

Methods:

Parasite culture maintenance. *Plasmodium falciparum* strain 3D7 (MR4, cat. no. MRA-102) was maintained under standard conditions at 2% hematocrit with O+ human erythrocytes in RPMI1640 (Thermo Fisher Scientific) containing 10 mg/L hypoxanthine, 2 g/L sodium bicarbonate (Sigma-Aldrich), 15 mM HEPES (Sigma-Aldrich), 50 mg/L Gentamicin sulfate (Sigma-Aldrich) and 2.5 g/L AlbuMAX II (Thermo Fisher Scientific). Intraerythrocytic developing parasites were synchronized with 5% sorbitol (Sigma-Aldrich) over three subsequent developmental cycles to synchronize parasites within 4-6 hours, as previously described. Briefly, *P. falciparum* cultures were collected by centrifugation at 1500 g for 5 min and the parasitized RBC pellet was resuspended in 10x volume of 5% sorbitol, followed by incubation at 37°C for 10 min. Following incubation, the cells were pelleted and washed in 50 mL of complete medium, prior to placing the parasites back into culture flasks at 2% hematocrit and 2% parasitemia.

Parasite cultures were maintained mycoplasma-free and were confirmed (by PCR) prior to metabolomic analysis. Separate mycoplasma-free culture apparatus and solutions were used for all metabolomics culturing to prevent contamination. Cultures were checked for mycoplasma contamination weekly using IntronBio *e-Myco*TM, Mycoplasma PCR Detection kit (Boca Scientific; cat. no. 25235).

Method optimization using atovaquone. Stage: At 10 (ring), 24 (trophozoite), and 38 (schizont) hours post-invasion (hpi), parasites were treated with atovaquone (kindly provided by Dr. Akhil B. Vaidya, Drexel University College of Medicine, Philadelphia,

PA) at 10x IC₅₀ (10nM) for 2.5 hours in parallel with an untreated parasite culture and an uninfected erythrocyte control. Post-treatment, 1.5 mL of parasites were saponin (Acros Organics) lysed (0.02%) for 30sec, collected by centrifugation, and resuspended in ice-cold 1x PBS for metabolite extraction.

Parasite preparation: At 24 hpi parasites were treated with atovaquone at 10x IC₅₀ (10 nM) for 2.5 hours parallel to an untreated parasite culture, an uninfected erythrocyte control, and magnetically purified parasites (both treated and untreated). Following treatment, 1.5 mL of treated and untreated parasite culture suspension was centrifuged in an Eppendorf tube for 30sec. The supernatant was removed and the parasitized RBC pellet was then resuspended in 1.0 mL of 1x PBS. For measurement of metabolites in “bulk culture”, these parasite suspensions were then placed on ice for metabolite extraction. Alternatively, to remove background erythrocyte metabolite contamination, the parasites were lysed with 0.02% saponin for 30 seconds followed by centrifugation and supernatant aspiration. Isolated parasite pellets were then resuspended in 1.0 mL of ice-cold 1x PBS for metabolite extraction. Magnetic purification was performed as described below and extraction performed following centrifugation and supernatant removal. The various extraction methods were performed in triplicate for two biological replicates.

Concentration: At 24 hpi parasites were synchronized and magnetically purified as described below. After a 1-2 hour period of recovery, parasites were then treated with atovaquone at 1x (1.0 nM), 2x (2.0 nM), 5x (5.0 nM), 10x (10 nM), 20x (20 nM) and 50x

IC₅₀ (50nM) for 2.5 hours or no drug was added. All treatments were carried out in triplicate and performed for two biological replicates.

Time: At 24 hpi parasites were synchronized and magnetically purified as described below. After a 1-2 hour period of recovery, parasites were then either untreated or treated with atovaquone at 10x (10 nM) IC₅₀ for 0.5, 1.0, 2.0, 4.0, and 8.0 hours. All time-points were collected in triplicate and performed for two biological replicates.

Magnetic column separation. Magnetic separation of mature trophozoites (24-36 hpi) was carried out as previously described using an in-house constructed magnetic cell fractionation system. Upon purification, parasites were allowed to recover under standard culture conditions at 0.5% hematocrit for 1-2 hours before experimentation.

Sample preparation for antimalarial drug screening. Drug treatments were typically performed on six compounds, with an atovaquone treatment as a positive control and a paired untreated control. Briefly, magnetically purified parasites (20-30 μ L pellet) were incubated in 6-well plates at 0.5% hematocrit with antimalarial compounds at >2x IC₅₀ for 2.5 hours. The concentration used was subject to compound availability and not all Malaria Box drug-like compounds could be tested due to limited quantities. Infected erythrocytes were quickly pelleted by centrifuging for 15 seconds at 15,000 RPM at 4°C, media was removed, and cells were extracted as described below.

Metabolite Extraction. Extractions were performed as described previously. Briefly, a ~20 μL pellet of cells was resuspended in 1.0 mL of pre-chilled 90:10 methanol:water and placed at 4°C. The internal standard $^{13}\text{C}_4\text{-}^{15}\text{N}_1\text{-aspartate}$ was spiked into the extraction methanol solution to control for sample preparation and handling. Samples were vortexed, resuspended, and centrifuged for 10 min at 15000 rpm and 4°C. Supernatants were collected and stored at -80°C or dried down immediately under nitrogen flow. The dried metabolites were resuspended in HPLC-grade water (Sigma, CHROMASOLV) to between 1.0×10^5 and 1.0×10^6 cells/ μL , based on hemocytometer counts of purified parasites. All samples were processed in technical triplicate with method blanks to reduce unwanted variation and account for background signal, respectively. Samples were randomized and 10 μL of resuspended metabolite extract or method blank was injected for UHPLC-MS analysis.

Gametocyte Culturing. Culturing of 3D7 for gametocyte production was performed using established methods. Gametocyte induction of an asexual ring stage culture was performed through volume expansion (2x) without the removal of the “spent” media, resulting in a hematocrit reduction and 50/50 media mix (Day -1). On Day +1 the media was replaced on cultures now containing a mixture of asexual and sexual stage rings. On Day +2 cultures were given fresh media containing 50 mM *N*-acetyl-glucosamine (NAG) to block subsequent asexual replication and enrich for non-replicating gametocytes. Geimsa-stained thin blood smears were prepared and examined on Day +3 to ensure the absence of ring stage asexual parasites and the media was changed daily in the absence of NAG. On Day +6 the culture contained mostly stage III-IV gametocytes, which were

magnetically purified and processed following the protocol described above. These gametocytes were treated with 1.0 μ M atovaquone for 2.5 hours followed by metabolite extraction as described above.

Ultra-High Performance Liquid-Chromatography Mass-Spectrometry (UHPLC-MS) measurement of whole-cell metabolic extracts. Extracts were analyzed using reversed phase Ultra-High Performance Liquid Chromatography Mass-Spectrometry (UHPLC-MS) on a Thermo Exactive Plus Orbitrap™. Metabolite separation was performed with a C18 column (Phenomenex Hydro-RP; cat. No 00D-4387-B0) using a 25 minute gradient of A: 3% aq. methanol/15 mM acetic acid/10 mM tributylamine ion pairing agent and B: 100% MeOH. Detection was performed in negative ion mode, using a scan range of 85-1000 m/z and a resolution of 140,000 @ m/z 200. Calibration was performed prior to every acquisition batch, using Pierce Negative ESI calibration solution (ThermoFisher Scientific), and glucose-free RPMI without Albumax II was routinely run as a quality assurance sample to monitor analytical performance. Additional sample randomization was also performed to reduce within batch variability. To aid in the detection of cellular metabolites, a database was generated from 242 pure metabolite standards using the same instrument and method to determine detection capability, mass/charge ratio (m/z), and retention time for each metabolite.

Data analysis. Raw data files from the Thermo Exactive Plus Orbitrap™ (.raw) were converted to a format compatible with our analysis software (.raw \rightarrow .mzXML). Spectral data (.mzXML files) were visualized in MAVEN, heavy-labeled aspartate internal

standard intensity was assessed for technical reproducibility and peaks for each metabolite in the targeted library were inspected and demarcated as good or bad based on peak shape, proximity to standard retention time, and signal/blank ratio. Peak areas were exported into an R working environment (<http://www.R-project.org>) for calculation of \log_2 fold changes for each sample as compared to an untreated control. Metabolites that were not reliably detected across 90% of the all the trials were removed prior to additional analysis to minimize subsequent imputation bias. Any remaining metabolites not detected were imputed as 10,000 and metabolites detected below background levels (negative after blank subtraction), were maintained as “0” prior to averaging and \log_2 calculation. Since our metabolite extraction method did not include a wash step, metabolites found in the RPMI medium were excluded.

The \log_2 fold changes of detected metabolites from the validation drugs were used to train a self-organizing map (SOM) and a 2-dimensional hexagonal fingerprint was generated for each drug by projecting onto the trained map with the supraHex package for R/Bioconductor. These supra-hexagons display related metabolites within nodes or small hexagons that are arranged radially outward from the center based on vector weight. This organizational pattern places the most influential metabolite nodes on the outer edge of the supra-hexagon, while preserving the input data information such as the dimensionality, distribution, distance, clusters, and identity of metabolites. The validation dataset was used to generate the base Metabolic fingerPrint or ‘MetaPrint’, against which all additional metabolite data from the Malaria Box and gametocytes was projected onto. A subset of compounds from the validation set that gave a strong

signature was used to assist in the classification of parasite metabolic perturbation to MMV Malaria Box compounds. Hierarchical clustering was performed on the \log_2 fold change values using Pearson-Ward clustering.

All processed metabolomics spectral data and analytical meta-data from this study will be deposited to the NIH Metabolomics Workbench (Accession #XXX).