A new aminomethylphenol, JPC-2997, with high *in vitro* and *in vivo* antimalarial activity against blood stages of Plasmodium

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Supplemental Material

Analysis of JPC-2997 in Blood and Plasma

Blood and plasma JPC-2997 concentrations were measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS), with a lower limit of quantification (LLOQ) of 0.5 ng/mL with 50 µL of blood or plasma. Blood or plasma samples (50 µL aliquots in microfuge tubes) were thawed on ice and were vortex-mixed prior to analysis. To these tubes was added 200 µL Internal Standard solution (2 ng/mL JPC-3211, a staple deuterated form of JPC-2997 in acetonitrile) and the tubes were mixed by vortexing for 1 min. For the blood

assay, the tubes were vigorously mixed immediately after adding the Internal Standard solution to dissociate the blood sample. This had the effect of increasing the assay precision significantly. After vortexing, the tubes were centrifuged (16,000 x *g*, 5 min) and 100 µL organic layer was subsequently transferred to a 96 well assay plate containing 100 µL of 0.1% formic acid.

Samples (10 µL) were analysed on a 4000 Q Trap LC-MS/MS analyser (Applied Biosystems) preceeded by a Prominence liquid chromatography system (Shimadzu), including a temperature-controlled autosampler as follows:

Chromatographic Conditions:

Column: Gemini-NX C18 (50 x 2.0 mm; 5 µm), Phenomenex.

Column oven: 40°C

Mobile Phase: 31% Acetonitrile in 0.1% Formic Acid.

Chromatography: Isocratic, 3 minute run, 10 µL injection volume.

Mass Spectrometry Conditions:

Curtain gas	30
CAD gas	Medium
IS (V)	5500
Temp (˚C)	500
GS1	50
GS2	50
IHE	On

Mass Spectrometry Transitions:

Analyte	Q1 (Da)	Q3 (Da)	Time (msec)	DP (v)	CE (v)	CXP (v)
JPC-2997	381.094	308.100	150.00	91.00	31.00	22.00
JPC-3211	392.078	319.200	150.00	86.00	33.00	20.00

The peak area ratios of the NH^+-adduct of JPC-2997 (product at *m*/*z* 308.10 from the parent ion at *m*/*z* 381.09) to the internal standard JPC-3211 (product at *m*/*z* 319.20 from the parent ion at *m*/*z* 392.08) were calculated for each sample from the measured peak areas obtained by selected reaction monitoring. The retention times of the Internal Standard and JPC-2997 were both 1.0 min. Quadratic regression of the concentration data (range; 0.5 to 4,000 ng/mL) with 1/concentration^2 (χ^2) weighting yielded a correlation coefficient of >0.998 for JPC-2997.

Species Matrix Comparison

Preliminary experiments comparing both fresh and frozen human blood and plasma with fresh and frozen mouse blood and plasma found no discernible difference. Fresh human blood and plasma were used to prepare standards and quality controls (QCs) for mouse and Aotus monkey studies due to its availability and suitability after pre-screening for ion suppression (matrix) effect.

Matrix Effect Screening

Fresh ACD anticoagulated blood samples were collected from six healthy subjects. Extractions were performed on all six using acetonitrile extraction methods described above. Extracted samples were injected in a LC/MS batch run while continuously infusing of 10 ng/mL JPC-2997. The resultant chromatograms were carefully examined for ion suppression effect on the continuous JPC-2997 ion signal. There was no significant ion suppression effect from any of the samples. All standards and QC samples were subsequently prepared using frozen human drug-free blood and plasma, with no ion suppression effect detected.

Quality Control (QC) Samples

A quantity of JPC-2997 separate to that used to prepare calibration standards, was weighed and the amount of base compound calculated. Methanol was added to make a stock solution of 1 mg/mL. The stock was further diluted in 50% methanol to prepare intermediate solutions with concentrations of 100, 10, 1 and 0.1 µg/mL. QC samples were prepared in drug-free human plasma and blood covering a low, mid and high range. For the *Aotus* monkey blood and mouse plasma assay, the actual QC concentrations prepared were; low (4 ng/mL), mid (100 ng/mL), and high (2,000 ng/mL). For the mouse blood assay, the actual QC concentrations prepared were; low (4 ng/mL), mid (120 ng/mL), and high (1,500 ng/mL). Aliquots of the QC samples were stored at -80ºC. The QC samples were analysed in duplicate in an identical manner to the calibration and unknown plasma samples. A quantitation run was considered invalid if more than two QC sample observed values were out by >15% of their

calculated value, or if two QC samples from the same group, low, mid or high, were out by >15%.

Inter-day precision and accuracy of JPC-2997 mouse plasma assay

JPC-2997 Nominal	Mean	SD	CV (%)		Accuracy (%)
0.5	0.48	0.02	3.80	4	96.00
1	1.02	0.06	5.43	10	102.10
2.5	2.56	0.24	9.36	5	102.56
10	10.41	0.27	2.56	5	104.14
40	38.53	1.55	4.02	5	96.32
200	195.46	4.75	2.43	5	97.73
1,000	959.08	14.23	1.48	5	95.91
4,000	4,050.00	198.50	4.90	7	101.25

The LLOQ for JPC-2997 using 50 µL of mouse plasma was 0.5 ng/mL with a coefficients of variation (CV) of 3.8%, and an inaccuracy of 4.0%. The quadratic regression equation $y = ax^2 + bx + c$ where x is the amount of drug and y is the ratio of drug area to internal standard area was used to determine the concentrations of unknowns and QC samples for JPC-2997. A typical regression equation of a calibration curve for JPC-2997 was $y = -1.304\ e\text{-}4x^2 + 1.356x + 0.0400$ with $r^2 = 0.9967$.

Inter-day precision and accuracy of JPC-2997 mouse blood assay

JPC-2997 Nominal	Mean	SD	CV (%)		Accuracy (%)
0.5	0.50	0.02	3.05	9	100.90
1	1.02	0.03	3.26	7	98.45
2.5	2.55	0.12	4.59	5	98.12
10	9.90	0.35	3.51	5	101.03
40	39.72	1.38	3.47	5	100.69
200	196.98	7.78	3.95	5	101.53
500	501.80	18.70	3.73	4	99.64
2000	2000.50	31.98	1.60	10	99.98

The LLOQ for JPC-2997 using 50 µL of mouse blood was 0.5 ng/mL with a CV of 3.1% and an inaccuracy of 0.9%. A typical regression equation of a calibration curve for JPC-2997 was $y = -1.419\ e\text{-}4x^2 + 1.161x + 0.0153$ with $r^2 = 0.9992$.

Inter-day precision and accuracy of JPC-2997 Aotus monkey blood assay

JPC-2997 Nominal Concentration (ng/mL)	Mean	SD	CV (%)	*n*	Accuracy (%)
1	1.01	0.05	4.76	7	101.29
2.5	2.41	0.16	6.83	6	96.47
10	9.72	0.57	5.81	6	97.23
40	41.43	3.64	8.80	6	103.56
200	196.35	10.54	5.37	6	98.18
500	519.13	23.66	4.56	6	103.83
2,000	1,984.21	87.65	4.42	7	99.21

The LLOQ for JPC-2997 using 50 µL of *Aotus* monkey blood was 1 ng/mL with a CV of 4.8%, and an inaccuracy of 1.3%. A typical regression equation of a calibration curve for JPC-2997 was $y = -6.317\ e\text{-}6x^2 + 0.112x + 0.0131$ with $r^2 = 0.9969$.

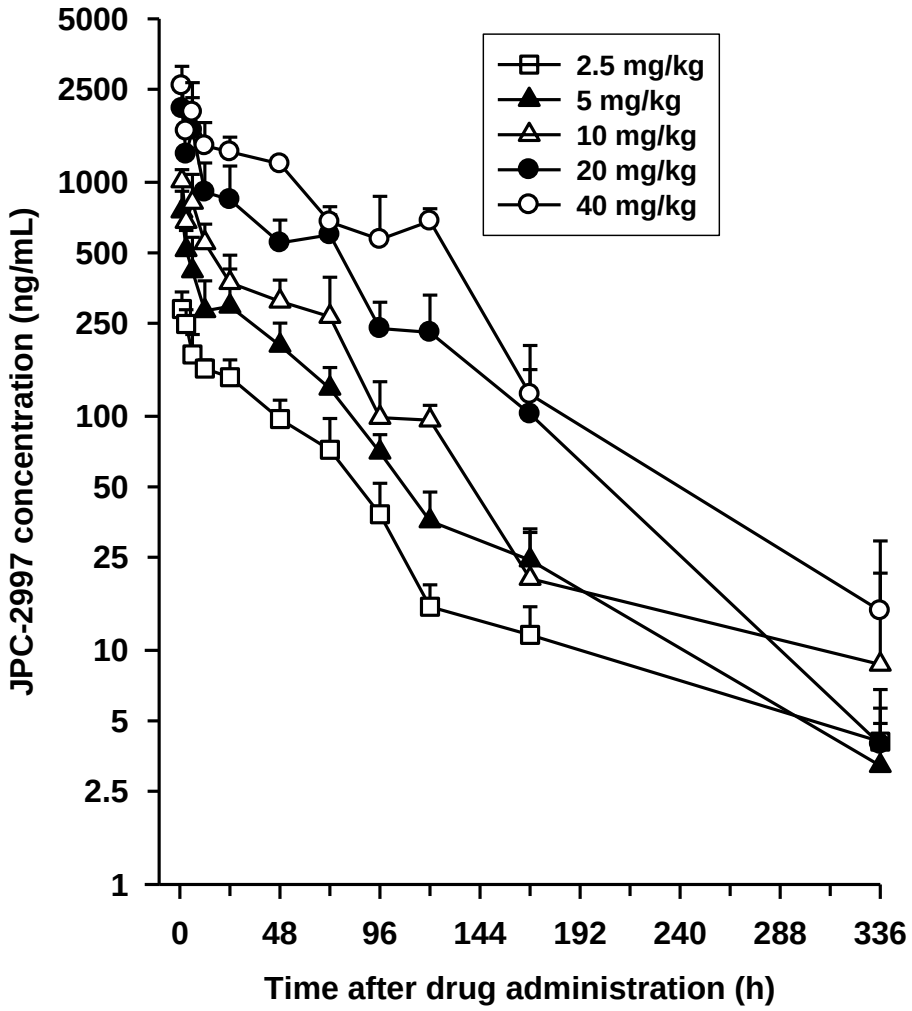

FIG. S1 Mean (SD) plasma concentration-time profile of JPC-2997 in healthy mice following escalating single oral doses (2.5 mg to 40 mg/kg) of JPC-2997. Each data point represents the mean of plasma JPC-2997 concentrations from 5 mice.

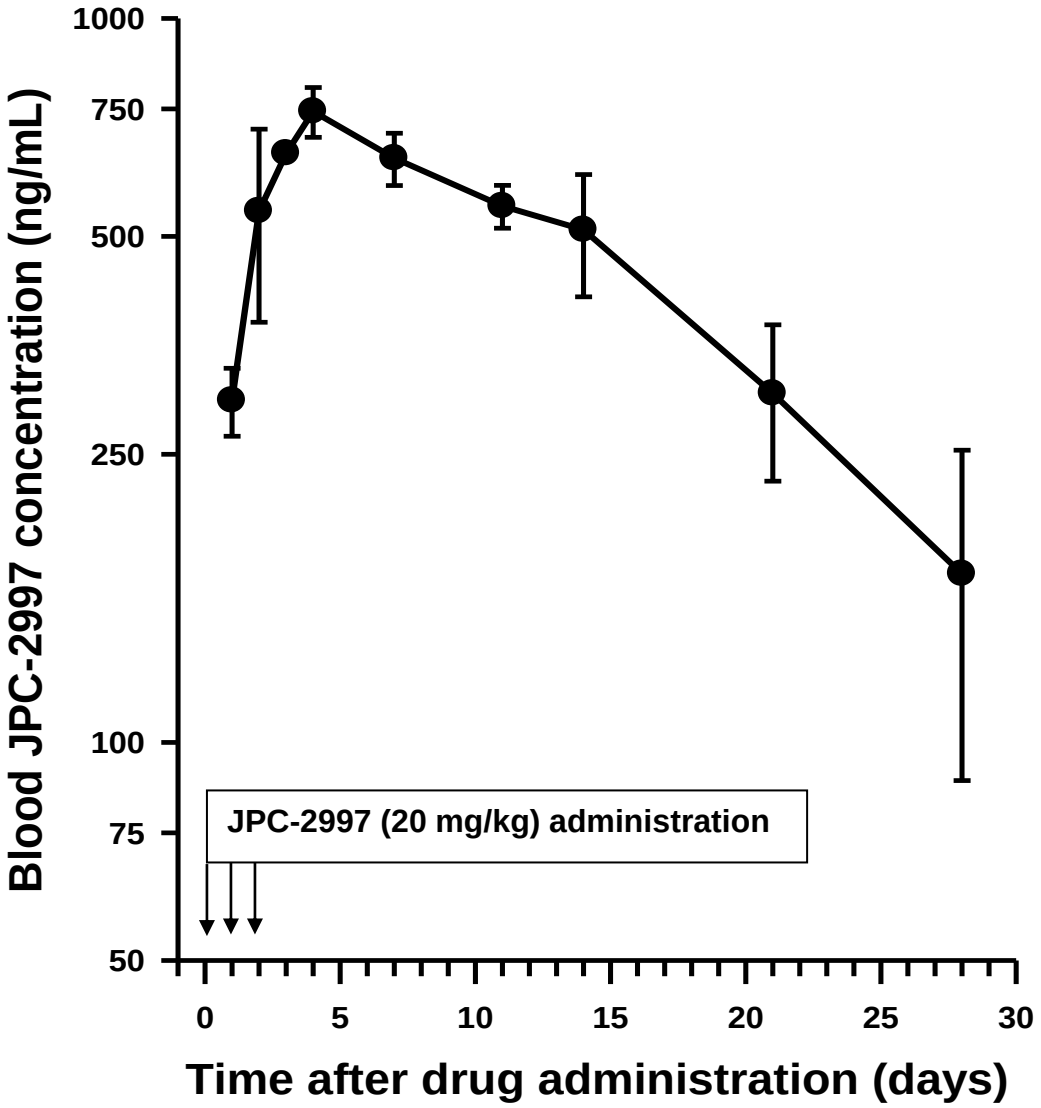

FIG. S2 Mean (±SD) blood JPC-2997 concentration-time profile in *Aotus* monkeys following oral administration of 20 mg/kg JPC-2997 daily for 3 days at 24 h intervals for the treatment of the *Plasmodium vivax* AMRU1 strain. Heparinized blood samples (20 µL) were collected pre-dose on day 0 (i.e. immediately before JPC-2997 administration), on days 1 and 2 before the next dose, and then on days 3, 4, 7, 11, 14, 21, and 28 at the same time of day after starting treatment.