1 **Supplemental Material**

2 MATERIALS AND METHODS

3 ***Parasites and tissue culture maintenance*** – We obtained *P. falciparum* strain 3D7 for growth in human
4 erythrocytes from Walter and Eliza Hall Institute (Melbourne, Australia). We previously reported the *P. knowlesi*
5 strain yH-1, adapted for growth in human erythrocytes (1), and the C9.1 line of *B. bovis* for growth in bovine
6 erythrocytes (2). *B. divergens strain* Rouen 1987 for growth in human erythrocytes was a kind gift from Drs.
7 Kirk Deitsch and Laura Kirkman (Weill Cornell Medical College). Human erythrocytes were purchased from
8 Research Blood Components (Boston, USA) and cow erythrocytes from Lampire Biologicals Laboratories
9 (Pipersville, USA). We maintained all parasite lines in standard hypoxic conditions [1% O_2, 5% CO_2, 94% N_2;
0 e.g. (3, 4)], typically at ~2%-hematocrit, in tissue culture media RPMI-1640 supplemented with 25 mM HEPES,
1 50 mg/l hypoxanthine, 2.42 mM sodium bicarbonate, and 4.31 mg/ml AlbuMAX II (Invitrogen). Before addition
2 of AlbuMAX-II and sodium bicarbonate, we adjusted the pH of the media to 6.75.

3 ***The Malaria Box*** – We obtained the Open Access Malaria Box antimalarial library (5), 5x 96 well plates with
4 80 compounds per plate (>10 µl/well) at 10 mM in DMSO (rows A-H and columns 2-11), from the Medicine for
5 Malaria Ventures (http://www.mmv.org) in October of 2012. We stored the library long-term at -80º C.

6 ***The 3H-hypoxanthine uptake assay to measure parasite growth*** – We performed the 3H-hypoxanthine
7 uptake essentially as described elsewhere for *P. falciparum* (6, 7) with some modifications for the various
8 parasite species tested here. All samples for measurement with or without drugs were prepared in 96-well flat
9 bottom plates at 1 culture volume of 100 µl, 1%-hematocrit, and starting parasitemia of 1%, in RPMI-1640 as
0 described above but with reduced hypoxanthine: 2.5 mg/l for *Plasmodium* spp. parasites and 0.1 mg/l for
1 *Babesia* spp. parasites. We extensively washed synchronized ring-stage *Plasmodium* spp. parasites or
2 asynchronous *Babesia* spp. parasites in hypoxanthine-free RPMI-1640 before resuspension in hypoxanthine-
3 reduced media, and addition to concentrated compounds in 96-well flat bottom plates. We synchronized *P.*
4 *falciparum* by treatment with sorbitol (5% w/v in deionized water) (8), and *P. knowlesi* by centrifugation through
5 a Percoll gradient (60% in PBS) (9). For *P. falciparum*, we supplemented cultures after ~44-48 hrs in standard
6 culture conditions, the duration of one blood-stage cell cycle in this species, with typically 1 (up to 1.5) µCi of
7 3H-hypoxanthine monochloride at 0.2 culture volumes, prepared in RPMI-1640 without unlabeled
8 hypoxanthine. We similarly added 1-2 µCi of 3H-hypoxanthine monochloride to *P. knowlesi* samples after ~24-

30 hrs, and 2 µCi to *Babesia* parasite species after ~24-30 hrs following initiation of inhibitor susceptibility assays. For all species, we froze cultures at -80º C after an additional 24 hrs of incubation in standard culture conditions. Following thawing to lyse cells, we collected radioactive biomass on 96-well filter plates for quantification by liquid scintillation counting.

Screening bioactive compounds in the Malaria Box in Plasmodium and Babesia spp. parasites – We performed all automated liquid handling of the Malaria Box compounds with the assistance of the ICCB Longwood Screening Facility at the Harvard Medical School (Boston, USA). All dilutions of compounds were prepared in AlbuMAX II-free RPMI-1640 with reduced or no hypoxanthine. We ultimately plated inhibitors with parasites at positions on 96 well plates corresponding to the positions of inhibitors in the source Malaria Box plates. We added uninfected erythrocytes with media to Column 1 (negative controls for parasite growth), and parasite-infected erythrocytes to media without inhibitors in Column 12 (positive controls for parasite growth). During incubations at standard culture conditions, we kept an open dish with sterile water in the sealed modular incubator chamber with the assay plates to humidify the environment and prevent evaporation from samples. We performed all measurements of growth inhibition with 2-3 technical replicates.

Following thawing at room temperature and centrifugation, we used automated liquid handling to transfer 10 µl of compound from the 96-well Malaria Box plates into 384 well-plates using the Vertical Pipetting Station (VPS) robot (Agilent). We began any other operations using the Malaria Box from compounds stored in these 384-well plates. For inhibitor-susceptibility assays with Plasmodium parasites, we used a custom SEIKO D-TRAN model XM3106-31PN robot fitted with a stainless steel pin array to take up compounds by surface tension from source plates and transfer into replicate assay plates. With this pinning procedure, a total of 400 nl of each DMSO-dissolved compound was diluted into 200 µl of AlbuMAX II-free RPMI-1640 media for a final compound concentration of 20 µM. We used the VPS to serially dilute compounds by ten-fold (20 µl compound with 180 µl media) twice from the 20 µM preparation. On a separate occasion, we prepared 6.32 and 0.632 µM compound by serial dilutions from 20 µM compound. Following transfer of 25 µl of 4x concentrated compound to 96 well assays plates with the VPS, we added *P. falciparum* or *P. knowlesi* parasites in RPMI-1640 with reduced hypoxanthine (1.33x with respect to Albumax and hematocrit) to initiate inhibitor-susceptibility assays as described above. We used the results of these assays at 5, 1.58, 0.5, 0.158, and 0.05 µM compound to

assess the IC50 values for inhibition of *Plasmodium* spp. parasites. For *P. falciparum*, the 5 μM data point was averaged with an additional biological replicate measurement.

For *Babesia* spp., we used the VPS to pipette and dilute 1 μl of compound from the 384-well plates containing the Malaria Box compounds into 200 μl media in 96 well plates for a final concentration of 50 μM. We thereafter used the VPS to serially dilute the compound by 5-fold (40 μl compound to 160 μl media) thrice. After transfer of 50 μl of 2x compound to 96 well plates with the VPS, we added *B. bovis* or *B. divergens* in RPMI-1640 with reduced hypoxanthine (2x with respect to Albumax and hematocrit) to initiate inhibitor susceptibility assays. We used the results of these assays at 25, 5, 1, and 0.2 μM compound, to assess the IC50 values for inhibition of *Babesia* spp. parasites. For *B. divergens*, inhibition at 5 μM is an average value with two other biological replicate measurements, in which compound was prepared through pinning as described for the *Plasmodium* spp. parasites. For *B. bovis*, inhibition at 5 μM is derived wholly from two distinct biological replicate measurements prepared through pinning. We stored diluted compounds in hypoxic conditions at 4º C for addition to parasites typically the same day, but no more than two days later.

Determination of inhibitory concentrations for Malaria Box inhibitors – We quantified inhibition by compounds with the 3H-scintillation counts used to assess parasite growth. To control for plate-to-plate variability, for each assay plate we considered 100% parasite growth as the average value of the 8 no-drug measurements in column 12, and zero growth as background 3H-scintillation associated with the average value of the 8 uninfected erythrocyte sample measurements in column 1. Percent inhibition by each compound on each plate was calculated accordingly. For further analysis, we calculated the average percent inhibition values across technical replicate measurements from different plates. For IC50 determination for each Malaria Box compound, we used non-linear regression analysis in the software Prism (Graphpad), fitting our dose-response data to the equation, $\%\ \text{Inhibition} = 100\%/[1+10^{\wedge(\text{LogIC50-X})*\text{HillSlope}}]$, where X is the log10-transform of the inhibitor concentration. For all datasets, we manually inspected the resulting data to assess confidence in the IC50 values. Criteria which raised confidence included clear dose-dependent inhibition of parasite growth with at least ~40% inhibition at the highest concentration, reproducibility between technical replicates and biological replicates, and goodness of fit (R^2). We assigned an IC50 value equal to the lowest concentrations tested, 0.05 μM for the *Plasmodium* species and 0.2 μM for the *Babesia* species, for strongly inhibitory compounds that blocked growth by more than 50% over all tested doses. Only Malaria Box compounds that we

1 found to be active in *P. falciparum* were considered for analysis in the other parasite species. We performed all
2 other statistical analyses, including Pearson correlation, Spearman correlation, and linear regression, in Prism
3 software. For Spearman correlations between any of the parasite species, all compounds that we found to
4 inhibit *P. falciparum* blood-stage proliferation were considered for analyses.
5 ***Parasite susceptibility to re-purchased compounds*** – We purchased compounds MMV667491 (Cat. No.
6 STK586450), MMV019266 (Cat. No. STK845176), MMV396693 (Cat. No.STL036739), MMV085203 (Cat.
7 No.STK565927), and MMV019690 (Cat. No. STK003728) from VitaScreen, LLC (Urbana-Champaign, USA);
8 compounds MMV666022 (Cat. No. 6412712), MMV665814 (Cat. No. 7014447), and MMV665943 (Cat. No.
9 7609381) from ChemBridge Corp. (San Diego, USA); and imidocarb/MMV665810 (Cat. No. I387550) from
0 Toronto Research Chemicals, Inc. (Toronto, Canada). We generated chemical structures in Table 1 with
1 PubChem Sketcher v2.4 (https://pubchem.ncbi.nlm.nih.gov/edit2/index.html), with input of the SMILES strings
2 from the original publication of the Malaria Box (5), or from the PubChem database from the National Center
3 for Biotechnology Information. Except for imidocarb and atovaquone, which we prepared at 50 and 1 mM in
4 DMSO, respectively, we prepared all concentrated stocks of inhibitors at 10 mM in DMSO. For confirmation of
5 susceptibility of parasites to inhibitors (Table 1), we measured parasite growth by 3H-hypoxanthine uptake
6 (see above) over 8 serially-diluted concentrations with a high concentration of 25 µM. We prepared 2x stocks
7 of all test concentrations, designed to capture the full dose-response, in reduced hypoxanthine RPMI-1640.
8 We assessed inhibitory concentration values from resulting dose-response data, as described above.
9

1 SUPPLEMENTAL REFERENCES

2

3 1. **Lim C**, **Hansen E**, **Desimone TM**, **Moreno Y**, **Junker K**, **Bei A**, **Brugnara C**, **Buckee CO**, **Duraisingh**
4 **MT**. 2013. Expansion of host cellular niche can drive adaptation of a zoonotic malaria parasite to humans.
5 Nature Communications **4**:1638.

6 2. **Allred DR**, **Cinque RM**, **Lane TJ**, **Ahrens KP**. 1994. Antigenic variation of parasite-derived antigens on
7 the surface of Babesia bovis-infected erythrocytes. Infection and Immunity **62**:91–98.

8 3. **Trager W**, **Jensen JB**. 1976. Human malaria parasites in continuous culture. Science **193**:673–675.

9 4. **Levy M**, **Ristic M**. 1980. Babesia bovis: continuous cultivation in a microaerophilous stationary phase
0 culture. Science **207**:1218–1220.

1 5. **Spangenberg T**, **Burrows JN**, **Kowalczyk P**, **McDonald S**, **Wells TNC**, **Willis P**. 2013. The Open
2 Access Malaria Box: A Drug Discovery Catalyst for Neglected Diseases. PLoS ONE **8**:e62906.

3 6. **Fidock DA**, **Nomura T**, **Wellems TE**. 1998. Cycloguanil and Its Parent Compound Proguanil
4 Demonstrate Distinct Activities against Plasmodium falciparumMalaria Parasites Transformed with Human
5 Dihydrofolate Reductase. Molecular Pharmacology **54**:1140–1147.

6 7. **Paul AS**, **Saha S**, **Engelberg K**, **Jiang RHY**, **Coleman BI**, **Kosber AL**, **Chen C-T**, **Ganter M**, **Espy N**,
7 **Gilberger T-W**, **Gubbels M-J**, **Duraisingh MT**. 2015. Parasite Calcineurin Regulates Host Cell
8 Recognition and Attachment by Apicomplexans. Cell Host Microbe **18**:49–60.

9 8. **Lambros C**, **Vanderberg JP**. 1979. Synchronization of Plasmodium falciparum erythrocytic stages in
0 culture. J Parasitol **65**:418–420.

1 9. **Fernandez V**, **Treutiger CJ**, **Nash GB**, **Wahlgren M**. 2000. Multiple Adhesive Phenotypes Linked to
2 Rosetting Binding of Erythrocytes in Plasmodium falciparum Malaria. Infection and Immunity **68**:2391–
3 2391.

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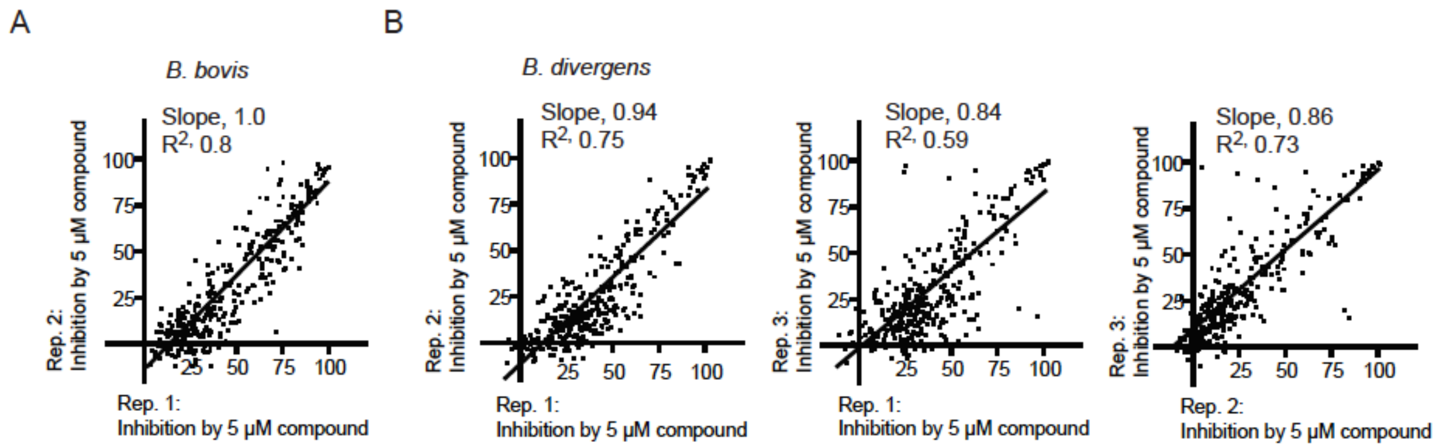
1 SUPPLEMENTAL FIGURE

2 FIG S1: *Reproducibility of chemical inhibition of growth in the Babesia species*.



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4 Scatter plots comparing absolute levels of growth inhibition between biological replicates of (A) *B. bovis*, or (B)

5 *B. divergens* by the Malaria Box compounds. Each data point reports the activity of a single compound dosed

6 at 5 µM (n=328 inhibitors active in *P. falciparum*, Figure 1B). For linear regression of each plot, we show the

7 best-fit line with its slope and R^2 value.