Supporting Information

Title: Pre-erythrocytic activity of M5717 in monotherapy and combination in preclinical *Plasmodium* infection models.

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Methods

Pharmacokinetic Analysis of *P. berghei* blood samples

C57BL/6J mice were infected with $3x10^4$ *Pb*-Luc sporozoites. Blood samples for pharmacokinetics analysis were collected from mice of the vehicle treated, M5717-treated, pyronaridine (Pyro)-treated and M5717+Pyro-treated groups at 28 and 48 hours post-infection (hpi). The quantitative determination of M5717/pPyronaridine in mouse whole blood and water samples was performed using an liquid chromatography (LC)-mass spectrometry (MS)/MS (Agilent1200 / Sciex API4000). Stock solutions were prepared by weighting M5717 and Ppyronaridine and dissolving it in DMSO (Dimethylsulfoxid Art. 1.09678.0100). Working solutions were created by diluting the stock solution with ACN/H_2O (Art. Acetonitrile 34998-4X2.5L / 1.15333.2500). The calibration standards were created by serial dilution of a working solution with CD1/H2O (CD1 mouse plasma provided by Biotrend). Samples and calibration standards were each precipitated in 1.5 mL Eppendorf tubes using MeOH (Methanol Art. 1.06007.2500). Aliquots were transferred to a deepwell-plate and diluted with H_2O/ACN. Detection was performed in positive ion mode. M5717 (Q1: 463, Q3:392) and Ppyronaridine (Q1: 518, Q3 447) selectivity was achieved using multiple reaction monitoring (MRM) for MS/MS detection of the compounds. LC used a gradient method using H_2O with FAC (Formic Acid Art. 1.00264.0100) and ACN as eluents. The concentrations of M5717 and Ppyronaridine in samples and calibration standards were calculated by interpolation of the peak area ratio of analyte versus the ratio of their nominal concentration into the regression line obtained from the calibration standards. Calibration curve calculation was performed applying weighted (1/x) quadratic regression.

In vitro assessment of HepG2 spheroids cell viability

To ascertain the lack of hepatotoxicity of the drug conditions employed *in vitro*, cell viability was assessed employing different analytical methods besides the resazurin reduction:

dsDNA quantification

Cell viability of hepatic spheroids was assessed by dsDNA quantification, following the manufacturer's instructions for the Quant-iT PicoGreen dsDNA Assay Kit (P7589, Thermo Fisher Scientific). Briefly, samples were diluted with Tris-HCl EDTA (TE) 1X buffer 1:10 and Picogreen working solution was added. After 5 min at room temperature, the absorbance was measured at 480 and 520 nm in a microplate reader TecanProInfinite200.

Lactate dehydrogenase release (LDH)

Lactate dehydrogenase (LDH), a cytosolic enzyme that is released from the cells upon damage was used to measure cell cytotoxicity. LDH activity in the cell supernatant was assessed following the manufacturer's instructions for the CyQUANT LDH Cytotoxicity Assay Kit (C20300, Thermo Fisher Scientific). Briefly, 50 µL of Reaction Mixture were added to 50µL of culture supernatant. After 30 min incubation 50 µL of Stop Solution were added and absorbance at 490 and 680 nm was measured using a microplate reader TecanProInfinite200.

The cell viability for all the experimental conditions was assessed by the methods described above and normalized to that of the spheroids exposed to DMSO (control).

Supplementary Figures

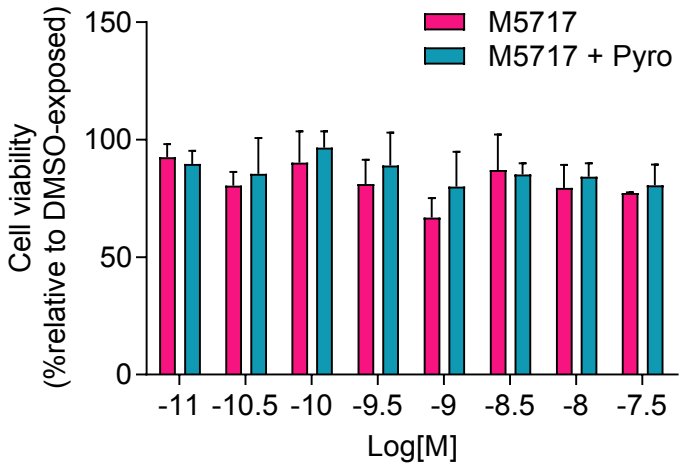

Figure S1 – Cell viability of *Pb*-infected HepG2 spheroids exposed to the M5717-pyronaridine combination. Cell viability upon exposure to M5717 (pink) or M5717-pyronaridine combination (blue) was assessed by fluorescence analysis and normalized to that of DMSO-exposed spheroids. Results are represented as mean ± SD of at least three independent experiments.

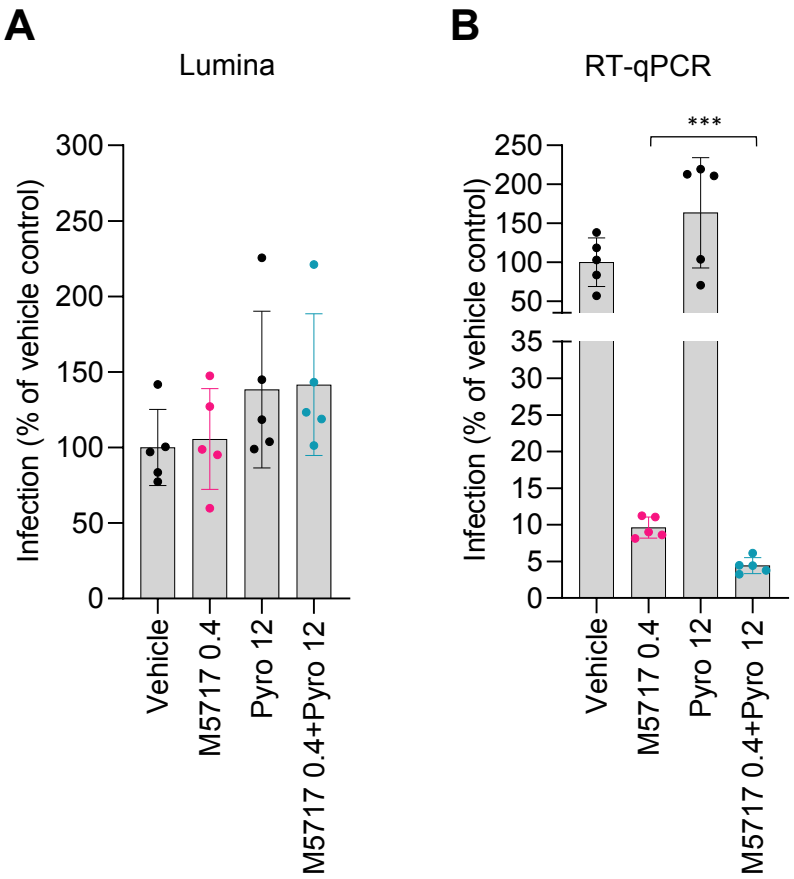

Figure S2 – *In vivo* assessment of the pre-erythrocytic activity of the M5717-pyronaridine combination. (A) The liver parasite load was assessed at 24 h post-infection (hpi) by live bioluminescence. Results are the mean ± SD relatively to the vehicle-treated control group of five mice from one independent experiment. Statistical analysis was performed by one-way ANOVA. (**B**) The impact of M5717 (pink) or M5717-pyronaridine combination (blue) in liver load was evaluated at 48 hpi by RT-qPCR analysis. Infection was normalized to that of the vehicle-treated control group and results are represented as mean ± SD of five mice from one independent experiment. Statistical analysis of RT-qPCR data was performed by a T test. *** P ≤ 0.001.

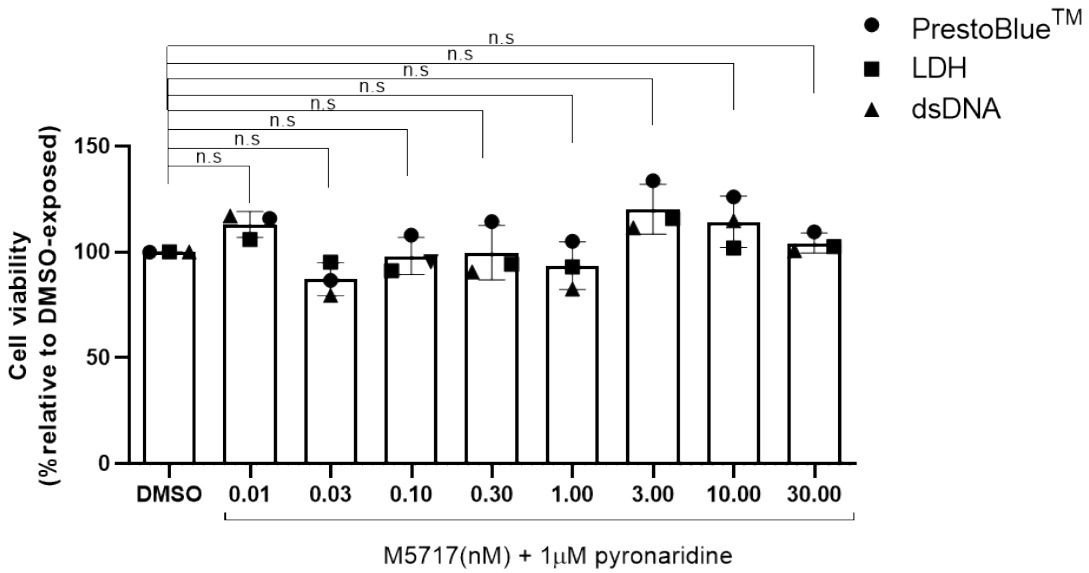

Figure S3 – Cell viability of *Pb*-infected HepG2 spheroids exposed to 1 μM ~~P~~pyronaridine combination by different analytical methods. Hepatotoxicity of the drug vehicle (DMSO) and 1 μM ~~P~~pyronaridine combination with M5717 (8 doses) was assessed by fluorescence as a measurement of: (i) resazurin reduction (PrestoBlue™, ●), (ii) Lactate dehydrogenase release (LDH, ■) and (iii) dsDNA quantification (dsDNA, ▲). Fluorescence values were normalized to those of DMSO-exposed spheroids and the results are represented as mean of technical replicates of one biological experiment. Dunnet's Multicomparison's test was performed for all samples relative to the control (n.s – non significant).

Table S1 - Pharmacokinetic analysis of *P. berghei* blood samples. The quantification of M5717/~~P~~pyronaridine in mouse whole blood and water samples was performed by LC-MS/MS at 28 and 48 h post-infection (hpi). The concentrations of M5717 and ~~P~~pyronaridine in unknown samples were determined employing calibration curves of the standard compounds. The results are presented as mean±S.D. of up to 9 mice from one independent experiment. Concentrations below the lower limit of quantification (LLOQ) of the method were identified as LLOQ.

Conditions	Time (hpi)	M5717 Concentration (µM)	Pyro Concentration (µM)
Vehicle (n=5)	28	<LLOQ	<LLOQ
Vehicle (n=5)	48	<LLOQ	<LLOQ
M5717 0.3 mg/kg (n=9)	28	<LLOQ	<LLOQ
M5717 0.3 mg/kg (n=9)	48	<LLOQ	<LLOQ
Pyronaridine 12 mg/kg	28	<LLOQ	0.0455 (n=3)
Pyronaridine 12 mg/kg	48	<LLOQ	0.0658±0.0518 (n=3)
M5717 0.3 mg/kg + Pyronaridine 12 mg/kg	28	<LLOQ	0.107±0.11 (n=5) 0.0700±0.09 (n=4)
M5717 0.3 mg/kg + Pyronaridine 12 mg/kg	48	<LLOQ	0.0902±0.08 (n=5) 0.0741±0.08 (n=4)