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Invited article

Repurposing the open access malaria box reveals compounds with activity against *Tritrichomonas foetus* trophozoites

Katy A. Martin, Jeba R.J. Jesudoss Chelladurai, Christopher Bader, Elizabeth Carreiro, Katelyn Long, Kylie Thompson, Matthew T. Brewer *

Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, IA, USA

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ABSTRACT

The protozoan parasite *Tritrichomonas foetus* causes early embryonic death in cattle which results in severe economic loss. In the United States, there are no drugs are approved for treatment of this pathogen. In this study, we evaluated *in vitro* anti-protozoal effects of compounds from an open access chemical library against *T. foetus* trophozoites. An initial high-throughput screen identified 16 compounds of interest. Further investigation revealed 12 compounds that inhibited parasite growth and 4 compounds with lethal effects. For lethal compounds, dose-response curves were constructed and the LD_{50} was calculated for laboratory and field strains of *T. foetus*. Our experiments revealed chemical scaffolds that were parasiticidal in the micromolar range, and these scaffolds provide a starting point for drug discovery efforts. Further investigation is still needed to investigate suitability of these scaffolds and related compounds in food animals. Importantly, open access chemical libraries can be useful for identifying compounds with activity against protozoan pathogens of veterinary importance.

1. Introduction

Tritrichomonas foetus is the etiologic agent of bovine trichomoniasis, a venereal disease of cattle with a cosmopolitan distribution. Flagellated, pear-shaped trophozoites are transmitted between bulls and cows during breeding. In bulls, trophozoites live on the mucosal surfaces within the preputial cavity and infections are generally asymptomatic (Yule et al., 1989). Although bulls typically lack overt clinical signs, they remain chronically infected and serve as a reservoir of infection for cows. Following coital exposure, motile trophozoites migrate into the uterine lumen via the cervix and the entire reproductive tract becomes colonized within 1–2 weeks (Skirrow and BonDurant, 1990). This colonization does not disrupt conception and few lesions are observed prior to 50 days of gestation (Parsonson et al., 1976; Yule et al., 1989). After day 50, infection induces mononuclear and neutrophilic inflammation which leads to varying intensity of endometritis, cervicitis, and vaginitis depending on the immune response of the animal. Subsequently, the majority of infected cows experience fetal loss at some point during the first five months of gestation, with the majority of losses occurring approximately 17 days after infection (Rhyan et al., 1988; Yule et al., 1989).

There are no treatments for *T. foetus* currently approved in the United States. Although metronidazole is efficacious against trophozoites *in vitro*, metronidazole use in food animals is illegal in the United States because of food safety concerns due to the carcinogenic potential of this drug (Bendesky et al., 2002). Because it is accepted that infected cows eventually clear the infection, the current control measures in the United States entails testing and culling infected bulls. Most states require negative culture or PCR prior to importation of bulls and some states prohibit the importation of non-pregnant females unless they are proceeding to terminal markets. Antigen detection tests are needed in order to perform better field screening, and such assays are currently in the research phase (Schaut et al., 2017). Vaccination against bovine trichomoniasis can reduce the time required for females to clear infections, but is not effective in eradicating the disease (Corbeil, 1994). Altogether, an effective treatment is needed to aid in control of bovine trichomoniasis.

The Medicines for Malaria Venture has compiled the Open Access Malaria Box which consists of 400 compounds with activity against *Plasmodium falciparum*. These compounds were selected from nearly 20,000 compounds identified during a high throughput assay for activity against intraerythrocytic stages (Gamo et al., 2010). More recently, the Malaria Box has been made available to investigators studying other protozoan parasites and has been assessed with

* Corresponding author. 2758 Vet Med, 1800 Christensen Dr, Ames, IA, 50011, USA.
E-mail address: brewermt@iastate.edu (M.T. Brewer).

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Table 1
Malaria box compounds identified as inhibitors of *T. foetus*.

Compounds inhibiting metabolism >50% at 50 μM	Compounds inhibiting metabolism >50% at 25 μM	Lethal compounds by hemocytometer counts
MMV020549	MMV020549	MMV020549
MMV306025	MMV306025	MMV306025
MMV665941	MMV665941	MMV665941
MMV009085	MMV009085	MMV009085
MMV020700	MMV020700	
MMV007977	MMV007977	
MMV020548	MMV020548	
MMV403679	MMV403679	
MMV019241	MMV019241	
MMV666599		
MMV666023		
MMV666054		

pathogens such as *Toxoplasma*, *Cryptosporidium*, *Trypanosoma*, and *Leishmania* (Bessoff et al., 2014; Khraiwesh et al., 2016; Spalenka et al., 2018; Sykes and Avery, 2015). In the present study, we assessed the activity of the Malaria Box library compounds against *T. foetus* in an effort to reveal new leads for potential chemotherapeutic approaches to controlling this parasite.

2. Materials and methods

2.1. Parasite cultures and compounds tested

T. foetus strains ATTC BP-4 Beltsville strain (ATTC) and an Iowa field strain (IA-1) were used for conducting parasite killing assays. Trophozoites were maintained in trypticase-yeast extract-maltose (TYM) medium supplemented with 10% adult bovine serum and 1% 100X penicillin-streptomycin (Clark and Diamond, 2002). Cultures were maintained under at 32 °C and regularly subcultured to maintain cell concentrations of approximately 4×10^5 trophozoites/mL. The cultures were maintained in sterile 15 mL centrifuge tubes filled completely with media and capped tightly to create an anaerobic environment. Test compounds (n = 390) were obtained from the Medicines for Malaria Venture and were supplied as 10 mM solutions in 10 mM DMSO in 96 well plates. Freshly synthesized compounds of interest were obtained from Medicines for Malaria Venture or commercial sources for additional testing.

2.2. High throughput screening

For initial compound screening, a resorufin metabolism assay was utilized (Bader et al., 2016). Assays were carried out in black-walled, clear bottom 96 well plates. 5000 mid-log phase cells were distributed in each well in a total volume of 200 μl containing 25 or 50 μM test compound for 24 h at 32 °C. Following incubation, 200 μM resorufin was added to each well and plates were incubated for 10 min at 32 °C under anaerobic conditions. Fluorescence was then immediately measured with a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA) (excitation: 544 nm, emission: 599 nm). All wells were also examined under an inverted microscope to confirm the results of the fluorescence assay.

2.3. Assessment of anti-T. foetus compounds

Following identification of compounds with activity against trophozoites, we obtained newly synthesized samples of available

Table 2
EC_{50} values for compounds killing *T. foetus* (μM).

	IA1	ATCC
MMV020549	25.69	10.89
MMV665941	7.332	15.51
MMV009085	1.641	2.137
MMV306025	0.37	1.36

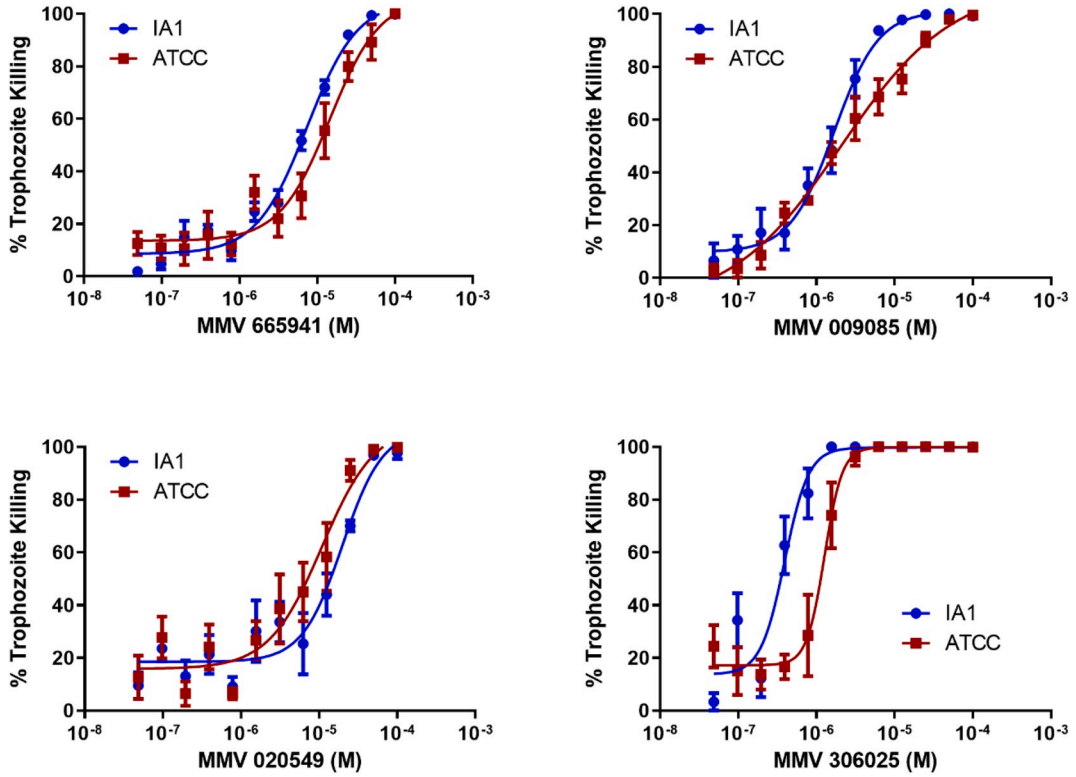

Fig. 1. Dose-response relationship of selected MMV compounds against *T. foetus* trophozoites *in vitro*. Points represent mean ± SE.

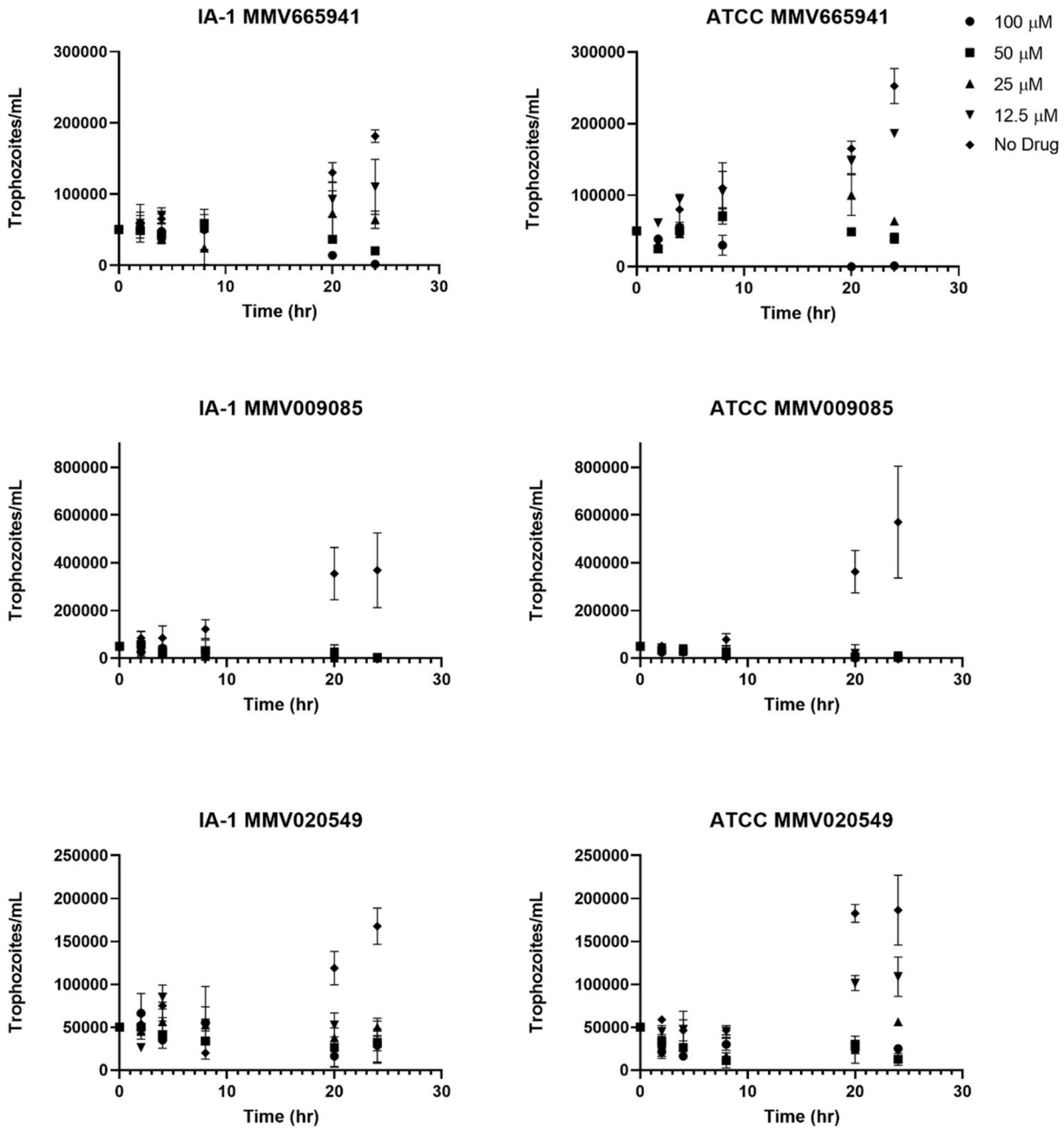

Fig. 2. Parasite counts (Mean ± SD) over time following coincubation with selected compounds.

compounds for further testing. Dilutions of each compound were co-incubated with 10^5 *T. foetus* trophozoites and maintained for 24 h at 32 °C under anaerobic conditions. Experiments were repeated in triplicate with two technical replicates for each trial. Time course experiments were also conducted to characterize the temporal effect of trophozoite killing. For time course experiments, trophozoites were treated dilutions of compounds and compared to untreated controls or those co-incubated with metronidazole.

Lethality of the compounds was assessed by performing recovery assays. Following 24 h exposure to the 100 μM MMV compound, the culture was diluted in 50 mL drug free media, resulting in a final MMV compound concentration of 3 μM. Hemocytometer counts were used to assess the ability of the trophozoites to recover and resume growth following dilution of the MMV compound. Graphpad Prism was used to generate dose-response curves and calculate EC_{50} values using a 4 parameter nonlinear regression model.

3. Results

3.1. High-throughput screening by resorufin metabolism

All Malaria Box compounds were initially screened against *T. foetus* trophozoites utilizing a high throughput resorufin assay. This assay measures the ability of trophozoites to metabolize fluorescent resorufin into the colorless compound dihydroresorufin (Natto et al., 2012). Therefore, a decrease in either metabolic activity or total numbers of trophozoites could be interpreted as trophozoite inhibition in the assay. Assay plates contained media, vehicle (DMSO), and positive (metronidazole) controls. Each assay also contained known dilutions of *T. foetus* trophozoites to create a standard curve and validate the relationship between live metabolically active trophozoites and resorufin metabolism. In our initial screen, we identified 12 compounds demonstrating greater than 50% inhibition at 50 μM. A secondary screen at a final

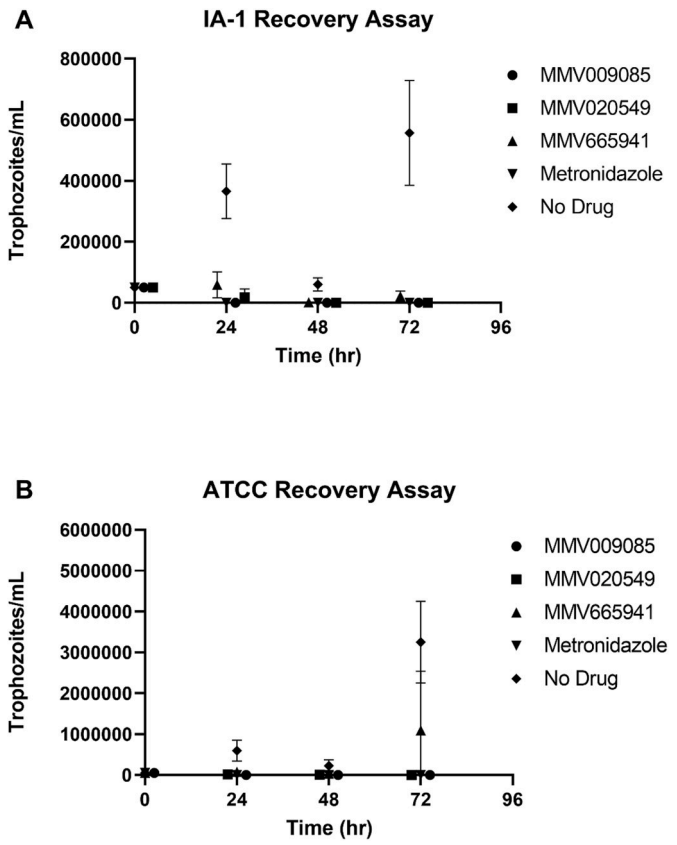

Fig. 3. Recovery assays for *T. foetus* strains IA1 (A) and ATCC (B). Parasites were treated with 100 μM drug, diluted in drug free medium to a final concentration of 3 μM, and observed for 3 days for signs of growth. Points represent mean parasite counts ± SD.

concentration of 25 μM identified 9 compounds with greater than 50% inhibition as determined by resorufin metabolism (Table 1). The structures of the compounds identified in these screens are provided in Table S1.

3.2. Validation of hits discovered during high throughput screening

Newly synthesized lead compounds were obtained and co-incubated with ATCC and IA-1 *T. foetus* trophozoites in order to verify the effects observed in the resorufin assays. In this phase of the study, trophozoite were measured directly by counting with a hemocytometer. MMV020700, MMV007977, MMV020548, MMV403679, MMV666599, MMV019241, MMV666023, and MMV666054 did not eliminate greater than 50% of cultured trophozoites. These compounds are potential inhibitors of *T. foetus* metabolism and could still be effective clinically, but they were not assessed further in the present study due to their lack of an overt parasiticidal effect.

Dose-response studies revealed four compounds with the ability to kill 100% of trophozoites for both laboratory (ATCC) and field (IA1) strains - MMV020549, MMV306025, MMV665941, and MMV009085 (Fig. 1). EC_{50} values for each of the four compounds are given in Table 2. Strain variation is likely contributing to the differences in drug response between IA1 and ATCC. Previous studies have also found differences in responses among various *T. foetus* strains in *in-vitro* drug assays (Kather et al., 2007).

3.3. Temporal nature of trophozoites killing by malaria box compounds

Temporal assays were conducted to determine the length of time needed to kill trophozoites. As shown in Fig. 2, trophozoite killing became apparent after 8 h of coincubation. Although MMV306025 could not be assessed in temporal assays due to availability, dose-response experiments had previously revealed that inhibitory effects on trophozoites could be observed following 24 h of incubation with this compound.

3.4. Evaluation of lethal effects of malaria box compounds on trophozoites

Recovery assays were performed to determine if the activity of the Malaria Box compounds was truly lethal or if trophozoites were able to recover after being transferred to drug free media. For both field and laboratory strains there is no recovery of trophozoites for MMV009085 or MMV020549 following 48 h of incubation in drug free media (Fig. 3). However, trophozoites incubated with MMV665941 recovered and had moderate growth upon addition of fresh culture medium.

4. Discussion

The economic losses attributed to *T. foetus* highlight the need for aggressive strategies designed to eliminate the parasite (Rae, 1989). Currently, a test and cull approach is the only option for control, so it is desirable to determine effective therapies that could be used to cure infections. In this study, we take a step toward identifying chemical scaffolds with efficacy against *T. foetus*. Although the compounds identified in this study may not be suitable for use in food animals for a variety of reasons, identification of these chemical scaffolds provides a starting point for drug discovery. For some pathogens, drugs that do not completely kill the organism *in vitro* are still clinically effective (Ersoy et al., 2017).

In this study, 16 compounds inhibited trophozoite growth at 50 μM, and 12 inhibited trophozoite growth at 25 μM Direct parasite counting revealed that 4 of these compounds killed trophozoites at micromolar concentrations. Some differences were observed between metabolic (resorufin) and direct parasite counting, which could represent compound batch variability as some compounds were re-synthesized during the study. The variation could also be a result of the mechanism of action of the compound, as direct counting measures parasiticidal activity, while resorufin provides a measure of redox potential.

Importantly, this study identified 4 compounds capable of eliminating trophozoites *in vitro*. MMV020549, 306025, 665941, and 009085 were the four top performing compounds. These compounds have been screened against many other pathogens and malignancies by researchers and have shown utility in several assays. MMV020549 was found to be a promising inhibitor of ezrin, a protein which acts as a linker between the plasma membrane and actin cytoskeleton and is a key driver of osteosarcoma progression and metastasis (Celik et al., 2015). However, this compound disturbs the lipid bilayer and therefore clinical utility may be limited as it would have significant effects on host cells (Ramsey, 2019). Both MMV306025 and MMV009085 have demonstrated activity against *Cryptosporidium* spp. (Bessoff et al., 2014; Egbe, 2014). While these studies identify chemical scaffolds with activity against trophozoites, further studies are needed to identify variations of these scaffolds that could be safely used in food animals.

MMV665941 showed was efficacious against some strains of *Perkinsus marinus*, a significant protozoal pathogen of oysters, however the efficacy was not found to be consistent among all strains of the parasite (Aleman Resto and Fernandez Robledo, 2014). This compound has also shown significant activity against *Brugia malayi* and *B. pahangi*, as well as *Cryptosporidium*, and *Trypanosoma cruzi* (Bessoff et al., 2014; Bilsland et al., 2016; Sykes and Avery, 2015). In combination with these studies, our result is particularly interesting as MMV665941 is structurally nearly identical to gentian violet. Gentian violet has been used in human medicine for infections with numerous microorganisms in addition to being used as a mold inhibitor in feed ingredients (Maley and Arbiser, 2013). However, gentian violet is specifically prohibited for use in any

food animal in the United States (Davis et al., 2009). Surprisingly, relatively few reasons have been given for the gentian violet ban, other than that the FDA has stated that the impact of drug residues has not been adequately assessed in human health (Davis et al., 2009).

This study demonstrated that the Malaria Box can be leveraged to discover chemical scaffolds with activity against veterinary parasites. Chemically modification of these scaffolds may further enhance the level of activity against trophozoites. In addition, investigating the mechanism of action for the promising compounds will identify assist in the identification of druggable targets.

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Declaration of competing interest

The authors have no conflicts of interest to report.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2020.06.003.

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