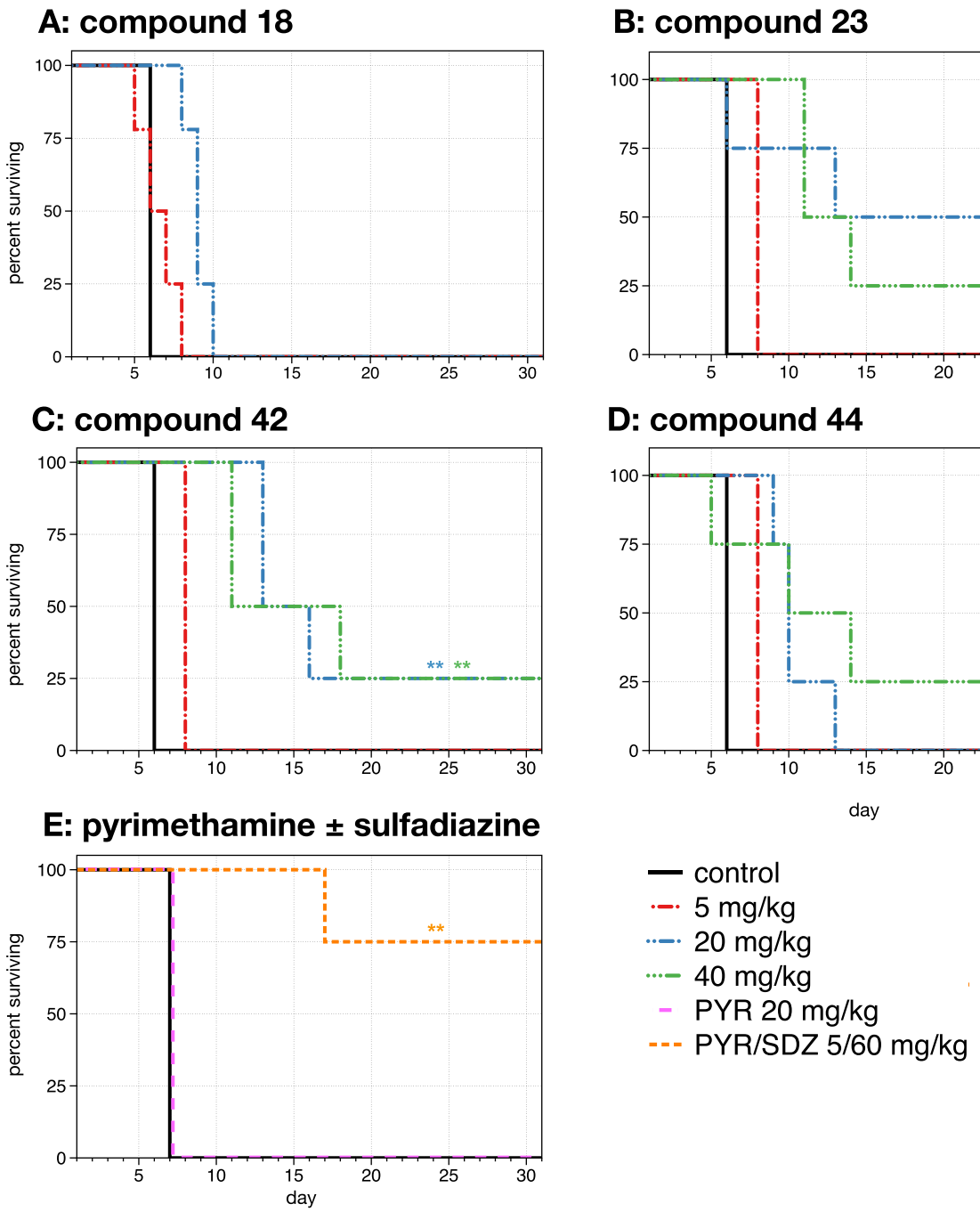

Figure 2. *In vivo* efficacy of compounds **18** (T36, A), **23** (T111, B), **42** (T121, C), **44** (T122, D) in a mouse model of acute infection with *T. gondii*. Compounds were dosed orally at 5 mg/kg (red), 20 mg/kg (blue), and 40 mg/kg (green) once daily. In E, the efficacy of oral pyrimethamine 20 mg/kg once daily (pink) and oral pyrimethamine 5 mg/kg plus sulfadiazine 60 mg/kg once daily (orange) were tested in this model as a basis for comparison. Compound **23** at 20 mg/kg ($p < 0.05$, *) and at 40 mg/kg ($p < 0.01$, **), compound **42** at 20 mg/kg and 40 mg/kg ($p < 0.01$ for both), and pyrimethamine/sulfadiazine 5/60 mg/kg ($p < 0.05$) demonstrated statistically significant survival benefit versus control by the log-rank test.

potent against *T. gondii*, demonstrating the importance of cytochrome bc_1 in the proliferation of *T. gondii*.

Halogen substitution at positions 1 and 3 of ring A was associated with the greatest potency. Substitutions at positions 1 and 2 or 2 and 3 were associated with compounds that were still highly potent but less so than those with position 1 and 3 substitutions. On ring B, substitutions at the 6-position seemed to be optimal for enhanced potency. 7-Aryloxy and other position 7 substitutions are associated with a relative loss of potency. However, all the tested acridones with 7-aryloxy/benzyloxy substitutions still display enhanced potency relative to compound **1**, which is an unsubstituted acridone scaffold. Interestingly, compound **23** (T111), which is the most potent acridone against *P. falciparum*,[6] showed the most potent activity against *T. gondii* (Table 2). In general, acridones with higher potency against *P. falciparum* had higher potency against *T. gondii*. Comparison of these EC_{50}'s is complicated by the fact that a beta-galactosidase assay was used for the *T. gondii* measurements while a SYBR green assay was used for the *P. falciparum* measurements. However, if the SYBR green

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assay was biased to generate lower EC_{50}'s, one would expect to see uniformly lower *P. falciparum* EC_{50}'s, but this is not observed. While there is a high degree of homology between the cytochrome bc_1 of both parasites, differences in amino acids in the active sites may account for the variation in EC_{50}'s.[9]

Several acridones, including compounds **3** (T45), **23** (T111), **25** (T113), and **27** (T65), are less potent against the T222P strain of *T. gondii*, consistent with preferential binding to the Q_i (quinone reduction) site of cytochrome bc_1. Further evidence for such site selectivity is provided by the observation that compound **27** (T65) was significantly less potent against T222P-derived cytochrome bc_1 versus RH-derived.

A strain of *T. gondii* with a Q_o (quinol oxidation) site mutation was not available for testing. However, owing to the high degree of homology between *T. gondii* and *P. falciparum* cytochrome bc_1, evidence of Q_o site specificity can be inferred by comparing the *in vitro* potency of acridones against D6 strain (pan-susceptible) *P. falciparum* versus Tm90−C2B strain (atovaquone resistant) *P. falciparum*.[6] A number of acridones (compounds **22** (T44), **23** (T111), **32** (T115), **40** (T156), **41** (T123), **43** (T126), **45** (T125), and **56** (T130)) have *in vitro* EC_{50}'s against Tm90−C2B that are over 100-fold greater than the corresponding EC_{50}'s against D6. Consistent with Q_o site inhibition in *P. falciparum*, the potency of compound **22** (T44) was similar against the T222P strain and the parental RH strain. Substitutions on ring A of the acridones appear to determine Q-site specificity in *T. gondii* in the same manner previously seen with ELQs wherein the 3-OMe substitution was associated with the Q_i site. Interestingly, however, the available *in vitro* data suggest that compound **23** (T111) may bind to both the Q_o and Q_i sites.

In addition to being potent inhibitors of cytochrome bc_1, acridones also inhibit *Tg*DHOD-VSSM with affinities in the nanomolar to low micromolar range. *Tg*DHOD catalyzes the fourth step in the *T. gondii* pyrimidine biosynthesis pathway, the oxidation of dihydroorotate to orotate with the concomitant reduction of ubiquinol to ubiquinone.[14] Several lines of evidence suggest that *TgDHOD* is an essential gene and a drug target in *T. gondii.* CRISPR (clustered regularly interspaced short palindromic repeats) phenotypic screening scores place it with other genes that confer a high degree of fitness.[15] *T. gondii*, unlike *P. falciparum*, is capable of both pyrimidine salvage and *de novo* biosynthesis, but the salvage pathway is not essential for acute[16] or chronic[17] infection by *T. gondii*. Deletion of the first, fifth, or sixth steps in the pyrimidine synthetic pathway in *T. gondii* results in uracil auxotrophy and severely attenuated virulence.[17−20] Attempted knockout of *TgDHOD* to create uracil auxotrophy has not been successful.[8] Creation of uracil auxotrophy by ablation of *Tg*DHOD activity required the presence of a catalytically deficient *DHOD* allele, suggesting an essential second function of this gene that is independent of its role in pyrimidine synthesis. Although the precise role of *TgDHOD* in *T. gondii* metabolism is an active area of investigation, *TgDHOD* has been identified as a drug target for HDQ and the related HDQ derivative **II**[21] (Figure 1) by the creation and analysis of HDQ resistant mutants. We show here that acridones can inhibit *Tg*DHOD in an enzymatic assay. The significance of this finding should be determined by further studies.

Conclusions. Although a number of questions remain regarding the mode(s) of action of acridones, these experiments indicate that acridones inhibit both cytochrome bc_1 and DHOD in *T. gondii*. Future studies will examine the relative contributions of cytochrome bc_1 versus DHOD inhibition and evaluate what other mechanisms of action the acridones may have in *T. gondii*.

Finally, we demonstrate that compounds **23** and **42** improve survival in a murine model of systemic *T. gondii* infection that is similar to that seen with pyrimethamine/sulfadiazine, the current first-line therapy for toxoplasmosis in humans. Compounds **23** and **42** are metabolically stable but have low oral bioavailability,[6] resulting in maximum plasma concentrations of 17.2 and 42 ng/mL, respectively, from a single oral dose of 80 mg/kg. The efficacy of these compounds despite the low plasma concentrations suggests that their efficacy could be markedly improved by incorporating promoieties to increase oral bioavailability. In particular, the ketone functional group of these compounds would be amenable to a similar prodrug strategy that has previously been used for ELQs.[22] Additionally, all of the acridones tested have been optimized against *Plasmodium*. Future *in vivo* studies will evaluate acridones and acridone prodrugs specifically optimized for *T. gondii*. The findings presented here demonstrate that acridones are a new class of anti-*Toxoplasma* compounds that act on a validated *T. gondii* drug target and are excellent candidates for future drug development efforts.

METHODS

Chemicals and *T. gondii* Strains. Acridones were synthesized as previously described,[5,6] identified by proton nuclear magnetic resonance (^{1}H NMR) and high-resolution mass spectrometer (HRMS), and determined to be >95% pure by reversed-phase high-performance liquid chromatography (HPLC). Atovaquone (ATV) was obtained from Sigma-Aldrich. Development of the T222P strain has been previously described.[7] All compounds were dissolved in DMSO. The final concentration of DMSO in all assays was 1% or less. 1% DMSO was found to have no significant effect in any of the assays presented here.

***T. gondii* Growth Inhibition.** Compounds were evaluated for their ability to inhibit the growth of *T. gondii* using a 96-well assay in which *T. gondii* stably expressing beta-galactosidase were cultured in an HFF cell monolayer and their growth quantified colorimetrically.[23,24] Compounds dissolved in DMSO were added to the first column of a plate to obtain a final concentration of 1 μM and then diluted serially across the plate by 4-fold dilutions. The final column of each plate was left empty as a growth control. 4000 *T. gondii* RH-strain tachyzoites were added to each well. After 3 days of incubation (37 °C, 5% CO_2), a solution of chlorophenol red-β-D-galactopyranoside (CPRG) and NP-40 was added. The absorbance of each well was measured at 575 nm in a Molecular Devices SpectraMax 190 plate reader. At least three replicates[7] were performed for each dilution series. Absorbance data were plotted against the base-10 log of compound concentration in GraphPad Prism v8 and fit to a four-parameter model of the Hill equation to estimate the EC_{50} for each compound.

Cytochrome bc_1 Inhibition. Cytochrome bc_1 was isolated from both RH and T222P strain tachyzoites as previously described.[7] Compounds were evaluated for their ability to inhibit cytochrome bc_1 activity using a previously described procedure[7] modified for use in 96-well plates. Assay buffer (2 mM KCN, 100 mM KCl, 50 mM tricine, pH 8.0) containing

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oxidized cytochrome c (horse heart, Sigma-Aldrich, final concentration 50 μM) and varying concentrations of decylubiquinol (final concentration either 50, 25, or 12.5 μM) was added to a single column of a 96-well plate. Decylubiquinone was reduced to decylubiquinol with an excess of sodium borohydride, which was subsequently quenched with 100 μM hydrochloric acid. Compounds dissolved in DMSO were added to the first well in each column to 5 μM final concentration and serially diluted 10-fold, skipping the next to last well so that it served as an uninhibited control. Isolated cytochrome bc_1 was mixed separately with assay buffer and added to all wells in the column except the last. An equal volume of assay buffer alone was added to the last well in a column to obtain a measurement of the background rate of cytochrome c reduction in the absence of cytochrome bc_1. Immediately after these additions, the absorbance of each well at 550 nm was measured at 2-s intervals in a Molecular Devices SpectraMax 190 plate reader.

The rate of cytochrome bc_1 reduction was measured as a function of compound concentration at three concentrations of decylubiquinol (50, 25, 12.5 μM) for both RH- and T222P-derived mitochondria. Rate data were corrected by subtracting the background rate of cytochrome bc_1 reduction. Corrected rate data for each compound were fit to global values of K_i, K_m, and V_{max} in GraphPad Prism v8.

Expression and Purification of *Tg*DHOD. The DNA sequence of the previously characterized amino-terminal truncation mutant *Tg*DHOD-VSSM[8] was codon-optimized for expression in *E. coli*, chemically synthesized and inserted into the plasmid pRSET A so as to place a 6x histidine tag at the carboxy terminus of the resulting protein (ThermoFisher). The *Tg*DHOD-VSSM-pRSET A plasmid was transformed into electrocompetent *E. coli* BL21(DE3) R3 pRARE2 (Source Bioscience) using a BTX Electro Cell Manipulator 600 (2.5 kV/resistance mode, 2.45 kV charging voltage, 129 Ω resistance, 2 mm chamber gap). Culture and purification methods were similar to those previously described[8] with modifications as follows. A 1 L culture of the transformed strain was grown in Studier autoinduction media[25] ZYP-5052 supplemented with 0.1 mM flavin mononucleotide (FMN, Sigma-Aldrich) at 20 °C for 20 h with shaking.

Cultures were pelleted by centrifugation, resuspended in minimal lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 2% Triton X-100, 1 mM PMSF), and sonicated to lyse cells (Branson Sonifier 250, 70% power, 20 s on, 9:40 min off, 1 min total sonication). Cell debris was pelleted by centrifugation (Beckman type 70Ti rotor, 42k rpm, 20 min, 4 °C). The supernatant was applied to a 1 mL HisTrap FF crude column (GE). The column was washed with wash buffer (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 2% Triton X-100) until the A_{280} of the eluent stabilized. Protein was eluted in elution buffer (wash buffer supplemented to 500 mM imidazole). Fractions were analyzed by SDS-PAGE. Sufficiently pure (>95%) fractions were pooled, concentrated in an Amicon Ultra 30k centrifugal filter (Merck Millipore), resuspended in storage buffer (50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, 2% Triton X-100), snap frozen in a dry ice-ethanol bath, and stored at −80 °C until further use. Total protein concentration was determined with the bicinchoninic acid method using the manufacturer's directions (Thermo Scientific).

***Tg*DHOD Inhibition.** The activity of *Tg*DHOD-VSSM was measured using previously described procedures[8,26] modified for a 96-well plate format. Assay buffer (50 mM Tris-HCl, 150 mM KCl, 0.1% Triton X-100, 10% glycerol, 1 mM L-DHO, 100 μM DCIP) (DCIP, 2,6-dichlorophenolindophenol) containing varying concentrations of decylubiquinone (final concentration of 50, 25, or 12.5 μM) was added to a single column of a 96-well plate. Compounds dissolved in DMSO were added to the first well to a final concentration of 500 nM and serially diluted 2-fold, skipping the last two wells in the column. Separately, recombinant *Tg*DHOD-VSSM was mixed with assay buffer and added to all wells in the column except the last, to which assay buffer alone was added. The final concentration of *Tg*DHOD-VSSM in wells A−G was 6.2 nM. Well G contained enzyme but no drug and provided a baseline for uninhibited enzyme activity. Well H contained no enzyme, allowing for the rate of nonenzymatic reduction of DCIP to be measured.

Immediately after the addition of enzyme, absorbance of each well at 600 nM was recorded every 2 s in a Molecular Devices SpectraMax 190 plate reader. Initial rate data were corrected by subtracting the rate of nonenzymatic DCIP reduction. The rate of DCIP reduction was measured at varying concentrations of decylubiquinol (50, 25, 12.5 μM) and test compound. All data for each compound were fit to global values of K_i using a model of competitive inhibition in GraphPad Prism v8. Other models (noncompetitive, uncompetitive, mixed mode) were attempted, but competitive inhibition gave the best fit to the data.

***In Vivo* Efficacy.** All mice used in this study were housed in an American Association for Accreditation of Laboratory Animal Care (AAALAC) approved facility at the Portland Veterans Affairs Medical Center (PVAMC). The Institutional Animal Care and Use Committee (IACUC) at PVAMC approved the use of all animals and procedures.

4−5 week old CF-1 mice (25−30 g) were purchased from Charles River Laboratories (Wilmington, MA) and allowed to acclimate for 1 week. Mice were inoculated intraperitoneally with 10,000 RH-strain tachyzoites in sterile PBS on day 1. Mice were given test compound dissolved in 100 μL of PEG-4000 by oral gavage once daily on days 2−6. A control group was given 100 μL of PEG-4000 alone. Four mice were included in each group. Mice were weighed and monitored daily throughout the course of the experiment. Mice displaying signs of severe infection (loss of more than 10% of body weight, poor grooming, and/or lethargy) and all mice alive at the 30-day end point were euthanized. Survival data were analyzed by the log-rank test in GraphPad Prism v9 and plotted in DataGraph (Visual Data Tools, Chapel Hill, NC).

A total of three *in vivo* efficacy experiments were done. In the first, compounds **18**, **23**, **42**, and **44** were given at 5 mg/kg and 20 mg/kg. On the basis of these results a second experiment was done in which compounds **23**, **42**, and **44** were given at 40 mg/kg. In both of these experiments all controls met end point criteria on the same day. To provide a basis for comparison in this model, we tested pyrimethamine at 20 mg/kg and the combination of pyrimethamine/sulfadiazine at 5/60 mg/kg in a third experiment.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

In honor of Dr. Jonathan Vennerstrom's 65th birthday, this manuscript is dedicated to his lifelong contributions to the field of malaria and neglected tropical diseases.

■ ABBREVIATIONS

CRISPR, clustered regularly interspaced short palindromic repeats; CPRG, chlorophenol red-β-D-galactopyranoside; DCIP, 2,6-dichlorophenolindophenol; EC_{50}, half-maximal inhibitory concentration; ELQ, endochin-like quinolone; HDQ, 1-hydroxy-2-dodecyl-4(1*H*)quinolone; HFF, human foreskin fibroblast; HPLC, high-performance liquid chromatography; HPRS, high-resolution mass spectrometer; ^{1}H NMR, proton nuclear magnetic resonance; K_i, enzyme−inhibitor dissociation constant; SAR, structure−activity relationship; *Tg*DHOD, *Toxoplasma gondii* dihydroorotate dehydrogenase.

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