

## Screening the Medicines for Malaria Venture Pathogen Box against piroplasm parasites



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### ABSTRACT

Diminazene aceturate (DA) and imidocarb dipropionate are commonly used in livestock as antipiroplasm agents. However, toxic side effects are common in animals treated with these two drugs. Therefore, evaluations of novel therapeutic agents with high efficacy against piroplasm parasites and low toxicity to host animals are of paramount importance. In this study, the 400 compounds in the Pathogen Box provided by the Medicines for Malaria Venture foundation were screened against *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi*. A fluorescence-based method using SYBR Green 1 stain was used for initial *in vitro* screening and determination of the half maximal inhibitory concentration (IC<sub>50</sub>). The initial *in vitro* screening performed using a 1 μM concentration as baseline revealed nine effective compounds against four tested parasites. Two “hit” compounds, namely MMV021057 and MMV675968, that showed IC<sub>50</sub> < 0.3 μM and a selectivity index (SI) > 100 were selected. The IC<sub>50</sub>s of MMV021057 and MMV675968 against *B. bovis*, *B. bigemina*, *T. equi* and *B. caballi* were 23, 39, 229, and 146 nM, and 2.9, 3, 25.7, and 2.9 nM, respectively. In addition, a combination of MMV021057 and DA showed additive or synergistic effects against four tested parasites, while combinations of MMV021057 with MMV675968 and of MMV675968 with DA showed antagonistic effects. In mice, treated with 50 mg/kg MMV021057 and 25 mg/kg MMV675968 inhibited the growth of *Babesia microti* by 54 and 64%, respectively, as compared to the untreated group on day 8. Interestingly, a combination treatment with 6.25 mg/kg DA and 25 mg/kg MMV021057 inhibited *B. microti* by 91.6%, which was a stronger inhibition than that by single treatments with 50 mg/kg MMV021057 and 25 mg/kg DA, which showed 54 and 83% inhibition, respectively. Our findings indicated that MMV021057, MMV675968, and the combination treatment with MMV021057 and DA are prospects for further development of antipiroplasm drugs.

### 1. Introduction

Piroplasmosis is a tick-transmitted disease caused by *Babesia* and *Theileria* parasites. Piroplasmosis affects humans, livestock, and wild animals worldwide. Generally, piroplasm infection is characterized by fever, icterus, hemolysis, hemoglobinuria, and death if treatment fails or is not attempted (Schnittger et al., 2012; Wise et al., 2013).

Significant economic impacts of bovine babesiosis and equine piroplasmosis on the cattle and horse industries have been reported, especially in piroplasmosis-endemic countries. Bovine babesiosis caused by *Babesia bovis* and *Babesia bigemina* decreases meat and milk production and leads to the death of infected cattle (Yusuf, 2017). Equine piroplasmosis caused by *Babesia caballi* and *Theileria equi* is associated with detrimental effects in horses. Once the horse is infected by

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either or both *Babesia caballi* and *Theileria equi*, that animal could remain a carrier for its entire life. Consequently, such horses are restricted with regard to international movement because they can transmit disease that affects trade and equestrian sport (Knowles, 1996).

There are three widely used strategies for piroplasmosis control: vaccination, the use of antipiroplasm drugs, and vector control measures. In the past several decades, antipiroplasm drugs, including diminazene aceturate (DA) and imidocarb dipropionate (ID), have played an important role in the prevention and control of piroplasmosis (Mosqueda et al., 2012). However, recent studies on *Babesia* have reported the development of resistance to DA and documented toxic side effects in ID-treated equines (Hwang et al., 2010; Tuntasuvan et al., 2003). Furthermore, high levels of ID and DA drug residue in edible tissue have been reported in treated animals (Belloli et al., 2007; Mdachi et al., 1995). Therefore, continuous efforts to discover and develop novel antipiroplasm drugs are urgently needed.

One alternative approach to fast-track the development of novel antiparasitic agents is large-scale screening of compounds from existing databases, such as the Medicines for Malaria Venture (MMV) Pathogen Box. The MMV foundation offers free access to compounds in the MMV Pathogen Box to researchers all over the world. The activity of compounds in the MMV Pathogen Box has been confirmed against several diseases, including tuberculosis, malaria, leishmaniasis, trypanosomiasis, helminthiasis, toxoplasmosis, and dengue. Additionally, the MMV Pathogen Box contains 26 reference compounds. Recently, several researchers have discovered effective compounds in the MMV Pathogen Box and repurposed them for treatment against other parasitic protozoa, including *Toxoplasma gondii*, *Cryptosporidium parvum*, *Giardia lamblia*, *Neospora caninum*, *Plasmodium falciparum*, and *Trypanosoma* spp. (Duffy et al., 2017; Hennessey et al., 2018; Müller et al., 2017; Spalenka et al., 2018). Moreover, all of the compounds have been tested for their cytotoxicity on mammalian cells with greater than fivefold selectivity indexes (<http://www.pathogenbox.org/about-pathogen-box/supporting-information>). The present study aimed to discover potent inhibitors against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* by screening 400 compounds from the MMV Pathogen Box.

## 2. Materials and methods

### 2.1. Pathogen Box compounds

The Pathogen Box consists of 400 compounds provided by the MMV foundation following a request from our laboratory. The compounds were delivered in five plates, each containing 80 compounds. Each compound had a 10  $\mu$ l volume diluted in 100% dimethyl sulfoxide (DMSO) to a concentration of 10 mM. In order to prepare a 1 mM stock solution, 90  $\mu$ l of DMSO was added to each well and divided into two identical plates in accordance with MMV instructions. Additionally, DA (Sigma-Aldrich, Tokyo, Japan) was diluted in Milli-Q water (MQW) to make a 10 mM stock solution. All compounds were stored at  $-30^{\circ}\text{C}$  until needed for the experiments. For *in vivo* studies, MMV021057 was purchased from Sigma-Aldrich, while MMV675968 was supplied from MMV in powder form. All compounds were diluted in normal saline with 4% DMSO and 8% Tween 80 for *in vivo* studies.

### 2.2. Reagents

A lysis buffer containing Tris (130 mM at pH 7.5), EDTA (10 mM), saponin (0.016%; w/v), and Triton-X 100 (1.6%; v/v) was prepared and stored at  $4^{\circ}\text{C}$ . The 10,000 x SYBR Green 1 nucleic acid stain (Lonza Rockland Inc., Rockland, USA) was stored at  $-30^{\circ}\text{C}$ . All reagents were purchased from Sigma-Aldrich (Tokyo, Japan).

### 2.3. Parasites

Four strains of parasites were used for *in vitro* studies, including *B.*

*bovis* (Texas strain), *B. bigemina* (Argentine strain), and a USDA strain of *B. caballi* and *T. equi*. The Munich strain of *B. microti* was used for *in vivo* studies.

### 2.4. In vitro cultures

*Babesia bovis* and *B. bigemina* were cultured in purified bovine red blood cells (RBCs) using Medium 199 (M199) supplemented with 40% bovine serum. *Babesia caballi* and *T. equi* were cultured in purified horse RBCs. The medium for *B. caballi* was GIT supplemented with 40% horse serum, while M199 supplemented with 40% horse serum and hypoxanthine (MP Biomedicals, USA) at a final concentration of 13.6  $\mu\text{g}/\text{ml}$  was used for *T. equi* cultivation. An antibiotic-antimycotic solution containing 60 U/ml penicillin G, 60  $\mu\text{g}/\text{ml}$  streptomycin, and 0.15  $\mu\text{g}/\text{ml}$  amphotericin B (Sigma-Aldrich, Tokyo, Japan) was added to all of the media.

### 2.5. In vitro inhibition assay

Initially, all 400 compounds were tested against *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* at a single concentration of 1  $\mu\text{M}$ . Each compound was tested in duplicate and repeated in three separate experiments. Compounds that showed over 60% inhibition against all of the parasites tested were selected for further analysis to determine their half maximal inhibitory concentration ( $\text{IC}_{50}$ ).

To determine the  $\text{IC}_{50}$ , each compound was diluted by a twofold serial dilution ranging from 0.001 to 1  $\mu\text{M}$ . DA was also included for each experiment as a comparator drug. The medium containing 0.1% DMSO with infected RBCs (iRBCs) and uninfected RBCs were used as positive and negative controls, respectively. This experiment was repeated three times in separate experiments.

*In vitro* inhibition assays were carried out as previously described (Guswanto et al., 2014; Rizk et al., 2015). Briefly, the parasites were harvested after 4 days of the initial culture, when the parasitemia reached more than 3%. The iRBCs were harvested and diluted with fresh RBCs to make a 1% parasitemia. The experiment was conducted using 2.5  $\mu\text{l}$  (for *B. bovis* and *B. bigemina*) or 5  $\mu\text{l}$  (for *B. caballi* and *T. equi*) of iRBCs. The iRBC was added to each well in triplicate. The volume of culture medium containing the drug was added, up to a total reaction volume of 100  $\mu\text{l}$ . The plates were incubated at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ , 5%  $\text{O}_2$ , and 90%  $\text{N}_2$ . After 96 h, 100  $\mu\text{l}$  of lysis buffer containing SYBR Green 1 was added to each well and mixed gently by pipetting. The plates were wrapped in aluminum foil to protect them from light and kept at room temperature for 6 h. The relative fluorescence values were measured using a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo Fisher Scientific, USA) excitation and emission wavelength of 485 nm and 518 nm, respectively. The relative fluorescence value was set to percentages after subtracting the mean values of the negative control.

### 2.6. Combination treatment with MMV675968, MMV021057, and DA

A drug combination assay was performed using a 96-well culture plate with a single drug assay at the constant ratio in accordance with Chou (2006). The two-drug combination therapy (MMV675968 + MMV021057, MMV675968 + DA, and MMV021057 + DA) was performed at concentrations of 0.25 x  $\text{IC}_{50}$ , 0.5 x  $\text{IC}_{50}$ ,  $\text{IC}_{50}$ , 2 x  $\text{IC}_{50}$ , and 4 x  $\text{IC}_{50}$ . The degree of synergism was determined as the weighted average of CI values using the formula  $((1 \times \text{IC}_{50}) + (2 \times \text{IC}_{75}) + (3 \times \text{IC}_{90}) + (4 \times \text{IC}_{95}))/10$ . The values with  $< 0.9$ , 0.90 to 1.10, and  $> 1.10$  indicated synergistic, additive, and antagonistic effects, respectively. Each experiment was repeated in three separate trials.

## 2.7. *In vivo* inhibition assay

The growth-inhibitory effect of the hit compounds was determined using a mouse model infected with *B. microti*, as previously described (Tuvshintulga et al., 2016). Thirty-five BALB/c mice (CLEA Japan Inc., Tokyo, Japan) were divided into seven groups, each consisting of five mice. Groups 1 and 2 were kept as a negative control (uninfected and untreated) and positive control (infected and untreated), respectively. Groups 3 and 4 were treated by intraperitoneal (i.p.) injection of 25 mg/kg body weight (BW) MMV675968 and subcutaneous (sc) injection of 50 mg/kg BW MMV021057, respectively. Groups 5 and 6 were treated by i.p. injection of 6.25 mg/kg and 25 mg/kg BW DA, respectively. Group 7 was injected with a combination of 25 mg/kg BW MMV021057 + 6.25 mg/kg BW DA by sc and i.p. injection, respectively.

Prior to the start of the *in vivo* experiments, a frozen stock of *B. microti* was recovered from  $-80^{\circ}\text{C}$  and injected intraperitoneally into a mouse. Subsequently, the parasitemia was monitored every 2 days in a Giemsa-stained blood smear. When 30% parasitemia was observed in mice, the mouse was anesthetized, and blood was collected through cardiac puncture. The blood was then diluted by  $1 \times \text{PBS}$  to acquire  $2 \times 10^7/\text{ml}$  *B. microti* iRBCs. Subsequently, all mice in the six groups except the negative control group were injected intraperitoneally with 0.5 ml of inoculum to achieve  $1 \times 10^7/\text{ml}$  iRBCs.

Parasitemia was monitored by counting iRBCs every 2 days among 10,000 RBCs in Giemsa-stained blood smears. When approximately 1% parasitemia was achieved on day 4 post-infection (p.i.), the treatment was initiated and continued for 5 consecutive days (day 4 to day 8). To analyze the effects of the treatment, 10  $\mu\text{l}$  of blood from the tail was collected every 4 days and used to determine the hematological profiles using an automatic hemocytometer (Celltac  $\alpha$  MEK-6450, Nihon Kohden, Tokyo, Japan). All parameters were monitored until day 30, and the experiment was repeated twice. The animal experiment was conducted in accordance with the Regulations for Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine, Japan (accession number of Animal experiment: 18-120; Pathogen experiment: 201826).

## 2.8. Statistical analysis

The percentage of inhibition on the *in vitro* studies was calculated in Microsoft Excel. Data were calculated for the  $\text{IC}_{50}$  using a nonlinear regression sigmoidal dose-response curve fit, available in GraphPad Prism (GraphPad Software Inc., USA). For combination therapy, the combination indexes (CIs) were calculated using CompuSyn software. Statistical analysis of the parasitemia and hematological profiles was done using Student's *t*-test. The difference in parasitemia between untreated and drug-treated groups was considered statistically significant if  $P < 0.05$ .

## 3. Results

### 3.1. *In vitro* inhibition assay

The initial screening was performed to identify compounds showing at least 60% growth inhibition at the initial concentration of  $1 \mu\text{M}$  against the four parasites tested, namely, *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*. Out of the 400 compounds, six were active against *B. bovis* and *B. bigemina* (Supplementary Fig. S1). Four compounds were active against *B. caballi* and *T. equi*. Nine compounds were active against all tested parasites (Table 1). The compound structures of active compound against four tested parasites shown in Fig. 1.

The  $\text{IC}_{50}$ s of all nine compounds were determined as shown in Table 2 and Supplementary Figs. S2–S11. Two compounds, MMV021057 and MMV675968, were selected as hit compounds because they showed an  $\text{IC}_{50} < 0.3 \mu\text{M}$  and a selectivity index

(SI)  $> 100$ . The  $\text{IC}_{50}$ s of MMV021057 on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi* were  $23 \pm 8$ ,  $39 \pm 11$ ,  $229 \pm 62$ , and  $146 \pm 36 \text{ nM}$ , respectively. The  $\text{IC}_{50}$ s of MMV675968 on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi* were  $2.9 \pm 0.3$ ,  $3 \pm 0.8$ ,  $25.7 \pm 6.4$ , and  $2.9 \pm 0.1 \text{ nM}$ , respectively.

### 3.2. Combination treatment

The combination of two drugs including MMV021057, MMV675968, and DA was performed as described section in section 2.6. A combination of MMV021057 and DA showed synergistic effects against *B. caballi* and *B. bovis*, while an additive effect was observed against *B. bigemina* and *T. equi*. On the other hand, drug combinations of MMV021057 and MMV675968 and of MMV675968 and DA showed antagonistic effects in all tested parasites (Table 3 and Supplementary Fig. S12).

### 3.3. *In vivo* inhibition assay

*In vivo* experiments were conducted to evaluate the chemotherapeutic efficacy of single treatments with 50 mg/kg BW MMV021057, 25 mg/kg BW MMV675968, 25 mg/kg BW DA, and 6.25 mg/kg BW DA and combination treatments with MMV021057 + DA (25 + 6.25 mg/kg BW) against *B. microti*. The groups treated with 50 mg/kg BW MMV021057 and 25 mg/kg BW MMV675968 showed a higher inhibition level and more rapid reduction of parasitemia than the untreated group (Fig. 2A). The peak parasitemia in MMV021057- and MMV675968-treated mice was reached on day 10 p.i., while that in the untreated and DA-treated group was reached on day 8 p.i. The percentage of growth inhibition of 50 mg/kg BW MMV021057, 25 mg/kg BW MMV675968 and 25 mg/kg BW DA, compared with that of the untreated group, was 54, 64, and 83%, respectively. Furthermore, the inhibitory effect was improved to 91.6% when a combination of 25 mg/kg BW MMV021057 and 6.25 mg/kg BW DA was used (Fig. 2B). The hematocrit indexes were stable in the treated groups as compared to the positive control group (infected and untreated) (Fig. 3).

## 4. Discussion

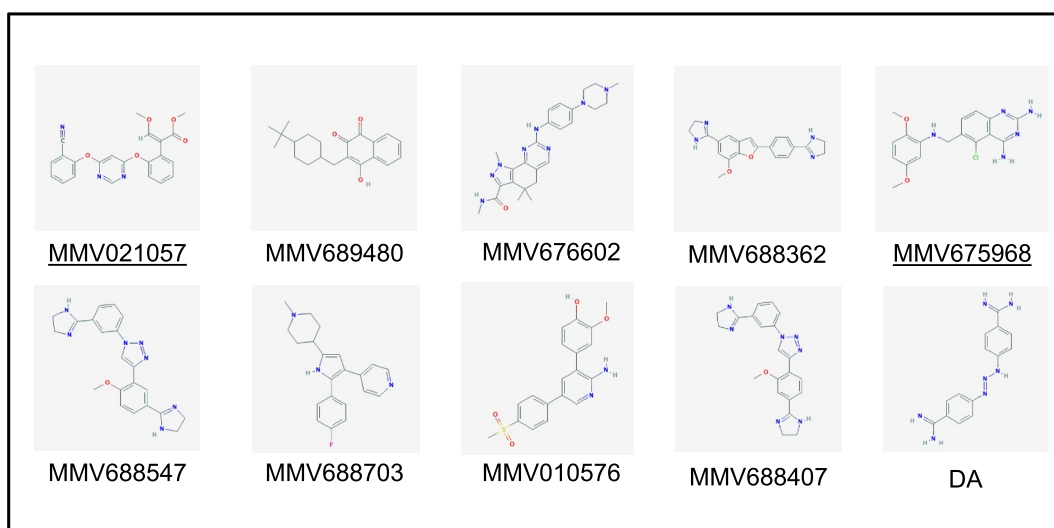
Although diminazene aceturate and imidocarb dipropionate are still readily available for use against piroplasmidosis, it has been reported that they have toxic side effects and leave residue in animal products and that parasites are developing resistance (Belloli et al., 2007; Tuntasuvan et al., 2003). Thus, there is a need to find new drugs with potent anti-*piroplasm* activity and low toxic side effects to the host.

In the present study, 400 compounds from the MMV Pathogen Box were screened against *Babesia* and *Theileria* parasites. Nine hit compounds were identified as effective against *Babesia* and *Theileria* parasites with  $\text{IC}_{50}$ s of less than  $1 \mu\text{M}$ . The most interesting hit compounds were MMV021057 and MMV675968, with  $\text{IC}_{50}$ s of less than  $0.3 \mu\text{M}$  and an SI of more than 100. The two hit compounds identified from the Pathogen Box were fewer than the four hits identified from the Malaria Box in a previous study under the same conditions (Van Voorhis et al., 2016). Interestingly, one of the reference compounds, known as buparvaquone, showed a lower  $\text{IC}_{50}$  on *T. equi* than on *Babesia* parasites. This finding was comparable to that of a previous report showing that buparvaquone could be useful for the treatment of equine theileriosis in the field. However, this drug was unable to clear carrier infections alone (Zaug and Lane, 1992).

MMV675968 was the most effective anti-*piroplasm* compound, with  $\text{IC}_{50}$ s of less than  $10 \text{ nM}$  in *B. bovis*, *B. bigemina*, and *B. caballi*, but not in *T. equi*. This result revealed that MMV675968 is more effective against *Babesia* than *Theileria*. Moreover, the  $\text{IC}_{50}$  of this compound was 5.6 times lower than that of DA. MMV675968 is known as an inhibitor of the dihydrofolate reductase (DHFR) enzyme in *Cryptosporidium*. The mechanism of action (MOA) of MMV675968 has been reported the

**Table 1**The nine lead compounds in all tested parasites after primary screening at 1  $\mu\text{M}$ .

Compound ID	Disease set within the Pathogen Box	Compound class	Mechanism of action in other organisms
MMV021057	Malaria	$\beta$ -Methoxyacrylate analogue (azoxystrobin)	Mitochondrial cytochrome <i>bc</i> <sub>1</sub> complex
MMV689480	Reference compound (buparvaquone)	Hydroxynaphthoquinone	Q <sub>o</sub> quinone-binding site of mitochondrial cytochrome <i>b</i>
MMV676602	Kinetoplastids	Milciclib	Cyclin-dependent kinase 2 inhibitor
MMV688547	Kinetoplastids	Bisarylamidine	DNA minor groove binding at AT-rich DNA sequences
MMV688703	Toxoplasmosis	Trisubstituted pyrrole	cGMP-dependent protein kinase
MMV010576	Malaria	2-Amino-3,5-diaryl pyridine	Kinase
MMV688362	Kinetoplastids	Bisarylamidine	DNA minor groove binding at AT-rich DNA sequences
MMV688407	Kinetoplastids	Triazole	Not available
MMV675968	Cryptosporidiosis	Quinazoline-2,4-diamine	Dihydrofolate reductase

**Fig. 1.** Structures of the nine hit compounds identified by the initial *in vitro* screening using 1  $\mu\text{M}$  as the highest concentration. The most effective compounds are indicated by underlined letters. These compound structures were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>).**Table 2**Half maximum inhibition concentrations (IC<sub>50</sub>s).

Compound ID	IC <sub>50</sub> (nM)				CC <sub>50</sub> ( $\mu\text{M}$ )	SI <sup>f</sup>
	<i>B. bovis</i>	<i>B. bigemina</i>	<i>T. equi</i>	<i>B. caballi</i>		
MMV021057 <sup>h</sup>	23 $\pm$ 8	39 $\pm$ 11	229 $\pm$ 62	146 $\pm$ 36	> 28 <sup>a</sup>	> 1217; > 717; > 122; > 192
MMV689480	135 $\pm$ 41	488 $\pm$ 30	96 $\pm$ 19	237 $\pm$ 93	8.66 <sup>d</sup>	64; 18; 90; 37
MMV676602	243 $\pm$ 45	327 $\pm$ 31	468 $\pm$ 122	510 $\pm$ 145	1.1 <sup>b</sup>	5; 3; 2; 2
MMV688547	143 $\pm$ 16	694 $\pm$ 173	219 $\pm$ 97	562 $\pm$ 33	> 32 <sup>b</sup>	> 224; > 46; > 146; > 57
MMV688703	796 $\pm$ 57	804 $\pm$ 27	751 $\pm$ 70	583 $\pm$ 199	> 50 <sup>c</sup>	> 63; > 62; > 67; > 86
MMV010576	65 $\pm$ 10	30 $\pm$ 8	480 $\pm$ 140	610 $\pm$ 173	> 10 <sup>a</sup>	> 153; > 333; > 21; > 16
MMV688362	74 $\pm$ 62	566 $\pm$ 118	338 $\pm$ 99	262 $\pm$ 96	> 32 <sup>b</sup>	> 432; > 57; > 95; > 122
MMV688407	92 $\pm$ 33	188 $\pm$ 20	263 $\pm$ 106	204 $\pm$ 113	> 32 <sup>b</sup>	> 348; > 170; > 122; > 157
MMV675968 <sup>h</sup>	2.9 $\pm$ 0.3	3.0 $\pm$ 0.8	25.7 $\pm$ 6.4	2.9 $\pm$ 0.1	5.5 <sup>d</sup>	1896; 1833; 214; 1896
DA <sup>g</sup>	215 $\pm$ 3.6	1050 $\pm$ 61	140 $\pm$ 30	23 $\pm$ 6	> 40 <sup>c</sup>	> 186; > 38; > 286; > 1739

The cytotoxicity values on HepG2, MRC5, and HL60 were obtained from (<http://www.pathogenbox.org/about-pathogen-box/supporting-information>).<sup>a</sup> The CC<sub>50</sub> values on HepG2 cells.<sup>b</sup> The CC<sub>50</sub> values on MRC5 cells.<sup>c</sup> The CC<sub>50</sub> values on HL60 cells.<sup>d</sup> The CC<sub>50</sub> values on Vero cells (Spalenka et al., 2018).<sup>e</sup> The CC<sub>50</sub> values on MDBK cells (Guswanto et al., 2018).<sup>f</sup> Selectivity index (SI) = CC<sub>50</sub>/IC<sub>50</sub> on *B. bovis*, *B. bigemina*, *B. caballi* and *T. equi* ratio.<sup>g</sup> Diminazene aceturate (DA) as a control drug.<sup>h</sup> Two selected compounds which showed IC<sub>50</sub> < 0.3  $\mu\text{M}$  and SI > 100 in four tested parasites.

substituted two-atom linker interacts with Cys 113 of *C. hominis*-DHFR enzyme and extends the phenyl group into two different hydrophobic pockets, therefore, inhibited an enzyme activity (Popov et al., 2006). DHFR is an essential enzyme in nucleic acid and amino acid and also is highly conserved in all protozoan parasites, including *Babesia* and *Theileria* (Anderson, 2005; Begley et al., 2011). MMV675968 was also

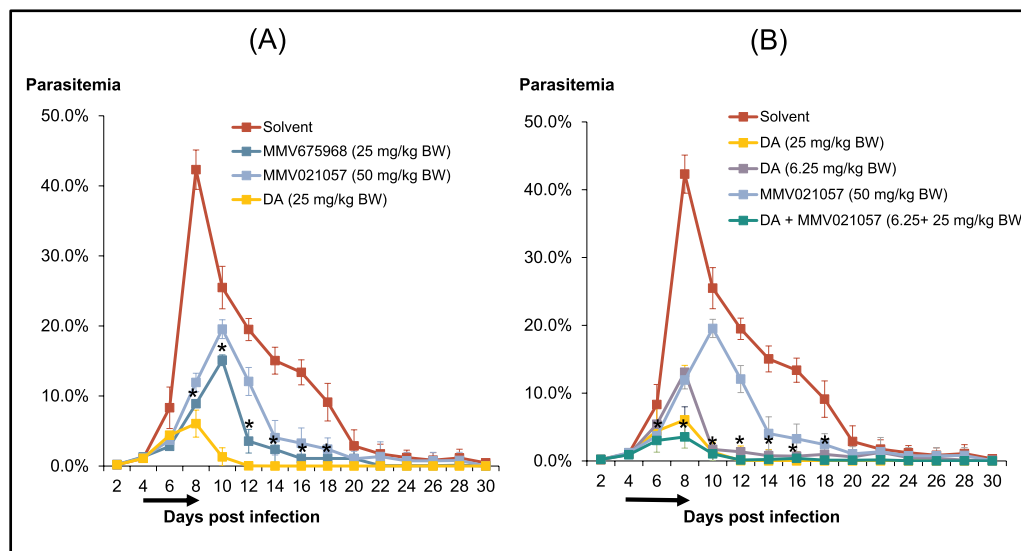
identified as a potent inhibitor against *Plasmodium falciparum* and *Toxoplasma gondii*, with IC<sub>50</sub>s of 0.03  $\mu\text{M}$  and 0.02  $\mu\text{M}$ , respectively (Duffy et al., 2017; Spalenka et al., 2018). In concordance with previous studies, we assumed that the findings in the current study indicate that MMV675968 also inhibited the DHFR enzyme in *Babesia* and *Theileria* parasites.

**Table 3**  
Combination indexes among MMV675968, MMV021057, and DA.

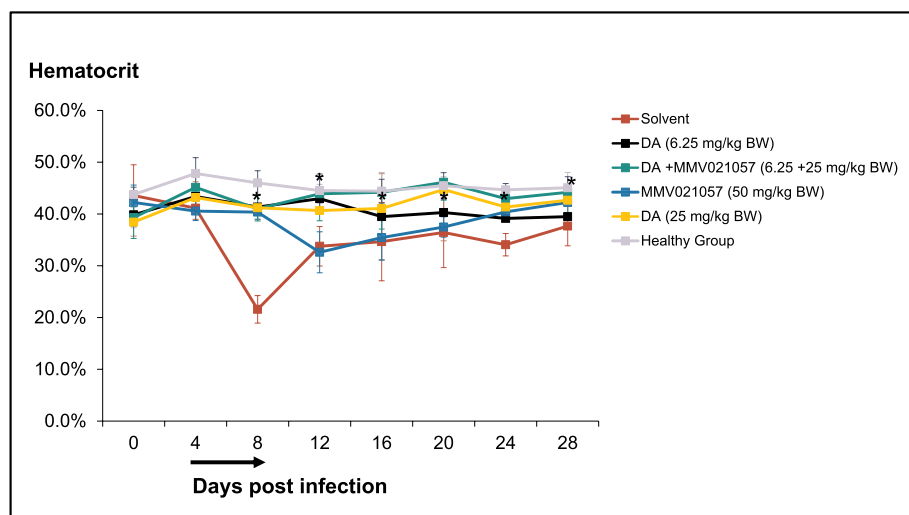
Parasites	Drug combinations	CI value at				WI <sup>a</sup>	Degree of synergism <sup>b</sup>
		IC <sub>50</sub>	IC <sub>75</sub>	IC <sub>90</sub>	IC <sub>95</sub>		
<i>B. bovis</i>	MMV675968 + MMV021057	12.872	7.214	4.293	3.135	5.272	Antagonistic
	MMV675968 + DA	5.795	3.255	2.009	1.568	2.460	Antagonistic
	MMV021057 + DA	0.691	0.646	0.615	0.602	0.624	Synergistic
<i>B. bigemina</i>	MMV675968 + MMV021057	20.143	7.681	3.102	1.756	5.184	Antagonistic
	MMV675968 + DA	15.61	6.727	2.908	1.648	4.438	Antagonistic
	MMV021057 + DA	2.05	1.303	0.872	0.68	0.999	Additive
<i>B. caballi</i>	MMV675968 + MMV021057	1.66	1.652	1.674	1.697	1.677	Antagonistic
	MMV675968 + DA	0.493	1.109	2.649	4.88	3.018	Antagonistic
	MMV021057 + DA	1.162	0.989	0.842	0.755	0.869	Synergistic
<i>T. equi</i>	MMV675968 + MMV021057	27.663	21.66	16.996	14.433	17.970	Antagonistic
	MMV675968 + DA	6.877	9.54	13.357	16.878	13.354	Antagonistic
	MMV021057 + DA	0.913	0.953	0.994	1.025	0.990	Additive

<sup>a</sup> Weight average (WI) of combination index (CI) values.

<sup>b</sup> WI values: synergistic (< 0.90), additive (0.90–1.10), antagonistic (> 1.10).



**Fig. 2.** The growth-inhibitory effects of *B. microti* in mice treated with the test compounds MMV675968, MMV021057, and DA and monitored for 30 days. (A) The parasitemia in mice administered with single treatments of MMV675968, MMV021057, and diminazene aceturate (DA) at doses of 25, 50, and 25 mg/kg BW, respectively. (B) The parasitemia in mice administered with combination treatments of 25 mg/kg BW MMV021057 and 6.25 mg/kg BW DA via subcutaneous and intraperitoneal injections, respectively. The arrow indicates the 5 consecutive days of treatment beginning on day 4 and continuing through day 8 p.i. Each value represents the mean and standard deviation (S.D.) from two separate experiments of five mice per experimental group. The significant differences ( $P < 0.05$ ) between untreated and MMV021057 or combination-treated mice are indicated with asterisks.



**Fig. 3.** The hematocrit levels in uninfected and untreated mice and in *B. microti*-infected and treated mice administered with 6.25 mg/kg BW DA, 25 mg/kg BW DA, 50 mg/kg BW MMV021057, and the combination of DA and MMV021057 (6.25 + 25 mg/kg BW). Drug treatment was performed on days 4–8 p.i. (arrow). The hematocrit levels were the mean and S.D. from two separate experiments of five mice in each experimental group. Asterisks indicate statistically significant differences ( $P < 0.05$ ) between infected and treated mice and uninfected and untreated mice.



The compound MMV021057, also known as azoxystrobin, was the second best after MMV675968. MMV021057, also known as a broad-spectrum fungicide, has been reported to function as an inhibitor of the mitochondrial respiration process by binding at the Q<sub>o</sub> site of cytochrome *b*. Eventually, there is blockage of the electron transfer between cytochrome *b* and *c*, which blocks the production of ATP (Bartlett et al., 2004). In addition, the MOA of MMV021057 (azoxystrobin) has been reported binding at the Q<sub>o</sub> site in the haem *b*<sub>1</sub>-proximal region or in the distal site of cytochrome *b* in *Plasmodium falciparum* (Vallieres et al., 2013). Furthermore, MMV021057 that targeted *bc 1* complex was a potent inhibitor of *P. falciparum*, with ranged IC<sub>50</sub> 15–30 nM (Duffy et al., 2017; Witschel et al., 2012). Although further studies are needed to elucidate the exact mode of action, it is possible that MMV021057 exerted this effect against the growth of *Babesia* and *Theileria*.

The present study investigated the growth-inhibitory effects of MMV675968 and MMV021057 against *B. microti* in mice. The findings showed that MMV675968 and MMV021057 inhibited the growth of *B. microti* by 64% and 54%, respectively, compared to the untreated group. Furthermore, the mice treated with MMV675968 and MMV021057 showed peak parasitemia on day 10, whereas the untreated mice and DA-treated group had peak parasitemia on day 8. Even though the MMV675968 and MMV021057 single treatments were less effective than DA against *B. microti* in mice, they delayed the peak of parasitemia by 2 days (Fig. 2A). This implied that MMV675968 and MMV021057 slowed the growth of *B. microti* and could be potentiated to increase their efficacy.

To test the effect of potentiation with an effective drug (DA) and the possibility of reducing the therapeutic dose of DA in the field, we conducted a combination treatment of 25 mg/kg BW MMV021057 with a lowered dose of DA 6.25 mg/kg BW. Importantly, the combination treatment showed a stronger inhibitory effect than single treatments with 50 mg/kg BW MMV021057 and 25 mg/kg BW DA. Comparatively, the MMV021057 and DA combination was more effective than combination treatments of DA with clofazimine, 17-DMAG, allicin, and thymoquinone (El-Sayed et al., 2019; Guswanto et al., 2018; Salama et al., 2014; Tuvshintulga et al., 2017). This emphasized that MMV021057 in combination with DA could reduce the toxic effect of DA, since only 6.25 mg/kg BW was used in the combination therapy. The synergistic and additive effect of other known cytochrome *b* inhibitor (atovaquone) also observed when combined with DA against piroplasm parasites (Guswanto et al., 2018). This finding strongly suggest that drug targeted to cytochrome *b* could be develop for combination treatment with DA as approved drug against piroplasm parasites. Moreover, MMV021057 was reported to be nongenotoxic, nonteratogenic, and nonneurotoxic by the Codex Committee on Pesticide Residues (CCPR). In addition, MMV021057 is metabolized quickly and excreted in both feces and urine (FAO, 2005).

The current study identified two hit compounds from the MMV Pathogen Box that possess potent growth-inhibitory effects against *Babesia* and *Theileria* parasites *in vitro*. Further analysis *in vivo* confirmed the effectiveness observed *in vitro*. Furthermore, one of the compounds, MMV021057, produced outstanding growth-inhibitory effects when combined with DA, which was superior to the previously reported combination of DA and other test compounds, namely, clofazimine, 17-DMAG, allicin, and thymoquinone. Taken together, this study has identified compounds that could be repurposed for treatment against piroplasmosis. Most importantly, the combination of MMV021057 and DA could be an alternative treatment with higher chemotherapeutic efficacy and could reduce the side effects of single treatment with DA. Future studies are needed to evaluate the mode of action of MMV675968 and MMV021057 and to evaluate the efficacy of derivatives related to these compounds against *Babesia* and *Theileria* parasites.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpddr.2019.06.004>.

## Conflicts of interest

The authors declare that they have no competing interests.

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