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Evaluation of current and emerging anti-malarial medicines for inhibition of *Toxoplasma gondii* growth in vitro

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Abstract

Toxoplasma gondii is a common zoonotic infection of humans and estimates indicate that 1-2 billion people are chronically infected. Although largely asymptomatic, chronic infection poses risk of serious disease due to reactivation should immunity decline. Current therapies for toxoplasmosis only control acute infection caused by actively proliferating tachyzoites but do not eradicate the chronic tissue cyst stages. As well, there are considerable adverse side effects of the most commonly used therapy of combined sulfadiazine and pyrimethamine. Targeting the folate pathway is also an effective treatment for malaria, caused by the related parasites *Plasmodium* spp., suggesting common agents might be used to treat both infections. Here we evaluated currently approved and newly emerging medicines for malaria to determine if such compounds might also prove useful for treating toxoplasmosis. Surprisingly, the majority of anti-malarial compounds being used currently or in development for treatment of malaria were only modestly effective at inhibiting in vitro growth of *T. gondii* tachyzoites. These findings suggest that many essential processes in *P. falciparum* that are targeted by anti-malarial compounds are either divergent, or non-essential in *T. gondii,* thus limiting options for repurposing of current antimalarial medicines for toxoplasmosis.

Introduction

Toxoplasma gondii is a common parasite of animals that causes zoonotic infections in humans. It has diverged from its closest relatives by adopting a broad host range re-enforced by flexible modes of transmission (1). *Toxoplasma gondii* is transmitted by cats, where sexual reproduction in the intestine results in shedding of highly resistant oocysts into the environment (2, 3). Ingestion of oocysts by rodents, and many other intermediate hosts, results in acute infection that is characterized by initial expansion of a fast growing tachyzoite form that disseminates widely throughout the body. Following a vigorous immune response the parasite differentiates into a slow growing, semi-dormant form called the bradyzoite, which inhabits tissue cysts in the muscle and brain (4-6). Human infections are acquired by ingestion of oocysts that contaminate food or water, or by eating undercooked meat that harbors tissue cysts. *Toxoplasma gondii* is a

significant cause of serious food borne infection in the USA (7), and it has also been associated with waterborne outbreaks in North and South America (8). Global serological studies indicate that ~25% of humans are chronically infected, although prevalence rates vary widely in different locations (7). Most human infections with *T. gondii* are relatively benign, although they are persistent, as the chronic stages of infection (i.e. bradyzoites within tissue cysts) are not eliminated by the immune response. Additionally, toxoplasmosis can cause serious disease due to congenital infection (9) and in immuncompromised patients (10, 11). Additionally, even healthy adults are at risk due to highly pathogenic strain types that are found some regions such as South America (12, 13).

Toxoplasma is a member of the phylum Apicomplexa, a group of more than 10,000 known species, most of which are parasitic (14). Other apicomplexan parasites of medical importance include *Plasmodium* spp., the causative agent of malaria (15), and *Cryptosporidium* spp., a frequent cause of severe diarrheal disease in young children in developing countries (16). Apicomplexans are most closely related to ciliates and dinoflagellates, but only distantly related to humans, hence they share many key differences from their hosts (17). Although members of the phylum Apicomplexa span 400 mya of evolution (18), they contain many orthologous genes and much of their biology is conserved (19). Among their similar features, *Plasmodium* and *Toxoplasma* contain intact pathways for pyrimidine biosynthesis, while they are purine auxotrophs, and these pathways have been the focus of development of inhibitors to combat both infections (20).

Current therapies for treatment of toxoplasmosis rely primarily on inhibition of the folate pathway in the parasite, although antibiotics developed for treating bacterial infections have also been used with some success (21, 22). The most commonly used treatment is a combination of sulfa drugs with pyrimethamine (i.e. sulfadiazine and pyrimethamine or trimethoprim with sulfamethoxazole). This combination is highly synergistic as the sulfa drug inhibits dihydropteroate synthase (DHPS) while pyrimethamine inhibits dihydrofolate reductase (DHFR), together disrupting tetrahydrofolate levels and blocking DNA synthesis. The combination of pyrimethamine and sulfa drugs is highly effective in blocking replication of tachyzoites but has no

activity on bradyzoites within tissue cysts and therefore does not eliminate chronic infection (23). As well, there are significant adverse effects of this treatment regime due to intolerance or allergic reaction to the sulfa component and bone marrow suppression that requires co-administration of leucovorin (24, 25). Additionally, due to potential for inducing developmental defects, this combination is contraindicated during the first two trimesters of pregnancy, but can be effective in reducing clinical severity when given in the third trimester (26). Although alternative therapies such as clindamycin, azithromycin, and other antibiotics, have also been used to treat acute toxoplasmosis, they also do not clear chronic infection (21).

There have been several efforts to identify new drugs for toxoplasmosis based on FDA-approved drugs. Screening of FDA approved drugs has revealed several inhibitors of tachyzoite growth in vitro, most of which were initially developed to treat inflammation (27). Guanabenz, which targets alpha-2 adrenergic receptors and is used for hypertension, additionally shows efficacy in mouse models of toxoplasmosis (28, 29). Finally, treatment of infected cells with tamoxifen, an inhibitor of the estrogen receptor, leads to parasite clearance due to an autophagy-related process (30). Although such compounds provide promising leads, they do not allow selective inhibition of the parasite since they were originally optimized to modulate host processes. Hence, there is a need for new treatments that are more selective, less toxic, and effective at eliminating chronic infection by *T. gondii*.

One potential source for new drugs to treat toxoplasmosis would be repurposing of medicines that have been developed for malaria, a concept that is based on their shared ancestry and similar biology. One example is the use of pyrimethamine-sulfa drug combinations to treat toxoplasmosis. Similarly, Fansidar (sulfadoxine and pyrimethamine) was historically effective against *P. falciparum*. However, due to the global spread of anti-folate (31) and chloroquine resistance (32), the first line of treatment for malaria has shifted to the natural product artemisinin, which is a sequiterpene lactone that contains an endoperoxide bridge that is key to its activity (33). A number of semi-synthetic variants have been produced including artesunate, artemether, and artemisone, which are more soluble prodrug forms that are rapidly converted to dihydroartemisinin in plasma (31). Artemisinin derivatives also inhibit

replication of *T. gondii* in vitro (34-36) and are partially effective in murine infection models, although they do not eliminate chronic infection (37, 38).

Considerable effort has been expended to develop new generation anti-malarials based on large-scale phenotypic high throughout screens (HTS) for inhibition of asexual blood stage forms of *P. falciparum* (39). A number of the resulting hits were prioritized by the Medicines for Malaria Venture (MMV) based on chemical properties as well as activity to define a core set of compounds for inclusion in the Malaria Box and Pathogen Box projects (https://www.pathogenbox.org/). Combined with genomic analyses of evolved resistant mutants, these screening efforts have led to identification of new leads that target essential steps in the parasite (39). One of the first new active malarial compounds to be identified by a screening/genomics approach was the class of compounds known as spiroindolones (40), including the analog KAE609 that proved effective in curing mice of *P. berghei* infection with a single oral dose (41). Whole genome sequencing of resistant mutants, and subsequent genetic confirmation, indicated that spiroindolones target the cation transporter PfATP4 (42), thereby disrupting sodium transport in the parasite (43). A similar strategy of whole genome sequencing of resistant mutants has led to the identification of several tRNA synthases as targets of potent antimalarial candidate compounds and mutations in the PfCarl (cyclic amine resistance locus) gene that medicates resistance to potent imidazolopiperizines (39).

The availability of the Malaria and Pathogen Box (https://www.pathogenbox.org/) collections has made it possible to expand the analysis of these compounds to other pathogens (44). Analysis of compounds in the Malaria Box for inhibition of in vitro growth of *T. gondii* identified seven compounds with $EC_{50} \leq 5$ μM including a piperazine acetamide with an $EC_{50} < 0.19$ uM (45). The hit rate of ~ 2% observed in this study is higher than typically seen in typical HTS; however, it might be considered low based on the premise that *T. gondii* and *P. falciparum* are members of the same phylum and share much of their underlying biology. Several of the active compounds contain a quinolone moiety, suggesting they may be active due to their resemblance to endochin-like quinolones (46) and atovaquone (47), which act on the bc1 complex and that are active against *T. gondii*. A second study that also employed the Malaria Box reported a

much higher hit rate, with 49 compounds out of ~400 showing EC_{50} values ≤ 1 μM when tested for inhibition of tachyzoite growth in vitro (48); the difference in hit rate being attributed to methodology. Although a number of these compounds were also inhibitors of *P. falciparum*, the overall correlation in potency between these two parasites was low (48). A similar screen for tachyzoite in vitro growth inhibition by compounds in the Pathogen Box identified four compounds with EC_{50} values ≤ 1.0 μM and selectivity indices of > 4 (49). Among the more potent compounds identified was buparvaquone, which is a naphthoquinone that also inhibits mitochondrial electron transport. This study also reported that many compounds active against *Plasmodium* did not show appreciable inhibition of *T. gondii*.

To complement previous efforts that have focused on the early preclinical leads found in the Malaria and Pathogen Box collections, we focused here on approved medicines for malaria, new anti-malarial candidates or emerging leads that are in the global malaria portfolio, many of which with Medicines for Malaria Venture (MMV) (39, 50). Many of these compounds show excellent potential for treatment of malaria, and have advanced through a number of preclinical safety checks and in some cases clinical studies, and include a number of currently used medicines. The rationale for this project was that if active compounds were found among this set, they might be readily repurposed for treatment of toxoplasmosis or give information on biological pathways to target in *T. gondii*.

Results and Discussion

We tested 81 compounds including a number of current medicines used for treatment of malaria and candidates that are in late preclinical development or undergoing current clinical trials. Although some individual compounds had been tested on *T. gondii* previously, many are new compounds and this represents the first time this set of compounds has been compared side by side in the same assay. We tested them in parallel using a multi-well plate assay that monitors in vitro growth of *T. gondii* tachyzoites based on firefly luciferase expression. Initially, we evaluated each of the compounds at a single concentration (10 μM) in triplicate assays to define those that showed > 50 % growth inhibition. Based on this cutoff, 52 compounds were chosen for

further analysis based on duplicate 10-point dilution series that were used to define the EC_{50} values for growth inhibition. Results of the screen are summarized in Table 1 and Figure 1, where compounds are ranked by relative potency.

Among the set of 52 compounds, 18 of them showed EC_{50} values of < 1 μM, including pyrimethamine (Figure 1, red dot), consistent with previously reported values for the activity of this DHFR inhibitor on growth of *T. gondii* in vitro (51, 52). Other inhibitors of DHFR were considerably less potent, including methotrexate (53, 54) and cycloguanil (55, 56), consistent with previous reports (Table 1). As expected, sulfa drugs were also less potent including sulfadiazine, sulfamethoxoazole, and dapsone (Table 1), consistent with previous reports of their in vitro activity (52, 57, 58). The low activity of these molecules vitro may reflect high levels of *p*-araminobenzoic acid in culture medium, as this metabolite acts competitively with these inhibitors of DHPS. Although sulfa drugs are not effective when used alone, as part of the current combination therapy they are highly synergistic with pyrimethamine (52, 59). Among the potent compounds that have not been reported previously was methylene blue, a phenothiazin dye that is being evaluated as a transmission blocking compound for malaria (50). Also included among the most actives were several antibiotics that target prokaryotic protein synthesis by disrupting ribosomes including the lincosamides clindamycin and mirincamycin, and the macrolide azithromycin (Table 1). Interesting, although doxycycline was active in inhibiting *T. gondii* growth, tetracycline showed almost no activity, consistent with a previous report (60). A number of antibiotics have previously been shown to be active on *T. gondii* (59, 61-64) and their mechanism of action is likely due to inhibition of protein synthesis in the apicoplast (65). The main limitation to use of broad-spectrum antibiotics for treatment of toxoplasmosis is their potent activity on the endogenous bacterial flora in the microbiome leading to disbiosis and gastrointestinal distress, thus increasing the risk of *C. difficile* infection (66). The use of such agents that target bacteria also increases the risk of unwanted emergence of resistance among other classes of pathogens.

Other potent inhibitors include the endochin-like quinolone ELQ-300 that targets the Qi site in the cytochrome bc1 complex (67) and atovaquone that targets the Qo site (68) in the cytochrome bc1 complex of the mitochondrial respiratory chain (Table 1).

Previous studies have shown that atovaquone is effective in blocking *T. gondii* replication in vitro and in reducing cyst numbers in chronically infected mice (52, 62), as well as suppressing reactivation of chronic infection in an immunocomprmised mouse model (69). However, prior experience with atovoquone in humans with toxoplasmosis includes several examples of therapeutic failure (47, 70), possibly due to resistance arising, although the mechanism was not confirmed at the molecular level. Similar to atovoquone, ELQ-300 is potent in inhibiting parasite growth in vitro and in reducing cyst numbers in the brains of chronically infected mice (46). The main issue with ELQ-300 (46), and related quinolone compounds (71), is their low solubility that reduces oral bioavailability. Consequently, efficacy trials in murine models of toxoplasmosis have relied on parenteral administration of the compounds. This limitation has been partially mitigated by production of esterified pro-drugs that get activated in vitro, allowing for oral treatment that was protective in a mouse model for *P. yoelli* (72). Given that multiple quinolone containing compounds that affect mitochondrial electron transport are active against *T. gondii*, including against bradyzoites in tissue cysts, this pathway remains an important target for further investigation.

Drugs that have traditionally been used to treat malaria were much less potent in inhibiting *T. gondii* including both 4-amino and 8-amino quinolines (Table 1). Chloroquine and a variety of related 4-aminoquniolines are active against asexual parasite stages of *Plasmodium* that replicate in red blood cells, where these compounds are thought to inhibit hemozoin formation within the parasite's digestive food vacuole (73). Among this class of compounds, bisquinoline and benzylquine were the most active with EC_{50} values of less than 1 μM, while other derivatives were less potent against *T. gondii* (Table 1). These compounds are thought to target hemozoin formation during hemoglobin degradation in *Plasmodium* (73), and the lack of an analogous digestive pathway in *T. gondii* may explain the lack of potency of most members of this class. However, the fact that several 4-amino quinolones and 4-anilino compounds (i.e. pyronaridine, amodiaquine) showed modest potency in inhibiting *T. gondii*, suggests that they target another important process (Table 1).

A number of 8-aminoquinolones are also effective for treatment of malaria, although their mechanism of action remains uncertain. Primaquine has traditionally been used to

treat the dormant hypnozoite stage of *P. vivax* and it is also effective against *P. falciparum* gametocytes. The main deficiency of this compound is its toxicity in patients with G6PD deficiency. A number of 8-aminoquinoline derivatives, such as tafenoquine, lack some of the undesirable effects of primaquine and are also being advanced for preventing relapse of *P. vivax* (74). Unfortunately, the 8-aminoquinolines as a class were largely inactive against *T. gondii* tachyzoites (Table 1). However, given their differential activity on semi-dormant stages of *Plasmodium* development (i.e. gametocytes and hypnozoites) it would be interesting to test these compounds on bradyzoites of *T. gondii*.

Artemisinin derivatives have become the mainstay of combined therapy against severe and uncomplicated malaria (31). Artemsinin is potent across the stages of intraerythrocytic development and this activity has been attributed to hemoglobin degradation and release of free heme, which is thought to activate the endoperoxide bridge, likely forming adducts with multiple targets (33). More recent efforts have focused on completely synthetic endoperoxides, some of which show greater metabolic stability in vivo, and which could reduce reliance on the natural product produced from *Artemesia* cultivation (75). Consistent with prior studies (35, 37), a number of artemisinin derivatives were modestly active in inhibiting *T. gondii* growth (Table 1, Figure 2). Artemisone and artemether were among the most active derivatives, while deoxyartemesinin was inactive, indicating that activity is dependent on the endoperoxide moiety. Unfortunately, more stable trioxolane synthetic peroxides such as OZ439 and OZ277 were much less active on *T. gondii* (Table 1, Figure 2). Even the most potent artemisinin derivatives are several orders of magnitude less effective against *T. gondii* compared to *P. falciparum*, thus limiting their potential as therapeutic options for toxoplasmosis.

Several different chemical scaffolds have been shown to inhibit the P-type cation translocating ATPase in *P. falciparum* known as PfATP4, which resides in the parasite plasma membrane where it modulates cytosolic sodium levels by active extrusion of Na^+ in exchange for H^+ (76). Among the most potent PfATP4 inhibitors are the spiroindolones that are currently in clinical trials for treatment of infection by *P. falciparum* (50). The spiroindolone KAE609 (also known as NITD609), which is highly

active on *P. falciparum* (41-43), was among the more active molecules studied here for inhibition of *T. gondii* growth (EC_{50} ~ 0.25 μM) (Table 1, Figure 3). KAE609 was substantially more potent than a second spiroindolone analog KAF246 (EC_{50} = 1.4 μM) (Table 1, Figure 3), although these two compounds differ only by substitution of Cl for F on the indole group (Figure 3). KAE609 was previously reported to inhibit *T. gondii* growth in vitro with a 50% decrease in parasite growth at 1 μM (reported as MIC_{50}) and in a mouse model for acute toxoplasmosis when the compound was administered at 100 mg/kg (given orally on the day of infection and the day after infection) (77). Compound 21A092, which belongs to a different scaffold known as a pyrazoleamide, also targets PfATP4 and is highly active on *P. falciparum* (78), but was much less potent on *T. gondii* (Table 1). Unfortunately, even potent spiroindoles like KAE609 show much greater potency on *P. falciparum* (EC_{50} ~1 nM) (42) than *T. gondii*, despite the fact that the proposed binding site in PfATP4 is highly conserved, including sites that result in resistance when mutated (77). Interestingly, the relative potency between the ATP4 inhibitors (highest to lowest) KAE609, PA92 and SJ733 on *P. falciparum* appears to match that in *T. gondii*. A number of other chemical scaffolds have also been shown to affect PfATP4 in *P. falciparum* and a previous screen of the Malaria Box identified a number of compounds that likely target PfATP4 (76) as inhibitors of *T. gondii* (48). As many analogs of the spiroindolones, and other scaffolds that act on PfATP4, are available, it may be worth further investigation of this target to identify more potent inhibitors of *T. gondii*.

Additionally, potent new antimalarial compounds that have been recently identified showed only modest activity against *T. gondii* with EC_{50} values that ranged from 1 – 5 μM (Table 1). Included in this group was KAF156, an imidazolopiperazine that showed promising results in a recent clinical trial of *P. falciparum* malaria (79). Additionally, several inhibitors of phosphoinositol 4 kinase (PI4K) showed modest (i.e. UTC944), or no appreciable (i.e. KDU691), activity against *T. gondii* growth, despite having excellent potency against *P. falciparum* (80). Similarly, cladosporin, which targets lysyl tRNA synthase in *P. falciparum* (81, 82), showed only modest activity against *T. gondii* (Table 1). The reasons for the much lower activities observed on *T. gondii* is uncertain but it may reflect differences in the molecular targets of these compounds or differences in

the extent with which these targets play essential roles in the biology of *T. gondii* vs. *P. falciparum*. Alternatively, these differences might arise from differences in the intracellular compartment that affect access of the compounds or from a greater number of efflux mechanisms in *T. gondii*. Regardless of the precise reasons, these differences in sensitivity provide a rationale to explore a more diverse collection of compounds than studied here, with the potential that other analogs within these chemical scaffolds will be found to be more effective on *T. gondii.*

Targeting conserved and essential pathways may thus offer greater advantage for finding compounds with a broader spectrum of activity. One potential example is pyrimidine biosynthesis that is conserved in both *T. gondii* and *P. falciparum*. In particular, *Plasmodium* lacks pyrimidine salvage enzymes and thus is reliant on biosynthesis for RNA and DNA synthesis. Targeted screens have advanced new triazolopyrimidine compounds as inhibitors of *P. falciparum* dihydroorotate dehydrogenase (DHODH), thus blocking pyrimidine biosynthesis (83). One such analog DSM265 is active against both liver and blood stages of *P. falciparum*, shows excellent pharmacokinetic and safety properties, and has advanced to clinical trials for *P. falciparum* malaria (84). DHODH is also essential in for pyrimidine biosynthesis in *T. gondii*, as well as performing another essential function in mitochondria (85). However, DSM265 was not effective at inhibiting *T. gondii* growth in vitro (Table 1). This difference may reflect that fact that DSM265 has been carefully selected for potency on *P. falciparum* hence this may reflect a difference in the molecular target, suggesting that other analogs may be more effective. Alternatively this result that may be due to the capacity of *T. gondii* to salvage uracil (86). Hence, even apparently conserved pathways present challenges for identification of potent inhibitors both due to potential molecular differences in the target and/or alternative metabolic routes in these two parasites.

Conclusions

We evaluated 80 compounds that are used as current therapy for malaria, or which are in late stage development, for their ability to inhibit the growth of the related apicomplexan parasite *T. gondii*. The most active compounds identified were previously

known agents including lincosamide and macrolide antibiotics that target the apicoplast and quinolones that target the bc1 complex in the mitochondria. Consistent with this pattern, artemisinin and related analogs, were modestly potent, while several new generation trioxanes showed very little activity. Moreover, traditional drugs used against malaria including 4-amino and 8-aminoquinolines showed very little activity against *T. gondii*. Similarly, a number of newly identified compound classes that target novel pathways in *Plasmodium* showed limited activity against *T. gondii* including spiroindolones, which inhibit PfATP4, as well as compounds that target PI4K, lysyl tRNA synthase, and others. These findings may suggest that current malaria drugs target pathways that are not conserved in these two parasites, or alternatively that differences in the molecular target will require different analogs to effectively target each of these parasites. Hence, identifying new treatments for toxoplasmosis will require a concerted effort to identify potent inhibitors of essential targets in this organism.

Materials and Methods

Cell culture and parasite propagation

Tachyzoites of ME49 strain encoding a transgenic copy of firefly luciferase (type II, ME49-FLuc) (87) were continually passaged in confluent monolayers of human foreskin fibroblasts (HFF) cultured in DMEM supplemented with 10% fetal bovine serum (FBS), glutamine (10 mM) and gentamycin (10 µg/mL). To isolate parasites, heavily infected cultures of late-stage vacuoles containing replicating tachyzoites were scraped, force lysed through a 23g needle and residual host cell material removed using a polycarbonate filter (3 micron pore). The parasites were then counted, diluted in fresh culture medium and added to 96-well plates as described below. All HFF and parasite cultures were grown in a 37°C incubator supplemented with 5% CO_2 and were verified to be mycoplasma free using the e-Myco Plus kit (Intron Biotechnology).

Luciferase based growth inhibition assays

HFF cells were plated in white, clear-bottom 96-well plates (Costar #3610) and incubated to confluency. Only the inner 60 wells were used to reduce variability due to edge affect with outside wells. Compounds KAF246 and KDU691 were obtained from Novartis Tropical Research Institute. The remaining compounds were provided by

MMV. All compounds were prepared as 10 mM stock in 100% DMSO and stored at -80^{o}C until use. After dilution, the final DMSO concentration for all experimental wells was 0.1%. Pyrimethamine (Sigma-Aldrich, #46706) was reconstituted in 100% DMSO at 5 mM stock concentration and stored at -20^{o}C until use.

For the single point assay, 5x10^{3} ME49-FLuc parasites (in 100 µL volume) were inoculated into plates containing 100 µL of 2x compound (10 µM final concentration, 200 µL final well volume), incubated for 72h at 37°C and luciferase activity evaluated using the Luciferase Assay System (Promega, E1501) according manufacturers protocol. Briefly, culture medium was aspirated and replaced with 40 µL of 1x Passive Lysis buffer (1xPLB, Promega, E1531) and incubated for 5 min at room temperature (RT). Luciferase activity was measured on a Beckman Coulter integrated and automated platform using the following protocol: Inject 100 µL of Luciferase Assay Reagent (LAR), shake 1 sec and read 10 sec post-injection. Only compounds that showed greater than 50% growth inhibition over DMSO control were selected for EC_{50} determination (average of 3 biological replicates). Liquid handling steps (media exchange, compound dilution and addition and luciferase assay steps) were performed on a Biomek Dual Pod FX system using the SAMI EX software as part of the High Throughput Screening Center at Washington University School of Medicine.

For compounds demonstrated >50% growth inhibition at 10 µM, EC_{50} values were determined form a 10-point dose-response curve. Briefly, 5x10^3 ME49-FLuc parasites (100 µL/well) were added to a 96-well plate that contained 100 uL of 2x compound (1x final compound concentration, 200 µL total well volume,) and allowed to replicate for 72h prior to preparation for luciferase assay. All experimental steps, growth conditions and luciferase assay protocols were completed as described above. Compounds were tested using a 3-fold dilution series from 10 µM to 0.001 µM with all wells containing a final concentration of 0.1% DMSO. Pyrimethamine (2.5 µM, positive control) and DMSO (vehicle) controls were added to the outside wells of all plates as controls. The EC_{50} data are presented as the average of two biological replicates.

Statistics

All results are presented as the average of two or more biological replicates. Linear regression analysis and dose-response inhibition (Log (inhibitor) vs. normalized

response – variable slope) or (Log (inhibitor) vs. normalized response) were performed in Prism 7 (GraphPad Software, Inc.).

Ancillary Information

Supporting Information:

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Author contributions: JBR designed or performed screening assays; JBR and LDS analyzed primary screening data and generated figures; JB and DEG reviewed the findings and provided scientific advice; JBR and LDS wrote the manuscript with input from all authors.

Abbreviations used: ART, artemisinin; DHFR, dihydrofolate reductase; DHODH, dihydroorotate dehydrogenase; PfATP4, adenosine triphophosphatase 4; HFF, human foreskin fibroblasts; HTS, high throughput screen; PI4K, phosphotidylinositol 4 phosphate kinase; EC, effective concentration;

Figure legends

Figure 1 Rank order of compounds in terms of EC_{50} values for inhibition of *T. gondii* tachyzoite growth in vitro. Values represent mean of two independent 10-point titrations that were used to derive EC_{50} values by dose- response curve fits (see methods). Compounds of interest are highlighted including pyrimethamine (red dot), artemisinin and related compounds (yellow), inhibitors of PfATP4 (orange), and antibiotics (green). See Table 1 for complete EC_{50} values.

Figure 2 Structures and EC_{50} values for artemesinin and related analogs artusunate and artemether. Deoxyartemesinin, which is inactive, is shown for comparison. Several synthetic trioxanes are also illustrated. See Table 1 for complete EC_{50} values.

Figure 3 Structures and EC_{50} values for several PfATP4 inhibitors including two spiroindolones (KAE609 and KAF246) and a structurally unrelated pyrazoleamide (21A092). See Table 1 for complete EC_{50} values.

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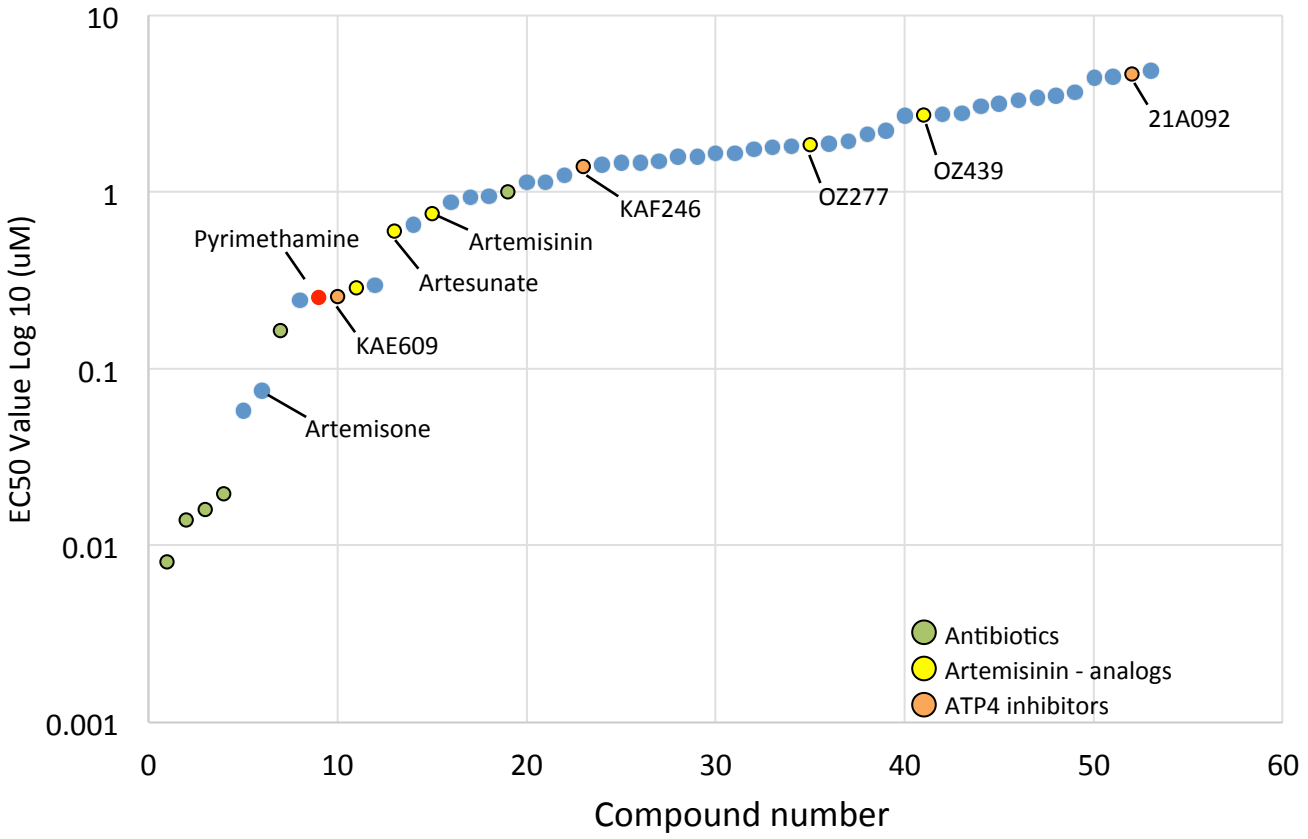
Figure 1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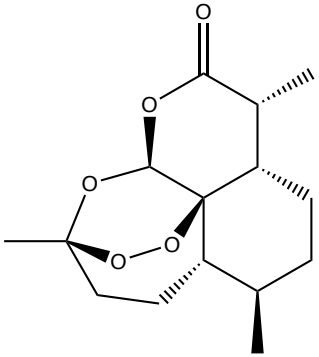
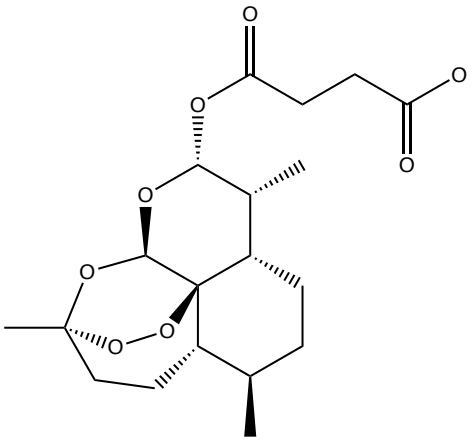
ATP4 inhibitors

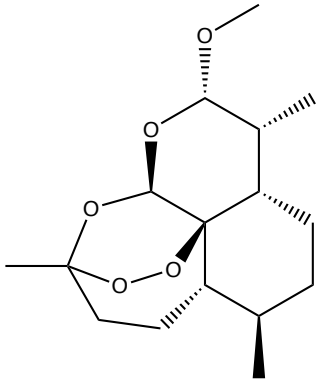
Figure 2



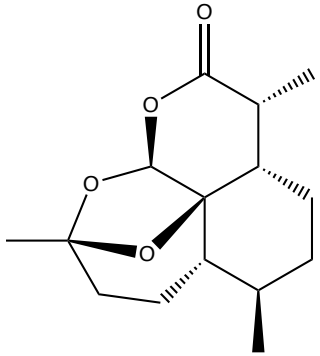
Artemisinin
EC_{50} = 0.7579



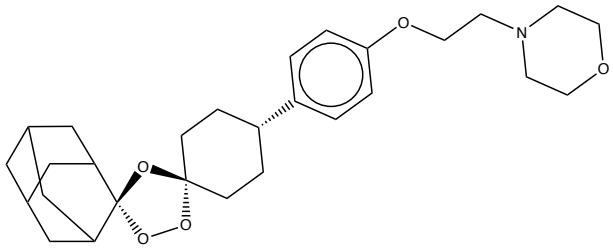
Artusunate
EC_{50} = 0.5989



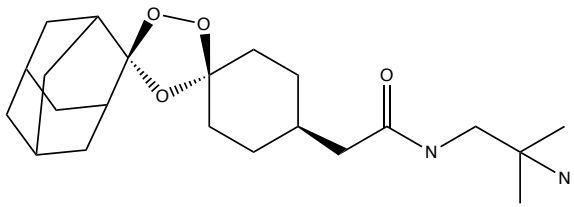
Artemether
EC_{50} = 0.2861



Deoxyartemesinin?
EC_{50} = 10.00



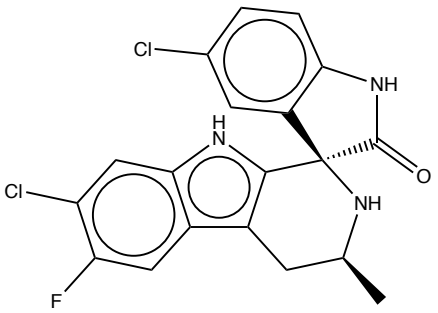
OZ439
EC_{50} = 2.7220



OZ277
EC_{50} = 1.8570

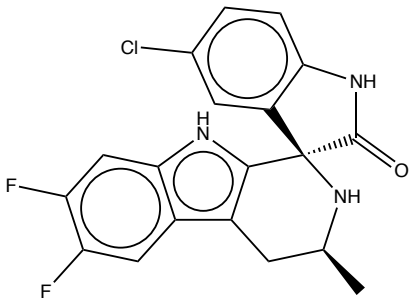
Figure 3

A



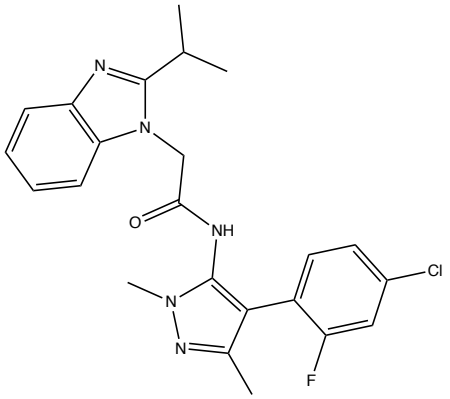
KAE609
EC_{50} = 0.256

B



KAF246
EC_{50} = 1.400

C



21A092
EC_{50} = 4.64

Table 1 Sumary of EC_{50} values for inhibtion of *T. gondii* growth in vitro

Compound Name	Chemical class	Known or suspected mechanism of action (resistance mechanism)	EC_{50} log inhib vs normalized response (μM)
Clindamycin	macrolide - lincosamide	Protein synthesis - bacterial 50S ribosomal subunit	0.0081
Trans-Mirincamycin	macrolide - lincosamide	Protein synthesis - bacterial 50S ribosomal subunit	0.0140
Cyclohexamide	glutarimide	Protein synthesis - eukaryotic - elongation	0.0161
Cis-Mirincamycin	macrolide - lincosamide	Protein synthesis - bacterial 50S ribosomal subunit	0.0197
ELQ-300	quinolone	Mitochondrial bc1 complex- Qi	0.0577
Artemisone	sesquiterpene lactone	peroxide-mediated, oxidative damage (Kelch 13)	0.0755
Azithromycin	macrolide - azalide	Protein synthesis - bacterial 50S ribosomal subunit	0.1654
Atovaquone	hydroxynapthylquinone	Mitochondrial bc1 complex- Qo	0.2441
Pyrimethamine	pyrimidine derivative	DHFR	0.2538
KAE609	spirotetrahydro β-carboline	ATP4	0.2561
Artemether	sesquiterpene lactone	peroxide-mediated, oxidative damage (Kelch 13)	0.2861
Methylene blue	phenothiazin	Uncertain	0.2958
Artesunate	sesquiterpene lactone	peroxide-mediated, oxidative damage (Kelch 13)	0.5989
Ro 47-7737	4-aminoquinoline (bis)	Hemozoin formation	0.6508
Artemisinin	sesquiterpene lactone	peroxide-mediated, oxidative damage (Kelch 13)	0.7595
BIX-01294	diaminoquinazoline	Histone methyl transferase	0.8792
Phenylequine	4-aminoquinoline	Hemozoin formation	0.9362
MMV688558	pantothenamide	Co-enzyme A	0.9526
Doxycycline	macrolide - tetracycline	Protein synthesis - bacterial	1.0080
Thiostrepton	cyclic oligopeptide - thiopeptide	Protein synthesis - bacterial 50S ribosomal subunit	1.1310
Dihydroartemisinin	sesquiterpene lactone	peroxide-mediated, oxidative damage (Kelch 13)	1.1390
Pyronaridine	4-anilino-quinoline	Hemozoin formation and novel	1.2430
KAF246	spirotetrahydro β-carboline	ATP4	1.4000
Chlorproguanil	biguanide - prodrug	DHFR after cyclization - and unique	1.4330
2k	4-anilino-quinoline	Hemozoin formation	1.4560
NPC-1161B	8-aminoquinoline	Uncertain	1.4680
Mefloquine (racemic)	quinoline amino-alcohol	Uncertain (Pfmdr1)	1.4900
Sitamaquine	8-aminoquinoline	Uncertain	1.5800
Cladosporin	isocoumarin	Lysyl t-RNA synthase	1.5820
(+)-Mefloquine	quinoline amino-alchohol	Uncertain (Pfmdr1)	1.661
AQ-13	4-aminoquinoline	Hemozoin formation	1.6460

AZ412	triaminopyrimidine	Vacuolar ATPase synthase / V-type H+ ATPase	1.7560
Pamaquine	8-aminoquinoline	Uncertain	1.7840
Halofantrine	amino alcohol	Hemozoin formation	1.8140
OZ277	trioxolane - synthetic endo-peroxide	peroxide-mediated, oxidative damage	1.8570
N-desethyl amodiaquine	4-anilino-quinoline	Hemozoin formation	1.8670
Dapsone	sulfone	Dihydropteroate synthesis	1.9430
Tafenoquine	8-aminoquinoline	Uncertain	2.1160
Amodiaquine	4-anilino-quinoline	Hemozoin formation	2.2260
AN13762	oxaborole	Uncertain	2.7030
OZ439	trioxolane - synthetic endo-peroxide	peroxide-mediated, oxidative damage	2.7220
Cycloguanil	cyclic-biguanide	DHFR	2.7580
UCT944	aminopyrazine	PI4K	2.7900
KAF156	imidazolopiperazine	(PfCarl)	3.0620
Sulfamethoxazole	sulfonamide	Dihydropteroate synthesis	3.1530
MK-4815	aminocresol	Hemozoin formation	3.3010
UCT048	aminopyridine	PI4K	3.4340
Primaquine	8-aminoquinoline	Uncertain	3.5140
Pentamidine	bisamidine derivative	Uncertain	3.6790
Proguanil	biguanide - prodrug	DHFR after cyclization - and unique	4.4700
Sulfadiazine	sulfonamide	Dihydropteroate synthesis	4.5220
21A092	pyrazoleamide	ATP4	4.6370