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Extensive Shared Chemosensitivity between Malaria and Babesiosis Blood-Stage Parasites

Aditya S. Paul,[a] Cristina K. Moreira,[a] Brendan Elsworth,[a] David R. Allred,[b,c] Manoj T. Duraisingh[a]

Department of Immunology and Infectious Diseases, Harvard T. H. Chan School of Public Health, Boston, Massachusetts, USA[a]; Department of Infectious Diseases and Pathology, University of Florida, Gainesville, Florida, USA[b]; Emerging Pathogens Institute, University of Florida, Gainesville, Florida, USA[c]

The apicomplexan parasites that cause malaria and babesiosis invade and proliferate within erythrocytes. To assess the potential for common antiparasitic treatments, we measured the sensitivities of multiple species of *Plasmodium* and *Babesia* parasites to the chemically diverse collection of antimalarial compounds in the Malaria Box library. We observed that these parasites share sensitivities to a large fraction of the same inhibitors and we identified compounds with strong babesiacidal activity.

The apicomplexan phylum of eukaryotic microbial parasites is important in human and veterinary medicine. Apicomplexans cause malaria (*Plasmodium* spp.), babesiosis (*Babesia* spp.), toxoplasmosis (*Toxoplasma gondii*), and cryptosporidiosis (*Cryptosporidium* spp.), among other diseases. The *Plasmodium* and *Babesia* genera are relatively closely related among the apicomplexans (1) (last common ancestor, ~55 million years ago [2]) (Fig. 1A) and share similar features in their biology, including mechanisms for host cell invasion and metabolism (3–6). Both *Plasmodium* and *Babesia* spp. are pathogenic during the stage of infection when parasites colonize host erythrocytes. Historically, drug development has focused more strongly on inhibitors for *Plasmodium* sp. parasites (7). Researchers have found that some antimalarial drugs also reduce proliferation of *Babesia* sp. parasites in erythrocytes as well (8, 9). The antimalarial atovaquone, a ubiquinone analog, is the preferred clinical treatment for human babesiosis in combination with azithromycin (10) and is used also in veterinary practice for babesiosis in dogs (11).

A renewed focus on malaria eradication has led to the identification of an unprecedented number of bioactive compounds that block proliferation of *Plasmodium falciparum* in erythrocytes (12). In 2011, the nonprofit group Medicines for Malaria Venture (MMV) made available to the research community the Malaria Box, a collection of 400 chemically diverse, previously uncharacterized blood-stage antimalarials (13). Researchers have screened the antiparasitic activities of the Malaria Box compounds in nonerythrocytic host cells for the apicomplexans *T. gondii*, *Cryptosporidium parvum*, and *Theileria annulata* and identified a limited number of inhibitors (<3% of the library) active against each of these species (14–16). Here, we measured the susceptibilities of multiple blood-stage *Plasmodium* and *Babesia* parasite species to the Malaria Box compounds and found that erythrocyte-specific apicomplexans share considerable chemical sensitivities during the clinically relevant stages of parasitic infection.

To determine the species-specific action of the Malaria Box compounds, we measured the chemical susceptibility of *Plasmodium knowlesi* in parallel with the reference species *P. falciparum* (13) (see Dataset S1 in the supplemental material). Endemic to macaque monkeys in southeast Asia and an emerging zoonosis in humans, *P. knowlesi* is distinguished from *P. falciparum* by its shorter blood-stage cell cycle and reduced rate of parasite multiplication per cycle (17). Additionally, *P. knowlesi* is closely related to the second most important human malaria parasite, *Plasmodium vivax*, for which it is a useful experimental model parasite (18). We used a metabolic assay to measure biosynthetic incorporation of ^{3}H-labeled hypoxanthine and parasite growth in the presence of Malaria Box compounds (19) and observed that 90% of inhibitors active against *P. falciparum* are also active against a human erythrocyte-adapted line of *P. knowlesi* (Fig. 1B). For 72 Malaria Box compounds, we observed limited or negligible activity against *P. falciparum*, and these molecules were excluded from all analyses. Compounds active against both *P. falciparum* and *P. knowlesi* exhibited similar well-correlated 50% inhibitory concentration (IC_{50}) values up to ~7 μM (Pearson's $r = 0.53$), with both species exhibiting sensitivity to ~30% to 40% of the small molecules at submicromolar IC_{50} values (Fig. 1C and D). These results argue strongly that the majority of Malaria Box inhibitors are directed toward well-conserved targets in the blood stages of infection by divergent *Plasmodium* species.

To determine the efficacy of the Malaria Box inhibitors toward the *Babesia* parasite spp., we measured the chemical susceptibilities of the parasite species *Babesia bovis* and *Babesia divergens* growing in erythrocytes (see Data Set S1 in the supplemental material). Both species are cow parasites and cause major economic losses in the livestock industry in various parts of the world (20, 21). *B. divergens* occasionally causes severe zoonotic infections in splenectomized individuals (20). We used the [^{3}H]hypoxanthine assay to measure growth of *B. bovis* in bovine erythrocytes and *B. divergens* in human erythrocytes (8, 22, 23). Of the 328 Malaria Box compounds that inhibit *P. falciparum* with an IC_{50} value of <7 μM, we observed that 65, or ~20% of the total, inhibit growth of both *B. bovis* and *B. divergens* with an IC_{50} of <7 μM. An additional 65 molecules inhibit *B. bovis* selectively, and 28 molecules inhibit *B. divergens* selectively, perhaps reflecting species-

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Address correspondence to Manoj T. Duraisingh, mduraisi@hsph.harvard.edu.

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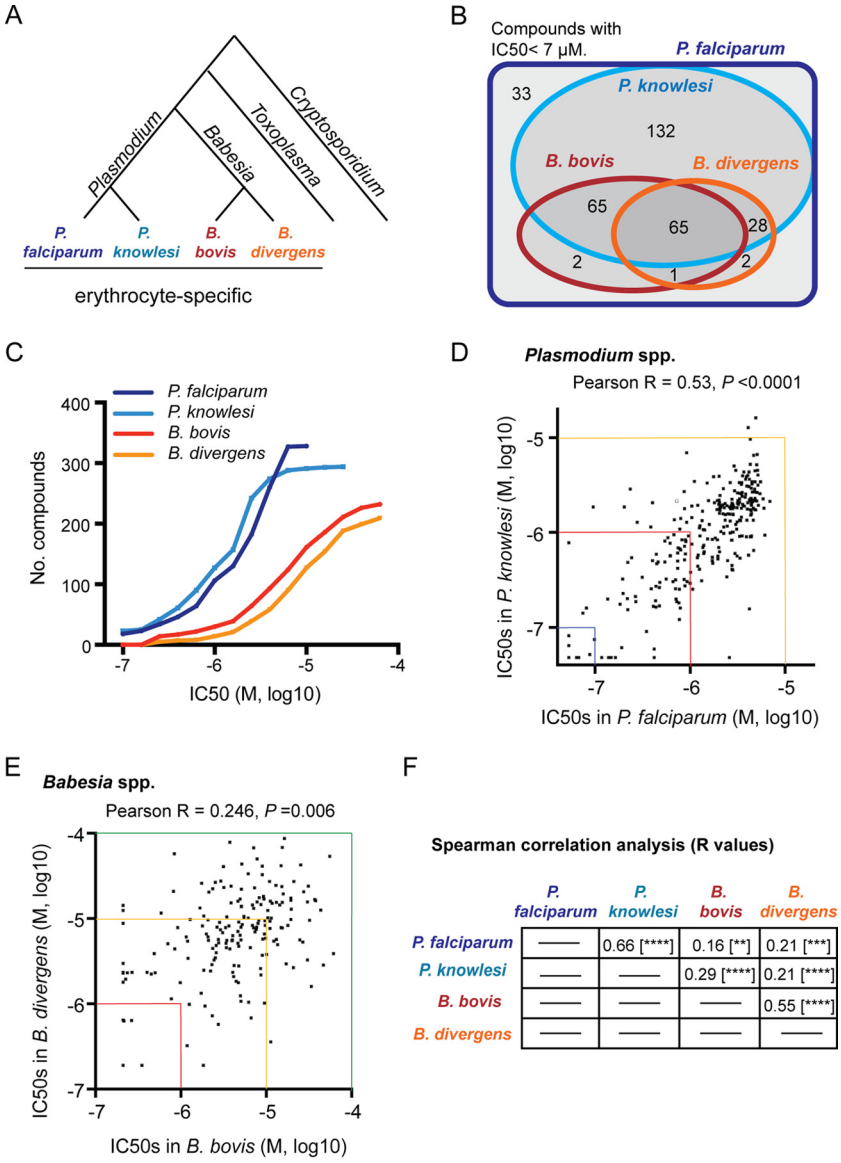
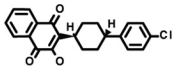
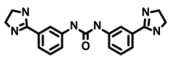
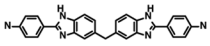
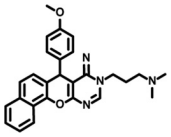
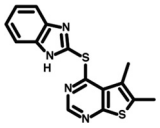
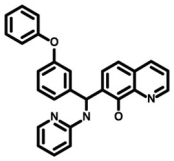
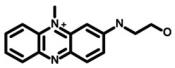
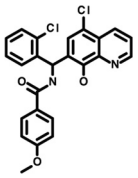
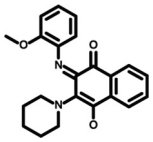
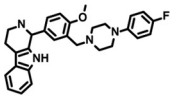


FIG 1 Comparative chemosensitivity analysis of *Plasmodium* and *Babesia* parasite species with the Malaria Box inhibitors. (A) Phylogeny of selected genera of apicomplexan parasites, including *Plasmodium*, *Babesia*, *Toxoplasma*, and *Cryptosporidium* (31). The erythrocyte-specific *Plasmodium* and *Babesia* species examined in this study are indicated. (B) Venn diagram summarizing the species specificity of Malaria Box compounds against the *Plasmodium* and *Babesia* parasite species tested. The number of compounds with an IC_{50} of <7 μM in each category is indicated. (C) For each of the *Plasmodium* and *Babesia* parasite species tested, the number of Malaria Box compounds with an IC_{50} value less than or equal to the indicated values on the *x* axis is shown. (D) Scatter plot comparing the IC_{50} values for Malaria Box compounds in *P. falciparum* (*x* axis) to *P. knowlesi* (*y* axis). Pearson's *r* and *P* values are shown ($n = 294$). (E) Scatter plot comparing the IC_{50} values for Malaria Box compounds in *B. bovis* (*x* axis) to *B. divergens* (*y* axis). Pearson's *r* and *P* values are shown ($n = 190$). In panels D and E, the axes are colored at specific IC_{50} values to permit comparison of scale between the two plots. (F) Summary of all Spearman correlation-based analyses between the parasite species tested. All 328 small molecules found to be inhibitory toward *P. falciparum* growth were included for each analysis. *r* values are indicated. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

specific differences between the *Babesia* species. Growth inhibition by the Malaria Box compounds administered at a single concentration (5 μM) is reproducible for either *Babesia* species (see Fig. S1A and B in the supplemental material).

Both *Babesia* parasite species tested are less sensitive than the *Plasmodium* species to the Malaria Box inhibitors, with blood-stage growth of either *B. bovis* or *B. divergens* sensitive to <10% of the small molecules at a submicromolar IC_{50} value compared to ~30% to 40% sensitivity for the *Plasmodium* species (Fig. 1C). At an IC_{50} of <25 μM, each *Babesia* species is susceptible to ~60% to 70% of the *Plasmodium*-active molecules. The lower sensitivities of the *Babesia* species to inhibitors compared to those of the *Plasmodium* species suggest variation in general features of these parasites (e.g., solute permeability of infected erythrocytes) (24). Significant correlation in the susceptibilities of *B. bovis* and *B. divergens* to Malaria Box compounds (Pearson's $r = 0.246$) (Fig. 1E) suggests frequent activity of these molecules against targets conserved within the *Babesia* genus. Additionally, the high number of Malaria Box compounds with inhibitory activity against all apicomplexan species tested (Fig. 1B and C) and significant cor-

TABLE 1 Confirmed inhibitory concentrations for selected Malaria Box compounds against *P. falciparum* and *Babesia* spp.

Structure and compound identification no.[a]	Molecular wt	IC_{50}/IC_{90} (μM) for[b]:			
		P. falciparum	*B. bovis* (cow)	*B. divergens* (human)	*B. divergens* (cow)
 Atovaquone	366.8	$3.8 \times 10^{-4}/1.8 \times 10^{-3}$	0.018/0.23	0.032/0.13	0.012/0.056
 MMV665810 (imidocarb)	348.4	0.11/0.44	0.66/1.9	0.69/3.4	0.23/0.99
 MMV665943	430.5	0.39/1.2	0.38/2.7	0.51/1.5	0.39/0.82
 MMV667491	440.5	0.31/0.51	0.52/1.9	1.1/2.7	1.0/2.0
 MMV019266	312.4	0.11/0.22	0.59/3.3	0.73/0.83	1.1/2.7
 MMV665814	419.5	0.46/1.1	1.1/2.1	0.88/2.2	0.70/2.2
 MMV396693	254.3	0.11/0.28	2.4/6.3	0.64/7.9	0.31/1.38
 MMV666022	453.3	0.15/0.28	1.1/1.4	0.63/1.7	0.74/2.5
 MMV085203	362.4	$8.6 \times 10^{-3}/0.058$	0.31/1.6	0.16/1.3	0.030/0.090
 MMV019690	470.6	0.37/0.55	1.1/8.7	2.1/6.9	1.1/3.6

[a] From the supplemental material of the original report of the Malaria Box (13).
[b] Determined from 2 to 7 biological replicates.

relation in the potencies of the compounds between the *Plasmodium* and *Babesia* parasite species (Fig. 1F) suggest targeting of features of blood-stage parasite biology common to the *Plasmodium* and *Babesia* genera.

To confirm the activities and determine the potencies of select babesiacidal Malaria Box compounds identified in our screen, we purchased nine compounds from commercial vendors and conducted dose-response susceptibility assays (Table 1). The compounds include imidocarb (Malaria Box compound MMV665810), which is used for treatment of babesiosis in livestock (20). We tested these small molecules in *P. falciparum* proliferating in human erythrocytes, *B. bovis* in cow erythrocytes, and *B. divergens* in both human and cow erythrocytes. Imidocarb exhibited IC_{50} values of 230 to 690 nM against the *Babesia* parasite species, and we observed IC_{50}

values ranging from 30 nM to 2.4 μM for the other Malaria Box compounds in the *Babesia* species. In comparison, atovaquone demonstrated IC_{50} values of 12 to 32 nM in the *Babesia* species. Consistent with our primary screening data, the compounds are typically severalfold more potent against *P. falciparum* than against the *Babesia* species. Many of the compounds we tested exhibit IC_{90} values in all parasite species 4-fold or more lower than published IC_{50} values for inhibition of a human cell line (25) and do not violate the Lipinski rule of five parameters for the prediction of drug-like pharmacokinetics (13).

The breadth of babesiacidal Malaria Box inhibitors is striking in relation to the comparatively few Malaria Box inhibitors reportedly active against nonerythrocyte apicomplexans, such as *T. gondii*, *C. parvum*, and *T. annulata* (14–16). We speculate that this difference may reflect the existence of conserved targets required for proliferation within a similar erythrocytic niche for diverse apicomplexan hemoprotozoan parasites and/or the close phylogenetic relatedness of *Plasmodium* and *Babesia* spp. Our results suggest that, with the discovery of novel antimalarial chemotypes at the blood stage (26–30), a substantial fraction is likely also to be babesiacidal and potentially lead to compounds to be repurposed for the treatment of babesiosis. The work discussed here has implications for chemotherapeutic strategies regarding malaria and babesiosis and should inspire more detailed investigation of the comparative biology of these parasites.

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A.S.P., C.K.M., and B.E. performed all the experiments. A.S.P. analyzed data and prepared figures. D.R.A. assisted with establishment of *B. bovis* cultures and with manuscript preparation. A.S.P. and M.T.D. designed experiments, interpreted data, and wrote the manuscript.

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