

# Drugs in development for toxoplasmosis: advances, challenges, and current status

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**Abstract:** *Toxoplasma gondii* causes fatal and debilitating brain and eye diseases. Medicines that are currently used to treat toxoplasmosis commonly have toxic side effects and require prolonged courses that range from weeks to more than a year. The need for long treatment durations and the risk of relapsing disease are in part due to the lack of efficacy against *T. gondii* tissue cysts. The challenges for developing a more effective treatment for toxoplasmosis include decreasing toxicity, achieving therapeutic concentrations in the brain and eye, shortening duration, eliminating tissue cysts from the host, safety in pregnancy, and creating a formulation that is inexpensive and practical for use in resource-poor areas of the world. Over the last decade, significant progress has been made in identifying and developing new compounds for the treatment of toxoplasmosis. Unlike clinically used medicines that were repurposed for toxoplasmosis, these compounds have been optimized for efficacy against toxoplasmosis during preclinical development. Medicines with enhanced efficacy as well as features that address the unique aspects of toxoplasmosis have the potential to greatly improve toxoplasmosis therapy. This review discusses the facets of toxoplasmosis that are pertinent to drug design and the advances, challenges, and current status of preclinical drug research for toxoplasmosis.

**Keywords:** *Toxoplasma gondii*, therapeutics, preclinical medicine, experimental medicine, mechanism of action, Apicomplexa

## *Toxoplasma gondii*

*Toxoplasma gondii* is a protozoan parasite that belongs to the phylum Apicomplexa. Apicomplexa also includes the medically important genera, *Plasmodium*, *Babesia*, and *Cryptosporidium*. The eponymous organelle of Apicomplexa, the apical complex, is used to invade the host cell. Biological similarities among apicomplexans are the basis for shared susceptibility to drugs such as the antifolate drugs pyrimethamine and sulfonamides and the anti-respiratory drug atovaquone. The current treatments for *T. gondii* and *Babesia microti* are drugs that were used as anti-malarials prior to being repurposed. However, an examination of parasite genomes, routes of infection, life cycle stages, hosts, and disease manifestations reveals diversity in the underlying biology of apicomplexan pathogens. Drugs that are specifically designed to optimize the efficacy against *T. gondii* hold potential for improving the treatment of toxoplasmosis.

The unique pathogenesis of *T. gondii* also presents challenges for drug therapy. Unlike many apicomplexans, *T. gondii* crosses the blood–brain barrier and establishes persistent infection in a drug-resistant bradyzoite stage. An ideal medicine for toxoplasmosis would achieve therapeutic, systemic, brain and eye concentrations to be effective in the organs where the majority of disease occurs and would be active against both the acute replicating tachyzoite and latent bradyzoite stages of the parasite.

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New drugs should also prioritize having fewer, milder side effects, a significant problem with the current first-line drugs. Current research into new drugs developed specifically for toxoplasmosis has led to promising preclinical compounds. This review discusses the aspects of toxoplasmosis that are germane to drug development and ongoing preclinical drug research.

*T. gondii* is a remarkably successful parasite that is broadly distributed throughout the world and is capable of infecting both mammals and birds. Up to one-third of the human population is estimated to have been infected.<sup>1</sup> The great majority of human *T. gondii* infection occurs either by ingestion of oocysts that are generated in the felid intestine and spread throughout the environment via feces or ingestion of *T. gondii* tissue cysts in undercooked meat. Congenital infection occurs through vertical transmission when a previously uninfected mother is infected during pregnancy. Otherwise, uncommon means of transmission include transplantation of infected organs, blood transfusion, or inhalation of oocyst-contaminated dust.<sup>2</sup> The resiliency of *T. gondii* oocysts in the environment contributes to the high rates of *T. gondii* infection in humans, and the risk of toxoplasmosis outbreaks, as evidenced by large waterborne outbreaks of *T. gondii* infection from oocyst-contaminated drinking water in Canada and Brazil.<sup>3,4</sup> Although preventive measures focused on hygiene and sanitary meat production may have reduced the prevalence of human *T. gondii* infection, these measures will not reduce the overall burden of human *T. gondii* infection enough to decrease the need for better anti-*Toxoplasma* therapies in the near future.

## Prevalence of *T. gondii* infection and disease

The seroprevalence of *T. gondii* antibodies varies significantly worldwide as rates of human infection are influenced by climate, the consumption of undercooked meat, hygiene, and exposure to cats.<sup>5</sup> Direct comparisons of seroprevalence studies are limited by heterogeneous methodologies, but have been important in identifying specific high prevalence populations. For example, seropositivity for *T. gondii* antibodies in Brazil ranges from 20% to >90% among different groups.<sup>6</sup> In the US, the seroprevalence among people aged 12–49 years has declined from 14.1% to 6.7% between 1994 and 2010.<sup>7</sup> However, prevalence in the US was reported to be 29.9% in people aged >70 years and 25.1% in US residents born outside of the US.<sup>7</sup> In studies of pregnant women and women of child bearing age, seroprevalence in Europe, Asia, and Africa ranges from 20% to 60%.<sup>5</sup>

Although studies of seroprevalence provide valuable insight into *T. gondii* transmission and the underlying risk for the development of toxoplasmosis in a population, the worldwide incidence of disease caused by *T. gondii* is less understood. In addition to the risk of acquisition of *T. gondii* infection, the development of symptomatic toxoplasmosis is influenced by the prevalence of immunosuppressive conditions such as AIDS and may be influenced by differences in the virulence of *T. gondii* strains found all over the world. The hypothesis that certain strains of *T. gondii* are more virulent is most evident in Brazil where non-archetypal strains are associated with symptomatic ocular toxoplasmosis<sup>8</sup> and in French Guiana with severe disseminated disease in the immunocompetent.<sup>9</sup> By comparison, 1%–2% of persons infected with *T. gondii* develop eye disease in the US, whereas eye disease can approach up to 20% of seropositive persons in highly endemic areas of Brazil.<sup>10</sup> The broad distribution of *T. gondii* disease in populations with limited medical resources indicates that a new anti-*T. gondii* drug should be orally bioavailable, have a chemically stable formulation, and be inexpensive to produce.

## Toxoplasmosis

*T. gondii* infection in immunocompetent hosts rarely requires drug therapy. Over 80% of primary *T. gondii* infections in immunocompetent hosts are asymptomatic.<sup>11</sup> Symptomatic infection typically consists of self-limited bilateral nontender cervical lymphadenopathy, which may be accompanied by fevers and myalgias. Following primary infection, *T. gondii* establishes latent infection, converting to the quiescent bradyzoite form within tissue cysts. Although the host immune system is capable of controlling most *T. gondii* primary infections, ocular cysts may be reactivated and lead to vision loss from retinal scarring. In the US, 21,000 persons per year are estimated to develop ocular lesions and 4,800 persons per year develop symptomatic ocular lesions.<sup>10</sup> The effect of latent *T. gondii* brain infection on human behavior and mental health in immunocompetent individuals is an active area of research, but a causal relationship between latent infection and psychiatric disease or changes in human behavior has not been established.<sup>12–14</sup> Unlike *T. gondii* in most healthy people, infection in the immunocompromised is often debilitating or fatal.

Severe manifestations of *T. gondii* most often occur when tissue cysts reactivate in the setting of immunosuppression due to AIDS or medical immunosuppression. The most frequent severe clinical presentation is *Toxoplasma* encephalitis, which typically consists of multiple discrete brain

lesions. Ocular and pulmonary diseases are the most common extra-cerebral sites of infection; however, disease involving other organs has been reported as well as sepsis due to disseminated disease.<sup>15</sup> In individuals with AIDS and a CD4 T-cell count <100 cells/ $\mu$ L, the incidence of encephalitis is 28% in seropositive patients not taking prophylaxis.<sup>16</sup> In allogeneic stem cell transplant patients, the 6-month incidence of symptomatic toxoplasmosis has been reported to be as high as 6% in a prospective multicenter study of seropositive patients, although prior retrospective studies have reported a lower incidence.<sup>17–20</sup> Of note, patients who developed symptomatic toxoplasmosis in this study were not taking standard prophylaxis. Toxoplasmosis has been reported in autologous stem cell transplant patients as well, although the risk for disease is lower.<sup>21</sup> Despite the relatively low prevalence of *T. gondii* infection in the US, in 2008, there were ~3,585 toxoplasmosis-related hospitalizations.<sup>22</sup> The cost of illness in the US caused by *T. gondii* has been estimated to be ~\$3 billion and an annual loss of 11,000 quality-adjusted life years.<sup>23</sup> Severe toxoplasmosis in the US is reduced from the height of the AIDS epidemic prior to highly active antiretroviral therapy, but remains a significant source of morbidity and mortality in the US and worldwide.

Severe toxoplasmosis also results from congenital infection and may occur rarely in healthy individuals. Congenital toxoplasmosis ranges from subclinical to severe disease that consists primarily of brain and eye manifestations, but may also include extracranial pathology in up to half of neonates.<sup>24</sup> Although rare, severe toxoplasmosis in the immunocompetent has been reported, and in a notable cluster of cases in French Guiana, it seems to be due to virulent, non-archetypal strains.<sup>9,25–27</sup> Unlike typical toxoplasmosis, patients in these series presented with disseminated infection involving multiple organs, frequently had pulmonary involvement, and required intensive care.

## Current medicines for toxoplasmosis

First-line therapy consists of the combination of pyrimethamine and sulfadiazine with leucovorin added to prevent hematologic toxicity. In observational studies and controlled trials for *Toxoplasma* encephalitis, this regimen has been found to have high rates of toxic side effects leading to discontinuation of therapy. A review of 115 patients with *Toxoplasma* encephalitis found toxicity in 62% of patients and severe side effects requiring a change in therapy in 44% of patients.<sup>28</sup> Similarly, in treatment trials of *Toxoplasma* encephalitis, toxicity led to discontinuation of pyrimethamine-sulfadiazine

in one-third of patients.<sup>29,30</sup> Sulfadiazine may be replaced with clindamycin if the patient has an allergy to sulfa drugs; however, the clindamycin-containing regimen is less effective in preventing relapse and had similar rates of toxicity.<sup>29</sup> Trimethoprim-sulfamethoxazole has been shown to have efficacy similar to pyrimethamine-sulfadiazine and may be used as an alternative if patients do not have sulfa allergy and pyrimethamine is not tolerated or is not available.<sup>31–33</sup> Atovaquone or azithromycin may be used as alternate therapy in combination with pyrimethamine or sulfadiazine for the treatment and prophylaxis of toxoplasmosis when first-line therapy is contraindicated. However, use of these alternate therapies is supported by less clinical data, and these regimens have similar rates of patient intolerance.<sup>34–36</sup> In addition to frequent side effects, pyrimethamine and sulfadiazine are associated with rare severe reactions that may be fatal, including agranulocytosis, Stevens–Johnson syndrome, toxic epidermal necrolysis, and hepatic necrosis. Drugs that are less toxic would greatly improve the care of patients with toxoplasmosis.

The need for nontoxic medicines is further emphasized by the prolonged courses of therapy required for treatment and suppression of infection. Initial treatment duration for *Toxoplasma* encephalitis is at least 6 weeks followed by secondary suppression until sufficient immune reconstitution; duration for congenital infection is at least 1 year; ocular infection is 4–6 weeks with the consideration of continued suppression to prevent relapse.<sup>24,37,38</sup> Prolonged courses are required, in part because current clinical medicines are unable to eliminate the tissue-cyst stage of *T. gondii*. A promising aspect of several experimental compounds is activity against *T. gondii* tissue cysts that are established in mice 5 weeks prior to treatment.<sup>39,40</sup> Previous studies have shown that atovaquone possesses activity in experimental models in vivo against tissue cysts; however, clinical studies of ocular infection and encephalitis have not shown an advantage in preventing relapse, which may be related to subtherapeutic drug concentrations in infected tissue compartments or translational limitations of toxoplasmosis animal models.<sup>34,38,41</sup>

## Challenges for anti-*T. gondii* drugs

The biology of *T. gondii* bradyzoite containing tissue cysts has been poorly understood and is an active area of research that will be important for the development of drugs that eliminate *T. gondii* infection from the host. Tissue cysts are made up of bradyzoites surrounded by a thick glycan-rich cyst wall. Animal models have provided important insights into

drugs that have the potential to cure latent *T. gondii* infection. However, virulence and cyst formation vary depending on the *T. gondii* strain and the host species and strain. It is unclear which animal model most resembles human infection. In addition to uncertainty regarding the most appropriate translational animal models, the key biological questions of the metabolic pathways that are essential for bradyzoite survival in the tissue cyst and which of these are viable drug targets remain mostly unanswered. McPhillie et al<sup>42</sup> made recent progress in understanding bradyzoite biology, demonstrating that the cyst-forming EGS strain possesses a mutation in the transcription factor *Apetala 2*, a known repressor of bradyzoite genes. Provocative studies of the ME49 *T. gondii* strain in CBA/J mice have shown heterogeneous levels of replication among bradyzoites within cysts and variation between cysts.<sup>43</sup> Future studies probing the biology and drug susceptibility of latent *T. gondii* infection should provide more effective strategies for eliminating *T. gondii* tissue cysts from the host, preventing relapsing disease and potentially shortening the required duration of drug administration.

Drug resistance in *T. gondii* is suspected to contribute to treatment failures that have occurred in ~10% of patients during initial therapy and 10%–20% of patients who relapse during suppressive therapy. However, the contribution of drug resistance to treatment failure is difficult to quantify because *T. gondii* is not routinely recovered from patients with toxoplasmosis. Moreover, treatment failures of toxoplasmosis during clinical studies have been difficult to characterize due to diagnostic limitations, drug intolerance, nonadherence, variable drug absorption, and the complex morbidities of patients.<sup>29,30,32,34,44</sup> High-level sulfonamide resistance has been found in clinical isolates without known sulfonamide exposure and after sulfadoxine exposure.<sup>45,46</sup> The resistant *T. gondii* strain in which there was no drug exposure possessed a mutation in the dihydropteroate synthase (DHPS) gene known to confer resistance in both *T. gondii* and *Plasmodium falciparum* and demonstrated cross-resistance to several sulfonamides including sulfadiazine and sulfamethoxazole.<sup>43</sup> However, genetic mutations in DHPS do not account for sulfonamide resistance in all clinical isolates, and research into alternate mechanisms is ongoing.<sup>45,47,48</sup>

The range of sulfonamide susceptibility seems to be greater than that of pyrimethamine or atovaquone. Genetic evidence of atovaquone resistance has not been found in clinical isolates, but has been obtained in vitro with chemical mutagenesis.<sup>49</sup> Variations in atovaquone susceptibility among different *T. gondii* genotypes have been observed but

subsequent experiments did not find significant differences in genotype susceptibility.<sup>45,50</sup> Similarly, type I *T. gondii* strains have been shown to be less susceptible to pyrimethamine in vitro compared to type II and type III strains; however, subsequent studies found that decreased susceptibility to pyrimethamine correlated with the replication rate rather than genotype and decreased susceptibility was not great enough to be considered significant in pyrimethamine resistance.<sup>45,51</sup> Differences in in vitro susceptibility for pyrimethamine and atovaquone found in these studies likely reflect the differences in methodologies, and the translational significance of these findings is not clear. However, given that sulfonamides are part of first-line therapy, variations in sulfonamide susceptibility, whether preexisting or occurring after drug exposure are concerning and further accentuate the need for new drugs for toxoplasmosis.

## Preclinical testing of anti-toxoplasma compounds

Well-established in vitro assays and animal models exist for preclinical anti-*T. gondii* drug development, but there is no standard testing cascade. Most often, anti-*Toxoplasma* compounds are initially evaluated in vitro in tachyzoite replication assays. Common assays use *T. gondii* that expresses  $\beta$ -galactosidase or fluorescent proteins, or plaque formation or [<sup>3</sup>H] uracil uptake in non-transgenic *T. gondii*. *T. gondii* requires a host cell to replicate; therefore, it is also important to assess the effects of the compounds on the viability of the host cells while assessing the degree of compound inhibition. Although *T. gondii* inhibition assays may be performed on different strains of *T. gondii*, rates of replication vary among different strains, and the type I RH strain is often used initially because it is well adapted to in vitro culture and replicates relatively quickly and reliably.

The RH strain is typically fatal in mice within 10 days providing a model that reflects fulminate, disseminated infection that is not controlled by the host immune system. This strain is useful to evaluate acute infection; however, this model does not evaluate activity against brain tissue cysts, and type I strains are not the dominant strain in most of the areas of the world. Type II strains are more prominent in North America and Europe, are less virulent in mice, and establish tissue cysts in the mouse brain. Susceptibility to infection with type II strains differs among mouse strains, and the degree to which tissue cysts in the mouse brain resemble latent human *T. gondii* infection is unknown. Recent experiments have indicated that the ongoing replication of



bradyzoites occurs within the established brain tissue cysts as long as 8 weeks after infection drawing into question the presumption that latent bradyzoite infection is metabolically dormant.<sup>43,52</sup> Models of latent infection that started treatment with experimental compounds after 5 weeks of sublethal infection with the ME49 strain have demonstrated brain cyst reduction by experimental compounds.<sup>39,53</sup> In addition to acute and latent murine models of infection, congenital and ocular toxoplasmosis models as well as other animal species have been evaluated, but the majority of experimental compounds are initially tested in models wherein mice are infected orally or by intraperitoneal injection, and parasite burden in tissues or survival is measured as outcome.<sup>54</sup>

Future testing of preclinical compounds against genetically diverse, non-archetypal strains of *T. gondii* both in vitro and in vivo should prove insightful in that virulence and pathogenesis vary between strains. Recently characterized parasite strains, such as the EGS strain that forms thick-walled cysts spontaneously in tissue culture allows for the study of inhibitors against cysts in vitro.<sup>42</sup> In addition, this strain has been genetically modified to express stage-specific fluorescence to further characterize the activity against bradyzoites and tissue cysts.<sup>55</sup> Beyond improvements in testing cascades, new methods of target identification are likely to contribute to drug discovery. Future investigation of drug targets in *Toxoplasma* has recently been advanced by a genome-wide screen using CRISPR/Cas9 to identify essential genes.<sup>56</sup> Using the phenotypic data from this screen, which is available through [www.toxodb.org](http://www.toxodb.org), researchers are able to investigate fitness-conferring proteins for potential drug targets.<sup>57</sup>

Basic research in *T. gondii* biology and drug screening has identified a diverse array of drug targets. Over 20 preclinical drug development projects have been described in publications over the past decade. The research studies selected in this review reflect the advances, challenges, and current status of drug development for toxoplasmosis. Table 1 presents the classes of compounds in alphabetical order as they appear in the text and with the chemical structures.

## Artemisinin derivatives

Derivatives of artemisinin, an endoperoxidase, are the backbone of first-line treatment for malaria. Artemisinin and its derivatives also have in vitro efficacy against *T. gondii*.<sup>58–60</sup> Anti-parasitic activity of these compounds requires a 1,2,4-trioxane moiety and is enhanced by substitutions at the C-10 position.<sup>61</sup>

Schultz et al<sup>62</sup> tested two artemisinin derivatives, CPH4–136 and LEW3–27, in murine models of acute and chronic

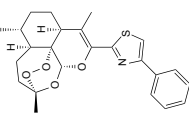
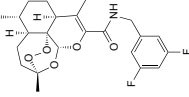
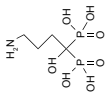
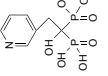

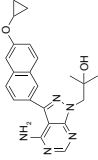
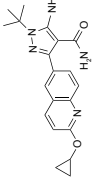
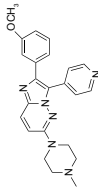
infection. CPH4–136 and LEW3–27 contain thiazole and carboxamide substitutions at their respective C-10 positions and were previously found to have in vitro activity against tachyzoites (340 and 360 nM, respectively).<sup>63</sup> Mice were infected intraperitoneally (IP) with RH strain tachyzoites and then treated with 3–50 mg/kg of either compound IP for 7 days. Both the compounds prolonged survival to a modest extent. Approximately 20% of infected mice treated with LEW3–27 at 30 mg/kg/day were alive at day 20, and all the mice treated with CPH4–136 at 10 mg/kg/day were dead by day 18. All the infected mice treated with atovaquone (10 mg/kg/day) survived until day 20, and the infected, untreated mice died by 12 days post-infection. In addition, CPH4–136 at doses of 30 and 10 mg/kg was associated with early mortality in the treatment group compared to the control group treated with vehicle alone, raising questions regarding toxicity at these doses. However, in a murine model of infection with the ME49 strain, a lower 3 mg/kg dose of CPH4–136 reduced cyst burden by ~40% when given for 32 days. Neither LEW3–27 nor artemether reduced the cyst burden.

## Bisphosphonates: farnesyl diphosphate inhibitors

Bisphosphonates are used for the treatment of osteoporosis and other diseases of bone resorption. Nitrogen-containing bisphosphonates, such as, alendronate or risedronate, inhibit farnesyl pyrophosphate synthase in the mevalonate pathway in osteoclasts.<sup>64</sup> Many parasitic protozoa, including *T. gondii*, possess a mevalonate pathway for the synthesis of sterols and polyisoprenoids. A key branch of this pathway is the synthesis of farnesyl diphosphate, a precursor to ubiquinone, sterols, and prenylated proteins. Synthesis of farnesyl diphosphate in *T. gondii* is catalyzed by the bifunctional enzyme farnesyl diphosphate/geranylgeranyl-diphosphate synthase<sup>65</sup> and is inhibited by nitrogen-containing bisphosphonates.<sup>66</sup>

In an early study,<sup>66</sup> risedronate was shown to have an in vitro IC<sub>50</sub> of 490 nM against *T. gondii*. In vivo experiments<sup>67</sup> demonstrated that risedronate (10 mg/kg/day) improved 30-day survival by 35% in Swiss Webster mice infected orally with 10 cysts of the C56 strain of *T. gondii*. An amount of 20 mg/kg/day improved 30-day survival by 55%. Later work<sup>67</sup> further improved the potency of bisphosphonate derivatives. Compound 1 in the series described in Ling et al is an n-alkyl derivative that has an in vitro IC<sub>50</sub> of 280 nM. When administered to mice infected with 5 cysts of C56 *T. gondii* at 10 mg/kg IP daily for 10 days, compound 1 improved 30-day survival to 80%.

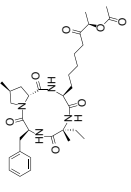
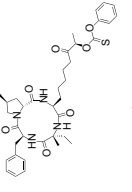
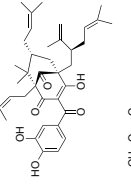
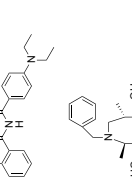
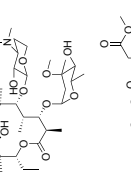
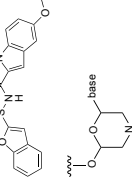
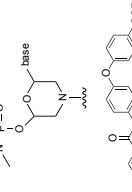
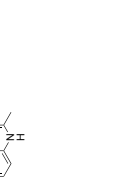
Table 1 Experimental compounds for toxoplasmosis

Compound	In vitro IC <sub>50</sub>	In vitro IC <sub>50</sub> method	Target	In vivo data	In vivo toxicity	Structure	Reference
Artemisinin derivative CPH4-136	340 nM	β-Galactosidase in HFF culture	Unknown	10 mg/kg IP twice daily ×7 days starting day after RH infection showed trend toward improved survival vs control 3 mg/kg/day IP × 32 days reduced cyst formation by 40%	Early mortality seen at 30 and 10 mg/kg/day		62, 63
Artemisinin derivative LEW3-27	360 nM	β-Galactosidase assay in HFF culture	Unknown	30 mg/kg IP twice daily ×7 days starting day after RH infection improved survival vs control	None observed		62, 63
Alendronate	25 μM	[ <sup>3</sup> H] uracil uptake in HFF culture	Farnesyl diphosphate synthetase	20 mg/kg/day IP given days 3–10 after RH infection not effective in prolonging survival	–		66, 67
Risedronate	490 nM	–	Farnesyl diphosphate synthetase	10 mg/kg/day IP given days 3–10 post- RH infection improved 30 days survival by 35% and 20 mg/kg/day IP improved survival by 55%	Apparent at 20 mg/kg IP ×2 days		66, 67
N-alkyl bisphosphonate derivative (compound 1)	280 nM	β-galactosidase in HFF culture	Farnesyl diphosphate synthetase	10 mg/kg/day IP given days 3–10 post oral C56 infection improved 30 days survival by 80%	Not reported		65
Bumped kinase inhibitor Pyrazolopyrimidine scaffold (compound 32)	60 nM	β-Galactosidase in HFF culture	Calcium-dependent protein kinase I (TgCDPK I)	20 mg/kg orally ×5 days started 2 days post-infection reduced acute RH infection by 95%–99%. 30 mg/kg orally ×14 days started 5 weeks post-infection reduced ME49 brain cysts by 89%	None observed up to 100 mg/kg		53
Bumped kinase inhibitor 5-Aminopyrazole-4-carboxamide scaffold (compound 35)	89 nM	β-Galactosidase in HFF culture	Calcium-dependent protein kinase I (TgCDPK I)	20 mg/kg orally ×5 days started 2 days post-infection reduced acute RH peritoneal infection to undetectable level	None observed up to 100 mg/kg		79
Bumped kinase inhibitor 14-Imidazo[1,2-b]pyridazine scaffold (compound 1)	50 nM	β-Galactosidase in HFF culture	Calcium-dependent protein kinase I (TgCDPK I)	–	–		83

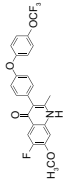
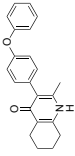
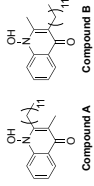
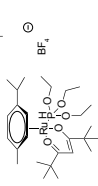
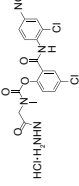
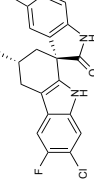
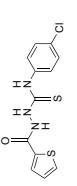
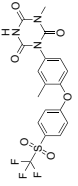
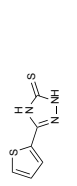
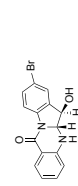
JPC-2056	ND	–	Dihydrofolate reductase	40 mg/kg given orally twice daily for 3 days reduced RH infection peritoneal parasite burden	None observed up to 98 mg/kg	87	
JPC-2067-B	20 nM	[ <sup>3</sup> H] uracil uptake in HFF culture	Dihydrofolate reductase	Reduced burden of infection by 2 logs when given IP 1.25 mg/kg ×4 days starting on day of infection	Not reported	87	
NITD731	71 nM	β-Galactosidase in U-2 OS culture	Signal peptide peptidase	–	–	89	
Tricosan derivative (compound 16c)	250 nM	Fluorescence of RH-YFP parasites in HFF culture	Enoyl-acyl carrier protein	75 mg/kg IP ×2 days started day of infection reduced peritoneal burden of RH infection	Not reported	98	
Tricosan-octoarginine	–	–	Enoyl-acyl carrier protein	40 mg/kg/day IP ×2 days started day of infection reduced peritoneal burden of RH <i>T. gondii</i>	None observed	101	
Tricosan (liposomal)	–	–	Enoyl-acyl carrier protein	100 mg/kg/day orally divided twice daily ×4 days started day after infection reduced peritoneal burden of RH <i>T. gondii</i> by 98%	None observed	102	
Thiolactomycin (analog 5)	1.6 μM	[ <sup>3</sup> H] uracil uptake in LLC-MK <sub>2</sub> culture	Acetoacetyl-acyl carrier protein synthase	–	–	106	
Trovafoxacin derivative (Analog 1)	530 nM	[ <sup>3</sup> H] uracil uptake in HFF culture	Uncertain, likely targets apicoplast DNA synthesis	–	–	113	
Ciprofloxacin ester prodrug (compound 2)	420 nM	Measured % inhibition by counting RH proliferation in LLC-MK2 culture	Uncertain, likely targets apicoplast DNA synthesis	50 mg/kg/day orally ×7 days started 1 day post-infection improved 60-day survival to 18% in acute RH infection	None observed up to 200 mg/kg	116, 117	
Ciprofloxacin adamantanyl derivative (compound 5)	460 nM	Measured % inhibition by counting RH proliferation in LLC-MK2 culture	Uncertain, likely targets apicoplast DNA synthesis	100 mg/kg/day ×7 days orally started 1 day post-RH infection improved 60-day survival to 25%. 50 mg/kg was not effective	None observed up to 200 mg/kg	116, 117	
FR235222	7.6 nM	[ <sup>3</sup> H] uracil uptake in HFF culture	Histone deacetylase TgHDAC3	Cysts treated ex vivo with 200 nM compound ×7 days were incapable of infecting mice	Not reported	120, 121	

(Continued)

Table 1 (Continued)

Compound	In vitro IC <sub>50</sub>	In vitro IC <sub>50</sub> method	Target	In vivo data	In vivo toxicity	Structure	Reference
W363	10.2 nM	[ <sup>3</sup> H] uracil uptake in HFF culture	Histone deacetylase TgHDAC3	–	–		121
W399	11.3 nM	[ <sup>3</sup> H] uracil uptake in HFF culture	Histone deacetylase TgHDAC3	–	–		121
Garcinol	1.8 μM	RT-qPCR of parasites in HFF culture	Histone acetyltransferase TgGCN5	–	–		122
QQ-437	16 nM (IC <sub>90</sub> )	[ <sup>3</sup> H] uracil uptake in HFF culture	Adaptin-3β	20 mg/kg/day IP reduced burden of Prugnau strain <i>T. gondii</i>	None observed		124
Azithromycin derivative (compound 11)	2 μM	Fluorescence of RH-YFP parasites in HFF culture	Suspected 50S ribosomal subunit	–	–		132
SW404	130 nM	Fluorescence of RH-YFP parasites in HFF culture	Pantothenic acid biosynthesis	–	–		134
Peptide-conjugated phosphorodiamidate morpholino (anti-DHFR)	–	–	DHFR	12.5 mg/day IP ×2 days started day of infection reduced peritoneal burden of RH <i>T. gondii</i> by 83% vs control	Not reported		137
Endochin-like quinolone 271	0.1 nM	β-Galactosidase in HFF culture	Q <sub>i</sub> site of cytochrome bc <sub>1</sub>	ED <sub>50</sub> = 0.14 mg/kg orally against RH <i>T. gondii</i> when given orally ×5 days started 2 days post-infection. Reduced latent ME49 strain brain cysts by 87% at 5 mg/kg IP ×16 days started 5 weeks post-infection	None observed up to 50 mg/kg orally ×5 days		39



Endochin-like quinolone 316	0.007 nM	$\beta$ -Galactosidase in HFF culture	$Q_i$ site of cytochrome $bc_1$	ED <sub>50</sub> = 0.08 mg/kg against RH <i>T. gondii</i> when given orally $\times 5$ days started 2 days post-infection. Reduced ME49 brain cysts by 88% at 25 mg/kg IP $\times 6$ days started 5 weeks post-infection	None observed up to 50 mg/kg orally $\times 5$ days	39	
MJM170	30 nM	Fluorescence of RH-YFP parasites in HFF culture	$Q_i$ site of cytochrome $bc_1$	25 mg/kg IP $\times 5$ days started day of infection significantly reduced peritoneal burden of RH and Prugnau infection. Reduced ME49 brain cysts at 12.5 mg/kg $\times 17$ days	None observed at 25 mg/kg IP $\times 5$ days	42	
Hydroxydodecylquinone derivative (compounds A and B)	A 0.4 nM B 0.8 nM	$\beta$ -Galactosidase assay in HFF culture	NADH dehydrogenase and dihydroorotate dehydrogenase	32 mg/kg/day IP $\times 5$ days started 3 days post-infection reduced peritoneal, liver, and lung burden of RH infection	Not reported	152, 154	
Ruthenium complex (compound 18)	41 nM	$\beta$ -Galactosidase assay of RH strain parasites in HFF culture	Unknown	–	–	157	
Salicylanilide derivatives (compound 14a)	16–31 nM	Fluorescence of RH-YFP parasites in HFF culture	Unknown	100 mg/kg orally once following TgGoatUS4 infection extended average survival by 1 day vs control	–	158	
NITD609	1 $\mu$ M	Fluorescence of RH-YFP parasites in HFF culture	Suspected TgA TPase4	100 mg/kg/day orally $\times 2$ days starting day of infection reduced IP parasite burden by 90%	Not reported	138	
Thiosemicarbazide derivative (compound 1g)	107 $\mu$ M	RT qPCR of parasites in L929 cell culture	Unknown	–	–	172	
Ponazuril	Not reported	1 mg/mL (2.2 $\mu$ M) reduced RH proliferation in HFF culture	Unknown	20 mg/kg/day $\times 10$ days started 6 days post-infection resulted in 100% 8-week survival vs 0% for controls in acute RH infection	Not reported	169	
s-Triazole	207 $\mu$ M	RT qPCR of parasites in L929 cell culture	Suspected 1-deoxy-D-xylulose-5-phosphate reductoisomerase based on in silico modeling	–	–	167	
Trypanthrin derivative (compound 15b)	2 nM	$\beta$ -Galactosidase assay in HFF culture	Unknown	–	–	174	

**Abbreviations:** GFP, green fluorescent protein; HFF, human foreskin fibroblasts; IP, intraperitoneal; ND, not determined; OS, osteosarcoma; qPCR, quantitative polymerase chain reaction; RH-YFP, RH strain *T. gondii* that expresses yellow fluorescent protein; RT, real time; PPMO, peptide-conjugated phosphorodiamidate morpholino oligomers.

## Bumped kinase inhibitors (BKIs)

BKIs inhibit the *T. gondii* calcium-dependent protein kinase 1 (TgCDPK1), a member of the serine/threonine protein kinase family.<sup>68,69</sup> TgCDPK1 regulates the calcium-dependent pathway of *T. gondii* microneme secretion and is required for gliding motility, host-cell invasion, and egress.<sup>70</sup> BKIs inhibit *T. gondii* replication by blocking host-cell invasion and egress.<sup>71,72</sup> *T. gondii* mitogen-activated protein kinase like 1 (TgMAPKL1) has also been suggested as a secondary target for the BKI, 1NM-PP1, as a mutation in TgMAPKL1, was associated with decreased susceptibility to 1NM-PP1 and similar BKIs.<sup>73,74</sup>

A key structural difference between TgCDPK1 and human kinases occurs at the “gatekeeper residue” in the ATP-binding pocket. TgCDPK1 contains a small glycine residue at this position, whereas human kinases have larger residues. The additional space afforded by the glycine residue in TgCDPK1 has been exploited for the design of potent and selective ATP-competitive TgCDPK1 inhibitors.<sup>68,75</sup> Numerous BKI analogs have been developed around different core scaffolds that have promising in vitro and in vivo activities.<sup>53,76–79</sup>

Potent TgCDPK1 inhibitors have been synthesized from pyrazolopyrimidine (PP) and 5-aminopyrazole-4-carboxamide (AC) scaffolds.<sup>53,71,72,74,79,80–82</sup> The BKI scaffold binds in the ATP-binding pocket of TgCDPK1 and the addition of a 6-alkoxy-2-naphthyl group at the C-3 position, and a 4-piperidinylmethylene group at the N-1 position of the PP scaffold further increases selectivity.<sup>75,76</sup> These BKIs were >15,000-fold more active against TgCDPK1 compared to the human kinases SRC and ABL, with no inhibition at 20  $\mu$ M. BKI 1294, which was synthesized with N-methylation of the 4-piperidinyl-methylene substituent to improve metabolic stability, had an in vitro IC<sub>50</sub> of 140 nM and reduced acute *T. gondii* infection by 93% when given orally at 30 mg/kg.<sup>76</sup> Later, BKI 1294 was found to inhibit the human Ether-à-go-go-Related Gene (hERG) ion channel preventing further advancement due to the risk of cardiotoxicity. Vidadala et al investigated the modifications of PP scaffold that maintained efficacy and selectivity for TgCDPK1 while eliminating hERG liability.<sup>53</sup> Compound 32 in their series of BKIs with reduced hERG activity had a >10  $\mu$ M IC<sub>50</sub> against hERG, a 60 nM in vitro IC<sub>50</sub> against *T. gondii* and was effective in vivo. Compound 32 administered orally to mice at 20 mg/kg for 5 days eliminated acute RH strain peritoneal infection. In the latent *T. gondii* infection model using the ME49 strain, 30 mg/kg of compound 32 given orally reduced the number of brain cysts by 88.7%.

Pharmacokinetic studies in noninfected mice demonstrated a brain to plasma concentration ratio of 0.33.<sup>53</sup> Lourido et al<sup>77</sup> described a series of PP analogs. Compounds 11 and 24 were metabolically stable and had in vitro IC<sub>50</sub>s of 250 and 610 nM. These compounds increased 30-day survival in mice infected with type II Pru-LUC strain and decreased brain cysts when administered IP prior to infection at 5 mg/kg for 10 days.<sup>77</sup>

Development of the AC scaffold by Huang et al identified compounds 34 and 35 as lead compounds. Compound 35 had an in vitro IC<sub>50</sub> of 89 nM against *T. gondii*, and in an acute in vivo model using the RH strain, compound 35 reduced infection in the peritoneum below the limits of detection when given orally at 20 mg/kg.<sup>79</sup> Compounds 34 and 35 had a brain to plasma concentration ratio of 0.16 and 0.43, respectively.

In addition to the PP and AC scaffolds, Moine et al identified biphenylimidazoazines as *T. gondii* growth inhibitors by screening a library of compounds.<sup>82</sup> Later, a series of 14 imidazo[1,2-b]pyridazines based on the biphenylimidazoazines were found to inhibit TgCDPK1 in enzymatic studies at EC<sub>50</sub>s of <1  $\mu$ M and 7 compounds inhibited *T. gondii* in vitro at IC<sub>50</sub>s of <1  $\mu$ M. Compound 1, 16a and 16f demonstrated in vitro IC<sub>50</sub>s of 50 nM, 100 nM and 70 nM, respectively, with minimal host cell toxicity.<sup>83</sup>

## Dihydrofolate reductase (DHFR) inhibitors

*T. gondii*, like several other protozoal parasites, has a unique bifunctional DHFR–thymidylate synthase (TS) that contains both catalytic sites on the same protein. DHFR is an extensively studied drug target that is inhibited by pyrimethamine, one of the first-line agents currently used against toxoplasmosis. Clinical use of pyrimethamine is primarily limited by the inhibition of host folate metabolism, which may cause neutropenia. DHFR inhibitors that have greater selectivity for *T. gondii*<sup>84</sup> could potentially be less toxic and more effective.

The dihydrotriazines are a new class of DHFR inhibitors originally developed for use against malaria<sup>85,86</sup> that have also been found to be effective against *T. gondii*. The dihydrotriazine JPC-2067-B<sup>87</sup> inhibited in vitro growth of *T. gondii* with an IC<sub>50</sub> of 20 nM. JPC-2067-B has poor oral bioavailability but was effective in a murine model of acute toxoplasmosis when given IP at a dose of 1.25 mg/kg/day for 4 days. Likewise, the orally available prodrug of JPC-2067-B, JPC-2056, was effective in the same murine model at an oral dose of 40 mg/kg twice daily for 3 days.

## Endoplasmic reticulum-associated degradation (ERAD) inhibitors

Proteins that are inserted into a membrane or secreted from the cell undergo folding and post-translational modification in the ER. A small fraction of proteins become irreversibly misfolded, and these proteins are recycled by a ubiquitin- and proteasome-mediated process called ERAD.<sup>88</sup> Most eukaryotic cells possess an extensive system for detecting misfolded proteins and target them to the ERAD pathway. However, the ERAD system in *T. gondii*, *P. falciparum*, and other protozoan parasites is limited relative to other eukaryotes<sup>89</sup> rendering these parasites more susceptible to interference with this pathway. Harbut et al<sup>89</sup> screened inhibitors known to target various proteins in the ERAD pathway for activity against *P. falciparum*. The authors determined that inhibitors of signal peptide peptidase (SPP) were the most effective and nontoxic agents based on in vitro IC<sub>50</sub>s against *P. falciparum* and human hepatocytes. One of these compounds, NITD731, inhibited *T. gondii* in human U-2 osteosarcoma (OS) cell culture at an IC<sub>50</sub> of 71 nM. No toxicity to host cells was noted at a compound concentration of 10 μM. These results demonstrate that SPP is a potential target for apicomplexan inhibitors.

## Fatty acid synthesis inhibitors

Synthesis of fatty acids in *T. gondii* takes place in the apicoplast.<sup>90–92</sup> Consistent with the evolutionary origin of this organelle, iterative elongation of nascent fatty acids is catalyzed by the multienzyme fatty acid synthase II (FAS II) pathway also found in bacteria and plants.<sup>91,93,94</sup> This is in contrast to the FAS I pathway found in animals and fungi that consists of a single large multifunctional polypeptide that catalyzes the steps in fatty acid elongation.<sup>95</sup>

The FAS II enzyme enoyl-acetyl carrier protein reductase (ENR), which catalyzes the last step in fatty acid elongation, has been the focus of a number of drug development efforts. ENR is inhibited by the antibacterial compound triclosan, which inhibits the in vitro growth of *T. gondii* at low micromolar to nanomolar concentrations.<sup>96,97</sup> Triclosan has a very low solubility in water and poor oral bioavailability and a number of triclosan derivatives have been synthesized to increase potency and solubility.<sup>98–100</sup> The most promising triclosan derivatives are described by Stec et al;<sup>98</sup> compound 16c of this series had an in vitro IC<sub>50</sub> of 250 nM compared to 3 μM for triclosan. Compound 16c also demonstrated improved solubility over triclosan, with a computational log P of 3.9 versus 5.5 for triclosan. Compound 16c, when given at an IP dose of 75 mg/kg/day

for 2 days starting on the day of infection, decreased *T. gondii* proliferation in vivo compared to vehicle controls. Doses of ≤50 mg/kg were not effective.

Samuel et al<sup>101</sup> demonstrated delivery of fluorescently labeled triclosan-octoarginine conjugates to intracellular and extracellular tachyzoites as well as encysted bradyzoites. Fluorescent triclosan-octoarginine conjugates were observed to localize around but not clearly enter the apicoplast. However, incubation of the RH strain in cell culture with a hydrolyzable triclosan-octoarginine conjugate resulted in concentration-dependent inhibition of *T. gondii* growth with maximum effect seen at 12 μM. Furthermore, administration of 40 mg/kg/day triclosan-octoarginine conjugate IP to mice infected IP with the RH strain reduced parasite counts over threefold after 5 days of treatment, compared to treatment with vehicle or unconjugated triclosan.

In another approach to improving the drug properties of triclosan, El-Zawawy et al<sup>102</sup> created triclosan-loaded liposomes and compared their efficacy against triclosan in a murine model of acute toxoplasmosis. Liposomal triclosan at an oral dose of 100 mg/kg/day reduced the parasite burden by 96% in peritoneal fluid compared to a reduction of 74% with standard triclosan at an oral dose of 150 mg/kg/day. There was no evidence of drug toxicity in uninfected controls treated with the same doses.

The benzimidazoles are a class of compounds known to target bacterial ENRs.<sup>103</sup> These compounds inhibit in vitro growth of *T. gondii* with IC<sub>50</sub>s in the low micromolar range.<sup>104</sup> Based on co-crystallization studies, these compounds do not seem to bind TgENR tightly, raising questions as to their primary mode of action.

Another validated drug target in the FAS II pathway is the enzyme β-ketoacyl-acyl carrier protein synthase, which is inhibited by thiolactomycin, a naturally occurring thiolactone.<sup>105</sup> Martins-Duarte et al<sup>106</sup> tested eight thiolactomycin analogs against RH strain *T. gondii* growing in LCC-MK<sub>2</sub> cell culture and noted IC<sub>50</sub>s between 1.6 and 29.4 μM. Electron microscopy of treated parasites revealed swollen mitochondrial, enlarged Golgi complex cisternae, and incomplete separation of dividing daughter cells.

## Fluoroquinolone derivatives

The fluoroquinolones are inhibitors of DNA gyrase and DNA topoisomerase IV widely used in human and veterinary medicine as antibacterial agents.<sup>107–109</sup> Trovafloxacin was found to have activity against *T. gondii*;<sup>110</sup> however, this drug is no longer used because of the risk of liver failure. Subsequent studies have examined fluoroquinolone derivatives and

veterinary fluoroquinolones against *T. gondii*. Although the mechanism of action of fluoroquinolones against *T. gondii* is unknown, they are presumed to inhibit DNA synthesis in the apicoplast.

The veterinary fluoroquinolone enrofloxacin was found to have a modest protective effect against vertical transmission of the apicomplexan parasite *Neospora caninum* in a mouse model.<sup>111</sup> When tested against in vitro replication of *T. gondii* in human foreskin fibroblast (HFF) cell culture, enrofloxacin at 25 µg/mL reduced the number of cells infected by 59% versus an untreated control.<sup>112</sup> It was more effective than sulfadiazine at 100 µg/mL, which reduced the number of infected cells by 27%. Enrofloxacin also demonstrated efficacy in a murine model of infection with the ME49 strain, reducing brain cysts in *Calomys callosus* by 68%, similar to sulfadiazine (79%).

A number of fluoroquinolone derivatives have been synthesized in an effort to create more potent inhibitors of *T. gondii*.<sup>113–115</sup> Khan et al<sup>113</sup> evaluated six compounds with in vitro IC<sub>50</sub>s below that of trovafloxacin. Analogs 1 and 2 of this series both showed IC<sub>50</sub>s of 530 nM, compared to 2.93 µM for trovafloxacin. Dubar et al<sup>116</sup> synthesized several derivatives of ciprofloxacin and noted in vitro IC<sub>50</sub>s of 420 nM for compound 2, an ester prodrug, and 460 nM for compound 5, an adamantyl derivative. Neither compound was observed to be toxic to host cells at 30 µM. In a murine model of acute infection, compound 2 at 50 mg/kg/day orally for 7 days improved 60-day mortality to 18% versus 0% for ciprofloxacin-treated controls.<sup>117</sup> In the same model, compound 5 at 100 mg/kg/day orally for 7 days improved 60-day mortality to 25%.

## Histone acetyltransferase/histone deacetylase inhibitors

The epigenetic control of gene expression by post-translational modification of histone proteins is a key process by which many organisms including *T. gondii* modulate transcription. Acetylation of conserved histone lysine residues by histone acetyltransferases (HATs) creates the post-translation modification, generally increasing transcription of the target gene. Conversely, de-acetylation by histone deacetylases (HDACs) removes the modification, generally decreasing the transcription of the target gene. The multiple stages in the *T. gondii* life cycle require significant changes in gene expression, and several groups have suggested developing novel therapeutic compounds that target epigenetic modification.<sup>118,119</sup>

The cyclic tetrapeptide FR235222 causes hyper-acetylation of histone H4 in *T. gondii*, inhibits growth of the parasite

with an in vitro EC<sub>50</sub> of 7.6 nM, and is associated with the conversion from the tachyzoite to bradyzoite stage.<sup>120</sup> Electron micrographs of bradyzoites formed during treatment with FR235222 show altered morphology and multiple nuclei. *T. gondii* resistant to FR235222 has T99A and T99I mutations in the HDAC TgHDAC3. Introducing either mutation into the parental strain reproduced the FR235222-resistant phenotype, demonstrating that TgHDAC3 is the target of FR235222. Interestingly, T99 is part of a two amino acid insertion (A98T99) that is unique to the members of the apicomplexan HDAC3 family, making this enzyme an attractive drug target.

Treatment of bradyzoites with FR235222 prevents differentiation into tachyzoites. Pretreated bradyzoites are incapable of infecting either an HFF monolayer in vitro or infecting mice.<sup>121</sup> However, host cell toxicity was a concern for FR235222 in that it inhibited HFF cells at an IC<sub>50</sub> of 128 nM. Two derivatives of FR235222, W363 and W399, display IC<sub>50</sub>s against *T. gondii* equivalent to that of the parent compound but less toxic against HFF cells (HFF IC<sub>50</sub>: W363 = 632 nM and W399 = 539 nM).

A recently described HAT inhibitor is garcinol, a polyisoprenylated benzophenone derivative extracted from the kokum fruit (*Garcinia indica*) that targets the HAT TgGCN5b.<sup>122</sup> TgGCN5b is essential for tachyzoite replication.<sup>123</sup> *T. gondii* exposed to garcinol showed decreased transcription of genes regulated by TgGCN5b. Compared to other histone deacetyl transferase inhibitors the in vitro IC<sub>50</sub> of garcinol was less potent at 1.8 µM.

## N-benzoyl-2-hydroxybenzamides

In an effort to identify novel leads effective against *T. gondii*, Fomovska et al<sup>124</sup> screened a library of 6,811 synthetic compounds. The most promising compound to emerge from this screen, MP-IV-1, was found to have an IC<sub>90</sub> of 31 nM against RH strain tachyzoites and no observed toxicity against host cells at 10 µM. A chemically diverse series of MP-IV-1 derivatives were synthesized and evaluated. Of these, the compound QQ-437 was the most potent with an in vitro IC<sub>90</sub> of 16 nM and no apparent toxicity to host cells at 250 nM. Treatment with MP-IV-1 at a dose of 50 mg/kg/day IP reduced RH strain peritoneal fluid parasite counts. In an experiment using type II Prugnialud strain, QQ-437 at a dose of 20 mg/kg/day decreased parasite burden compared to MP-IV-1.

Using an insertional mutagenesis library, the authors identified four clones, all with insertions in the gene coding for adaptin-3β. Although little is known about adaptin-3β



in *T. gondii*, the related protein adaptin-1 is known to be involved in sorting proteins from the Golgi complex to rhoptries.<sup>125</sup> Rhoptries, along with micronemes and dense granules, are secretory organelles found in *T. gondii* whose biogenesis requires correct trafficking of proteins from the Golgi complex.<sup>126</sup> Electron microscopy of parasites treated with either MP-IV-1 or QQ-437 demonstrated distortion of micronemes, dense granules, and rhoptries along with the absence of acidocalcisome, another secretory organelle. These results demonstrate that the N-benzoyl-2-hydroxybenzamides are potent inhibitors of secretory processes in *T. gondii*.

## Macrolide derivatives

The apicoplast is a plastid organelle present in most of the members of the phylum *Apicomplexa* acquired from endosymbiosis.<sup>127</sup> The *T. gondii* apicoplast is the site of several essential metabolic pathways, many of which contain potential drug targets.<sup>128</sup> Molecules that inhibit protein synthesis in the apicoplast have been shown to be effective agents against *T. gondii*. Clindamycin inhibits the bacterial 50S ribosomal subunit and is used clinically as an antibacterial and for the treatment of toxoplasmosis. The macrolides erythromycin and azithromycin also target the 50S subunit and are routinely prescribed for the treatment of bacterial infections. Azithromycin prevents death from acute toxoplasmosis in a murine model<sup>129</sup> and has several advantageous properties as a drug including high oral bioavailability and a long half-life. Similar to clindamycin, azithromycin causes a delayed death phenotype<sup>130</sup> in which parasites are only modestly inhibited during the first round of replication, but all daughter parasites are severely impaired in their ability to replicate further, even after the removal of azithromycin.<sup>131</sup>

Lee et al<sup>132</sup> synthesized a series of erythromycin and azithromycin analogs with the goal of improving antiparasitic efficacy. Compound 11 of their series is an azithromycin derivative in which the ring nitrogen was alkylated with a benzyl substituent. Compound 11 had an  $IC_{50}$  of 2  $\mu$ M. Compound 11 displayed a similar delayed death phenotype as azithromycin with an  $IC_{50}$  of 5  $\mu$ M against the first cycle of replication and an  $IC_{50}$  of 500 nM against the second cycle. Interestingly, in contrast to azithromycin, compound 11 significantly reduced the number of parasitophorous vacuoles formed on initial infection, suggesting an alternate mode of action.

## Pantothenate synthetase inhibitors

All organisms that require coenzyme A and acyl carrier protein have pathways for synthesizing or acquiring these

cofactors from pantothenate (vitamin B<sub>5</sub>). In plants, fungi, and bacteria, pantothenate is synthesized de novo from pyruvate via a conserved three-enzyme pathway;<sup>133</sup> however, animals must acquire pantothenate through their diet or from gastrointestinal bacteria. By searching the *T. gondii* genome for orthologs of the pantothenate synthesis pathway, Mageed et al<sup>134</sup> identified three conserved genes coding for de novo pantothenate biosynthesis in *T. gondii*. Among these genes is the terminal enzyme in the pathway, pantothenate synthetase that converts pantoate to pantothenate. Consistent with this finding, *T. gondii* did not exhibit a requirement for exogenous pantothenate when grown in culture.

Mageed et al<sup>134</sup> tested a series of pantothenate synthetase inhibitors originally developed for *Mycobacterium tuberculosis*<sup>135</sup> and found that two compounds, SW413 and SW404, inhibited in vitro growth of *T. gondii* with  $IC_{50}$ s at 20 and 130 nM, respectively. Supplementing the growth media with 50 mg/L pantothenate decreased drug susceptibility to SW404 216-fold. In silico modeling placed SW404 in the pantoate-binding site of pantothenate synthetase and predicted binding to two conserved glutamine residues of this enzyme.

These results suggest that de novo pantothenic acid biosynthesis is an attractive target for drug development due to the lack of a homologous pathway in the human host. The rapid metabolism of free pantothenate by host cells to coenzyme A would likely prevent *T. gondii* from scavenging pantothenate from the host and overcoming pantothenate synthetase inhibition; however, this would need to be verified by in vivo experiments.

## Peptide-conjugated phosphorodiamidate morpholino oligomers (PMOs)

PMOs are synthetic oligomers that bind complementary mRNA sequences and interfere with gene expression.<sup>136</sup> PMOs are potentially a highly specific means of disrupting the translation of key parasitic proteins. The challenge for developing effective PMOs is delivering the PMO to the target. Using a similar approach to that of Samuel et al,<sup>105</sup> Lai et al<sup>137</sup> conjugated various PMOs to arginine octomers, creating peptide-conjugated PMOs (PPMOs). The authors demonstrated specific knockdown of *T. gondii* DHFR, enoyl-acyl carrier protein reductase, and the transcription factor AP2XI-3, a key regulator of bradyzoite differentiation. Knocking down expression of each protein with 3–5  $\mu$ M of the targeted PMO partially inhibited the replication of RH strain tachyzoites without toxicity to the host HFF cells at 20  $\mu$ M. Growth of parasites treated with an anti-DHFR

PPMO was rescued by supplementation with exogenous folic acid.<sup>137</sup> When a dose of 12.5 mg/kg was given IP for 2 days, the anti-DHFR PPMO reduced parasite count by 83% after 96 h in mice infected with RH strain *T. gondii* compared to treatment with vehicle or an off-target PPMO. PPMOs targeting the 5' end of the *T. gondii* cytochrome *b* gene partially inhibited *T. gondii* replication at 10  $\mu$ M.<sup>42</sup> In principal, PPMOs could be extended to target many essential genes.<sup>137,138</sup>

## 4-(1H)-quinolones

The cytochrome *bc*<sub>1</sub> complex (*bc*<sub>1</sub>) is a drug target in several apicomplexan pathogens, including *T. gondii*, *P. falciparum* and *B. microti*. The *bc*<sub>1</sub> reduces cytochrome *c* as part of the electron transport chain and generates an electrochemical gradient by transferring protons to the intermembrane space. The *bc*<sub>1</sub> also creates ubiquinone for pyrimidine biosynthesis. The *bc*<sub>1</sub> Qo site oxidizes ubiquinol and the *bc*<sub>1</sub> Qi site reduces ubiquinone. Atovaquone is a Qo-site inhibitor that is used as an alternate treatment and prophylactic for toxoplasmosis, and in drug-combination regimens for malaria and mild to moderate babesiosis.<sup>37,139</sup> Qi site inhibitors are not currently in clinical use; however, pyridones, which are Qi site inhibitors, were advanced to human studies for malaria, but were found to have cardiotoxicity in rats and activity against human *bc*<sub>1</sub>.<sup>140</sup> The endochin-like-quinolone (ELQ) series of 4(1H)-quinolone-3-diarylethers are derived from endochin, target the Qi site and have been designed to avoid human *bc*<sub>1</sub> inhibition.<sup>141,142</sup>

Endochin was initially investigated as an antimalarial drug in an avian model of malaria by Salzer et al in 1948.<sup>143</sup> Gingrich and Darrow later found endochin to be active against avian and murine toxoplasmosis in 1951.<sup>144</sup> A library of 4(1H)-quinolone-3-diarylethers was made to improve metabolic stability, solubility and the in vivo efficacy of endochin. ELQ-316 and ELQ-271 were found to be highly effective against acute and latent toxoplasmosis.<sup>39</sup> The in vitro IC<sub>50</sub> values of ELQ-271 and ELQ-316 were 0.1 and 0.007 nM, respectively. The ED<sub>50</sub> values of ELQ-271 and ELQ-316 were 0.14 and 0.08 mg/kg when administered orally against acute toxoplasmosis with the RH strain in mice. ELQ-271 and ELQ-316 reduced the cyst burden in a mouse model of latent infection with the ME49 strain by 76%–88%.<sup>39</sup> ELQ-271 was found to inhibit the human *bc*<sub>1</sub> at 800 nM, whereas ELQ-316 did not inhibit the human *bc*<sub>1</sub> and was not toxic to human fibroblasts or human hepatocarcinoma cells (HepG2) at 10  $\mu$ M, the highest concentration tested.<sup>141</sup> Despite being

highly efficacious, the bioavailability of ELQs was limited by crystallinity and a lack of aqueous solubility, which was viewed as a liability for further clinical development. Improved bioavailability was achieved by the synthesis of ethyl carbonate prodrugs in which the ethyl carbonate moiety disrupts crystal lattice formation.<sup>145</sup> Like atovaquone, ELQ-316 has broad anti-apicomplexan activity and is also highly effective against *P. falciparum* and *B. microti* and offers a novel approach to prevent the development of resistance when combined with atovaquone for dual inhibition of the parasite *bc*<sub>1</sub>.<sup>141,146</sup>

The 4-(1H)-quinolone scaffold was also recently investigated by McPhillie et al.<sup>42</sup> A screen of 5,6,7,8-tetrahydroquinolin-4-one derivatives identified MJM170 as a lead compound that inhibits RH tachyzoites in vitro with an IC<sub>50</sub> of 30 nM and EGS encysted bradyzoites with an IC<sub>50</sub> of 4  $\mu$ M. A 25 mg/kg daily dose given for 5 days IP reduced the acute infection in mice with RH and Prugniald parasites, and a 12.5 mg/kg dose given for 17 days reduced ME49 brain cysts. Inhibition of *Saccharomyces cerevisiae* *bc*<sub>1</sub> Qi-site mutants and co-crystallography with bovine *bc*<sub>1</sub> indicate that MJM170 binds to the *bc*<sub>1</sub> Qi site.

The compound 1-hydroxy-2-dodecyl-4(1H)quinolone (HDQ), a structural analog of ubiquinone, has been identified as an inhibitor of type II NADH dehydrogenase isolated from the yeast *Yarrow lipolitica*, *S. cerevisiae* *bc*<sub>1</sub> Qi site and *T. gondii* dihydroorotate dehydrogenase.<sup>147–149</sup> The electron transport chain of *T. gondii* contains a single-component type II NADH dehydrogenase instead of the multisubunit type I NADH dehydrogenase found in mammals.<sup>150,151</sup> Hegewald et al<sup>152</sup> investigated HDQ in *T. gondii* by generating RH-strain mutants resistant to HDQ. Sequencing of genes coding for ubiquinone-binding proteins showed a N302S point mutation in dihydroorotate dehydrogenase. Enzymatic studies with purified wild-type and mutant enzyme confirmed that HDQ inhibited dihydroorotate dehydrogenase. HDQ was shown to inhibit the in vitro growth of RH strain *T. gondii* with an IC<sub>50</sub> of 3.7 nM.<sup>153</sup> Later work<sup>154</sup> explored the activity of four HDQ derivatives, two of which were more potent than the parent molecule with in vitro IC<sub>50</sub>s of 0.4 and 0.8 nM (compounds A and B, respectively). The in vivo efficacy of these compounds was examined in murine models of acute and chronic infection. Compounds A and B showed similar efficacy as atovaquone in controlling parasite replication in the peritoneal cavity, but HDQ was less effective than atovaquone. HDQ, compound A, and compound B were effective against parasite replication in liver and lung,



but not as effective as atovaquone. In a model of chronic infection with the ME49 strain, compound B showed a trend toward reduced brain cyst burden; however, this result did not reach statistical significance. Similar to the above-mentioned 4-(1*H*)-quinolones, HDQ derivatives are limited by low aqueous solubility via  $\pi$ -stacking of aromatic rings as Pidathala et al<sup>155</sup> describe in their investigation of HDQ derivatives.

## Ruthenium complexes

Ruthenium-based complexes have been investigated for various purposes including the treatment of cancer and bacterial infections.<sup>156</sup> Barna et al<sup>157</sup> examined 18 ruthenium-containing compounds for activity against *T. gondii*, and they found that two compounds, 16 and 18 of their series, had IC<sub>50</sub>s of 18.7 and 41.1 nM, respectively. Treated parasites displayed empty or lipid-filled inclusions after 12 h of incubation with compound 16, and by 36 h, the parasite cytoplasm was disorganized. Both compounds 16 and 18 are hydrolytically stable ruthenium phosphite complexes with hydrocarbon exteriors. The authors hypothesize that the hydrocarbon exterior facilitates movement across lipid bilayers while shielding the core of the molecule from nucleophilic attacks and subsequent degradation.

## Salicylanilides

Fomovska et al<sup>158</sup> synthesized 39 derivatives of the antihelminthic drug niclosamide and examined their in vitro efficacy against RH strain tachyzoites. Six derivative compounds had IC<sub>50</sub>s below 250 nM, and of these, four were tested further to assess whether they killed the parasites or the parasites resumed replication after removal of the compound (compounds 3i, 3j, 7a, and 14a). Parasites resumed replication after removal of compound 3i. Parasites did not recover after removal of 3j, 7a, or 14a. Two compounds, 14a and 14b, were tested for in vivo efficacy by challenging mice with oral infection of either ME49 or TgGoatUS4 oocysts and then treating orally with 100 mg/kg/day, 25 mg/kg/day, or vehicle control. All the infected mice died, but a mild protective effect was seen on treated animals as they died on average 1 day later. No gross toxicities were noted in animals treated with 100 mg/kg of either compound. The mechanism of action of salicylanilides is unknown despite efforts using random insertional mutagenesis to identify a target.

## Spiroindolones

The spiroindolone NITD609 was originally discovered in a drug screen for new antimalarial agents<sup>159</sup> and later found to

be effective against *T. gondii* in vitro and in vivo. The target of NITD609 in *P. falciparum* is a P-type cation-transporting adenosine triphosphatase, PfATP4. In *T. gondii*, NITD609 has an in vitro IC<sub>50</sub> of 1  $\mu$ M against RH strain tachyzoites. In vivo, NITD609 at an oral dose of 100 mg/kg/day for 5 days decreased the parasite burden by 90% in mice infected IP with RH strain parasites. Although the *T. gondii* ATP4 has a high degree of sequence homology to PfATP4, further work is needed to definitively identify the target of NITD609 in *T. gondii*.

## Thiosemicarbazones

Hydroxyurea, or hydroxycarbamide, is an antineoplastic drug primarily used in the treatment of polycythemia vera as well as other myeloproliferative disorders. In mammalian cells, it acts by inhibiting ribonucleotide reductase,<sup>160</sup> which catalyzes the rate-limiting step in DNA synthesis. Inhibition of ribonucleotide reductase leads to cell cycle arrest at the G1/S juncture. de Melo et al<sup>161</sup> demonstrated that incubation of infected Vero cells in 4 mM hydroxyurea for 5 h interfered with intracellular parasite replication. Electron microscopy showed that hydroxyurea caused severe morphologic alterations to intracellular parasites followed by the elimination of parasites from parasitophorous vacuoles.

Hydroxyurea is limited by its relatively low affinity for ribonucleotide reductase as well as its short half-life in humans.<sup>160</sup> However, the thiosemicarbazones have higher affinity for ribonucleoside reductase<sup>162</sup> and are active against *P. falciparum*.<sup>163</sup>

Tenório et al<sup>164</sup> synthesized a series of thiosemicarbazones as well as the structurally related 4-thiazolidinones and screened the resulting compounds for activity. Compounds were found to have IC<sub>50</sub>s ranging from 80 to 500  $\mu$ M based on microscopy of infected host cells. By comparison, both hydroxyurea and sulfadiazine were found to have IC<sub>50</sub>s of 100  $\mu$ M in this assay. The authors did not note any morphologic alterations to the Vero cells at the highest compound concentration of 30 mM. Of all the compounds tested, 4-thiazolidinones with phenyl substituents on the N-3 position showed the lowest IC<sub>50</sub>s. With this in mind, de Aquino et al<sup>165</sup> made an additional set of 4-thiazolidinones with the phenyl group fixed at the N-3 position and a set of thiosemicarbazones with the phenyl at the N-4 position (4-arylthiazolidinones). The two compounds with the lowest IC<sub>50</sub>, 2i and 2k, were 4-arylthiazolidinones with IC<sub>50</sub>s of 50  $\mu$ M against intracellular tachyzoites and 1 mM against the host Vero cells.

Liesen et al<sup>166</sup> continued this work by synthesizing additional 4-thiazolidinones and 1,3,4-thiadiazoles from acylthiosemicarbazides with imidazole substituents at their N-1 position. Several members of this series showed equal efficacy to compounds from previous series but with less toxicity toward the host cells. Dzitko et al<sup>167</sup> expanded on this series and examined how replacement of the imidazole ring with other heteroaryl rings might alter the activity. The most potent compound in their series 1g had an  $IC_{50}$  of 33.2  $\mu\text{g/mL}$  (107  $\mu\text{M}$ ) against RH tachyzoites in L929 fibroblast culture as measured by the incorporation of [<sup>3</sup>H]uracil. The authors conducted in silico studies in which these molecules were docked against possible target enzymes; however, no clear target was identified.

## Triazines

The anticoccidial triazine toltrazuril is widely used in the poultry industry for its activity against *Eimeria spp.* This drug is not used in humans due to its potential to cause developmental malformations in a rat model.<sup>168</sup> The major metabolite of toltrazuril is ponazuril. Mitchell et al<sup>169</sup> demonstrated that ponazuril reduced the proliferation of *T. gondii* grown in African green monkey kidney cells at a concentration of 1 mg/mL (2.2  $\mu\text{M}$ ). The authors also found that oral ponazuril (10 mg/kg/day for 10 days) given 6 days after subcutaneous infection with RH parasites reduced mortality at 8 weeks (5/5 control mice dead versus 3/5 treated). With a higher dose (20 mg/kg/day for 10 days) given 6 days post-infection, all treated mice survived to 8 weeks.

## Triazole derivatives

Itraconazole, a member of the triazole family of antifungal agents, is known to have in vitro activity against *T. gondii* tachyzoites.<sup>170</sup> However, in vivo studies of its protective effect against mortality and brain cyst formation in a mouse model of infection with the ME49 strain failed to demonstrate efficacy.<sup>171</sup> In the same study, the authors showed that the related compound fluconazole reduced 45-day mortality from 50% in untreated controls to 5% when given at 20 mg/kg/day to mice infected IP with ME49. However, fluconazole treatment did not reduce the formation of brain cysts when compared to untreated animals.

The idea of optimizing triazoles for better antiparasitic activity is a particularly attractive one, as these compounds are well tolerated and have high oral bioavailability. Dzitko et al<sup>172</sup> synthesized two such compounds, namely 3-(thiophen-2-yl)-1,2,4-triazole-5-thione and 4-ethyl-3-(4-methyl-1,2,3-thiadiazol-5-yl)-1,2,4-5-thione. The first of

these, which the authors describe as “s-triazole” showed in vitro activity against the RH strain of *T. gondii* grown in L929 mouse fibroblast culture.  $IC_{50}$ s measured by incorporation of <sup>3</sup>H-uracil and quantitative real-time PCR were 42.46  $\mu\text{g/mL}$  (235.6  $\mu\text{M}$ ) and 37.33  $\mu\text{g/mL}$  (207.1  $\mu\text{M}$ ), respectively. These compared favorably to those obtained for sulfadiazine by the same methods, which were 2,225.25  $\mu\text{g/mL}$  (8.9 mM) by <sup>3</sup>H-uracil incorporation and 1,217.62  $\mu\text{g/mL}$  (4.86 mM) by quantitative real-time PCR. Cytotoxic effects on host cells were measured using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay and calculated as the concentration needed to cause a 30% maximal effect ( $CC_{30}$ ). The  $CC_{30}$  for s-triazole was 194.46  $\mu\text{g/mL}$  (1.08 mM) and that of sulfadiazine was 537.06  $\mu\text{g/mL}$  (2.15 mM), demonstrating a better therapeutic index for s-triazole as calculated by  $IC_{50}/CC_{30}$ .

The target of s-triazole and other triazole compounds in *T. gondii* is unclear. Through a literature search, Dzitko et al<sup>172</sup> identified six likely enzymes and attempted in silico docking of s-triazole. Out of three potential targets, the authors suggested that Protein Data Bank number 3AU9 (1-deoxy-D-xylulose-5-phosphate reductoisomerase) was the most likely, followed by 4M84 (calmodulin-domain protein kinase 1) and 3MB8 (purine nucleoside phosphorylase).

## Tryptanthrin derivatives

Tryptanthrin (indolo[2,1-b]quinazoline-6,12-dione) is a compound found in several natural sources, among them the plant *Isatis indigotica*. Krivogorsky et al<sup>173</sup> examined the activity of tryptanthrin and derivative molecules in vitro. Several compounds, in particular derivatives with halogen substituents at the 8 position, had  $IC_{50}$ s under 10 nM. Further work<sup>174</sup> investigated the structure-activity relationship of the 6-keto group by replacing it with oximes, hydrazones, or alcohols. The oxime and alcohol derivatives of 8-bromotryptanthrin had excellent activity, with  $IC_{50}$ s of 3 nM (for the oxime, compound 6b) and 2 nM (for the alcohol, compound 15b). Cytotoxic effects were not seen at concentrations as high as 100  $\mu\text{M}$ . The target of these compounds was not determined.

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## Disclosure

The authors report no conflicts of interest in this work.

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