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Review article

Discovering the *in vitro* potent inhibitors against *Babesia* and *Theileria* parasites by repurposing the Malaria Box: A review

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ABSTRACT

There is an innovative approach to discovering and developing novel potent and safe anti-*Babesia* and anti-*Theileria* agents for the control of animal piroplasmosis. Large-scale screening of 400 compounds from a Malaria Box (a treasure trove of 400 diverse compounds with antimalarial activity has been established by Medicines for Malaria Venture) against the *in vitro* growth of bovine *Babesia* and equine *Babesia* and *Theileria* parasites was performed, and the data were published in a brief with complete dataset from 236 screens of the Malaria Box compounds. Therefore, in this review, we explored and discussed in detail the *in vitro* inhibitory effects of 400 antimalarial compounds (200 drug-like and 200 probe-like) from the Malaria Box against *Babesia* (*B.*) *bovis*, *B. bigemina*, *B. caballi*, and *Theileria* (*T.*) *equi*. Seventeen hits were the most interesting with regard to bovine *Babesia* parasites, with mean selectivity indices (SIs) greater than 300 and half maximal inhibitory concentration (IC_{50}s) ranging from 50 to 410 nM. The most interesting compounds with regard to equine *Babesia* and *Theileria* parasites were MMV020490 and MMV020275, with mean SIs > 258.68 and > 251.55, respectively, and IC_{50}s ranging from 76 to 480 nM. Ten novel anti–*B. bovis*, anti–*B. bigemina*, anti-*T. equi*, and anti–*B. caballi* hits, MMV666093, MMV396794, MMV006706, MMV665941, MMV085203, MMV396693, MMV006787, MMV073843, MMV007092, and MMV665875, with nanomole levels of IC_{50} were identified. The most interesting hits were MMV396693, MMV073843, MMV666093, and MMV665875, with mean SIs greater than 307.8 and IC_{50}s ranging from 43 to 630 nM for both bovine *Babesia* and equine *Babesia* and *Theileria* parasites. Screening the Malaria Box against the *in vitro* growth of *Babesia* and *Theileria* parasites helped with the discovery of new drugs than those traditionally used, diminazene aceturate and imidocarb dipropionate, and indicated the potential of the Malaria Box in finding new, potent antibabesial drugs.

1. Introduction

Animal piroplasmosis is a tick-born parasitic infection caused by hemoprotozoan parasites belonging to phylum Apicomplexa that infect the erythrocytes of a wide range of economically valuable animals, such as cattle and horses (Uilenberg, 2006; Rizk et al., 2017a, 2017b). *Babesia* (*B.*) *bovis* and *B. bigemina* have considerable effects on cattle health and productivity (Uilenberg, 2006; El-Sayed et al., 2017). *Babesia bovis* is more pathogenic than other bovine *Babesia* parasites (Criado et al., 2006). Although the virulence of *B. bovis* is high, the maximum parasitemia in infected blood is less than 1% in acute disease, while in *B. bigemina* infections, the parasitemia often exceeds 10% and may be as high as 30% (Uilenberg, 2006; Dalgliesh, 1993). The pathogenic effects of *B. bigemina* infection are directly associated with erythrocyte destruction; therefore, hemoglobinuria is observed earlier than in *B. bovis* infections (Uilenberg, 2006).

Theileria (*T.*) *equi* and *B. caballi* are considered the most important hemoparasites affecting horses, donkeys, and mules, causing equine piroplasmosis (EP) (Uilenberg, 2006; El-Sayed et al., 2015). The infection is transmitted *via* tick vectors (Scoles and Ueti, 2015), transplacentally, or through iatrogenic blood transfer (Allsopp et al., 2007; Wise et al., 2013). Of note, *Babesia* and *Theileria* spp. other than *T. equi* and *B. caballi*, including *B. bovis*(Criado et al., 2006), B. *capreoli*(Zanet et al., 2017), *B. canis*(Criado-Fornelio et al., 2003; Zanet et al., 2017), *T.*

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annae, *T. sergenti*, and *T. buffeli* (Moretti et al., 2010), were detected molecularly in horses. EP is of international concern, as the infection has been reported in many parts of the world, including Europe, Asia, Russia, Africa, and the United States of America (Kouam et al., 2010; El-Sayed et al., 2017). Consequently, the infection is considered one of the biggest problems in equid trade worldwide. *T. equi* causes greater mortality (50%) than *B. caballi* (10%), whereas locomotor-system disorders and posterior paralysis are observed in equines infected with *B. caballi* (Wise et al., 2013; Scoles and Ueti, 2015).

Generally speaking, animal piroplasmosis is manifested in fever, hemoglobinuria, hemolytic anemia, jaundice, and death, causing serious economic losses for the livestock industry worldwide (Rizk et al., 2016, 2018). In few cases, nervous manifestations, such as the paddling of limbs, ataxia, convulsion, and posterior paralysis, might occur, which can be attributed to the sequestration of infected erythrocytes in the cerebral capillaries (Dalgliesh, 1993). The severe clinical signs of the disease in the absence of an effective and safe vaccine make this endemic disease very important.

2. Treatment of animal piroplasmosis

In the past, tick vector eradication was the main strategy in piroplasmosis control (Kuttler, 1981). More recently, however, chemotherapy for piroplasmosis has become important in controlling the disease, whether treating field cases or controlling artificially induced disease (Rodriguez and Trees, 1996). A combination of both approaches is recommended (Suarez and Noh, 2011; Rizk et al., 2018). Moreover, blood transfusions, iron preparations, fluid replacement, and anti-inflammatory drugs are necessary supportive therapy in severe piroplasmosis (Zintl et al., 2003). In the last few years, large numbers of antibabesial drugs have proven their efficacy in the field (Vial and Gorenflot, 2006; Rizk et al., 2016, 2017a, 2017b), although many have been withdrawn from the veterinary market for various reasons (Food Security and Public Health, 2011).

Trypan blue was the first specific drug used against bovine babesiosis; however, it was specific and effective only against *B. bigemina* infections and caused discoloration of the animal's flesh. Therefore, it is rarely used (Kuttler, 1981). Next, quinuronium sulfate, amicarbalide, diminazene aceturate, and imidocarb dipropionate were used to treat babesiosis (Vial and Gorenflot, 2006). Quinuronium sulfate and amicarbalide were withdrawn because of manufacturing safety issues, while diminazene was withdrawn from the market in Europe and Japan for marketing reasons (Zintl et al., 2003; Vial and Gorenflot, 2006). Additionally, diminazene has not been approved by the Food and Drug Administration in the USA (Food Security and Public Health, 2011).

Imidocarb is the only antibabesial drug on the market that provides protection from clinical diseases for 3–6 weeks and builds solid, sterile immunity (Zintl et al., 2003). Unfortunately, a sufficient level of parasitemia is developed in animals treated by imidocarb to allow the development of subclinical disease. In addition, some animals with acute babesiosis were not responsive to treatment with imidocarb, and resistance can be developed (Mosqueda et al., 2012). Consequently, an innovative approach should be adopted to discover and develop new potent and safer babesicidal agents. In this review, we investigated and discussed in detail the anti-*Babesia* and anti-*Theileria* inhibitory effects of 400 blood-stage active anti-*Plasmodia* hits, either drug-like or probe-like compounds, from the Malaria Box (Spangenberg et al., 2013) to explore the most potent Medicines for Malaria Venture (MMV) chemicals against *Babesia* and *Theileria* parasites to use for future anti-*Babesia* and anti-*Theileria* drug discovery.

3. Malaria Box as a valuable source for anti-*Babesia* and anti-*Theileria* therapies

Medicines for Malaria Venture, a public–private partnership, has been established to overcome the high costs of drug-discovery research. One advantage of such partnerships is the development of novel antimalarial agents, such as the commonly used pediatric formulation of Coartem Dispersible (artemether-lumefantrine) (Abdulla et al., 2008). The MMV recently established the Malaria Box, a collection of 400 compounds, divided into 200 diverse drug-like compounds and 200 diverse probe-like ones. The drug-like compounds are the compounds that have physicochemical properties, are acceptable for oral absorption and were subjected to substructure filters (Rishton, 1997; Lipinski et al., 2001), REOS (Rapid Elimination Of Swill) and the PAINS (Pan Assay Interference Compounds) filters (Flower, 1999; Baell and Holloway, 2010) to remove known toxicophores. While the hits that failed one or more of these filters were assigned to the probe-like category (Spangenberg et al., 2013). This collection was selected from 19,000 structurally unique molecules that had previously shown activity against the erythrocytic stage of *Plasmodium* (*P.) falciparum* in three large phenotypic high-throughput screening (HTS) campaigns reported by GlaxoSmithKline (GSK) (Gamo et al., 2010), St. Jude Children's Research Hospital (Guiguemde et al., 2010), and the Genomics Institute of the Novartis Research Foundation (GNF) (Meister et al., 2011). The Malaria Box is designed to be a starting point for drug discovery and identifying drug targets and pathways for *P. falciparum* and other medically important pathogens (Spangenberg et al., 2013). All of these compounds are commercially available, which adds further merit to this huge library (Spangenberg et al., 2013).

The MMV Malaria Box is a valuable source for previously examined compounds against the *in vitro* growth of *P. falciparum* (Spangenberg et al., 2013). The Malaria Box was established to open a new door for apicomplexan parasite drug discovery. The successful application of antimalarial agents in *Babesia* and *Theileria* parasite treatments (Guswanto et al., 2014; Rizk et al., 2015, 2017a, 2017b, 2018) and the promising candidates identified from the screening of Malaria Box compounds against the *in vitro* growth of other parasites, including *Toxoplasma gondii* (*T. gondii*) and *Entamoeba histolytica* (*E. histolytica*) (Hotzel et al., 1997), *Schistosoma mansoni* (*S. mansoni)* (Avarzed et al., 1997), and *Cryptosporidium parvum* (*C. parvum*) (Bork et al., 2004), encourage us to discuss in detail the results of *in vitro* screening of Malaria Box compounds against *Babesia* and *Theileria*.

In vitro drug screening assays are used to monitor the susceptibility of the parasite to the inhibitory effect of different antiparasitic drugs through direct exposed the parasite to known concentrations of drugs. From these methods, there are assays based on measurement of parasitic nucleic acid as an indicator to the drug inhibitory effect as real-time quantitative PCR (qPCR) (Gomes et al., 2012), and assays based on fluorescent nucleic acid intercalating dyes as a laser-based fluorescently activated cell sorters (FACS) (van Vianen et al., 1990; Bennett et al., 2004), or SYBR Green I (SG I)- based (Johnson et al., 2007; Rizk et al., 2015) assays. FACS, or SG I assays are used to detect and measure the DNA content of parasite-infected erythrocytes and the intensity of the emitted fluorescence is directly proportionate with the parasite development (Bennett et al., 2004; Rizk et al., 2015). Though these assays are rapid, accurate, highly sensitive, automated, and nonradioactive, there are challenges faced these methods such as using flow cytometry (FACS) or qPCR assays are costly, requires a highly skilled personnel, too sophisticated instruments and well-equipped laboratory (van Vianen et al., 1990; Gomes et al., 2012; Bennett et al., 2004). Beside, qPCR is a quite complicated technique since it requires specific primers, nucleic acid extraction step and other steps to complete the procedure (Gomes et al., 2012). Additionally, the use of SG I to stain the DNA either by flow cytometry or fluorescence spectrophotometer didn't allow the differentiation between living and dead parasites (Jang et al., 2014). However, when measurement conditions are optimized, SG I - based assays should be very useful in areas related to the *in vitro* large scale drug screening.

Indeed, a fluorescence-based assay using SG I is a one-plate/one-step method that makes the assay robust and amenable to high-throughput screening (HTS). Taken together the assay requires neither

highly expensive, specialized equipment nor highly trained researcher (Bennett et al., 2004; Johnson et al., 2007). Therefore, recently, we developed a fluorescence-based HTS assay for large-scale drug screening against *Babesia* and *Theileria* parasites, using SYBR Green I stain (Guswanto et al., 2014; Rizk et al., 2015) and used this assay to screen a library of 400 compounds (200 drug-like and 200 probe-like) from the MMV Malaria Box (Spangenberg et al., 2013) against the *in vitro* growth of *B. bovis* (Texas strain) (Hines et al., 1992), *B. bigemina* (Argentine strain) (Hines et al., 1992), *T. equi* (U.S. Department of Agriculture) (Avarzed et al., 1997; El-Sayed et al., 2015), and *B. caballi* (U.S. Department of Agriculture) (Bork et al., 2004). The obtained data were published in a brief with a complete dataset on 236 screens of the Malaria Box compounds (Van Voorhis et al., 2016). Promising hits for further development as novel therapies for bovine and equine piroplasmosis were identified through this study. This work constitutes the first detailed exploration of the Malaria Box's importance as a rich source of potential screening hits and biological probes for the *in vitro* growth of *Babesia* and *Theileria* parasites. The data obtained from this *in vitro* mass screening against *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi* parasites might be helpful in filling up the empty anti-*Babesia/Theileria* drug pipeline and could be used as a guide for *Babesia* and *Theileria* researchers to develop novel drug candidates.

Generally speaking, drug potency is an expression to measure the drug activity in terms of the concentration or amount required to produce a defined effect. The drug to be identified as a highly potent should evoke a given response at low concentrations (Waldman, 2002). The drug potency is usually measured *in vitro* using the half maximal inhibitory concentration (IC_{50}), which is generally defined as the quantitative measure of a particular substance potency in inhibiting 50% of a specific biological, biochemical functions, or growth of a certain microorganism *in vitro* (Aykul and Martinez-Hackert, 2016). The IC_{50} of specific drugs when divided on its cytotoxicity value (TOX) provides an indication about the selectivity index (SI) (Pritchett et al., 2014). The higher the SI ratio, the theoretically more effective and safe a drug would be during *in vivo* treatment for a given parasitic infection (Pritchett et al., 2014). The ideal drug would be cytotoxic only at very high concentrations and have antiparasitic activity (APA) at very low concentrations, thus yielding a high SI value (high APA/low TOX) and thereby able to eliminate the target parasite at concentrations well below its cytotoxic concentration (Pritchett et al., 2014; Aykul and Martinez-Hackert, 2016).

In fact, the *in vitro* potency of a given chemotherapeutic can be a good preclinical marker of the therapeutic potential *in vivo*, while the *in vitro* drug screening assays are completely free from host-related factors such as host immunity, poor absorption, biotransformation, concentration in certain tissues, and rapid clearance (Bennett et al., 2004). Subsequently, the therapeutic behavior of the *in vitro* potent candidates may be modulated into the animal body by pharmacokinetic and pharmacodynamic parameters and by further interactions at sites other than the target site (Waldman, 2002). Therefore, it does not necessarily follow that more potent drugs *in vitro* have greater clinical efficacy *in vivo*.

The screening of Malaria Box compounds against the *in vitro* growth of *Babesia* and *Theileria* parasites revealed variations in the *in vitro* inhibitory effects of MMV compounds on *Babesia* and *Theileria* parasites. Some hits exhibited the highest anti-*Babesia/Theileria* activities with nanomole levels of half maximal inhibitory concentration (IC_{50}), while other compounds showed intermediate activities with micromole levels of IC_{50}. On the other hand, some MMV compounds showed poor activity or were inactive against the *in vitro* growth of *Babesia* and *Theileria* parasites with IC_{50}s higher than 25 μM.

All experimental protocols in this study were approved by the Animal Care and Use Committee, Obihiro University of Agriculture and Veterinary Medicine (Approval No. 27-65). All experiments were conducted in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

3.1. Inhibitory effects of MMV Malaria Box compounds against bovine Babesia parasites

The *in vitro* inhibitory effects of MMV Malaria Box compounds against *B. bovis* parasite growth were varied, having high, moderate, poor, or no activity. Ninety-two MMV compounds (24 drug-like and 68 probe-like) showed the highest activity with nanomole levels of IC_{50} (Tables S4–S6 and S8–S21). From these compounds, 64 hits (16 drug-like and 48 probe-like) were more effective than diminazene aceturate (positive control drug and commonly used antibabesial drug) (Tables S4–S6 and S9–S21). On the other hand, 26 compounds (16 drug-like and 10 probe-like) showed IC_{50}s higher than 25 μM (Tables S2–S7, S10, S11, and S13). Other MMV compounds (n = 282) exhibited intermediate *in vitro* activities with micromole levels of IC_{50} (Tables S2–S21).

Serially diluted MMV compounds were screened for *in vitro* activity against *B. bigemina*. Of 200 drug-like and 200 probe-like compounds tested, 82 compounds showed the highest activity against *in vitro* growth of the *B. bigemina* parasite: 32 drug-like compounds and 50 probe-like compounds (Tables S3–S9 and S11–S21). From the compounds that exhibited the highest anti–*B. bigemina* activity, 45 hits (9 drug-like and 36 probe-like) were more effective than diminazene aceturate (commonly used antibabesial drug) with IC_{50}s < 0.24 μM (Tables S4–S7, S9, S11, S13–S19, and S21). In contrast, 11 compounds (8 drug-like and 3 probe-like) showed IC_{50}s higher than 25 μM (Tables S12–S14 and S16). Other screened compounds (n = 307) exhibited intermediate *in vitro* activities against *in vitro* growth of the *B. bigemina* parasite (Tables S2–S21).

For the *in vitro* inhibitory effects of MMV Malaria Box compounds against both *B. bovis* and *B. bigemina*, 39 compounds showed the highest activity against the *in vitro* growth of both parasites, including 9 drug-like compounds and 29 probe-like compounds (Table 1). The *in vitro* growth of both *B. bovis* and *B. bigemina* was significantly inhibited ($p < 0.05$) by 100 nM of MMV000304, MMV019741, MMV000617, MMV000444, MMV006764, MMV000340, MMV396723, MMV007020, MMV019881, MMV086103, MMV396663, and MMV020243 compound treatments (data not shown). Additionally, the *in vitro* growth of both parasites was significantly inhibited ($p < 0.05$) by 1 μM of MMV000788, MMV396633, MMV007577, MMV019199, and MMV665934 compound treatments (data not shown). Treatment by MMV665939, MMV666067, MMV665890, MMV000653, MMV666072, MMV665879, MMV019266, MMV001241, MMV666125, MMV667491, MMV011438, MMV666689, MMV007764, MMV006656, MMV007285, and MMV006825 compounds significantly inhibited ($p < 0.05$) the *in vitro* growth of *B. bovis* and *B. bigemina* by 100 nM and 1μM, respectively (data not shown). In contrast, the *in vitro* growth of *B. bovis* and *B. bigemina* was significantly inhibited ($p < 0.05$) by 1 μM and 100 nM, respectively, of the MMV665908 compound treatment. *In vitro* treatment with MMV665886 and MMV667488 compounds significantly inhibited ($p < 0.05$) the growth of *B. bovis* and *B. bigemina* parasites by 10 μM and 1μM, respectively (data not shown). The *in vitro* growth of *B. bovis* and *B. bigemina parasites* was significantly inhibited ($p < 0.05$) by 100 nM and 10 μM, respectively, of the MMV665875 compound treatment (data not shown). Finally, the *in vitro* growth of bovine *Babesia* parasites was significantly inhibited ($p < 0.05$) by 10 μM of the MMV084434 compound treatment (data not shown).

Seventeen hits, MMV665939, MMV019266, MMV000340, MMV007020, MMV019881, MMV086103, MMV396663, MMV020243, MMV084434, MMV665886, MMV011438, MMV666689, MMV006656, MMV007285, MMV667488, MMV665875, and MMV019199, were the most interesting with regard to bovine *Babesia* parasites, with mean SIs greater than 300 and IC_{50}s ranging from 50 to 410 nM (Table 1).

Table 1
IC_{50}s, CC_{50} (MRC-5) values and selectivity indices of hits that showed the highest activities against the *in vitro* growth of bovine *Babesia* parasites.

Compound ID[a]	Set	IC_{50} (nM)[b]		CC_{50} (nM)[c] against MRC-5	Mean SI[d]
		Parasite			
		B. bovis	*B. bigemina*		
MMV665939	Drug-like	80	100	> 32,000	> 360
MMV666067	Drug-like	100	850	> 32,000	> 178.82
MMV665890	Drug-like	78	690	> 32,000	> 228.32
MMV000653	Drug-like	830	100	> 32,000	> 179.27
MMV000788	Drug-like	590	920	1169.43	1.62
MMV666072	Drug-like	270	980	> 32,000	> 75.58
MMV665879	Drug-like	320	80	17911.21	139.9
MMV396633	Drug-like	81	910	> 32,000	> 215.11
MMV019266	Drug-like	50	410	> 32,000	> 359.02
MMV000304	Probe-like	720	100	8438.13	48.05
MMV007577	Probe-like	900	260	> 32,000	> 79.32
MMV001241	Probe-like	240	550	> 32,000	> 95.75
MMV665908	Probe-like	790	100	> 32,000	> 180.25
MMV019741	Probe-like	130	100	> 32,000	> 283.07
MMV665934	Probe-like	860	770	> 32,000	> 39.38
MMV000617	Probe-like	91	68	10825.51	139.08
MMV666125	Probe-like	360	490	> 32,000	> 77.1
MMV000444	Probe-like	80	100	15544.51	174.88
MMV006764	Probe-like	72	250	> 32,000	> 286.22
MMV000340	Probe-like	52	82	32,000	502.81
MMV396723	Probe-like	77	66	10,649.39	149.82
MMV007020	Probe-like	56	65	18,629.44	309.64
MMV019881	Probe-like	60	77	> 32,000	> 474.45
MMV086103	Probe-like	70	65	21,220.8	314.81
MMV396663	Probe-like	86	73	> 32,000	> 405.22
MMV020243	Probe-like	78	60	23,775.91	350.54
MMV084434	Probe-like	100	82	> 32,000	> 355.12
MMV667491	Probe-like	52	33	3494	86.53
MMV665886	Probe-like	100	81	> 32,000	> 357.53
MMV011438	Probe-like	63	86	> 32,000	> 440.01
MMV666689	Probe-like	81	50	> 32,000	> 517.53
MMV007764	Probe-like	87	59	19,329.43	274.9
MMV006656	Probe-like	91	100	> 32,000	> 335.82
MMV007285	Probe-like	56	68	> 32,000	> 521.01
MMV667488	Probe-like	130	52	> 32,000	> 430.76
MMV019199	Probe-like	120	57	> 32,000	> 414.03
MMV006825	Probe-like	54	67	13,543.17	226.47
MMV665875	Probe-like	71	81	> 32,000	422.88

[a] Compounds are designated by their MMV identifier codes.
[b] IC_{50} values for each drug were calculated on the fourth day based on the growth inhibitions determined using the fluorescence-based method. Results are means from duplicate experiments.
[c] Cytotoxicity data was obtained from the ChEMBL database (https://www.ebi.ac.uk/chembl/) and evaluated in a culture against human fibroblast (MRC-5) cells.
[d] Selectivity indices (SIs) were calculated based on the ratio CC_{50} (MRC-5)/IC_{50} of the drug and the mean SI for *B. bovis* and *B. bigemina* parasites.

3.2. Inhibitory effects of MMV Malaria Box compounds against equine Babesia and theileria parasites

Fifty-three MMV compounds (22 drug-like and 31 probe-like) showed the highest activity against *in vitro* growth of the *T. equi* parasite with nanomole levels of IC_{50} (Tables S2, S4–S16, and S18–S21). From the highest activity compounds, 49 hits (22 drug-like and 27 probe-like) were more effective than diminazene aceturate, with IC_{50}s < 0.77 μM (Tables S2–S16 and S18–S21). On the other hand, 10 compounds (4 drug-like and 6 probe-like) showed IC_{50}s higher than 25 μM (Tables S3–S5, S10, S13, S14, and S17). Other screened compounds (n = 337) exhibited intermediate *in vitro* anti-*T. equi* activities (Tables S2–S21).

Screening of 400 MMV compounds against *in vitro* growth of the *B. caballi* parasite revealed that 66 compounds showed the highest activity, including 34 drug-like compounds and 32 probe-like compounds (Tables S3–S11, S13, S14, S16–S19, and S21). Interestingly, no hits showed an IC_{50} value lower than that obtained by diminazene aceturate. Nine compounds (8 drug-like and 1 probe-like) showed IC_{50}s higher than 25 μM (Tables S5, S6, S8, and S12). However, other compounds (n = 325) exhibited intermediate *in vitro* activities (Tables S2–S21).

Table 2
IC_{50}s, CC_{50} (MRC-5) values and selectivity indices of hits that showed the highest activities against the *in vitro* growth of equine *Babesia* and *Theileria* parasites.

Compound ID[a]	Set	IC_{50} (nM)[b]		CC_{50} (nM)[c] against MRC-5	Mean SI[d]
		Parasite			
		T. equi	*B. caballi*		
MMV001255	Drug-like	570	70	5606.81	44.96
MMV666080	Drug-like	470	550	11,843.62	23.36
MMV000972	Drug-like	650	800	18,081.85	25.210
MMV020490	Drug-like	480	71	> 32,000	> 258.68
MMV403679	Drug-like	470	73	652.76	5.16
MMV020275	Drug-like	390	76	> 32,000	> 251.55
MMV006704	Drug-like	590	100	25,398.42	148.51
MMV007654	Probe-like	140	710	> 32,000	> 136.82
MMV009085	Probe-like	670	530	13,791.56	23.30
MMV667492	Probe-like	760	410	> 32,000	> 60.07
MMV665864	Probe-like	250	672	> 32,000	> 87.81

[a] Compounds are designated by their MMV identifier codes.
[b] IC_{50} values for each drug were calculated on the fourth day based on the growth inhibitions determined using the fluorescence-based method. Results are means from duplicate experiments.
[c] Cytotoxicity data was obtained from the ChEMBL database (https://www.ebi.ac.uk/chembl/) and evaluated in a culture against human fibroblast (MRC-5) cells.
[d] Selectivity indices (SIs) were calculated based on the ratio CC_{50} (MRC-5)/IC_{50} of the drug and the mean SI for *T. equi* and *B. caballi* parasites.

Eleven compounds (7 drug-like and 4 probe-like) with potential against the *in vitro* growth of both *T. equi* and *B. caballi* (Table 3) were identified from Malaria Box screening. *In vitro* treatment with these compounds significantly inhibited ($p < 0.05$) the growth of *T. equi* and *B. caballi* parasites by 100 nM (data not shown). The most interesting compounds with regard to equine *Babesia* and *Theileria* parasites were MMV020490 and MMV020275, with a mean SI > 258.68 and > 251.55, respectively, and IC_{50}s ranging from 76 to 480 nM (Table 2).

3.3. Inhibitory effects of MMV Malaria Box compounds against both bovine Babesia and equine Babesia and Theileria

Interestingly, the screening of 400 MMV compounds from the Malaria Box against the *in vitro* growth of *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi* parasites revealed that 11 compounds showed the highest activity with nanomole levels of IC_{50} as follows: 3 drug-like compounds (MMV666093, MMV396794, and MMV006706) and 8 probe-like compounds (MMV665941, MMV085203, MMV396693, MMV006787, MMV073843, MMV007092, MMV665875, and MMV665810) (Tables 3 and S18). The MMV020885 and MMV006457 probe-like compounds and the MMV020654 drug-like compound exhibited IC_{50}s higher than 25 μM against the *in vitro* growth of *B. bovis* and *T. equi* parasites. Additionally, the MMV007430 drug-like compound showed an IC_{50} higher than 25 μM against the *in vitro* growth of *B. bigemina* and *B. caballi* parasites (Tables S3, S12, and S13).

The *in vitro* growth of *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi* was significantly inhibited ($p < 0.05$) by treatment with 100 nM of MMV665941, MMV085203, and MMV396693 compounds (Fig. 1). Additionally, the *in vitro* treatment with MMV006706, MMV006787, and MMV665810 compounds significantly inhibited ($p < 0.05$) the growth of *B. bovis, T. equi*, and *B. caballi* by 100 nM and significantly inhibited ($p < 0.05$) the growth of *B. bigemina* by 1 μM (Figs. 2C and 3

Table 3
IC_{50}s, CC_{50} (MRC-5) values and selectivity indices of hits with potential identified from *in vitro* screening of Malaria Box for further medicinal chemistry/biological screenings.

Compound ID[a]	Set	IC_{50} (nM)[b]				CC_{50} (nM)[c] against MRC-5	Mean SI[d]
		Parasite					
		B. bovis	*B. bigemina*	*T. equi*	*B. caballi*		
MMV665941	Probe-like	56	87	47	45	353.55	6.43
MMV085203	Probe-like	80	180	49	230	9878.88	105.73
MMV396693	Probe-like	403	59	57	48	> 32,000	> 462.46
MMV666093	Drug-like	59	83	219	130	> 32,000	> 330.04
MMV396794	Drug-like	83	320	530	71	16,112.42	125.455
MMV006706	Drug-like	750	100	65	380	> 32,000	> 234.79
MMV006787	Probe-like	81	760	840	380	16,227.94	70.93
MMV073843	Probe-like	68	68	630	43	> 32,000	> 434.04
MMV007092	Probe-like	50	130	450	370	10,657.47	86.90
MMV665875	Probe-like	71	81	100	489	> 32,000	> 307.8

[a] Compounds are designated by their MMV identifier codes.
[b] IC_{50} values for each drug were calculated on the fourth day based on the growth inhibitions determined using the fluorescence-based method. Results are means from duplicate experiments.
[c] Cytotoxicity data was obtained from the ChEMBL database (https://www.ebi.ac.uk/chembl/) and evaluated in a culture against human fibroblast (MRC-5) cells.
[d] Selectivity indices (SIs) were calculated based on the ratio CC_{50} (MRC-5)/IC_{50} of the drug and the mean SI for *B. bovis, B. bigemina, T. equi* and *B. caballi* parasites.

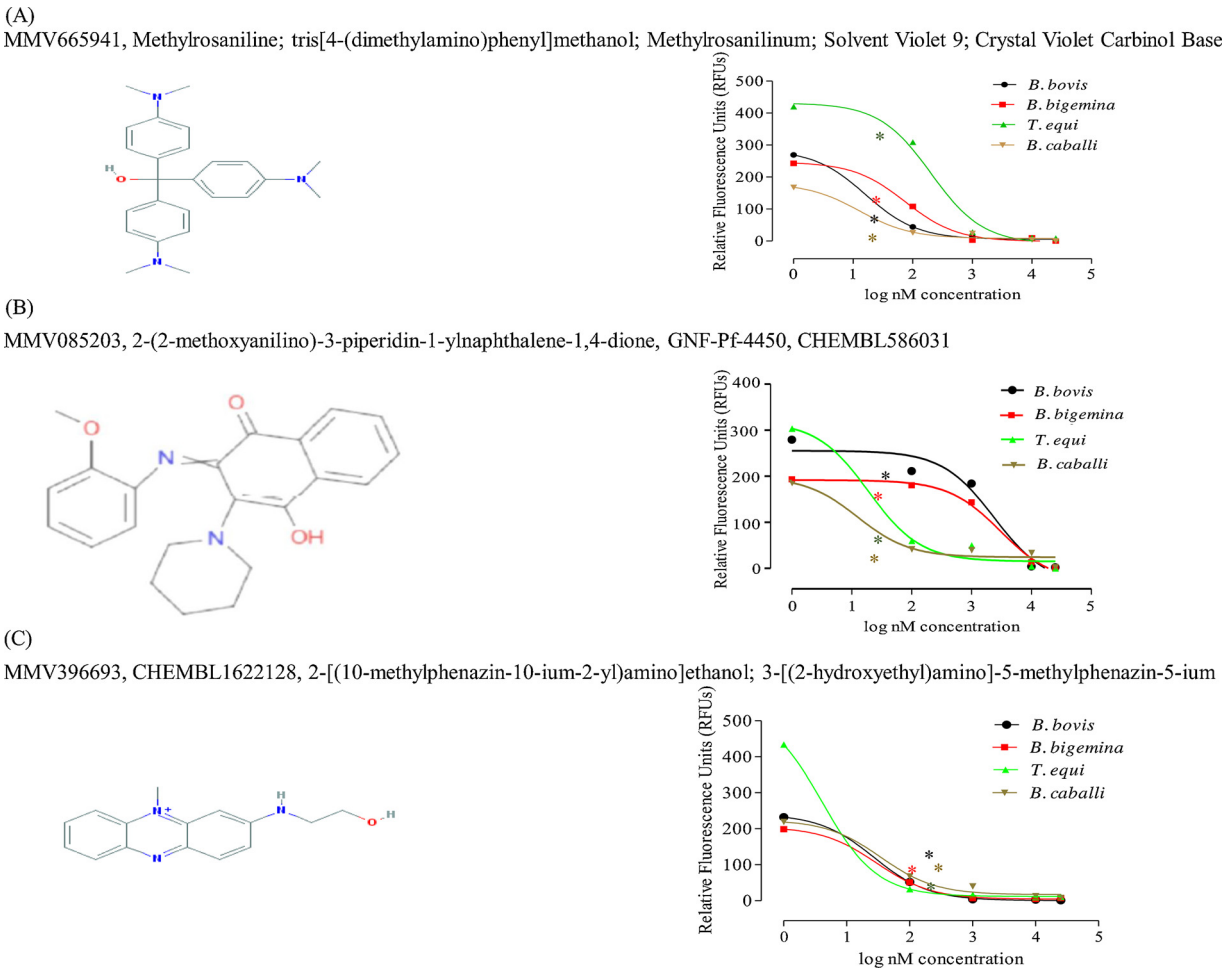
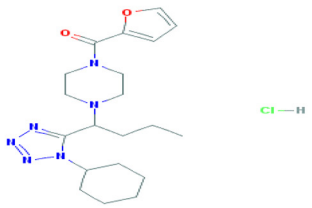
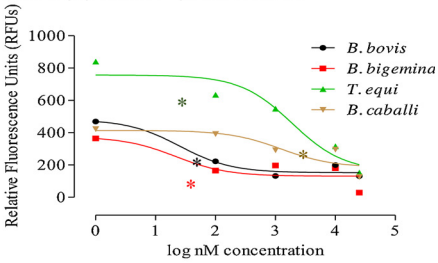


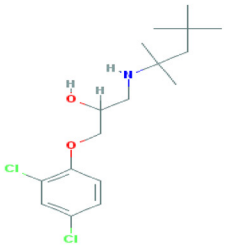
Fig. 1. Fluorescence-based monitoring of the hits with potential: MMV665941, MMV085203, and MMV396693 from the MMV Malaria Box induced growth inhibition of *Babesia* and *Theileria* parasites on the fourth day of treatment. (A) Correlation between MMV665941 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. (B) Correlation between MMV085203 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. (C) Correlation between MMV396693 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. Each value is presented as the mean of duplicates after subtraction of the background fluorescence for non-parasitized RBCs. MMV structures were provided by the MMV as part of the supporting information for the Malaria Box. Synonyms of MMV structures were obtained from the following website: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=2853195. Statistically significant differences between the drug-treated cultures and the control cultures are indicated by asterisks (*P < 0.05).

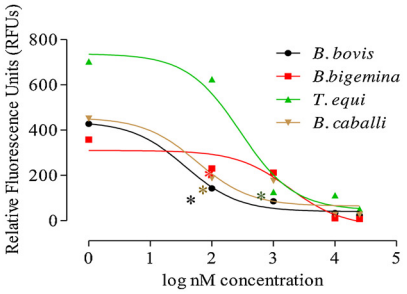
(A)
MMV666093, CHEMBL585686, {4-[1-(1-Cyclohexyl-1H-tetrazol-5-yl)-butyl]-piperazin-1-yl}-furan-2-yl-methanone



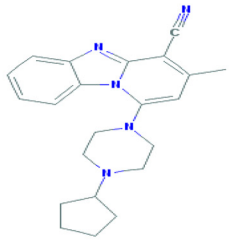


(B)
MMV396794, CHEMBL2028063; (2S)-1-(2,4-dichlorophenoxy)-3-(2,4,4-trimethylpentan-2-ylamino)propan-2-ol





(C)
MMV006706, GNF-Pf-3245; 1-(4-cyclopentylpiperazin-1-yl)-3-methylpyrido[1,2-a]benzimidazole-4-carbonitrile



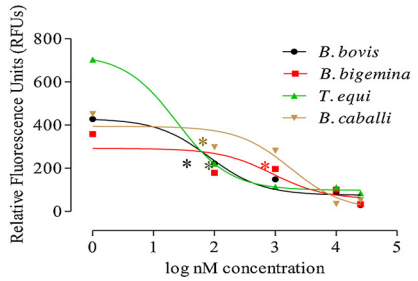


Fig. 2. Fluorescence-based monitoring of the hits with potential: MMV666093, MMV396794, and MMV006706 from the MMV Malaria Box induced growth inhibition of *Babesia* and *Theileria* parasites on the fourth day of treatment. (A) Correlation between MMV666093 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. (B) Correlation between MMV396794 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. (C) Correlation between MMV006706 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. Each value is presented as the mean of duplicates after subtraction of the background fluorescence for non-parasitized RBCs. MMV structures were provided by the MMV as part of the supporting information for the Malaria Box. Synonyms of MMV structures were obtained from the following website: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=2853195. Statistically significant differences between the drug-treated cultures and the control cultures are indicated by asterisks (*P < 0.05).

A, B).

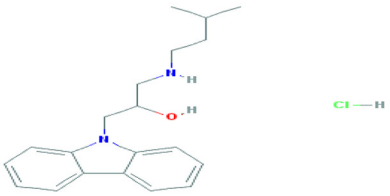
In vitro treatment with the MMV666093 compound significantly inhibited ($p < 0.05$) the growth of *B. bovis*, *B. bigemina*, and *T. equi* by 100 nM and significantly inhibited ($p < 0.05$) the growth of *B. caballi* by 10 μM (Fig. 2A). Moreover, *in vitro* treatment with the MMV396794 compound significantly inhibited ($p < 0.05$) the growth of *B. bovis*, *B. bigemina*, and *B. caballi* by 100 nM and significantly inhibited ($p < 0.05$) the growth of *T. equi* by 1 μM (Fig. 2B).

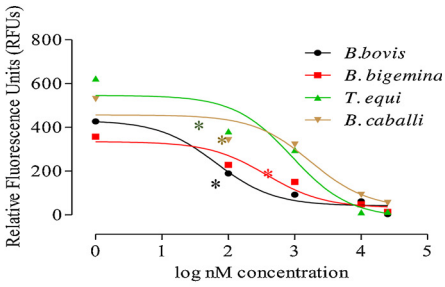
The *in vitro* growth of *B. bovis* and *B. bigemina* was significantly inhibited ($p < 0.05$) by treatment with 100 nM of MMV073843, whereas this compound significantly inhibited ($p < 0.05$) the *in vitro* growth of *T. equi* and *B. caballi* by 1 μM (Fig. 3C). Furthermore, *in vitro* treatment with the MMV007092 compound significantly inhibited ($p < 0.05$) the growth of *B. bovis* and *B. caballi* by 100 nM and significantly inhibited ($p < 0.05$) the growth of *T. equi* and *B. bigemina* by 1 μM (Fig. 4A). Moreover, the *in vitro* growth of bovine *Babesia* and equine *Babesia* and *Theileria* was significantly inhibited ($p < 0.05$) by 1 μM and 100 nM of the MMV665875 compound treatment, respectively (Fig. 4B). Collectively, the most interesting compounds were MMV396693, MMV073843, MMV666093, and MMV665875, which exhibited an excellent mean SI greater than 307.8 for both bovine *Babesia* and equine *Babesia* and *Theileria* (Table 3).

In vitro screening of Malaria Box compounds against *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi* aided in the discovery of 10 novel potent anti-*Babesia* and anti-*Theileria* hits, including MMV666093, MMV396794, MMV006706, MMV665941, MMV085203, MMV396693, MMV006787, MMV073843, MMV007092, and MMV665875. These 10 hits exhibited submicromolar potency against both bovine *Babesia* and equine *Babesia* and *Theileria* with IC_{50}s ranging from 40 to 840 nM. This represents a 2.5% hit rate, which is higher than those observed with the *in vitro* screening of Malaria Box compounds against either *T. gondii* and *E. histolytica* (1.75% hit rate) (Boyom et al., 2014) or *S. mansoni* (0.75% hit rate) (Ingram-Sieber et al., 2014). Our hit rate is similar to those obtained with the MMV compounds in *in vitro* screening against *Cryptosporidium parvum* (Bessoff et al., 2014). This may be attributed to the fact that these hits have properties that enable them to reach their biological target in a way similar to that against the *P. falciparum* blood-stage form *in vitro*. The high target similarity shared among these protozoan parasites might also be an explanation for the improved hit rate (Boyom et al., 2014).

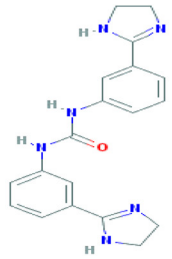
Of 10 identified hits, 7 compounds, including MMV665941, MMV085203, MMV396693, MMV666093, MMV073843, MMV007092,

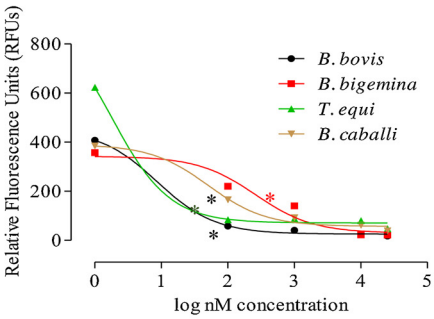
(A)
MMV006787, GNF-Pf-4600; AGN-PC-00VCN8; CHEMBL584235



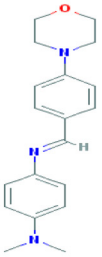


(B)
MMV665810, Imidocarb; Imidocarbe; Imidocarbo; Imidocarbum





(C)
MMV073843, GNF-Pf-2865; N,N-dimethyl-4-[(4-morpholin-4-ylphenyl)methylideneamino]aniline



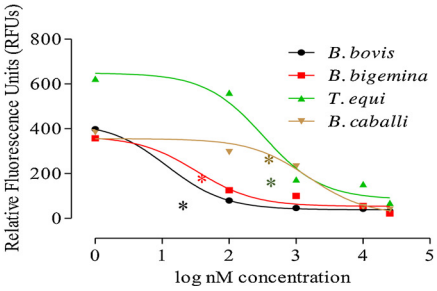


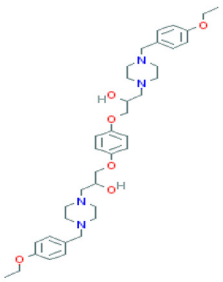
Fig. 3. Fluorescence-based monitoring of the hits with potential: MMV006787, MMV665810, and MMV073843 from the MMV Malaria Box induced growth inhibition of *Babesia* and *Theileria* parasites on the fourth day of treatment. (A) Correlation between MMV006787 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. (B) Correlation between MMV665810 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. (C) Correlation between MMV073843 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. Each value is presented as the mean of duplicates after subtraction of the background fluorescence for non-parasitized RBCs. MMV structures were provided by the MMV as part of the supporting information for the Malaria Box. Synonyms of MMV structures were obtained from the following website: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=2853195. Statistically significant differences between the drug-treated cultures and the control cultures are indicated by asterisks (*$P < 0.05$).

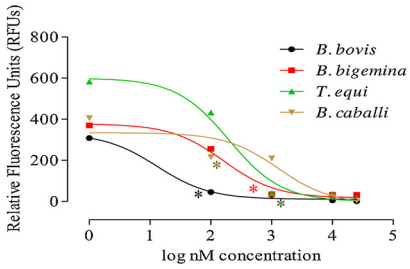
and MMV665875, showed activity against the *in vitro* growth of bovine *Babesia* and *T. equi* greater than those observed for diminazene aceturate, with IC_{50}s ranging from 0.04 to 0.63 μM. The structures of these compounds seemed different from those of currently used drugs. Moreover, the IC_{50} value obtained by MMV396794 compound treatment against the *in vitro* growth of *B. bovis* and *T. equi* was lower than that obtained with diminazene aceturate, which is currently used for chemotherapy against piroplasmosis. On the other hand, the *in vitro* treatment of *B. bigemina* and *B. caballi* with this compound revealed an IC_{50} value higher than that obtained with diminazene aceturate. Additionally, the IC_{50} value obtained by MMV006706 compound treatment against the *in vitro* growth of *B. bigemina* and *T. equi* was lower than that obtained with diminazene aceturate. In contrast, the *in vitro* treatment of *B. bovis* and *B. caballi* with this compound revealed an IC_{50} value higher than that obtained with diminazene aceturate. Meanwhile, the IC_{50} values obtained by MMV006787 compound treatment against the *in vitro* growth of bovine and equine *Babesia* and *Theileria* parasites were higher than those obtained with diminazene aceturate, except for the *in vitro* treatment of *B. bovis* parasite.

In vitro treatment with the 10 hits identified in this review for *B. bovis* and *T. equi* parasites revealed IC_{50}s lower than those obtained with luteolin, pyronaridine tetraphosphate, nimbolide, gedunin, and enoxacin treatment (Rizk et al., 2015; Omar et al., 2016). Moreover, the IC_{50} values of MMV666093, MMV006706, MMV665941, MMV085203, MMV396693, MMV073843, MMV007092, and MMV665875 compounds for *B. bigemina* were lower than those for luteolin, pyronaridine tetraphosphate, nimbolide, gedunin, and enoxacin (Rizk et al., 2015; Omar et al., 2016). The IC_{50} values of MMV396794 and MMV006787 compounds for *B. bigemina* were similar to those for luteolin and pyronaridine tetraphosphate, respectively (Rizk et al., 2015). On the other hand, the *in vitro* treatment of *B. bigemina* with MMV396794 and MMV006787 compounds revealed IC_{50}s lower than those obtained with nimbolide, gedunin, and enoxacin treatment (Rizk et al., 2015). Additionally, the IC_{50}s of MMV666093, MMV396794, MMV665941, MMV085203, MMV396693, and MMV073843 for *B. caballi* were lower than those for luteolin, pyronaridine tetraphosphate, nimbolide, gedunin, and enoxacin (Rizk et al., 2015; Omar et al., 2016). The IC_{50}s of MMV006706, MMV006787, MMV007092, and MMV665875

(A)

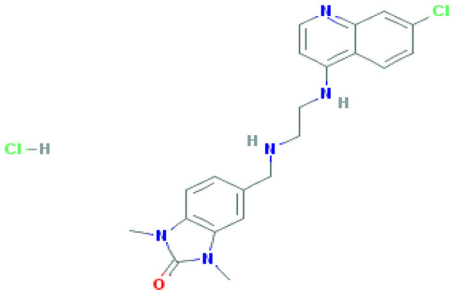
MMV007092, GNF-Pf-2381; STK699776; 1-[4-[(4-ethoxyphenyl)methyl]piperazin-1-yl]-3-[4-[3-[4-[(4-ethoxyphenyl)methyl]piperazin-1-yl]-2-hydroxypropoxy]phenoxy]propan-2-ol





(B)

MMV665875, CHEMBL548469, 5-[[2-[(7-chloroquinolin-4-yl)amino]ethylamino]methyl]-1,3-dimethylbenzimidazol-2-one



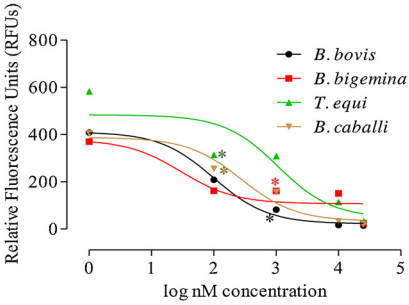


Fig. 4. Fluorescence-based monitoring of the hits with potential: MMV007092 and MMV665875 from the MMV Malaria Box induced growth inhibition of *Babesia* and *Theileria* parasites on the fourth day of treatment. (A) Correlation between MMV007092 compound concentrations and RFUs on *B. bovis*, *B. bigemina*, *T. equi*, and *B. caballi*. (B) Correlation between MMV665875 compound concentrations and RFUs on *B. bovis, B. bigemina*, *T. equi*, and *B. caballi*. Each value is presented as the mean of duplicates after subtraction of the background fluorescence for non-parasitized RBCs. MMV structures were provided by the MMV as part of the supporting information for the Malaria Box. Synonyms of MMV structures were obtained from the following website: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=2853195. Statistically significant differences between the drug-treated cultures and the control cultures are indicated by asterisks (*$P < 0.05$).

compounds for *B. caballi* were similar to those for pyronaridine tetraphosphate (Rizk et al., 2015) and lower than those for luteolin, nimbolide, gedunin, and enoxacin (28, 39).

The 10 potent hits identified in this review exhibited IC_{50}s for bovine *Babesia* and equine *Babesia* and *Theileria* lower than those obtained with *N*-acetyl-L-cysteine (Rizk et al., 2017a, 2017b), nitidine chloride (Tayebwa et al., 2018), clofazimine (Tuvshintulga et al., 2017), apicoplast-targeting antibacterials (ciprofloxacin, thiostrepton, and rifampin) (AbouLaila et al., 2012), miltefosine (AbouLaila et al., 2014), fusidic acid (Salama et al., 2013), and allicin (Salama et al., 2014).

In a recent study, a strategy similar to ours was adopted to repurpose the Malaria Box to identify the active chemical series against *T. gondii* (Boyom et al., 2014). The authors showed that the MMV085203 probe-like compound was also a potent inhibitor of the *in vitro* growth of *T. gondii*, with an IC_{50} value 4.54 μM. The IC_{50} values obtained by the *in vitro* treatment of this compound for bovine *Babesia* and equine *Babesia* and *Theileria* were lower than those for *T. gondii.* Additionally, the MMV665941 probe-like compound was examined against the *in vitro* growth of *S. mansoni* cercariae, adult worms (Ingram-Sieber et al., 2014), and *C. parvum* (Bessoff et al., 2014) and revealed IC_{50} values of 3.6, 9.7, and 0.83 μM, respectively. The IC_{50} values obtained by the *in vitro* treatment of this compound for bovine *Babesia* and equine *Babesia* and *Theileria* were lower than those for either *S. mansoni* cercariae/adult worms or *C. parvum*.

Other screened compounds showed different anti-*Babesia* and anti-*Theileria* activities ranging from intermediate activities with micromolar IC_{50} levels to poor or inactive compounds on different species of *Babesia* and *Theileria* parasites. These variations in compound activities might be due to differences in the *Babesia* and *Theileria* species size, levels of parasitemia, or the compound's mechanism of action. Nevertheless, follow-up studies to clarify these issues are warranted.

Although the MMV665941 compound showed the lowest IC_{50} values for both bovine *Babesia* and equine *Babesia* and *Theileria* parasites, its mean SI was equal to 6.43. This compound has a great variety of synonyms, including methylrosaniline, crystal violet, gentian violet, methyl violet 10B, hexamethyl pararosaniline chloride, and pyoctanin (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=103714763). The compound is a triarylmethane dye possessing several medical uses: antibacterial, antifungal, antiseptic, anthelmintic, antitrypanosomal, anti-angiogenic, and antitumor (Docampo and Moreno, 1990; Gloor and Wolnicki, 2001; Kondo et al., 2006; Maley and Arbiser, 2013). The results of the current study add a new property of methylrosaniline as an antibabesial agent.

Of note, 5 hits (MMV665941, MMV396794, MMV006706, MMV007092, and MMV665875) showed the highest anti-*Babesia* and anti-*Theileria* activities and exhibited IC_{50} values lower than those previously obtained for *P. falciparum* (Spangenberg et al., 2013), whereas other identified hits showed IC_{50}s higher than those for *P. falciparum* either in one *Babesia* sp. or in the four *Babesia* and *Theileria* spp. In detail, the IC_{50} values of the MMV085203 compound for bovine *Babesia* and equine *Babesia* and *Theileria* were higher than those obtained for *P. falciparum* (Spangenberg et al., 2013). In contrast, *in vitro* treatment with MMV396693 and MMV006706 compounds for bovine *Babesia* and equine *Babesia* and *Theileria* parasites revealed IC_{50} values lower than those for *P. falciparum* (Spangenberg et al., 2013), except for *B. bovis*. The IC_{50} values of the MMV006787 compound for *B. bigemina*

and *T. equi* parasites were higher than those for *P. falciparum* (Spangenberg et al., 2013).

P. falciparum, *Babesia*, and *Theileria* are all hemoprotozoa, within the apicomplexan family, most of which possess a unique organelle called an apicoplast (Barbrook et al., 2006). The apicoplast is involved in penetrating a host's cell and is essential for long-term parasite viability (Sullivan et al., 2000; He et al., 2001; Van Dooren et al., 2005). This organelle is currently an attractive target for antiparasitic drug discovery (Ralph et al., 2001; Vaishnava and Striepen, 2006; AbouLaila et al., 2012). The similarity between *P. falciparum*, *Babesia*, and *Theileria* parasites in this feature might explain the highest anti-*Babesia*/*Theileria* activities of the 92, 82, 53, and 66 MMV compounds from the Malaria Box *in vitro* treatment of *B. bovis*, *B bigemina*, *T. equi*, and *B. caballi*, respectively. Nevertheless, further studies are required to elucidate these compound pathways.

Finally, the results presented in the current review and other recent studies toward repurposing the Malaria Box to identify active hits against parasites other than *P. falciparum*, including *C. parvum* (Bessoff et al., 2014), *T. gondii*, and *E. histolytica* (Boyom et al., 2014), revealed the promising application of Malaria Box compounds for parasites other than *P. falciparum*, opening a new door for treatment of the most potent and economically significant parasites worldwide.

4. Conclusion

Screening the MMV Malaria Box compounds against the *in vitro* growth of *Babesia* and *Theileria* parasites aided in the discovery of new, more effective drugs than the traditionally used diminazene aceturate and imidocarb dipropionate. Through this study, 10 novel potent anti-*Babesia* and anti-*Theileria* hits, including MMV666093, MMV396794, MMV006706, MMV665941, MMV085203, MMV396693, MMV006787, MMV073843, MMV007092, and MMV665875, were identified. Four hits, MMV396693, MMV073843, MMV666093, and MMV665875, were the most interesting with regard to bovine *Babesia* and equine *Babesia* and *Theileria* parasites, with excellent SIs. Additionally, 17 and 2 hits were the most interesting with regard to bovine *Babesia* parasites and equine *Babesia* and *Theileria* parasites, respectively. Evaluation of the inhibitory effects of these antibabesial hits against the growth of *B. microti* or *B. rodhaini* in mice, and knowledge of their targets and mechanisms of action, might provide new insights into *Babesia* and *Theileria* biology. Moreover, further studies are required to analyze the synergistic or antagonistic effect of these hits when used in combination with each other and, subsequently, help determine the best effective composition ratio for the growth inhibition of bovine and equine hemoparasites for clinical application.

Competing interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.07.003.

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