Repurposing the Open Access Malaria Box to discover potent inhibitors of  
*Toxoplasma gondii* and *Entamoeba histolytica*

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Running Head: Malaria Box compounds hit *Toxoplasma* and *Entamoeba*

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Abstract

Toxoplasmosis and amoebiasis are important public health concerns worldwide. Currently available drugs to control these diseases have proven their limitations. Therefore, innovative approaches should be adopted to identify and develop new leads from novel scaffolds exhibiting novel modes of actions. This paper describes results from the screening of Medicines for Malaria Venture's Open Access Malaria Box compounds in a search for new anti-Toxoplasma and anti-Entamoeba agents. Standard in vitro phenotypic screening procedures were adopted to assess the biological activities. Seven anti-Toxoplasma hits with IC$_{50}$ < 5µM and selectivity indexes (SI) greater than 6 were identified. The most interesting hit compound was MMV007791, a piperazinacetamide, with an IC$_{50}$ of 0.19 µM and a Selectivity Index greater than 157. Also, two compounds MMV666600 and MMV006861 were identified with modest activity against E. histolytica with IC$_{50}$ values of 10.66 µM and 15.58 µM respectively. The anti-Toxoplasma hits identified in this study belong to different scaffold types than currently used drugs, underscoring their novelty and potential as starting points for the development of new antitoxoplasmosis drugs with novel modes of action.

Keywords: Toxoplasma gondii; Entamoeba histolytica; Malaria Box; Phenotypic screen; Drug Discovery; Repurposing.

Introduction

Infectious diseases exert a very heavy toll to populations at risk worldwide. Of particular note, infections caused by pathogenic protozoa affect the most vulnerable people, leading to a tremendous economic and social burden.
The diseases caused by protozoan parasites are responsible for considerable mortality and morbidity, affecting more than 500 million people in the world (1). Currently, chemotherapy remains essential component of both clinical management and disease control programmes in endemic areas. A number of factors limit the utility of anti/protozoan drugs such as high cost, poor compliance, drug resistance, low efficacy and poor safety.

Toxoplasmosis is globally an important zoonosis with potentially devastating health impacts both for humans and 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 over 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 a latent infection as tissue cyst in vital organs such as the brain, heart and kidneys. When a cyst ruptures, stage transition from latent bradyzoites back to rapidly growing tachyzoites occurs, causing destruction of surrounding tissue. Reasons for recrudescence of eye toxoplasmosis have not been completely defined, but it is a lifelong problem particularly in congenitally infected patients as well as 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 one 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 deaths 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 foetuses and newborns, and in most immunologically depressed subjects, particularly in 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 foetuses and in immune-compromised patients. Second, although available drugs can treat *Toxoplasma* infections, they are poorly tolerated, and are ineffective against chronic *Toxoplasma* infections. Clearly, all existing anti-toxoplasmosis therapies, including the antimalarial compound pyrithymethamine and the antibiotics clindamycin and sulfadiazine are ineffective against bradyzoites. In addition, resistance to some of these drugs has recently been reported (10, 11,12), highlighting the urgent need for new and improved anti-toxoplasmosis agents. An ideal anti-*Toxoplasma* drug would be potent, nontoxic, and eliminate latent infection (bradyzoites).

Amoebiasis caused by *Entamoeba histolytica* is often associated with high morbidity and mortality. It continues to be a major public health problem throughout the world, especially in sub-Saharan Africa where sanitation infrastructures and health facilities are usually inadequate (13, 14). Clinical features of amoebiasis range from asymptomatic colonization to amoebic colitis (dysentery or diarrhea) and invasive extra-intestinal amoebiasis which commonly manifest as liver abscesses (15). With 40-50 million cases of amoebic colitis and amoebic liver abscess, and up to 100,000 deaths annually, WHO estimates put amoebiasis as a major public health threat throughout the world, and second only to malaria in terms of mortality (16, 17, 18). Recent data have shown an increase in the occurrence of *E. histolytica* among...
HIV patients (19, 20, 21, 22). Although many drugs destroy *E. histolytica* within the colonic lumen, the number of tissue amoebicides used to treat invasive amoebiasis is still relatively limited. Metronidazole (MTZ), which is the drug of choice for invasive amoebiasis, and other nitroimidazoles have greatly simplified the chemotherapy of this disease. However, decades after the introduction of these drugs in the therapy of amoebiasis, there have been little 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 amoebicidal 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-*Plasmodia* hits from the Open Access Malaria Box compounds (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, 26, 27). Importantly, this fast-track approach has been successful and has resulted in some of the most important antiparasitic drugs in use today, 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, 29,30).

**Materials and methods**

The compounds were obtained from 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 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 original GSK/St Jude/Novartis compound number, structure, canonical SMILES, biological data, and select in silico physicochemical parameters is available [in pone.0062906.s001] as well as on the MMV website (http://www.mmv.org/research-development/malaria-box-supporting-information). List of vendors used to supply compounds for the Malaria Box, including vendor’s web address and the number of compounds from each vendor in the Malaria Box as of December 2011 [in pone.0062906.s002]. For compounds to be of most interest, a fresh solid sample was repurchased from vendors. They were used at 30µM top concentration, and diluted as needed in respective culture media for individual experiments.

Human Foreskin Fibroblasts (HFF; ATCC-HS68) cells were purchased from ATCC. Standard strains of *T. gondii* TS-4 and *E. histolytica* Rahnam were obtained from BEI Resources, 10801 University Blvd. Fax: 703-365-2898 Manassas, VA 20110-2209 USA (www.beiresources.org). According to the products sheets, *T. gondii* TS-4 (ATCC® 40050™) is a mutant of the RH strain, which was isolated in 1939 from a six year old boy with a lethal case of encephalitis in Cincinnati, Ohio, USA (31), and *E. histolytica* Rahnam (ATCC® 30886™) was isolated in 1972 from human feces of an adult male asymptomatic cyst passer in England (32).

**T. gondii cultures**

Following the product recommendations, *T. gondii* TS-4 parasites optimally grow in Human foreskin fibroblast cells (HFF), using Dulbecco’s Modified Eagle Medium (DMEM) supplemented with heat-inactivated fetal bovine serum (HIFBS).

Human foreskin fibroblast (HFF) HS68 cells (ATCC) were cultured in 75-cm² cell culture flasks (Corning Incorporated, USA) using Dulbecco’s Modified Eagle
Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 units and 10mg/ml, respectively). At approximately 90% confluency, the cells were trypsinized using a 1:3 dilution of 1X trypsin-EDTA+ 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 parasites vial was aseptically transferred to a tissue culture flask containing a fresh monolayer of HFF cells and 10 mL of growth medium containing 3% (v/v) HIFBS and 1% penicillin and streptomycin (Sigma, Germany) and incubated at 37°C in a humidified chamber containing 5% CO₂. *T. gondii* parasites were maintained in tissue culture by thrice-weekly passages in 75-cm² cell culture flasks (Corning Incorporated, USA) in DMEM (ATCC, USA) supplemented with 1% penicillin and streptomycin and 3% FBS (Sigma, Germany).

**In vitro cytotoxicity studies on human foreskin fibroblasts**

HFF cells 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% CO₂. Thereafter, 20µl of MTS/PMS (Promega) were introduced into each well and further incubated for 1.5h at 37°C. Negative control wells consisted of cultures with equivalent concentrations of DMSO. The total absorbance in each well was recorded at 490nm using a 96-well plate reader (Biotek EL800, USA). The percent growth inhibition was calculated from the optical densities relative to negative control, and CC₅₀ values determined using GraphPad Prism 5.0.
Selectivity indices of drugs were subsequently calculated on the basis of their anti-parasitic activities (IC$_{50}$) and host cell cytotoxicity (CC$_{50}$) profiles, and the results used to advise compound selection for progression into the hit optimization phase.

**Assessment of the anti-Toxoplasma activity in vitro**

Drugs were tested for anti-Toxoplasma activity in vitro targeting the parasite tachyzoite form as previously described (33), with some modifications. HFF cells were harvested during exponential growth (day 2) and cultured in 96-well plates (Costar, USA) (100µl suspension consisting of $6 \times 10^4$ cells/ml). 100µl of inoculum (6 $\times$ $10^5$ parasites/ml adjusted using haemocytometer and trypan blue exclusion dye) were added to each well (parasite/cells ratio $\sim$ 10/1, final volume 200µl). 6 h after inoculation, non-adherent parasites were removed and 100µl of complete DMEM (1% penicillin and streptomycin; 3% FBS) supplemented with inhibitors at different concentrations (2-fold serial dilution starting from 30 µM) added to each well except negative control wells. Positive controls, consisting of Pyrimethamine (PYR) and Sulfadiazine (SDZ) (Sigma-Aldrich) (20mg/ml stocks in DMSO), were tested at 2mg/ml final concentrations. Each test was performed in triplicate. Culture plates were incubated at 37°C under 5% CO$_2$ humidified atmosphere for 24 h. 20µl of MTS/PMS (Promega) were thereafter introduced into each well of the 96-well assay plate and incubated at 37°C for 1.5h under humidified, 5% CO$_2$ atmosphere. The absorbance were thereafter immediately recorded at 490nm using a 96-well plate reader (Biotek EL800, USA). These data were normalized to percent control activity and 50% inhibitory concentrations (IC$_{50}$) were calculated using Prism 5.0 software (GraphPad) as per the following variable slope sigmoidal dose–response formula with data fitted by nonlinear regression and the bottom and the top values...
constrained to 0 and 100 respectively, \( y = \frac{100}{1 + 10^{(\log_{10}IC_{50} - x)H}} \), where \( y \) = percent inhibition; \( 100 = \text{Bottom} + (\text{Top} - \text{Bottom}) \); \( x \) = drug concentration; and \( H \) = hill slope (largest absolute value of the slope of the curve). Sigmoidal dose-response analysis data are provided in supplemental information.

**Entamoeba histolytica cultures**

*E. histolytica* Rahman strain (ATCC® 30886™) was primarily cultured in Eagle's Minimum Essential Medium (EMEM) (Sigma, Germany) supplemented with varying concentrations of Heat-Inactivated Foetal Bovine Serum (HIFBS) and 1% (v/v) of the commercial penicillin/streptomycin solution (Sigma, Germany) in 15ml sterile screw-capped tubes, but showed poor growth and no significant difference. In the contrary, replacement of FBS with Adult Bovine Serum (ABS) resulted in optimal trophozoites growth. EMEM supplemented with Adult Bovine Serum (ABS) and 1% (v/v) of the commercial penicillin/streptomycin solution was then used successfully to maintain *E. histolytica* Rahman in culture. The parasites were sub-cultured twice weekly. Parasites at log phase of growth were used for drug susceptibility assays, and were grown for 1 day following regular sub-culturing 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 30µM final concentration, and in triplicate in sealed 96-well polystyrene microtiter plates (Costar, USA). Parasite inocula (100µl) comprising \( 2 \times 10^4 \) parasites/ml [adjusted using trypan blue exclusion dye (Sigma) and haemocytometer-based counts] were added to each well and grown at 37°C in a humidified atmosphere of 5% CO₂ for 72 h. Positive controls consisted of Metronidazole at 1mg/ml. Growth of *E. histolytica* trophozoites in each well was
determined microscopically by measuring the diameter of the confluent cells in drug-
containing wells relative to that in the negative control wells.

Based on the confluency and proportion of motile parasites, the growth
inhibition in each triplicate well was scored as follow:

   4+: <20% confluent cells and 100% parasite 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
   compounds with 4+, 3+, and 2+ scores were considered for IC50 determination to
   confirm the compound activities.

For the dose-response experiments, 100µl of a 2-fold serial dilution of each
selected compound were added to 100µl of E. histolytica trophozoites 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
above at 37°C for 72 h. Positive control wells contained metronidazole at the highest
concentration of 0.5mg/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
calculated with respect to the negative control. IC50 values were determined as
above using GraphPad prism 5.0. Sigmoidal dose-response analysis data are
provided in supplemental information.

Results

From the screening of the Malaria Box content, 7 compounds showed potent
anti-Toxoplasma gondii activity (IC50< 5µM) and 2 moderately inhibited Entamoeba
histolytica (IC\textsubscript{50}s ~ 10 to 15µM). The results are summarized below and discussed in the later sections. Additional information on moderately active compounds that represent alternative options for anti-Toxoplasma SAR-based optimization studies are also presented in a different section, supplemental materials.

**Cellular cytotoxicity of drugs**

From the cytotoxicity assay against HFF cells, none of the compounds showed significant toxic effects on cells after 72h of incubation. Few compounds showed cell growth inhibition (although < 40% inhibition) at 30µM final concentration, which was several orders of magnitude above the IC\textsubscript{50} values of the promising compounds. Therefore, CC\textsubscript{50} values were greater than 30µM against HFF cells (table 1).

**Anti-Toxoplasma activity of drugs**

Serially diluted compounds were screened for \textit{in vitro} activity against \textit{T. gondii}. Of the 200 drug-like and 200 probe-like compounds tested, 45 (27 drug-like and 18 probe-like) inhibited \textit{T. gondii} growth as determined by the MTS/PMS-based cell viability assay. Based on our activity cut-off criteria (IC\textsubscript{50}< 5µM, SI>6), 7 compounds showed potent activities (table 1 and figure 1), whereas 38 others moderately inhibited the parasites with IC\textsubscript{50} values ranging from 5.85-28.99µM (supplemental information- table S1 and fig. S2). The results indicate that amongst the most potent inhibitors (IC\textsubscript{50}< 5µM), the drug-like compound MMV007791 inhibited the parasites with an IC\textsubscript{50} of 190nM and a SI> 157. The 6 others exhibited anti-\textit{T. gondii} activities with 1<IC\textsubscript{50}<5 µM and SI> 6.

**Anti-Entamoeba activity**
The 400 Malaria Box compounds were primarily screened at 30µM single concentration to select promising candidates for IC₅₀ determination. *E. histolytica* trophozoite growth and viability were evaluated by microscopical estimation of the diameter of confluent cells and by standard trypan blue exclusion assays, respectively. Following our hit selection criteria, the following 5 compounds: MMV006861 and MMV666600 of score 4+, MMV006303 of score 3+, MMV006172 and MMV006087 of score 2+, were selected for IC₅₀ determination. By dose-response analyses, only two (MMV006861 and MMV666600) of the five compounds displayed promising potency with IC₅₀ values of 15.58 µM and 10.66 µM, respectively. These results are summarized in table 1 and their structures presented in figure 1.

**Discussion**

According to our hit criteria (IC₅₀ < 5 µM and and SI> 6), 7 anti-*Toxoplasma* hits were identified in this study (figure 1). This represents a 1.75% hit rate, a figure that seems to be above the standard 0.1% usually observed in other screens. A putative reason could be due to the fact that these hits have already favourable properties to reach their biological target in a whole cell assay as all compounds are known to be in vitro active against the blood-stage form of *Plasmodium falciparum*. Also the potential commonality of target shared between the two protozoan parasites might as well be an explanation for the improved hit rate.

First, 6 of them show an activity range similar to pyrimethamine with IC₅₀s going from 1.07 to 4.54 µM with moderate to good selectivities. However the structures seemed novel with a possible exception for MMV007881 that has a 4-aminobenzenesulfonamide group embedded in the structure similarly to...
sulfadiazine. MMV020548 is comprised of piperazine and a pyrroloquinoline carboxamide with an IC\textsubscript{50} of 3.85 µM and a SI greater than 7. Three hits, MMV007363, MMV006704 and MMV666095 contain a quinolone fragment with IC\textsubscript{50} values of 1.49µM, 1.95µM, 3.05µM and SI greater than 20, 15 and 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 and more interestingly, a recent study has described highly efficacious 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\textsubscript{50}s below 1nM \textit{in vitro} and are also effective in mice model. Overall, the quinolone scaffold also represents a target group for detailed medicinal chemistry for anti-\textit{Toxoplasma} drug discovery. MMV085203 is a 2,3-diaminonaphthoquinone with an IC\textsubscript{50} of 4.54µM and could be compared to \textit{P. falciparum} bc(1) inhibitor atovaquone which has known anti-\textit{Toxoplasma} activity (36).

More interestingly, MMV007791 showed a significantly higher potency with an IC\textsubscript{50} of 190 nM and an excellent selectivity index, SI >157, against HFF. This chemotype is novel for its anti-\textit{Toxoplasma} activity and the structure is comprised of a piperazinacetamide moiety. Additional data on this compound is reported in the Open Access database powered by the European Bioinformatics Institute (ChEMBL website: https://www.ebi.ac.uk/chembl/malaria) where the compound is reported to have good selectivity over other pathogens as it has no activity against kinetoplastids (IC\textsubscript{50}>32 µM), no effect on the non-replicating form of \textit{M. tuberculosis} at 25 µM, no activity against newly transformed schistosomosula at 2.5 µM and a good overall SI.
profiles as no overt toxicity was seen against MRC-5 (CC\(_{50}>32\mu M\)) and Huh7 (CC\(_{50}>100\mu M\)). 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. anti-parasitic (37), anticancer (38) and antiviral effects (39). Of particular interest is their activity against *Toxoplasma gondii* enoyl reductase (37). These derivatives have also proven P-glycoprotein and *Mycobacterium tuberculosis* H\(_37\)R\(_v\) inhibition *in vitro* (40, 41).

Also *in vitro* DMPK data suggest that this scaffold may have some liability as they have high intrinsic clearance in liver microsomes, CL\(_{int}\) of 46 and 906 μL/min/mg in human and mouse respectively. Efforts in the SAR to address the metabolism as well as the potentially genotoxicity risk represented by the aniline are considered.

In a very recent study, a strategy similar to ours was adopted to repurpose the Open Access Malaria Box to identify active chemical series 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 RH strain from which the TS-4 strain used in our study derived. Compounds of this series from commercial origin exerted anti-*Toxoplasma* activity with IC\(_{50}\) values ranging from 0.55-7.3 μM. From our findings, two derivatives (MMV019199 and MMV080034) of this series showed moderate activity (28.12μM and 28.99μM respectively) as compared to these authors’ 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 susceptibility of different *T. gondii* strains exist, and might justify the observed difference in activity level.
Currently recommended drugs in the treatment of toxoplasmosis act primarily against the tachyzoite form of *T. gondii*; thus, they do not eradicate the encysted form (bradyzoite) that maintains the parasite in 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 trisulfapyrimidines (e.g., a combination of sulfamerazine, sulfamethazine, and sulfapyrazine). These agents are active against tachyzoites and are synergistic when used in combination (43, 44, 45). These compounds belong to the pyrimidine scaffold. In contrast, all the hits identified in our study belong to other different 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 *T. gondii* RH-HX-KO-YFP2-DHFR(m2m3) strain. Similarly to our findings, the authors observed no effect of MMV008138 on *T. gondii* growth (46).

Other similar studies to identify novel hit compounds against *T. gondii* have been recently conducted. Amongst the findings, compounds of (benzaldehyde)-4-phenyl-3-thiosemicarbazone and (benzaldehyde)-(4 or 1)-phenylsemicarbazone scaffolds showed limited efficacy against intracellular tachyzoites (47). Also, a label extension strategy was adopted to assess the anti-*Toxoplasma* activity of nine antiretroviral drugs *in vitro*. Nucleoside analogs showed no effect on parasite growth, whereas ritonavir and nelfinavir were inhibitory for *Toxoplasma*, with IC$_{50}$ of 7.50 and 7.05 µM respectively (48). Fusidic acid also showed efficiency against *T. gondii in vitro*, but not in mice (49). In another study, 1-Hydroxy-2-Dodecyl-4(1H)Quinolone exhibited growth inhibition of *T. gondii* at nanomolar concentration, with the alternative (Type II) NADH Dehydrogenases as specific target (50). Recently, two
novel 1-hydroxyquinolones were shown to display low nanomolar anti-Toxoplasma activities (51).

From our results, two compounds were identified but with very modest activities against *E. histolytica*. They are MMV666600 (IC$_{50}=10.66 \mu M$) that is a 2-Methyl-1H-indole derivative, and MMV006861 (IC$_{50}=15.58 \mu M$) that is a Benzo[g]quinolin-4-ylamine derivative. Metronidazole which is the mainstay therapy for invasive amebiasis (52, 53) belongs to the imidazole class.

In a similar approach, some investigators have recently described the *in vitro* anti-*Entamoeba* activity of a FDA approved drug for use in rheumatoid arthritis (auranofin). The IC$_{50}$ of auranofin against *E. histolytica* trophozoites was lower than 2 µM. Of note, auranofin had much higher cysticidal activity on *Entamoeba invadens* cysts than the standard amoebicide, metronidazole, providing a potential therapeutic advantage (54, 55).

Our study has identified compounds with low micromolar anti-Toxoplasma activities (IC$_{50}<5 \mu M$), and acceptable physico-chemical drug profiles. Ongoing studies based on extensive medicinal chemistry and metabolism of these hits will identify related scaffolds with improved biological properties. Compounds that showed moderate activity might be potential options for SAR-based optimization studies. As well, 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 amoebiasis.

*Plasmodium falciparum, Toxoplasma gondii* and *Entamoeba histolytica* are all parasitic protozoa. Within this family, apicomplexan are a large group of parasitic protists, most of which possess a unique organelle, a type of plastid called
apicoplast, an apical complex structure most likely involved in penetrating a host's cell, and which is currently amongst the prioritized targets for drug discovery. *P. falciparum* and *T. gondii* share this feature in addition to 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* that does not possess any. Further studies on the rate and mechanisms of drugs action will elucidate these considerations.

This paper reports 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 afford drug candidates with innovative and acceptable profiles.

Acknowledgements

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References


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Figure 1's legend

Figure 1: Scaffolds and hits 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 identifier are shown for each listed compound. Scaffolds were afforded from SAR studies. Anti-Toxoplasma hits [MMV007791: N-(2-Ethoxy-phenyl)-2-[4-(furan-2-carbonyl)-piperazin-1-yl]-acetamide; MMV085203: 4-Hydroxy-2-(2-methoxy-phenylimino)-3-piperidin-1-yl-2H-naphthalen-1-one; MMV007881: Furan-2-carboxylic acid (4-dibutylsulfamoyl-phenyl)-amide; MMV020548: 1H-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)-1H-indole-3-carboxylic acid ethyl ester; MMV006861: Benzo[g]quinolin-4-yl-(4-morpholin-4-yl-phenyl)-amine].
Table 1: Hits with potential for further extensive medicinal chemistry/biological screenings against *T. gondii* and *E. histolytica*

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
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<th>IC₅₀ (µM)</th>
<th>CC₅₀ HFF (µM)</th>
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<th>*AlogP</th>
<th>IC₅₀ (µM)</th>
<th>CC₅₀ HFF (µM)</th>
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*Compounds are designated by their MMV identifier codes; *Compounds were serially diluted and tested in culture. Results are means from triplicate experiments; °Cell cytotoxicity was evaluated in culture against Human Foreskin Fibroblasts and results expressed as means of triplicate experiments; °Selectivity indices were calculated based on the ratio CC₅₀(HFF)/IC₅₀ Test drugs; °Molecular weights and AlogP values were gotten as supporting information to the Malaria Box. Positive controls (PYR: Pyrimethamine; SDZ: Sulfadiazine; MTZ: Metronidazole).