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Repurposing the Open Access Malaria Box To Discover Potent Inhibitors of *Toxoplasma gondii* and *Entamoeba histolytica*

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Toxoplasmosis and amebiasis are important public health concerns worldwide. The drugs currently available to control these diseases have proven limitations. Therefore, innovative approaches should be adopted to identify and develop new leads from novel scaffolds exhibiting novel modes of action. In this paper, we describe results from the screening of compounds in the Medicines for Malaria Venture (MMV) open access Malaria Box in a search for new anti-*Toxoplasma* and anti-*Entamoeba* agents. Standard *in vitro* phenotypic screening procedures were adopted to assess their biological activities. Seven anti-*Toxoplasma* compounds with a 50% inhibitory concentration (IC_{50}) of <5 μM and selectivity indexes (SI) of >6 were identified. The most interesting compound was MMV007791, a piperazine acetamide, which has an IC_{50} of 0.19 μM and a selectivity index of >157. Also, we identified two compounds, MMV666600 and MMV006861, with modest activities against *Entamoeba histolytica*, with IC_{50}s of 10.66 μM and 15.58 μM, respectively. The anti-*Toxoplasma* compounds identified in this study belong to scaffold types different from those of currently used drugs, underscoring their novelty and potential as starting points for the development of new antitoxoplasmosis drugs with novel modes of action.

Infectious diseases exert a very heavy toll on populations at risk worldwide. Of particular note is that infections caused by pathogenic protozoa affect the most vulnerable people, leading to tremendous economic and social burdens.

The diseases caused by protozoan parasites are responsible for considerable mortality and morbidity, affecting >500 million people worldwide (1). Currently, chemotherapy remains an essential component of both clinical management and disease control programs in areas where these diseases are endemic. A number of factors, such as high cost, poor compliance, drug resistance, low efficacy, and poor safety, limit the utility of antiprotozoan drugs.

Globally, toxoplasmosis is an important zoonosis with potentially devastating health impacts for humans and for a range of domestic and wild species (2). In humans, the causative agent (*Toxoplasma gondii*) has a simple life cycle consisting of two asexual parasite forms, tachyzoites and bradyzoites, that affect more than one-third of the world's population (3).Tachyzoites are rapidly growing obligate intracellular *T. gondii* forms that are commonly found in acutely infected individuals. Following a successful lytic phase in immunocompetent individuals, *T. gondii* tachyzoites differentiate into slowly growing bradyzoites that then establish latent infection as tissue cysts in vital organs, such as the brain, heart, and kidneys. When a cyst ruptures, a stage transition from latent bradyzoites back to rapidly growing tachyzoites occurs, causing destruction of the surrounding tissue. The reasons for recrudescence of eye toxoplasmosis have not been completely defined, but it is a lifelong problem, particularly in congenitally infected patients and in patients who acquired the infection shortly after birth (4, 5). Toxoplasmosis is a widely distributed parasitic disease, with seroprevalence rates as high as 98% in some regions of the world (6, 7). In the United States, for instance, about 1 million new infections occur each year, resulting in approximately 20,000 cases of retinal infection (8) and 750 deaths, making it the second most common cause of death related to food-borne diseases (9). Although infections in most animals and humans are asymptomatic, toxoplasmosis can cause severe illness in congenitally infected children, leading to severe sequelae that include complete blindness and various neurological impairments in developing fetuses and newborns, and in most immunologically depressed subjects, particularly organ transplant and AIDS patients (10).

Overall, studies on *Toxoplasma* should be spurred on for two important reasons. First, *Toxoplasma* can cause severe and life-threatening disease in developing fetuses and in immunocompromised patients. Second, although available drugs can treat *Toxoplasma* infections, they are poorly tolerated and are ineffective against chronic *Toxoplasma* infections. Clearly, all existing antitoxoplasmosis therapies, including the antimalarial compound pyrimethamine and the antibiotics clindamycin and sulfadiazine, are ineffective against bradyzoites. In addition, resistance to some of these drugs was recently reported (10–12), highlighting the urgent need for new and improved antitoxoplasmosis agents. An ideal anti-*Toxoplasma* drug would be potent and nontoxic and would eliminate latent infection (bradyzoites).

Amebiasis caused by *Entamoeba histolytica* is often associated with high morbidity and mortality. It continues to be a major

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public health problem throughout the world, especially in sub-Saharan Africa, where sanitation infrastructures and health facilities are usually inadequate (13, 14). The clinical features of amebiasis range from asymptomatic colonization to amebic colitis (dysentery or diarrhea) and invasive extraintestinal amebiasis, which commonly manifests as liver abscesses (15). With 40 to 50 million cases of amebic colitis and amebic liver abscess and up to 100,000 deaths annually, WHO estimates place amebiasis as a major public health threat throughout the world, second only to malaria in terms of mortality (16–18). Recent data showed an increase in the occurrence of *E. histolytica* among HIV patients (19–22). Although many drugs destroy *E. histolytica* within the colonic lumen, the number of tissue amebicides used to treat invasive amebiasis is still relatively limited. Metronidazole (MTZ), which is the drug of choice for invasive amebiasis, and other nitroimidazoles have greatly simplified chemotherapy for this disease. However, decades after the introduction of these drugs for the therapy of amebiasis, there have been few innovations in treating this infection. The toxic effects of MTZ and recent failures in the treatment of several intestinal protozoan parasites have led to a search for alternative amebicidal drugs (23).

Globally, the burden of infectious diseases is such that innovative drugs are greatly needed, especially for the control of protozoan infections. The aim of this work was to repurpose the 400 blood-stage-active anti-*Plasmodium* hits from the open access Malaria Box (24) to discover active chemical entities against *Toxoplasma gondii* and *Entamoeba histolytica* using standard *in vitro* drug susceptibility assays. This strategy is similar to the one used for extending the indications of existing treatments for other human and animal ailments to tropical diseases (25–27). Importantly, this fast-track approach has been successful, resulting in some of the most important currently used antiparasitic drugs, such as ivermectin for filariasis/onchocerciasis and praziquantel for schistosomiasis, and it continues to have a major role in the global drug discovery and development strategy for tropical diseases (28–30).

MATERIALS AND METHODS

The compounds were obtained from the Medicines for Malaria Venture (MMV) (Geneva, Switzerland) and used for the experiments. The Malaria Box was supplied in V-shaped 96-well plates in 20 μl of a 10 mM dimethyl sulfoxide (DMSO) solution and shipped frozen. The chemical purity based on liquid chromatography-mass spectrometry (LC-MS) was >90%. Plate mapping and full data on the Malaria Box with the original GSK/St Jude/Novartis compound numbers, structures, canonical simplified molecular-input line-entry system (SMILES), biological data, and selected *in silico* physicochemical parameters are available (see Table S1 in reference 24 and http://www.mmv.org/research-development/malaria-box-supporting-information). A list of vendors used to supply compounds for the Malaria Box, including their website addresses and the number of compounds from each vendor in the Malaria Box as of December 2011, is also available (see Table S2 in reference 24). For compounds to be of most interest, a fresh solid sample was repurchased from the vendors. The compounds were used at a 30 μM top concentration and diluted as needed in respective culture media for individual experiments.

Human foreskin fibroblasts (HFFs) were purchased from the ATCC (HS68). Standard strains *T. gondii* TS-4 and *E. histolytica* Rahman were obtained from BEI Resources (Manassas, VA). According to the product sheets, *T. gondii* TS-4 (ATCC 40050) is a mutant of the RH strain, which was isolated in 1939 from a 6-year-old boy in Cincinnati, Ohio, with a lethal case of encephalitis (31), and *E. histolytica* Rahman (ATCC 30886) was isolated in 1972 from the feces of an adult human male asymptomatic cyst passer in England (32).

***T. gondii* cultures.** *T. gondii* TS-4 parasites optimally grow in human foreskin fibroblasts (HFFs). Following the product recommendations, HS68 HFFs (ATCC) were cultured in 75-cm^2 cell culture flasks (Corning Incorporated, USA) using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) and 1% penicillin-streptomycin (10,000 units and 10 mg/ml, respectively). At approximately 90% confluence, the cells were trypsinized using a 1:3 dilution of 1× trypsin-EDTA plus magnesium in calcium-free phosphate-buffered saline (PBS) (Sigma, Germany) and passaged into a new culture flask or used in the drug susceptibility studies as described below.

To propagate the *T. gondii* parasites, the thawed content of a parasite's vial was aseptically transferred to a tissue culture flask containing a fresh monolayer of HFFs and 10 ml of growth medium containing 3% (vol/vol) HIFBS and 1% penicillin-streptomycin (Sigma, Germany) and incubated at 37°C in a humidified chamber containing 5% CO_2. *T. gondii* parasites were maintained in tissue culture by thrice-weekly passages in 75-cm^2 cell culture flasks (Corning Incorporated, USA) in DMEM (ATCC, USA) supplemented with 1% penicillin-streptomycin and 3% FBS (Sigma).

***In vitro* cytotoxicity studies on human foreskin fibroblasts.** HFFs were used for cytotoxicity profiling of the Malaria Box compounds. Cytotoxicity tests were performed in 96-well microtiter culture plates (Costar, USA) using serial dilutions of the compounds and incubation at 37°C for 24 h in a humidified chamber containing 5% CO_2. Thereafter, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium/phenazinemethosulfate (MTS/PMS) (Promega) was introduced into each well and further incubated for 1.5 h at 37°C. Negative control wells consisted of cultures with equivalent concentrations of DMSO. The total absorbance in each well was recorded at 490 nm using a 96-well plate reader (EL800; BioTek, USA). The percent growth inhibition was calculated from the optical densities relative to the negative control, and 50% cytotoxic concentration (CC_{50}) values were determined using GraphPad Prism 5.0. Selectivity indices (SIs) of the drugs were subsequently calculated on the basis of their antiparasitic activities (IC_{50}) and host cell cytotoxicity (CC_{50}) profiles, and the results were used to aid in compound selection for progression into the hit optimization phase.

Assessment of anti-*Toxoplasma* activity *in vitro*. Drugs were tested for anti-*Toxoplasma* activity *in vitro* by targeting the parasite tachyzoite form as previously described (33), with some modifications. HFFs were harvested during exponential growth (day 2) and cultured in 96-well plates (100-μl suspension consisting of 6×10^4 cells/ml; Costar, USA). A total of 100 μl of inoculum (6×10^5 parasites/ml adjusted using a hemocytometer and trypan blue exclusion dye) was added to each well (parasite/cell ratio, ~10:1; final volume, 200 μl). Six hours after inoculation, nonadherent parasites were removed, and 100 μl of complete DMEM (1% penicillin-streptomycin, 3% FBS) supplemented with inhibitors at different concentrations (2-fold serial dilutions starting from 30 μM) was added to all except the negative-control wells. Positive controls, consisting of pyrimethamine (PYR) and sulfadiazine (SDZ) (20-mg/ml stocks in DMSO; Sigma-Aldrich), were tested at a 2-mg/ml final concentration. Each test was performed in triplicate. Culture plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h. A total of 20 μl of MTS/PMS (Promega) was then introduced into each well of the 96-well assay plate and incubated at 37°C for 1.5 h in a humidified 5% CO_2 atmosphere. Thereafter, the absorbances were immediately recorded at 490 nm using a 96-well plate reader (EL800; BioTek, USA). These data were normalized to percent control activity, and 50% inhibitory concentrations (IC_{50}s) were calculated using Prism 5.0 software (GraphPad) per the following variable slope sigmoidal dose-response formula with data fitted by nonlinear regression and the bottom and top values constrained to 0 and 100, respectively: $y = 100/1 + 10(\log IC_{50} - x)H$ (where y = percent inhibition; 100 = bottom + [top − bottom]; x = drug concentration; and H = hill slope [largest absolute value of the slope of the

TABLE 1 Hits with potential for further extensive medicinal chemistry/biological screenings against *T. gondii* and *E. histolytica*

Compound[a]	Mol wt[b]	AlogP[b]	IC_{50} (μM)[c]	CC_{50} against HFFs (μM)[d]	SI[e]
Anti-*Toxoplasma gondii* TS-4					
MMV007791	357.40362	1.565	0.19	>30	>157
MMV007881	378.48574	3.784	1.07	>30	>28.04
MMV007363	232.70872	3.303	1.49	>30	>20.13
MMV006704	291.39014	3.91	1.95	>30	>15.38
MMV666095	280.16035	4.203	3.05	>30	>9.83
MMV020548	413.51478	3.765	3.85	>30	>7.79
MMV085203	362.42167	3.719	4.54	>30	>6.60
PYR			3.62		
SDZ			26.05		
Anti-*Entamoeba histolytica* Rahman					
MMV666600	459.92087	6.819	10.66	>30	>2.81
MMV006861	355.43233	4.324	15.58	>30	>1.92
MTZ			5.83		

[a] Compounds are designated by their MMV identifier codes. PYR, pyrimethamine; SDZ, sulfadiazine; MTZ, metronidazole.
[b] Molecular weight and AlogP values were obtained from Malaria Box supporting information.
[c] Compounds were serially diluted and tested in culture. Results are means from triplicate experiments.
[d] Cytotoxicity against HFFs was evaluated in culture. Results are means from triplicate experiments.
[e] Selectivity indices were calculated based on the ratio CC_{50} (HFF)/IC_{50} test drugs.

curve]). For sigmoidal dose-response analysis data, see Data S3 in the supplemental material.

***Entamoeba histolytica* cultures.** The *E. histolytica* Rahman strain (ATCC 30886) was primarily cultured in Eagle's minimum essential medium (EMEM) (Sigma, Germany) supplemented with various concentrations of heat-inactivated fetal bovine serum (HIFBS) and 1% (vol/vol) of the commercial penicillin-streptomycin solution (Sigma, Germany) in sterile 15-ml screw-cap tubes but showed poor growth and no significant difference. In contrast, the replacement of FBS with adult bovine serum (ABS) resulted in optimal trophozoite growth. EMEM supplemented with ABS and 1% (vol/vol) of the commercial penicillin-streptomycin solution was then used successfully to maintain *E. histolytica* Rahman in culture. The parasites were subcultured twice weekly. Parasites at the log phase of growth were used for drug susceptibility assays and were grown for 1 day following regular subculturing procedures.

***In vitro* anti-*Entamoeba* assay.** Anti-*E. histolytica* drug activities were determined as previously described (34) with the following modifications. Primary screening was performed with each compound at a 30 μM final concentration and in triplicate in sealed 96-well polystyrene microtiter plates (Costar, USA). Parasite inocula (100 μl) comprising 2×10^4 parasites/ml (adjusted using trypan blue exclusion dye [Sigma] and hemocytometer-based counts) were added to each well and grown at 37°C in a humidified atmosphere of 5% CO_2 for 72 h. Positive controls consisted of metronidazole at 1 mg/ml. The growth of *E. histolytica* trophozoites in each well was determined microscopically by measuring the diameters of the confluent cells in drug-containing wells relative to those in the negative-control wells.

Based on the confluence and proportion of motile parasites, the growth inhibition in each triplicate well was scored as follows: 4+, <20% confluent cells and 100% of parasites dead; 3+, <50% confluent cells and <50% parasite motility; 2+, >50% confluent cells and >50% parasite motility; 1+, 100% confluent cells and 100% parasite motility (0 growth inhibition). Only the compounds with scores of 4+, 3+, or 2+ were considered for IC_{50} determination to confirm the compound activities.

For the dose-response experiments, 100 μl of a 2-fold serial dilution of each selected compound was added to 100 μl of *E. histolytica* trophozoite inocula (2×10^4 cells/ml) in complete EMEM supplemented with 10% ABS in a 96-well microtiter plate. Plates were sealed with expanded polystyrene and incubated as described above at 37°C for 72 h. Positive-control wells contained metronidazole at the highest concentration (0.5 mg/ml), whereas the negative-control wells contained culture medium with equivalent numbers of trophozoites. The number of viable parasites in each well was determined using trypan blue exclusion dye, and the percent inhibition was calculated with respect to the negative control. IC_{50}s were determined as described above using GraphPad Prism 5.0. For sigmoidal dose-response analysis data, see Data S3 in the supplemental material.

RESULTS

In the screening of the Malaria Box content, 7 compounds showed potent anti-*Toxoplasma gondii* activity (IC_{50}, <5 μM) and 2 compounds moderately inhibited *Entamoeba histolytica* (IC_{50}s, ~10 to 15 μM). The results are summarized below and discussed in later sections. Additional information on the moderately active compounds that represent alternative options for anti-*Toxoplasma* structure-activity relationship (SAR)-based optimization studies are presented in the supplemental material.

Cellular cytotoxicity of drugs. From the cytotoxicity assays against HFFs, none of the compounds showed significant toxic effects on cells after 72 h of incubation. Few compounds showed limited cell growth inhibition (overall <40% inhibition) at a 30 μM final concentration, which was several orders of magnitude above the IC_{50}s of the promising compounds. Therefore, the CC_{50} values were >30 μM against HFFs (Table 1).

Anti-*Toxoplasma* activity of drugs. Serially diluted compounds were screened for *in vitro* activity against *T. gondii*. Of the 200 drug-like and 200 probe-like compounds tested, 45 (27 drug-like and 18 probe-like) inhibited the growth of *T. gondii* as determined by the MTS/PMS-based cell viability assay. Based on our activity cutoff criteria (IC_{50}, <5 μM; SI, >6), 7 compounds showed potent activities (Table 1 and Fig. 1), whereas 38 others moderately inhibited the parasites, with IC_{50}s ranging from 5.85 to 28.99 μM (see Table S1 and Fig. S2 in the supplemental material). These results indicate that among the most potent inhibitors (IC_{50}, <5 μM), the drug-like compound MMV007791 inhibited the parasites with an IC_{50} of 190 nM and an SI of >157. The 6 others exhibited anti-*T. gondii* activities with an IC_{50} of <5 μM and an SI of >6.

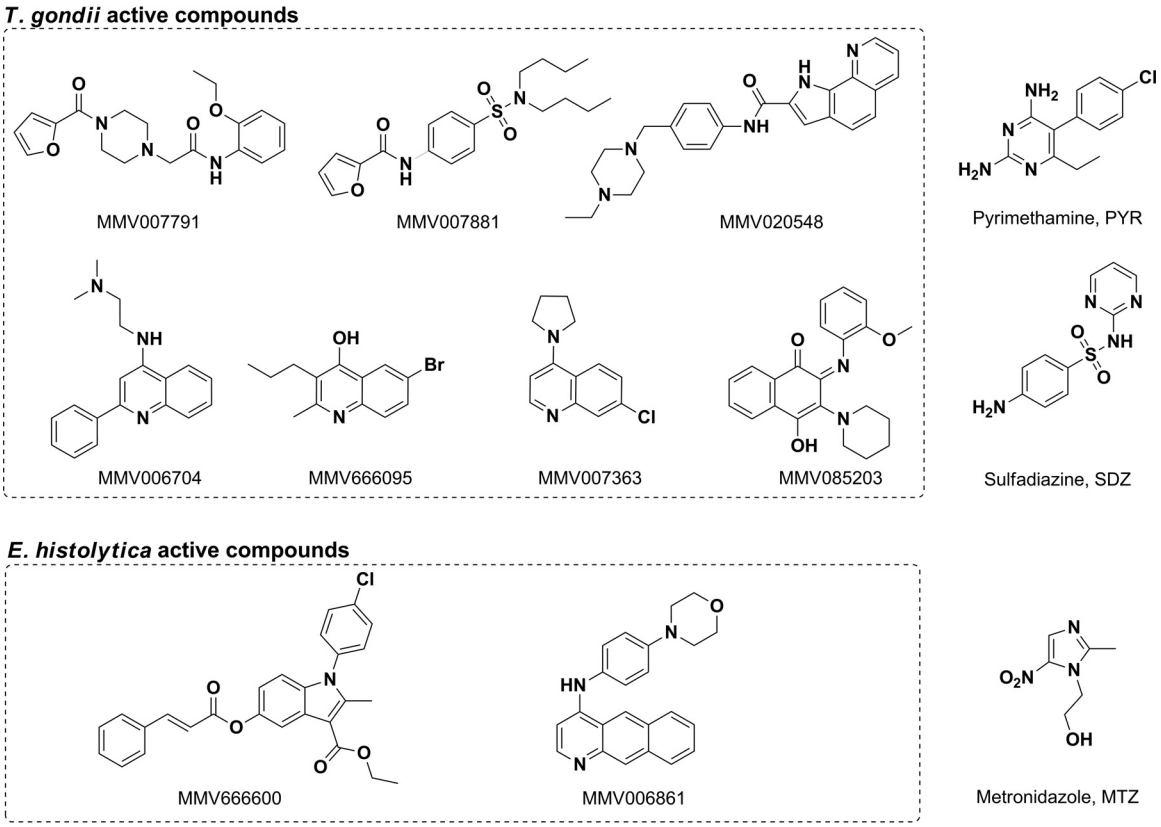

FIG 1 Scaffolds and compounds with high potential for anti-*Toxoplasma* drug discovery and anti-*Entamoeba* SAR-based studies. MMV identifiers and structures were provided by the MMV as part of the supporting information for the open access Malaria Box. Triplicate concentrations of serially diluted compounds were tested *in vitro* against *Toxoplasma gondii* TS-4 strain tachyzoites and *Entamoeba histolytica* Rahman strain trophozoites. IC_{50} (50% inhibitory concentration) values were calculated from sigmoidal dose-response curves. Structure and MMV identifiers are shown for each listed compound. Scaffolds were obtained from SAR studies. Anti-*Toxoplasma* compounds: MMV007791, *N*-(2-ethoxy-phenyl)-2-[4-(furan-2-carbonyl)-piperazin-1-yl]-acetamide; MMV085203, 4-hydroxy-2-(2-methoxy-phenyl imino)-3-piperidin-1-yl-2*H*-naphthalen-1-one; MMV007881, furan-2-carboxylic acid (4-dibutylsulfamoyl-phenyl)-amide; MMV020548, 1*H*-pyrrolo[3,2-*h*]quinoline-2-carboxylic acid [4-(4-ethyl-piperazin-1-ylmethyl)-phenyl]-amide; MMV007363, 7-chloro-4-pyrrolidin-1-yl-quinoline; MMV006704, *N*,*N*-dimethyl-*N*′-(2-phenyl-quinolin-4-yl)-ethane-1,2-diamine; MMV666095, 6-bromo-2-methyl-3-propyl-quinolin-4-ol. Anti-*Entamoeba* compounds: MMV666600, 1-(4-chloro-phenyl)-2-methyl-5-(3-phenyl-acryloyloxy)-1*H*-indole-3-carboxylic acid ethyl ester; MMV006861, benzo[*g*]quinolin-4-yl-(4-morpholin-4-yl-phenyl)-amine.

Anti-*Entamoeba* activity. The 400 Malaria Box compounds were primarily screened at a single concentration of 30 μM to select promising candidates for IC_{50} determination. *E. histolytica* trophozoite growth and viability were evaluated by microscopic estimation of the diameters of confluent cells and by standard trypan blue exclusion assays, respectively. Following our hit selection criteria, 5 compounds, MMV006861 and MMV666600 (scored 4+), MMV006303 (scored 3+), and MMV006172 and MMV006087 (scored 2+), were selected for IC_{50} determination. By dose-response analyses, only two (MMV006861 and MMV666600) of these five compounds displayed promising potency, with IC_{50}s of 15.58 μM and 10.66 μM, respectively. These results are summarized in Table 1, and their structures are presented in Fig. 1.

DISCUSSION

According to our hit criteria (IC_{50}, <5 μM and SI, >6), 7 anti-*Toxoplasma* compounds were identified in this study (Fig. 1). This represents a 1.75% hit rate, a value that seems to be above the standard 0.1% observed in other screens. A putative reason could be that these compounds already have properties favorable for them to reach their biological target in a whole-cell assay, as all compounds are known to be active against the blood-stage form of *Plasmodium falciparum in vitro*. The potential commonality of a target shared between the two protozoan parasites may be another explanation for the improved hit rate.

First, 6 of the compounds showed an activity range similar to that of pyrimethamine, with IC_{50}s going from 1.07 to 4.54 μM with moderate to good selectivities.

However, the structures seemed novel, with the possible exception of MMV007881, which has a 4-aminobenzenesulfonamide group embedded in the structure, similar to that in sulfadiazine. MMV020548 is composed of piperazine and a pyrroloquinoline carboxamide with an IC_{50} of 3.85 μM and an SI of >7. Three compounds, MMV007363, MMV006704, and MMV666095, contain a quinolone fragment with an IC_{50} of 1.49 μM, 1.95 μM, or 3.05 μM and an SI of >20, 15, or 9, respectively. Of interest is the close structural proximity of MMV007363 and MMV006704 to the marketed antimalarial chloroquine. Also, quinoline and derivatives tested with diverse pharmacological-activity functional groups constitute an important class of compounds for new drug development. For example, a recent study described highly effica-

cious endochin-like quinolones against acute and latent experimental toxoplasmosis (35). The study showed that this scaffold contains highly potent derivatives against acute and latent toxoplasmosis, with IC_{50}s of <1 nM *in vitro*, that are also effective in mouse models. Overall, the quinolone scaffold represents a target group for detailed medicinal chemistry for anti-*Toxoplasma* drug discovery. MMV085203 is a 2,3-diaminonaphthoquinone with an IC_{50} of 4.54 μM and can be compared to the *P. falciparum* mitochondrial cytochrome bc_1 complex (1) inhibitor atovaquone, which has known anti-*Toxoplasma* activity (36).

More interestingly, MMV007791 showed a significantly higher potency (IC_{50}, 190 nM) and an excellent selectivity index (SI, >157) against HFFs. This chemotype is novel for its anti-*Toxoplasma* activity, and the structure is composed of a piperazine acetamide moiety. Additional data on this compound are reported in the open access database powered by the European Bioinformatics Institute (ChEMBL website [see https://www.ebi.ac.uk/chembl/malaria]), where the compound is reported to have good selectivity over those of other pathogens, as it has no activity against kinetoplastids (IC_{50}, >32 μM), no effect on the nonreplicating form of *Mycobacterium tuberculosis* at 25 μM, no activity against newly transformed schistosomula at 2.5 μM, and good overall SI profiles, as no overt toxicities were seen against MRC-5 (CC_{50}, >32 μM) and Huh7 (CC_{50}, >100 μM) cells. Hence, compounds with the closely related 2-phenoxy-*N*-phenylacetamide core structure have attracted considerable research interest, as these entities have demonstrated a variety of biological activities, e.g., antiparasitic (37), anticancer (38), and antiviral (39) effects. Of particular interest is their activity against *Toxoplasma gondii* enoyl reductase (37). These derivatives have also shown P-glycoprotein and *Mycobacterium tuberculosis* H37Rv inhibition *in vitro* (40, 41). Also, *in vitro* drug metabolism and pharmacokinetic (DMPK) data suggest that these scaffolds may have some liability, as they have high intrinsic clearance rates (46 and 906 μl/min/mg in humans and mice, respectively) in liver microsomes. Efforts in SAR-based studies to address the metabolism and the potential genotoxicity risk represented by the anilines are considered.

In a very recent study, a strategy similar to ours was adopted to repurpose the open access Malaria Box to identify chemical series active against *Cryptosporidium parvum* (42). The authors showed in a subsidiary objective that the 2,4-diamino-quinazoline-based compounds were also potent growth inhibitors of the related apicomplexan parasite *T. gondii* of the RH strain from which the TS-4 strain used in our study was derived. Compounds of this series from commercial origin exerted anti-*Toxoplasma* activity, with IC_{50}s ranging from 0.55 to 7.3 μM. From our findings, two derivatives (MMV019199 and MMV080034) of this series showed moderate activity (28.12 μM and 28.99 μM, respectively) compared to the other study's results. Given that the *T. gondii* TS-4 strain used in our study is a mutant of the RH strain used by these authors, it is likely that significant differences in the susceptibilities of different *T. gondii* strains exist and might justify the observed differences in activity levels.

Currently recommended drugs for the treatment of toxoplasmosis act primarily against the tachyzoite form of *T gondii*; thus, they do not eradicate the encysted form (bradyzoites) that maintains the parasite in a latent state in tissues. Pyrimethamine is the most effective agent and is included in most drug regimens. The most effective available therapeutic combination is pyrimethamine plus sulfadiazine or the trisulfapyrimidines (e.g., a combination of sulfamerazine, sulfamethazine, and sulfapyrazine). These agents are active against tachyzoites and are synergistic when used in combination (43–45). These compounds belong to the pyrimidine scaffold. In contrast, all the compounds identified in our study belong to other scaffold types, underscoring their novelty and potential as starting points for the development of new anti-toxoplasmosis drugs with novel modes of action.

In a recent work, the potent Malaria Box compound MMV008138 was tested against the *T. gondii* RH-HX-KO-YFP2-DHFR(m2m3) strain. The authors observed no effect of MMV008138 on *T. gondii* growth (46), similar to what we found.

Other similar studies attempting to identify novel hit compounds active against *T. gondii* were conducted recently. Among their findings, compounds of (benzaldehyde)-4-phenyl-3-thiosemicarbazone and (benzaldehyde)-(4 or 1)-phenylsemicarbazone scaffolds showed limited efficacies against intracellular tachyzoites (47). Also, a label extension strategy was adopted to assess the anti-*Toxoplasma* activities of nine antiretroviral drugs *in vitro*. Nucleoside analogs showed no effects on parasite growth, whereas ritonavir and nelfinavir were inhibitory for *Toxoplasma*, with IC_{50} values of 7.50 and 7.05 μM, respectively (48). Fusidic acid also showed efficacy against *T. gondii in vitro* but not in mice (49). In another study, 1-hydroxy-2-dodecyl-4(1*H*)quinolone exhibited growth inhibition of *T. gondii* at a nanomolar concentration, with the alternative (type II) NADH dehydrogenases as the specific target (50). Recently, two novel 1-hydroxyquinolones were shown to display low nanomolar anti-*Toxoplasma* activities (51).

From our study, two compounds were identified, but they have very modest activities against *E. histolytica*. They are MMV666600 (IC_{50}, 10.66 μM), which is a 2-methyl-1*H*-indole derivative, and MMV006861 (IC_{50}, 15.58 μM), which is a benzo[*g*]quinolin-4-ylamine derivative. Metronidazole, which is the mainstay therapy for invasive amebiasis (52, 53), belongs to the imidazole class.

From a similar approach, some investigators recently described the *in vitro* anti-*Entamoeba* activity of an FDA-approved drug for use in rheumatoid arthritis (auranofin). The IC_{50} of auranofin against *E. histolytica* trophozoites was <2 μM. It is notable that auranofin had much higher cysticidal activity on *Entamoeba invadens* cysts than the standard amebicide metronidazole, providing a potential therapeutic advantage (54, 55).

Our study identified compounds with low micromolar anti-*Toxoplasma* activities (IC_{50}s, <5 μM) and acceptable physicochemical drug profiles. Ongoing studies based on the extensive medicinal chemistry and metabolism of these compounds will identify related scaffolds with improved biological properties. Compounds that showed moderate activity might be potential options for SAR-based optimization studies. In addition, detailed studies on the MMV Malaria Box novel scaffolds that were identified as anti-*Entamoeba* in this work might be fruitful in drug development against amebiasis.

Plasmodium falciparum, *Toxoplasma gondii*, and *Entamoeba histolytica* are parasitic protozoa. Within this family, apicomplexans are a large group of parasitic protists, most of which possess a unique organelle, a type of plastid called an apicoplast, an apical complex structure most likely involved in penetrating a host's cell that is currently among the prioritized targets for drug discovery. *P. falciparum* and *T. gondii* share this feature, as do many other metabolic drug targets. The Malaria Box compounds that inhibited *T. gondii* might therefore likely target the parasite apicoplast, in contrast to *E. histolytica*, which is not an apicomplexan. Further

studies on the rates and mechanisms of drug action will elucidate these considerations.

We report here the results from repurposing the Malaria Box to identify hit compounds against *T. gondii* and *E. histolytica*. The findings are encouraging and support further investigations to find drug candidates with innovative and acceptable profiles.

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