SUPPORTING INFORMATION

Identification and SAR evaluation of hemozoin-inhibiting benzamides active against *Plasmodium falciparum*

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S1. General

Commercially obtained chemicals (AR or higher grade) were purchased from Sigma-Aldrich or Kimix Chemicals. Nonidet P-40 was acquired from Pierce Biotechnology, Rockford, IL, USA. Compounds **1-10** were purchased from Vitas-M Laboratory. Water used in all experiments was double distilled deionised Millipore® Direct-Q water ($D.H_2O$). All pH measurements were carried out using a Crison 2000 MicropH meter or Jenway 3510 benchtop pH meter, calibrated using standard phosphate buffer solutions at pH 4.00 ± 0.02 and 7.00 ± 0.02 prior to use. Manual micro-volume additions were delivered using Eppendorf single-channel micropipettes or multichannel micropipettes. All reactions were monitored by thin layer chromatography (TLC) using silica gel plates (Merck F_{254} aluminum-backed). TLC plates were visualized with ultraviolet light (254 nm) and, where an amine was present, plates were stained with ninhydrin spray (0.2% w/w in EtOH). Silica gel flash column chromatography was carried out with Fluka 60: 70-230 mesh on either a Biotage Isolera One Flash Chromatography System or using a combination of a D-star DVW-10 variable wavelength detector with a Teledyne ISCO fraction collector. Melting points were measured using a Reichert-Jung Thermovar hot stage microscope. Proton (^1H) and carbon (^{13}C) NMR spectra were recorded using a Varian Mercury spectrometer (300 MHz for ^1H), a Bruker 300 (at 300.08 MHz for ^1H) or a Bruker Ultrashield 400 Plus (at 399.95 MHz for ^1H and 100.64 MHz for ^{13}C) spectrometer. NMR experiments were performed in deuterodimethyl sulfoxide (DMSO-d_6), deuterochloroform ($CDCl_3$), deuteromethanol (MeOD) or deuteroacetone (acetone-d_6) with internal standards at δ_H = 2.50, 7.26, 3.31 or 2.05 ppm respectively for ^1H NMR and δ_C = 40.05, 77.16, 49.00 or 29.8 and 206.26 ppm respectively for ^{13}C NMR. All chemical shifts were reported in ppm and *J* coupling values in Hz. Electron ionization mass spectrometry was recorded using a JEOL GC mate II single magnetic mass spectrometer. High resolution mass spectrometry (HRMS) was performed on a Time-of-flight (TOF)

Waters Synapt G2 instrument using leucine encephalin as a standard. All mass spectra for the benzamide series were recorded using the electrospray negative (ES^-) technique and introduction of the sample was via an ESI probe injected into a stream of MeOH. High performance liquid chromatography (HPLC) was carried out on an Agilent Technologies 1220 Infinity LC (G4288C) in UV mode at 250 nm with a C18 reverse phase column in HPLC grade acetonitrile (ACN) and double distilled deionized Millipore® Direct-Q water.

S2. Detergent mediated assay for β-hematin inhibition

The inhibition of βH formation assay method described by Carter *et al.*[1] was modified for manual liquid delivery. Samples were dissolved in DMSO to give 20 mM solutions and 20 µL of each was delivered in duplicate or triplicate to wells in the last column (column 12) of a 96-well plate together with distilled water (140 µL) and NP40 detergent (305.5 mM, 40 µL). A solution containing water/NP40 (305.5 mM)/DMSO at a v/v ratio of 70%/20%/10% respectively was prepared and then 100 µL was added to all other wells (columns 1-11). A serial dilution of each compound (100 µL) from column 12 down to column 2 was carried out. Column 1 served as a blank with 0 mM sample. A 25 mM hematin stock solution was prepared by sonicating hemin in DMSO for one minute and then suspending 178 µL of this in a 1 M acetate buffer (20 mL, pH 4.8). The homogenous suspension (100 µL) was then added to the wells to give final buffer and hematin concentrations of 0.5 M and 100 µM respectively. The plate was covered and incubated at 37 °C for 5-6 h in a water bath or incubator. Analysis was carried out using the pyridine-ferrichrome method developed by Ncokazi and Egan.[2] A solution of 50% (v/v) pyridine, 30% (v/v) H_2O, 20% (v/v) acetone and 0.2 M HEPES buffer (pH 7.4) was prepared and 32 µL added to each well to give a final pyridine concentration of 5% (v/v). Acetone (60 µL) was then added to assist with hematin dispersion. The UV-vis absorbance of the plate wells was read on a SpectraMax P340 plate

reader. Sigmoidal dose-response curves were fitted to the absorbance data using GraphPad Prism v3.02[3] to obtain a 50% inhibitory concentration (IC_{50}) for each compound.

S3. Heme binding

The association constant for the interaction between selected benzamides and Fe(III)PPIX was determined using a modified version of the titration method reported by Egan and co-workers.[4] Briefly, a stock solution of the test sample in either 40% aqueous DMSO and HEPES buffer (0.02 M, pH 7.4) or 100% DMSO (for the benzamides) was prepared. The initial concentrations the quinolines (CQ and QD) and benzamides were 2 mM and 20 mM respectively. A working solution of hematin (7.5 µM) and HEPES buffer (0.02 M, pH 7.4) in 40% aqueous DMSO was prepared and used immediately. Identical volumes of the stock solution were added with a Hamilton syringe into quartz cuvettes (Hellma) of 1 cm path length containing the hematin working solution or a blank solution of 40% aqueous DMSO and HEPES buffer (0.02 M, pH 7.4). The UV-vis absorbance values of the blank were automatically subtracted from that of the heme working solution. The UV-vis absorbance was monitored from 350 nm to 650 nm at 25 °C with each addition of the test sample on a Varian Cary 100 UV–Visible spectrophotometer. The data obtained were corrected for dilution and analyzed using non-linear least squares fitting methods. Titrations were performed in triplicate and the average association constant (*K*) values were reported with the standard error of the mean (SEM). The value of log*K* was calculated from the best fit model, which described a 1:1 association using *Eq. 2*, where A_0 is the initial absorbance (no test sample), A_∞ is the final limiting absorbance and [L] is the concentration of the free ligand, which in the case of weak association is approximately the total test sample concentration in the hematin working solution.

$$A = \frac{A_0 + A_\infty K[L]}{1 + K[L]} \qquad \textbf{Eq. 2}$$

S4. *P. falciparum* culturing and assays

S4.1 LDH malaria parasite survival assay

Cultures of CQ-sensitive D10 and NF54 and CQ-resistant K1 *P. falciparum* in the asexual erythrocyte stages were continuously maintained *in vitro* using a modified version of the method of Trager and Jensen.[5] The antimalarial activity of the purchased and synthesised benzamides were assessed in at least duplicate using the parasite lactate dehydrogenase (LDH) assay described by Makler *et al.*[6] *P. falciparum* was maintained in culture flasks containing O+ human erythrocytes (2% hematocrit, 1-10% parasitemia) suspended in RPMI 1640 medium (10.4 g/L) with glutamine, glucose (22 mM), HEPES buffer (25 mM), hypoxanthine (0.65 mM), Albumax (5 g/L), gentamicin (0.1 mM) and $NaHCO_3$ (32 mM). Flasks were gassed before incubation at 37 °C with a mixture of 3% O_2, 4% CO_2, and 93% N_2. CQ, tested up to 100 ng/ml maximum concentration, was used as a control on each plate. Parasitized red blood cells (pRBCs) were prepared via dilution from the culture flask to give a 2% hematocrit and 2% initial parasitemia. The samples were prepared as 2 mg/ml stock solutions in DMSO and diluted with complete cell culture medium before testing in triplicate in a 96-well plate. Row 1 of the plate was reserved as a blank with uninfected RBCs (200 μL 1% hematocrit). Row 2 contained the positive control with no test compound (200 μL, 1% hematocrit, 1% parasitemia). Rows 3-12 contained the inoculated pRBCs at a range of sample concentrations, prepared by adding 200 μL of the required sample stock to row 3 and performing a two-fold serial dilution across the plate to row 12. Finally, the pRBCs (100 μL) were added to rows 3-12 to give a final volume of 200 μL, hematocrit of 1% and parasitemia of 1%. The plate was incubated at 37 °C for 48 h in a gas chamber containing 3% O_2, 4% CO_2 and 93% N_2. Following resuspension of the plate contents in a non-sterile environment using a multichannel pipette, 15 μL from each well was transferred to the corresponding well of a new plate containing Malstat (100 μL per well). Nitroblue tetrazolium (NBT, 25 μL) was

added to each well and the plate was left to develop in the dark for 10 min before measuring the absorbance at 620 nm on a microplate reader. The highest concentration of solvent to which the parasites were exposed had no measurable effect on the parasite viability. The IC_{50} values were obtained from fitting analysis using a non-linear least-squares dose response curve in GraphPad Prism v.3.0 software.

S4.2 Cell fractionation

Target validation was carried out by measuring the increase in free heme and the decrease in hemozoin formation via cell fractionation studies using the procedure described by Combrinck at al.[7]

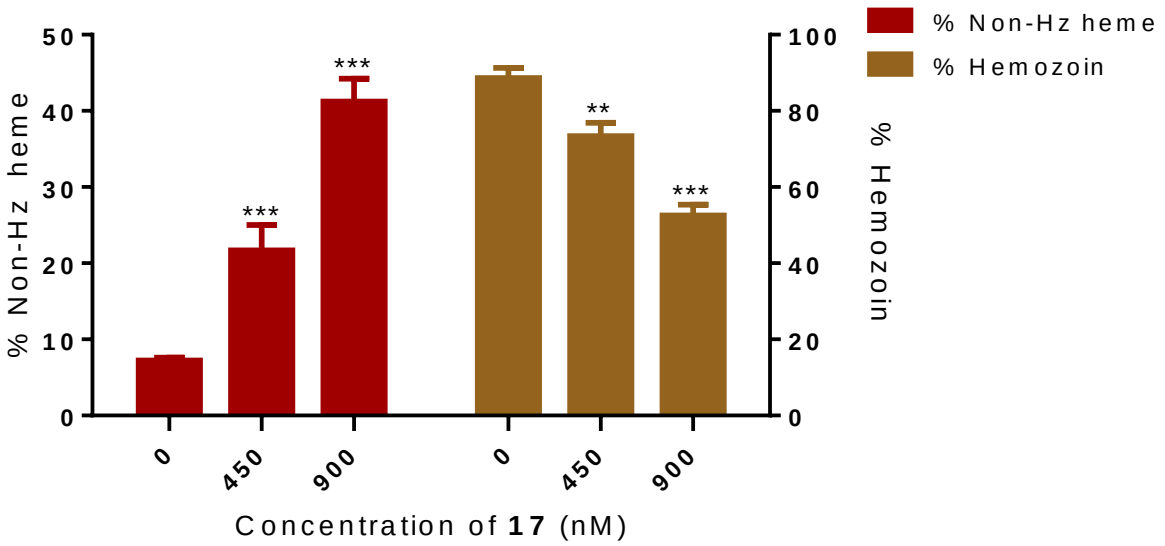

Figure 4.16 A sharp increase in the % non-Hz heme accompanied by a decrease in the % hemozoin within the CQ-sensitive D10 strain of *P. falciparum* for compound **17**. Asterisks indicate statistical significance relative to no drug (2-tailed t-test): **P < 0.01; ***P < 0.001, n = 3.

S4.3 TEM Images

Cells were dosed at 2.5 × the IC_{50} value (0.22 μg/ml for **15i**) at immature ring stage and incubated for 32 h until the cells were mature trophozoites. TEM was performed on the samples using a Tecnai G2 electron microscope (Fei) according to the procedure described by Egan *et al*.[8] Images were collected using a slit width of 18 eV on a Tiedtz charge coupled-device camera (1024 × 1024 pixels). The images were processed with analySIS software from Soft Imaging System.

S4.4 The inoculum effect

The relative accumulation was determined via the inoculum effect using a procedure similar to those previously reported.[9, 10] The LDH assay described above was employed to measured IC_{50} values in triplicate at inoculum sizes ranging from 1 to 10 and fractional volumes of parasitized erythrocytes from 0.0001 to 0.001. The relationship was extrapolated via linear regression to give the absolute IC_{50} from the y-intercept in Graph Pad Prism v3.0.

S5. *In vitro* ADMET assays

S5.1 Kinetic solubility

The kinetic solubility assay was performed using a miniaturized shake flask method. 10 mM stock solutions of each of the test compounds were used to prepare calibration standards (10- 220 µM) in DMSO, and to spike (1:50) duplicate aqueous samples of FaSSIF (simulating fasting state biorelevant media, pH 6.5), with a final DMSO concentration of 2%. After shaking for 2 hours at 25 °C, the solutions were filtered and analyzed by means of HPLCDAD (Agilent 1200 Rapid Resolution HPLC with a diode array detector). Best fit calibration curves were constructed using the calibration standards, which were used to determine the solubility of the aqueous samples.[11]

S5.2 Microsomal stability

The metabolic stability assay was performed in duplicate in a 96-well micro titer plate. The test compounds (0.1 µM) were incubated individually in mouse, rat and pooled human liver microsomes (0.4 mg/mL) at 37 °C for predetermined time points, in the presence and absence of the cofactor NADPH (1 mM). Reactions were quenched by adding 300 µL of ice cold acetonitrile containing internal standard (carbamazepine, 0.0236 µg/mL). Test compounds in the supernatant were analyzed by means of LC-MS/MS (Agilent Rapid Resolution HPLC, AB SCIEX 4000 QTRAP MS) for the disappearance of parent compound. Metabolite searches were not conducted during the metabolic stability assay.[12]

S5.3 Cytotoxicity

Compounds were screened for in vitro cytotoxicity against Chinese hamster ovarian (CHO) mammalian cell-lines, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)- assay. The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays.[13, 14] The tetrazolium salt MTT was used to measure all growth and chemosensitivity. The tetrazolium ring is cleaved in active mitochondria. Thus only viable cells are able to reduce the water-soluble yellow colored MTT to water insoluble purple colored formazan. Formazan crystals are dissolved in DMSO. The test samples were tested in triplicate on one occasion. The test samples were prepared to a 20 mg/mL stock solution in 100% DMSO. Stock solutions were stored at -20 ºC. Further dilutions were prepared in complete medium on the day of the experiment. Samples were tested as a suspension if not completely dissolved. Emetine was used as the reference drug in all experiments. The initial concentration of emetine was 100 µg/mL, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg/mL. The same dilution technique was applied to the all test samples. The highest concentration of solvent to which the cells were exposed had no measurable effect on the cell viability (data not shown). The 50% inhibitory concentration (IC50) values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4 software.

S6. References

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