Supporting information

Synthesis, Antiplasmodial Activity and β-hematin Inhibition of Hydroxypyridone -Chloroquine Hybrids

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1) General Methods of Synthesis

All reagents used in the synthesis were purchased from Sigma Aldrich as analytical grade reagents and were used without further purification. The solvents used were of AR quality. Column chromatography was performed using Merck Kieselgel 60: 70-230. Reactions were monitored by thin layer chromatography (TLC) using Merck F_{254} aluminium backed silica gel 60 pre-coated plates. Detection and visualization of the spots was by ultra violet light (254/366 nm), charring, 0.125 M ferric ammonium sulfate or 2% ninhydrin in methanol. All biologically tested

compounds attained a purity level of 95% or above by HPLC. The HPLC purity analysis was performed on a SPECTRA SYSTEM apparatus with XBridge columns (USA) C18, 5µ*m*, 4.6 mm x 150 mm 100A analytical column respectively. Eluents were acetonitrile and water, beginning with ratio of 10:90 (v/v) and reaching 100:0 (v/v) after a period of 15min. (flux rate, 20 or 10mL/min). Melting points were determined using a Reichert-Jung Thermovar hot stage microscope and are uncorrected. ^{1}H NMR spectra were recorded using a Varian Mercury spectrometer (300MHz) or on a Bruker spectrometer (400MHz). All spectra were recorded in deuterated chloroform or dimethylsulfate or methanol with tetramethylsilane as an internal standard. ^{13}C NMR was recorded with the same instruments at 75MHz or 100MHz. Chemical shifts (δ) are reported in parts per million (ppm) downfield from the internal standard tetramethylsilane (TMS). The following abbreviations are used to describe resonances in ^{1}H NMR spectra: br, broad; s, singlet; d, doublet; dd, doublet; q, quartet; m, multiplet; t, triplet. High-resolution Mass spectrometry (TOF MS ES or ESI) was performed using a Waters API Q-TOF Ultima at Stellenbosch University, Cape Town. Low-resolution mass spectrometry (EI+) was performed on a JOEL GC mate III instrument at the University of Cape Town. Microanalyses were performed on a Fisons EA 1108; C, H, N, S instrument. Infrared spectra were recorded on a Thermo Nicolete FT-IR instrument in the 4000-300 cm^{-1} range using KBr discs.

*2) Synthesis of Compounds **2d**, **3d** and **4***

2-methyl -3-(benzyloxy)-4-pyranone (benzylmaltol) 1a. NaOH (5ml; 10M) was added to a solution of 3-hydroxy-2-methylpyranone (maltol) (5.00g; 39.70mmol) in MeOH (38ml), followed by drop wise addition of benzylchloride (6.70g; 52mmol). The mixture was refluxed

for 22h at 92^{o}C. Complete removal of the solvent under reduced pressure yielded a residue, which was mixed with water (80mL) then extracted into CH_2Cl_2 (80mL×3). The combined organic extracts were washed with 5% (aq) NaOH (80ml×2) followed by water (80ml ×1). The extract was dried over anhydrous Na_2SO_4 and concentrated to give **1a** as orange oil: (9.45g, 69%). R_f (10% MeOH/CH_2Cl_2) 0.64; δ_H (300MHz, $CDCl_3$) 7.58 (1H, *d, J* 4.8, H-6), 6.36 (1H, *d,* 5.0 H-5), 7.27-7.25 (2H, *m*, H- 4', 6'), 7.42-7.30 (3H, *m*, H-3',5',7'), 5.16 (2H, *s*, H-1'), 2.09 (3H, *s*, CH_3-2); δ_c (75MHz, $CDCl_3$) 174.9, 159.5, 153.3, 143.7, 136.8, 128.9, 128.6 (2C), 128.3, 128.2, 117.0, 73.4, 14.6;

1-ethyl-2-methyl-3-(benzyloxy)-4(1H)-pyridinone 1b. The compound was prepared as **1a** above. orange oil: (10.335g, 80%), δ_H (400MHz, $CDCl_3$) 7.6 (1H, *d, J* 5.6, H-6), 7.39 (2H, *dd, J* 2, 7.8 H-4", 6"), 7.35-7.31 (3H, *m*, H-3", 5", 7"), 6.3 (1H, *d, J* 5.6, H-5), 5.2 (2H, *s*, H-1"), 2.5 (2H, *q, J* 7.6 CH_2-2), 0.99 (3H, *t, J* 7.6, CH_3-2); δ_C (100MHz, $CDCl_3$) 176, 164, 144, 138, 129 (2C) 128.4 (2C), 128.3 (2C), 117, 73, 21.8, 10.9.

Procedure 1: Synthesis of 1-methyl-2-methyl-3-(benzyloxy)-4(1H)-pyridinone hydrochloride 2a. 40% (aq) Methylamine (0.8ml; 9.00mmol) was added to a solution of benzylmaltol (1.25g; 5.80mmol) in 50% (aq) EtOH (20ml) followed by addition of a catalytic amount of 2M NaOH (0.05ml; 0.01mmol). The mixture was then refluxed for 18h at 105^{o}C. The pH of the resultant mixture was adjusted to 7 using NaOH (10M) then extracted into CH_2Cl_2 (50ml×3). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The oil obtained was dissolved in 35ml EtOH/HCl (1:1)), concentrated then recrystallized from EtOH/Et_2O. A similar procedure was used to prepare compounds **2b-d**

1,2-Dimethyl-3-(benzyloxy)-4(*1H*)-pyridinone hydrochloride 2a. White solid (793mg, 48%); m.p. 190-192; R_f (10% MeOH/CH_2Cl_2) 0.32; V_{max} (KBr) cm^{-1} 3448 (H_2O), 3280 (NH) 1628 (C=O), 843 (py C-H); δ_H (300MHz, $CDCl_3$) 7.38 (2H, *dd, J* 2.1, 7.3, H-4", 6"), 7.3-7.2 (3H, *m*, H-3", 5", 7") 7.14 (1H, *d, J* 7.5, H-6), 6.34 (1H, *d, J* 7.4, H-5), 5.16 (2H, *s*, H-1"), 3.48 (3H, *s*, NC**H**$_3$), 2.07 (3H, *s*, CH_3-2); Anal.Calcd (found) for $C_{14}H_{16}ClNO_2.0.5H_2O$: C, 61.2 (62.9); H, 6.24(5.8); N 5.1(5.1).

Octyl-2-methyl-3(benzyloxy)-4(*1H*)-pyridinone hydrochloride 2d. The compound was prepared according to general procedure 1 to give **2d** as colourless crystals: (630mg, 28%); m.p. 55-57^oC; R_f (5%MeOH/CH_2Cl_2) 0.36; δ_H (400MHz, $CDCl_3$) 8.38 (1H, *d, J* 7.02, H-6), 7.99 (1H, *d, J* 7.06, H-5), 7.36-7.31(5H, *m*, H-benzyl), 5.18 (2H, *s*, H-1"), 4.28(2H, *t, J 7.5*, H-1'), 2.38(3H, *s*, H-2), 1.8-1.67(2H, *m*, H-2'), 1.37-1.18(10H, *m*, H-3'- 7'), 0.87(3H, *t, J* 6.75, H-8'); δ_C (100MHz, $CDCl_3$) 164.7, 147.9, 143.4, 141, 135.8, 129.1, 128.8 (2C), 128.6 (2C), 113.5, 74.9, 56.95, 31.6, 30.5, 29.0, 28.9, 26.2, 22.5, 14, 13.3; Anal.Calcd (found) for $C_{21}H_{30}ClNO_2.H_2O$: C 67.63 (67.48); H 8.38 (8.16); N 3.76 (3.79).

Procedure 2: Hydrogenolysis of benzylprotected 3, 4-hydroxypyridinones A solution of compound **2a** (155mg, 0.57mmol) was dissolved in 90% (aq) EtOH and transferred into a 250ml hydrogenation flask. An 8g Pd/C (10%w/w) suspension in 90% EtOH (aq) was then added. The pH was adjusted to 1 (conc. HCl) before hydrogenation at 4bars (58psi) for 4hrs. Afterwards the mixture was filtered, concentrated and recrystalized from EtOH/Et_2O to give a white powder **3a.** A similar method was applied in the deprotection of the other *N*-alkyl-3, 4-HPOs (**3b - d**).

1,2-Dimethy-3-hydroxy-4-(*1H*)-pyridinone hydrochloride 3a. White solid (84mg, 75%); m.p. 163-165^{o}C; R_f (10%MeOH/CH_2Cl_2) 0.25; V_{max} (KBr) cm^{-1} 3448 (H_2O), 3300 (OH), 3287 (NH), 1632 (C=O), 809 (py C-H); δ_H (300MHz, $CDCl_3$) 8.21 (1H, *d, J* 6.95, H-6), 7.32(1H, *d, J* 6.95, H-5), 3.99(3H, *s,* NCH_3), 2.5(3H, *s,* CH_3-2); δ_C (75MHz, $CDCl_3$) 158.6, 142.5, 141.9, 138.3, 110.4, 43.7, 12.6. Anal. Calcd (found) for $C_7H_{10}ClNO_2.H_2O$: C 43.42(43.17); H 6.25(5.54); N 7.24 (7.03).

Octyl-2-methyl-3hydroxy-4(1H)-pyridinone hydrochloride 3d. The sample was prepared according to general procedure 2 to give 3d a white crystals: (155mg, 69%) from EtOH/Et_2O; m.p. 55-57^{o}C; R_f (50%MeOH/EtOAc) 0.73; δ_H (300MHz, CD_3OD) 8.15 (1H, *d, J* 6.9, H-6), 7.1 (1H, *d, J* 6.9 H-5), 4.80 (br.s H_2O), 4.37(2H,*t, J 7.5*, H-1'), 2.6(3H, *s*, CH_3-Py), 1.86 (2H, *sextet*, *J* 7.8 H-7'), 1.36 (10H, *m*, H-2'- 6'), 0.9(3H, *t, J* 6.9, H-8'); δ_C (75MHz, CD_3OD) 159.6, 145, 143, 139, 111, 57, 32.8, 31.3, 30.2, 30.1, 27.3, 23.6, 14.4, 12. Anal. Calcd. (found) for $C_{14}H_{24}ClNO_2.1H_2O$: C 57.62 (57.26); H 8.98 (8.97); 4.80 (3.34).

Tris (3-hydroxy-1,2-dimethyl-4-pyridinonato) gallium (III) 4. An aqueous solution of $Ga(NO)_3.9H_2O$ (27.9mg, 0.067mmol) was added to a solution of **3d**, (58.3mg, 0.2mmol) in MeOH (1ml) while stirring for 3min. The pH was adjusted to 8 (0.1M NaOH), before refluxing (4h at 80^{o}C). Evaporation to dryness under reduced pressure gave a pink resin, which was dissolved in 1:1 H_2O/MeOH and purified by gel filtration on Sephadex LH 20 with MeOH as the mobile phase. The homogeneity of the fractions was monitored on TLC and visualised by use of ferric ammonium sulphate. Combined fractions were evaporated to dryness to give the target complex **4** as a pink resin: (73.5mg, 96%) R_f (50%EtOAc/MeOH) 0.83; δ_H (300MHz, CD_3OD) 7.51 (1H, d, *J* 6.6, H-6), 6.51 (1H, *d, J* 6.6 H-5), 4.1 (2H, *t, J* 7.4, H-1'), 2.4 (3H, *s*, CH_3-2), 1.76

(2H, *m*, H-2'), 1.33 (10H, *m*, H-3'- 7'), 0.9 (3H, *t, J* 6.9, H-8'); δ_C (75MHz, CD_3OD) 168.6, 154, 134.8, 133, 108.6, 56.4, 32.9, 31.9, 30.3, 27.4, 23.7, 14.4 (2C), 12.; MS 541.27(ML_2), 778.46 (ML_3+H), Anal. Calcd. (found) for $C_{42}H_{66}GaN_3O_6H_2O$ [ML_3H_2O] C 63.62 (63.10); H 8.82 (8.20); N 5.18 (4.85).

3) Synthesis of Compounds ***7a***, ***7g***, ***7h***, ***8c***, ***8d*** *and* ***8d.2HCl***

Procedure 3: Synthesis of *N*-(7-chloro-4-quinolinyl)-diaminoalkanes. A mixture of 1,3-diaminopropane (3.7g, 50mmol) and 4,7-dichloroquinoline (2.293g, 11.6mmol) was heated to 80^{o}C for 1.5hrs under nitrogen atmosphere and subsequently at 145^{o}C for 4hrs with continuous stirring. After cooling, 2M NaOH (10ml) was added and stirred to precipitate the product, which was washed with water before drying to give **6b.** Analogous procedures were used to prepare compounds **6a**, **6c** and **6d**.

***N*-(7-chloro-4-quinolyl)-1,3-diaminopropane 6b**. A white solid: (2.83g, 90%); R_f (20%MeOH/ EtOAc) 0.15; V_{max} (KBr) cm^{-1} 3279w (NH), 1575s (C=N), 1532m (C=C), δ_H (400MHz, DMSO) 8.38, (1H, *d, J* 5.2, H-2'), 8.3(1H, *d, J* 8.8, H-5'), 7.7 (1H, *d, J* 2.4, H-8'), 7.38 (2H, *dd, J* 2.4, 9.0, H-6'), 6.47 (1H, *d, J* 5.6, H-3'), 3.36 (2H, *t, J* 6.8, H-1) 1.88 (2H, *t, J* 6.8, H-3), 1.6 (2H, *quintet, J* 6.8, H-2).

***N*-(7-chloro-4-quinolyl)-1,6-diaminohexane 6d.** The sample was prepared according to general procedure 3 to give **6d** as a white solid: (3.823g, 92%), $\delta_{H;}$ (400MHz, DMSO) 8.37 (1H, *d, J* 5.6, H-2'), 8.25 (1H, *d, J* 8.8, H-5'), 7.7 (1H, *d, J* 2.0, H-8'), 7.4 (1H, *dd, J* 2.4, 9.0, H-6'), 6.4 (1H, *d, J* 5.2, H-3'), 3.2 (2H, *q, J* 6.0, H-1), 1.67 (2H, *quintet, J* 6.4, H-6), 1.4 (8H, *m*, H-2,3,4,5); δ_C (100MHz, DMSO) 152.5, 150.9, 149.9, 134, 128.2, 124.7, 124.6, 118, 99.3, 43.2, 28.6, 27.2, 27.1, 26.9 (2C).

Procedure 4: Synthesis of N-(7-chloro-4-quinolinyl)-aminoalkyl-3-benzyloxy-2-alkyl-4 (1H)-pyridinones.. Ethylbenzylmaltol (**1b**), (552mg, 2.4mmol) and *N*-(7-chloro-4-quinolyl)-1,2-diaminobutane (**6c**, 1.0g, 3.7mmol) were dissolved in ethanol. Water (25ml) was added to the mixture to obtain a 50% aqueous EtOH solution. The pH of the solution was adjusted to 13 (2M NaOH) before refluxing at 110^{o}C for 24hrs. Afterwards the pH was adjusted to 1 (2M HCl) before washing with diethylether (50mlx2). On adjustment of the pH to 7 (2M NaOH), a yellow precipitate formed which was filtered and washed with water and dried under vacuum to give 425mg (36%) of the pure compound **8c**. Analogous procedures were used for preparation of the compounds **7a - d** and **8a - d**. It is important to note that most of the hybrid compounds had water of hydration as seen from proton NMR (singlets appearing at 1.40-1.50 for $CDCl_3$; 3.80-3.20 for $(CD_3)_2SO$ and 4.90 -4.81 for CD_3OD) and elemental analyses calculations.

3-(benzyloxy)-1-(2-(7-chloroquinolin-4-lyamino)ethyl)-2-methylpyridin-4(1H)-one 7a. The sample was prepared according to general procedure 4 to give **7a** as bright yellow crystals; (366mg, 93%), from EtOH/EtOAc; mp. 151-152 ^{0}C. R_f (50%MeOH/ CH_2Cl_2) 0.5; V_{max} (KBr) cm^{-1} 3448w (H_2O), 3280w (NH) 1628s (C=O), 1624s (C=N), 1600s (C=N), 1491m (C=C), 822m (py C-H); δ_H (400MHz, DMSO) 8.77 (1H, *d, J* 9.0, H-5"), 8.6 (1H, *d, J* 7.2, H-2"), 8.34 (1H, *d, J*

7.2, H-6), 8.16 (1H, *d*, *J* 2.0, H-8"), 7.65 (1H, *dd*, *J* 2.0, 9.0, H-6"), 7.45-7.28 (5H, *m*, H-benzyl), 7.27 (1H, *d*, *J* 7.27, H-5), 6.99 (1H, *d*, *J* 7.2, H-3"), 4.92 (2H, *s*, CH_2-Bn), 4.7 (2H, *t*, *J* 7.2, H-1'), 4.04 (2H, *q*, *J*, 7.2, H-2'), 2.5 (3H, *s*, CH_3-2); δ_C(100MHz, DMSO) 164, 156.9, 151.8 (2C), 139.5, 128.1 (2C), 128 (2C), 127.6, 127.5 (2C), 124.3, 124 (2C), 123.8, 123.6, 115.99, 110, 98.7, 71.8, 70.1 (2C), 21.6; HRMS m/z calculated for $C_{24}H_{23}N_3O_2Cl$ [M+H] 420.1490 found 420.1479.

3-(benzyloxy)-1-(4-(7-chloroquinolin-4-lyamino)butyl)-2-ethylpyridin-4(1H)-one 8c.

The sample was prepared according to general procedure 4 to give **8c** as a bright yellow powder: (403mg, 40%); Mp 112-116^{o}C R_f (Et_3N/MeOH/ CH_2Cl_2 ; 0.1:2:8) 0.36; V_{max} (KBr) cm^{-1} 3392w (H_2O), 3226w (NH) 1613s (C=O), 1575s (C=N), 1540m (C=C), 809m (py C-H); δ_H (400MHz, DMSO) 8.6 (1H, *d*, *J* 8.8, H-5"), 8.47 (1H, *d*, *J* 6.8, H-2"), 7.96 (1H, *d*, *J* 2, H-8"), 7.64. (1H, *d*, *J* 7.2 H-6), 7.63. (1H, *dd*, *J* 2, 9.2, H-6"),7.5-7.4 (5H, *m*, H- benzyl), 6.73 (1H, *d*, *J* 6.4, H-3"), 6.15 (1H, *d*, *J* 7.2, H-5), 5.1 (2H, *s*, C**H**$_2$-Bn), 3.92 (2H, *t*, *J* 6.4, H-1'), 3.46 (2H, *m*, H-4'), 2.9 (2H, *q*, *J* 7.2, CH_3C**H**$_2$ Py), 1.78-1.68 (4H, *m*, H-2', 3') 0.98 (3H, *t* , *J* 7.2, CH_2C**H**$_3$-2); δ_C (100MHz, DMSO) 174, 146, 145, 140, 137 (2C), 128.9, 128.84 (2C), 128.1 (2C), 128.3(2C), 126.6, 126.5, 126, 122, 116.9, 99 (2C), 72.4, 52.5, 43.3, 29.1, 25, 19.6, 14.1; HRMS *m/z* calculated for $C_{27}H_{29}N_3O_2Cl$ [M+H] 462.1948 found 462.1951; Anal. Calcd. (found) for C 65.12 (65.86), H 6.48 (6.35), N 8.44 (8.65) $C_{27}H_{28}ClN_3O_2$ $2H_2O$.

***N*-(7-chloro-4-quinolyl)-1-(6-aminohexyl)-3-(benzyloxy)-2-ethyl-4(1H)-pyridinone 8d.** The sample was prepared according to general procedure 4 to give **8d** a yellow powder (360mg, 34%); Mp 168-170^{o}C R_f (Et_3N/MeOH/ CH_2Cl_2 ; 0.1:2:8) 0.65; V_{max} (KBr) cm^{-1} 3302w (H_2O),

3103w (NH) 1609s (C=O), 1575s (C=N), 1532m (C=C), 870m (py C-H); δ_H (300MHz, $CDCl_3$) 8.47 (1H, *d, J* 6, H-2"), 7.92 (1H, *d, J* 3, H-8"), 7.78 (1H, *d, J* 9, H-5"), 7.41-7.13 (6H, *m*, H-benzyl, H-6"), 7.14 (1H, *d, J* 6, H-6), 6.38 (1H, *d, J* 6, H- 3"), 6.34 (1H, *d, J* 6, H- 5), 5.24 (2H, *s*, CH_2-Bn), 3.73 (2H, *t, J* 6, H-1'), 3.28 (2H, *m*, H-6'), 2.56 (2H, *q, J* 7.5, $CH_3C\mathbf{H}_2$-2), 1.78- 1.62 (4H, *m*, H-2', 5') 1.53 – 1.3 (4H, *m*, H-3',4'), 1.41-1.22 (2H, *m*, H-3', 4'), 1.01 (3H, *t, J* 7.5, $CH_2C\mathbf{H}_3$-2) δ_C (75MHz, $CDCl_3$) 173.5, 151.5, 150, 148.7, 145.9, 145.6, 138.1, 137.7, 134.9, 128.5(2C), 128.2 (2C), 127.9, 125, 121.4, 117.4, 117.2, 98.8 (2C), 72.8, 53.1, 43, 31.2, 29.6, 28.5, 26.7, 19.5, 13.3; HRMS *m/z* calculated for $C_{29}H_{33}N_3O_2Cl$ [M+H] 490.2261 found 490.2265.

Dihydrogenchloride salt of 8d (**8d.2HCl**) 0.6ml of 1.25 M HCl (0.75mmol) in MeOH solution was added to **8d** (101mg, 0.207mmol) and stirred overnight in a sealed flask. Ethyl acetate was added until precipitation was complete, solvent was removed and the white solid dried under vacuum for 2hrs to give the dry salt *N*-(7-chloro-4-quinolyl)-1-(6-aminohexyl)-3-(benzyloxy)-2-ethyl-4(1H)-pyridinone dihydrogenchloride) as a yellow deliquescent solid: (550mg, 86%) ; δ_H (300MHz, CD_3OD) 8.48 (1H, *d, J* 8.8, H-5"), 8.38 (2H, *m*, H-2", 6), 7.88 (1H, *d, J* 1.5, H-8"), 7.65 (1H, *dd, J* 2, 9.2, H-6"), 7.60 (5H, *m*, H-benzyl), 7.25 (1H, *d, J* 7.2, H- 3"), 6.88 (1H, *d, J* 7.2, H- 5), 5.25 (2H, *s*, CH_2-Bn), 4.37 (2H, *t, J* 7.6, H-1'), 3.62 (2H, *t, J* 7.2, H-6'), 2.92 (2H, *q, J* 7.6, CH_3 $C\mathbf{H}_2$-2), 1.85 (4H, *m*, H-2', 5') 1.51 (4H, *m*, H-3',4'), 1.48 (3H, *t, J* 7.6, $C\mathbf{H}_3CH_2$-2), δ_C (100MHz, CD_3OD) 155.8, 148.1, 146.1(2C), 135.2, 134.2, 133.7, 131.5, 130.5, 127.9, 120.4 (2C), 120.2 (2C), 119.1, 116.8, 110.7, 107.4, 104.8, 90.3, 66.7, 47.9, 35.2, 22.7, 19.4, 17.9, 17.4, 12.1, 3.45; HR-MS m/z calculated for 490.2557 $C_{29}H_{33}Cl_3N_3O_2$. [M + H] found 492.2278; Anal. Calcd. (found) for C 56.45 (56.77), H 6.53 (6.51), N 6.81 (6.41) $C_{29}H_{34}Cl_3N_3O_2.3H_2O$.

Procedure 5A: Synthesis of *N*-(7-chloro-4-quinolinyl)-diaminoalkyl-3-hydroxy-2-alkyl-4 (*1H*)-pyridinones; deprotection by hydrogenolysis. 237mg (0.48mmol) of compound **8d** was dissolved in 30ml 2M ethanolic-HCl then subjected to hydrogenation in the presence of Pd/C 40mg (10 %w/w), 4 atmospheres pressure for 6.5h. The catalyst was filtered off and the solvent removed by rotary evaporation to give 169mg (88%) of a brown resin, **8h**. Similar procedures were used to deprotect the other compounds followed by recrystallization where possible.

***N*-(7-chloro-4-quinolyl)-1-(6-aminohexyl)-3-(hydroxy)-2-ethyl-4(1H)-pyridinone dihydrogen chloride 8h.** The sample was prepared according to general procedure 5 to give **8h** as a brown resin: (169mg, 88%); R_f (50% MeOH/CH_2Cl_2) 0.64; V_{max} (KBr) cm^{-1} 3302br.w (OH), 3103w (NH) 1624s (C=O), 1616s (C=N), 1609m (C=C), 862m (py C-H)δ_H (400MHz, CD_3OD) 8.4 (1H, *d, J* 9.3, H-5"), 8.37 (1H, *d, J* 7.2, H-2"), 8.18 (1H, *d, J* 6.9, H-6), 7.87 (1H, *d, J* 1.5 H-8"), 7.68(1H, *d, J* 8.8, H-6"), 7.1 (1H, *d, J* 7.2, H-5), 6.94 (1H, *d, J* 7.2, H-3"), 4.39 (2H, *t, J* 7.5, H-1'), 3.62 (2H, *J* 7.5, H-6'), 3.06 (2H, *q, J* 7.5, CH_3CH_2 -2), 1.97-1.79 (4H, *m,* H-2', 5'), 1.55-1.15 (4H, *m,* H-3', 4'), 1.29 (3H, *t , J* 7.5, CH_2CH_3-2); δ_C (100MHz, DMSO) 159, 156, 146.7, 143.6, 143.4, 139.1, 138.9, 138.7, 127.5 (2C), 126.2, 119.8, 111.7, 99.3, 56.1, 43.9, 31.3, 28.2, 26.7, 26.2, 20.3, 12.6; MS. *m/z* calculated for $C_{22}H_{26}N_3O_2Cl$ [M+H] 400.18 found 400.2 (100%).

Procedure 5A: Synthesis of N-(7-chloro-4-quinolinyl)-diaminoalkyl-3-hydroxy-2-alkyl-4 (1H)-pyridinones; deprotection by acid hydrolysis.

***N*-(7-chloro-4-quinolyl)-1-(4-aminoethyl)-3-(hydroxy)-2-methyl-4(1H)-pyridinone 7f**. The benzylated analogue **7b**, (45mg 0.104mmol) was dissolved in a solvent mixture (water, conc.

HCl, EtOH, 1:3:2) and the resultant slurry refluxed at 74^{o}C, for 12hrs. Solvent removal by evaporation in vacuo and further drying in vacuum gave **7f** as a bright orange powder: (25mg, 50%); mp. 143-146 ^{0}C; R_f 0.24 (MeOH/ CH_2Cl_2 1:1), V_{max} (KBr) cm^{-1} 3422br.w (OH), 3226w (NH) 1636s (C=O), 1613s (C=N), 1590m (C=C), 759m (py C-H); δH (CD_3OD, 400MHz) 8.5 (1H, *d, J* 9.2, H-5"), 8.45 (1H, *d, J* 6.8, H-2"), 8.31 (1H, *d, J* 6.4, H-6), 7.9 (1H, *d, J* 2, H-8"), 7.71 (1H, *dd, J* 1.6, 9.0, H-6"), 7.1 (1H, *d, J* 6.4, H-3"), 6.99 (1H, *d, J* 6.4, H-5), 4.6 (2H, *t, J* 5.6, H-3'), 3.79 (2H, *t, J* 6.4, H-1') 2.66 (3H, *s,* CH_3-2), 2.39 (2H, *m,* H-2').; δ_C (CD_3OD, 100MHz), 158, 156, 142, 140 (2C), 136, 126 (2C), 123.6 (2C), 117.6, 109, 114, 97.3, 54, 39, 26.9, 10.1; MS. *m/z* calculated for $C_{18}H_{18}N_3O_2Cl$ [M+H] 344.11 found 344.1 (100%).

***N-(*7-chloro-4-quinolyl)-1-(4-aminobutyl)-3-(hydroxy)-2-methyl-4(1H)-pyridinone dihydrogen chloride 7g.**

The sample was prepared according to general procedure 5 to give **7g** as brown crystals: (100mg, 70%) from hot Et2O; R_f (50% MeOH/ CH_2Cl_2) 0.3; V_{max} (KBr) cm^{-1} 3399br.w (OH), 3239w (NH) 1632s (C=O), 1609s (C=N), 1577m (C=C), 813m (py C-H); δ_H (400MHz, DMSO) 9.9 (1H, *m,* NH), 8.87 (1H, *d, J* 9.3, H-5"), 8.5 (1H, *d, J* 7.2, H-2"), 8.36 (1H, *d, J* 6.9, H-6), 8.1 (1H, *d, J* 2.1, H-8"), 7.7 (1H, *dd, J* 1.8, 9.0, H-6"), 7.34 (1H, *d, J* 6.9, H-5), 6.86 (1H, *d, J* 6.9, H-3"), 4.43 (2H, *t, J* 7.2, H-1'), 3.58 (2H, *q, J* 6, H-4'), 2.5 (3H, *s,* C**H$_3$**-2) 1.92-1.77 (4H, *m,* H-2', 3'); δ_C (100MHz, DMSO) 159, 156, 143.6, 143.3 142, 139, 138.8, 127 (2C), 126.9, 119, 116, 111, 99, 70.8, 56, 42.9, 24.8, 13.2.; MS. *m/z* calculated for $C_{19}H_{21}N_3O_2Cl$ [M+H] 358.13 found 358.1 (100%).

***N*-(7-chloro-4-quinolyl)-1-(6-aminohexyl)-3-(hydroxy)-2-methyl-4(1H)-pyridinone dihydrogen chloride 7h.**

The sample was prepared according to general procedure 5 to give **7h** as a white powder: (179mg, 90%), from hot Et_2O; Mp 134-138^oC R_f (50% MeOH/ CH_2Cl_2) 0.61; V_{max} (KBr) cm^{-1} 3425br.w (OH), 3226w (NH) 1632s (C=O), 1613s (C=N), 1586m (C=C), 816m (py C-H); δ_H (400MHz, CD_3OD) 8.48 (1H, *d, J* 9.2, H-5"), 8.37 (1H, *d, J* 7.2, H-2"), 8.2 (1H, *d, J* 6.8, H-6), 7.88 (1H, *d, J* 2, H-8"), 7.66 (1H, *dd, J* 2.0, 9.2 H-6"), 7.1 (1H, *d, J* 6.8, H-5), 6.88 (1H, *d, J* 7.2 H-3"), 4.4 (2H, *t, J* 8.0, H-1'), 3.6 (2H, *t, J* 7.2, H-6'), 2.6 (3H, *s,* C**H**$_3$-2), 1.9 – 1.8 (4H, *m,* H-2',5') 1.56 – 1.48 (4H, *m,* H-3', 4'); δ_C (100MHz, CD_3OD) 158.3, 156.4, 143.9, 142.5, 142, 138.8, 138, 127.4 (2C), 125, 119.8, 115.7, 110.7, 98.6, 65.7, 56.7, 43.6, 30, 27.7, 25.8, 11.6.; MS. *m/z* calculated for $C_{21}H_{25}N_3O_2Cl$ [M+H] 386.16 found 386.0 (100%); Anal. Calcd. (found) for C54.97 (54.87), H 5.71 (5.94), N 9.16 (9.35), $C_{21}H_{26}Cl_3N_3O_2$.

*4) Synthesis of Compound **9b***

Procedure 6: Synthesis of pyranone intermediates

KOH (2.5M) was added to a solution of ethylmaltol (7.0g, 50mmol) in acetone (20ml) followed by methyliodide (7.76g, 55mmol) drop wise. The solvent was removed under pressure after 12hrs and product extracted into CH_2Cl_2. The organic extract was dried over anhydrous $MgSO_4$ then concentrated to give **5a.** Compound **5b** was prepared using a method similar to that of **5a** but using dimethylsulphate as the methylating agent.

2- Ethyl-2-methoxy-pyranone 5a

The sample was prepared according to general procedure 6 to give **3** as a pale orange oil: (4.46g, 58%) R_f 1.6 (CH_2Cl_2) δ_H ($CDCl_3$, 300MHz), 7.61 (1H, *d, J* 5.7, H-6), 6.3 (1H, *d, J* 5.7 H-5), 3.82 (3H, *s*, 3-OCH_3), 2.66 (2H, *q*, *J* 7.5, 2- C**H**$_2$$CH_3$) 1.19 (3H, *t, J* 7.5, 2-CH_2C**H**$_3$).

2-Methyl-2-methoxy-pyranone 5b

The sample was prepared according to general procedure 6 to give **4** as orange oil: (5.7g, 41%) R_f 0.44 (10% MeOH /CH_2Cl_2) δH (CD_3OD, 300MHz), 7.95 (1H, *d, J* 5.7, H-6), 6.41 (1H, *d, J* 5.7 H-5), 3.94 (3H, *s*, OCH_3-3), 2.35 (3H, *s*, CH_3-2).

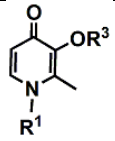
***N*-(7-chloro-4-quinolinyl)-1-(6-aminohexyl)-3-(methoxy)-2-ethyl-4(*1H*)-pyridinone 9b.** To a solution of **5a** (200mg, 1.3mmol) in EtOH was added *N*-(7-chloro-4-quinolinyl)-1,4-diaminobutane **6c**, (522mg, 1.85mmol) followed by water to make a 50% aq EtOH solution. After pH adjustment to 13 (2M NaOH) the mixture was refluxed for 24h. Removal of the solvent by evaporation, was followed by addition of an equal amount of water, pH ad*j*ustment to 1 (Concentrated HCl) and washing with Et_2O (50ml×2). Further pH adjustment to 8 resulted in precipitation of the product, which was filtered and washed with water and recrystallized from methanol/ ethylacetate. This method was used to synthesize compounds **9a**, **10a** and **10b.** Yellow powder (400mg, 70%); mp. 166 - 165 ^{0}C; R_f 0.45 (MeOH/ CH_2Cl_2 1:1), δ_H (DMSO d_6, 300MHz), 8.4 (1H, *d, J* 5.4, H-2’’), 8.26 (1H, *d, J* 9.0, H-5”), 7.78 (1H, *d, J* 2.1, H-8”), 7.58(1H, *d, J* 7.5 H-6 py), 7.44 (1H, *dd, J* 2.1, 9.0, H-6”), 7.34 (1H, *br.s*, N-H), 6.5 (1H, *d, J* 5.4, H- 3”), 6.1 (1H, *d, J*, 7.5, H-5), 3.93 (2H, t, *J*, 6.9, H-4’), 3.75 (3H, *s*, OCH_3-3), 2.65 (2H, *q, J*, 7.5, C**H**$_2$$CH_3$-2), 1.74 (6H, *m*, H-1’, 2’, 3’-H), 1.1 (3H, *t, J* 7.5, 2- CH_2C**H**$_3$); δ_C (DMSO d_6, 75MHz), 171.7, 151.1, 150.2, 148.4, 146.4, 144.7, 139.1, 133.5, 126.9, 124.0, 123.9, 117.2,

116.1, 98.6, 58.5, 51.8, 43.8, 28.4, 24.5, 18.7, 13.4; HR MS. *m/z* calculated for $C_{21}H_{23}$ Cl N_3O_2 [M-H] 384.1479 found 384.1482; Anal. Calcd (found) for $C_{21}H_{24}ClN_3O_2$ $1.5H_2O$, C 60.22 (60.15), H 6.32 (6.65), N 10.53 (10.11);

5) Biological assays

Antiplasmodial data of precursors

Table S1. Antiplasmodial activity of *N*-alkyl-3-hydroxypyridin-4-ones and a gallium(III) complex

Compd			*P. falciparum* IC_{50} (μM)[a]		
	R1	R^3	D10	3D7	K1
CQ			0.066	0.003	0.21
2a	Me	Bn	>377	>50	>87
3a	Me	H	142	49.1	114.2
2b	i-pr	Bn	>343	>35	>77
3b	i-pr	H	90.1	>77.8	>98.5
2c	n-hex	Bn	84	>98	>66
3c	n-hex	H	14.7	3.54	4.16
2d	n-oct	Bn	48	30.8	>61
3d	n-oct	H	5.49	2.38	1.8
4	n-oct	Ga^{3+}	14.9	2.37	>25.7

[a]**SE ≤ 7%, n = 3.**

Drug combination studies of 2d or 3d with chloroquine

Compounds **2d** and **3d** were combined with chloroquine diphosphate according to reported methods.[1, 2] The two compounds were chosen for combination studies based on their good activity in the K1 and 3D7 isolates. Fractional inhibitory concentrations (FIC_{50}) were used to determine their mode of interaction (synergistic, anatgoonistic or additive). The FIC_{50}s were calculated using the relationship below:

$$FIC_{50}(B_i) = \frac{IC_{50}(A_i + B_i)}{IC_{50}(B)} \times B_i$$

$$FIC_{50}(A_i) = \frac{IC_{50}(A_i + B_i)}{IC_{50}(A)} \times A_i$$

$$\Sigma FIC_{50} = FIC_{50}(A_i) + FIC_{50}(B_i)$$

Where A_i and B_i = fraction of A and B in a given combination respectively, IC_{50} (A_i+B_i) = IC_{50} of fraction combinations of two drugs A and B, IC_{50} (B) = IC_{50} of fraction containing 100% B, IC_{50} (A) = IC_{50} of fraction containing 100% A

Tables S2. *In vitro* antiplasmodial activity against *P. falciparum* and FIC_{50} for different combinations of **2d** or **3d** with chloroquine diphosphate (CQ-DP)

Table S2a. In vitro activity and FIC_{50} for different combinations of **3d** to CQ-DP

Fraction of 3d	**Fraction of CQDP**	**IC_{50} (µg/mL) against 3D7**	**IC_{50} (µg/mL) against K1**	**Σ FIC_{50} (3D7)**	**Σ FIC_{50} (K1)**
1	0	3.512	3.306	1	1
0.788	0.211	0.183	0.095	0.043	0.08
0.647	0.353	0.036	2.281	0.007	2.81
0.476	0.524	0.02	0.18	0.003	0.30
0.35	0.65	0.012	0.388	0.0015	0.78
0.261	0.739	0.015	0.009	0.0017	0.02
0.18	0.82	0.04	0.831	0.0037	2.04
0.115	0.885	0.007	0.273	0.0005	0.72
0	1	20	0.3413	1	1

Table S2b. In vitro activity and FIC_{50} for different combinations of **2d** to CQ-DP

Fraction of 2d	**Fraction of CQ-DP**	**IC_{50} (µg/mL) against 3D7**	**IC_{50} (µg/mL) against K1**	**Σ FIC_{50} (3D7)**	**Σ FIC_{50} (K1)**
1	0	20	20	1	1
0.833	0.277	0.32	2.659	11.09	2.70
0.7	0.3	0.045	1.778	1.69	1.94
0.524	0.476	0.058	1.608	3.46	2.74
0.455	0.545	0.033	3.028	2.25	5.88
0.283	0.717	0.009	0.586	0.81	1.49
0.18	0.82	0.003	0.343	0.31	0.99
0.11	0.89	0.013	0.311	1.45	0.98
0	1	0.008	0.284	1	1

Table S3. Antiplasmodial activity, resistance indices and cytotoxicity data for the hybrids[a]

	Compd.	*Antiplasmodial activity (μM)			Resistance Indices		**KB	Selective (SI)		Indices
		3D7	K1	W2	K1/3D7	W2/3D7	(μg/ml)	(μM)	KB/3D7	KB/K1
A	CQ	0.01	0.44	0.10	44.0	10.0	10.9	34.2	3420	77.7
	POD	-	-	-	-	-	0.0003	0.0007	ND	ND
B	7a	0.49	3.15	9.93	6.4	2.0	22.5	53.6	109	17
	8a	0.95	8.43	2.59	8.9	2.7	23.3	51.4	54	6.1
	7b	0.28	3.57	2.15	12.8	7.7	28.9	66.6	23.7	18.6
	8b	0.12	71.6	2.32	596	19.3	6.6	14.7	122	0.2
	7c	0.05	2.2	1.12	0.27	44.0	53.2	118.8	2376	54.0
	8c	0.004	0.13	0.10	32.5	25	4.16	9	2250	69.2
	7d	35.70	4.38	0.39	0.25	0.12	75.3	158.2	4.43	36.1
	8d	0.01	0.08	0.02	11.4	2.1	1.86	3.8	380	47.5
	8d.2HCl	0.01	0.1	ND	12.5	ND	3.27	6.7	670	67
C	7e	10.80	32.6	0.28	3.0	0.026	ND	ND	ND	ND
	8e	0.40	0.89	1.24	2.2	3.1	ND	ND	ND	ND
	7f	ND	ND	ND	ND	ND	ND	ND	ND	ND
	8f	0.12	0.38	0.54	3.2	4.5	19.5	54.5	454	143
	7g	0.03	0.07	0.08	0.66	2.3	66.3	185	6166	2642
	8g	0.05	0.27	0.16	5.4	3.2	28.3	76.1	1522	281
	7h	0.390	0.16	0.07	0.47	0.4	1.38	3.58	9.18	22.3
	8h	0.08	0.61	0.09	7.7	1.1	17.7	44.3	553	72.6
D	10a	0.13	2.8	0.09	-	-	18.6	46.7	359	16.6
	9a	0.07	0.22	0.76	-	-	10.3	27.8	397	126
	10b	ND	ND	0.22	-	-	ND	ND	ND	ND
	9b	ND	ND	0.21	-	-	ND	ND	ND	ND

IC_{50} (μM) antiplasmodial activity against *P. falciparum*, ** IC_{50} represent the molar equivalents of test compound relative to β-haematin required to inhibit β-haematin formation by 50%; A= controls; B= benzylated analogues, C= deprotected analogues, D = methoxy analogues, ND = not determined. [a]SE ≤ 7%, n = 3

.

Correlations

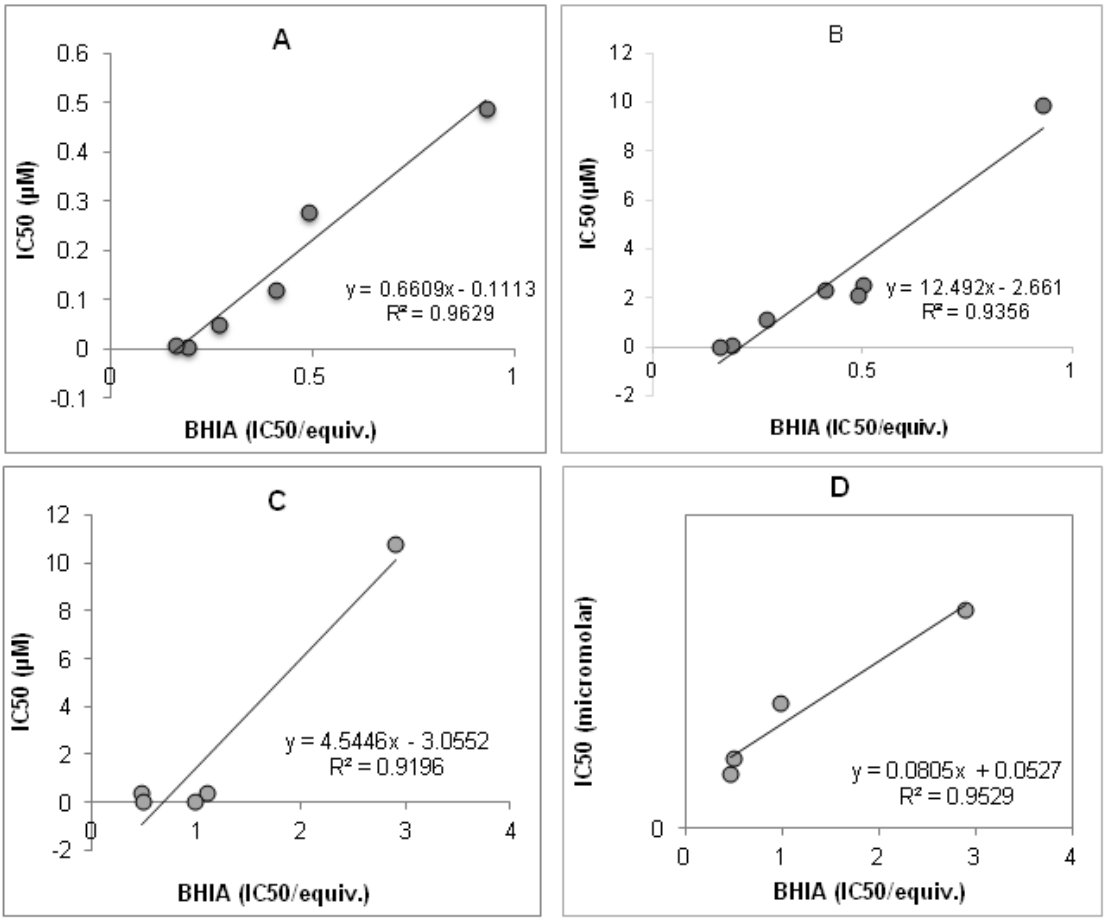
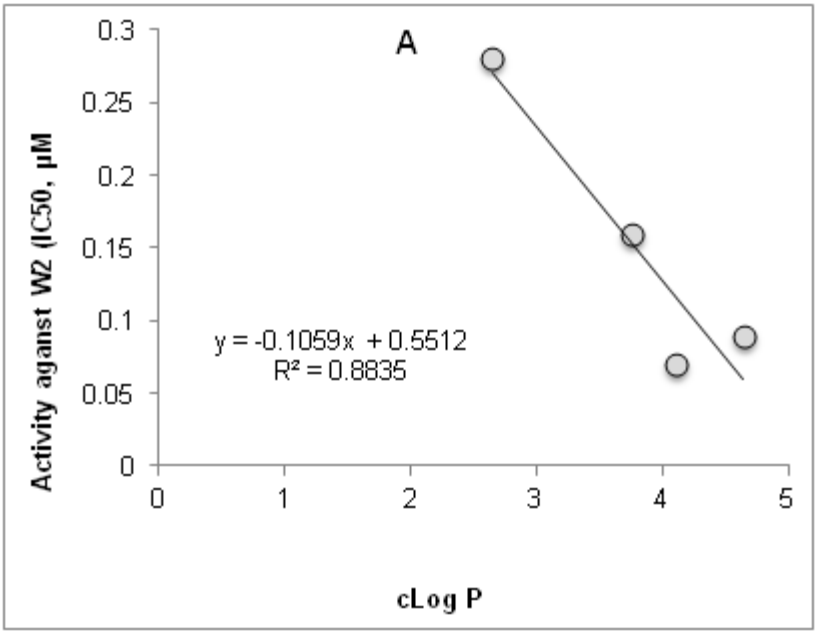


Figure S1: Correlation between β-haematin inhibition activity and antiplasmodial activity for the benzylated series [**A**]- against 3D7 and [**B**]- against W2 strains and deprotected analogues[**C**] - against 3D7 and [**D**]- against W2.



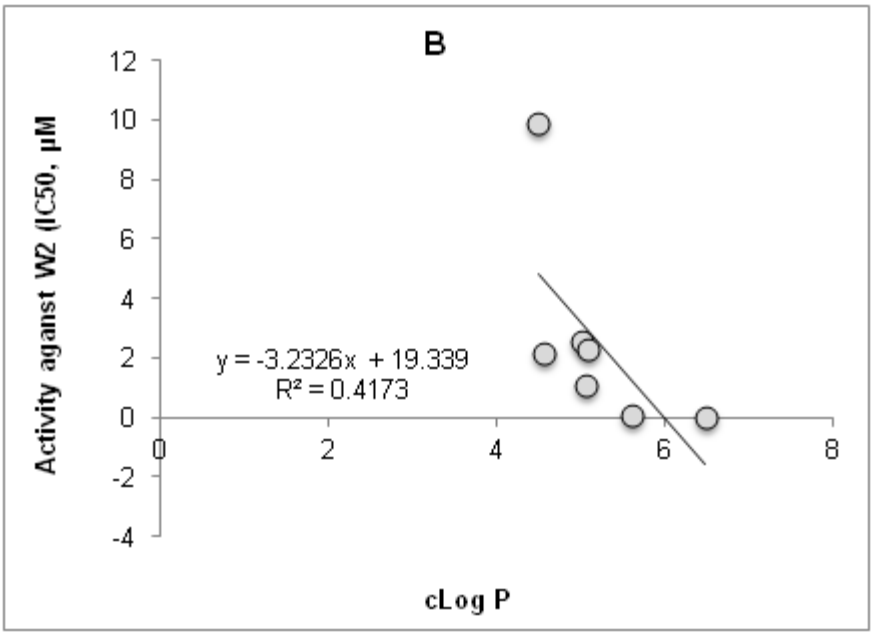


Figure S2: Correlation between lipophilicy (clog P) and *in vitro* antiplasmodial activity against W2 parasites for benzylated **(A)** and deprotected **(B)** compounds.

phiβ-Haematin Inhibition Assay

The assay is based on the ability of haematin, but not β-haematin to form a low spin complex with aqueous pyridine at pH 7.5 as described by Ncokazi and Egan.[3] It involves serial dilution of the drug solutions in triplicate in a 96 well plate using a multichannel pipette. The test drugs (compounds) were dissolved in DMSO or methanol. Drug concentrations varied from 1-10 equivalents relative to haematin, each well containing 10.12µL in the final mixture. 101.2 µL of haematin stock solution (1.68mM in 0.1M NaOH) was added to each well followed by 10.12 µL of 1.0M HCl. The 58.7 µL of acetate solution (12.9*M*, pH 5.0) which was preincubated at 60^{o}C was added and the plate incubated at 60^{o}C for 60 minutes. The mixture was quenched with 80 µL of 30% (v/v) pyridine solution in 20mM HEPE*S*, pH 7.5 and allowed to settle at ambient temperature. Afterwards 30 µL of the supernatant was transferred to another plate and diluted to 250 µL with 30% (v/v) pyridine solution (pH 7.5, 20mm HEPES). The absorbance was read at 450nm using an ASYS UVM 340 plate reader. The IC_{50} values for β-haematin inhibition were determined by fitting the absorbance data to a sigmoidal dose response curve by non-linear least squares fitting using GraphPadPrism software.

In vitro* Antiplasmodial assays: Dd2 and D10 strains of *Plasmodium falciparum

Compounds were tested in duplicate on one occasion against D10 Chloroquine sensitive (CQS) and Dd2 Chloroquine resistant (CQR) strains using a protocol described by Guantai *et .al.,* 2010.[4] Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (1976).[5] The quantitative assessment of antiplasmodial activity in vitro was determined via the parasite

lactate dehydrogenase assay using a modified method by Makler, 1993. [6] The samples were prepared to a 2mg/ml stock solution in 10% DMSO or 10% methanol and were sonicated to enhance solubility. The samples were tested as a suspension if not completely dissolved. The stock solutions were stored at -20^{o}C and further dilutions were prepared on the day of the experiment. Chloroquine (CQ) was used as the reference drug in all experiments. A full dose-response was performed for all compounds to determine the concentration inhibiting 50% parasite growth (IC_{50} value). Compounds were tested at a starting concentration of 100µg/ml, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.2µg/ml. The same dilution technique was used for all the samples. CQ was tested at a starting concentration of 100ng/ml. The highest concentration of solvent to which the parasites were exposed had no measurable effect on parasite viability (data not shown). The IC_{50} values were obtained using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4.0 software.

In vitro* Antiplasmodial assays: W2 strain of *Plasmodium falciparum

The protocol to this assay is as described by Sijwali and Rosenthal, 2004.[7] The W2 (CQ resistant) strain of *P. falciparum* (1% parasitemia, 2% hematocrit), were cultured in 0.5ml of medium in 48-well culture dishes. Stock solutions of inhibitors (10mM) in DMSO were added to cultured parasites to a final concentration of 20µM. From the 48-well plates, 125µM of culture was transferred to two 96-well plates (duplicates). Serial dilutions (1%) of inhibitors were made to final concentrations of 10µ*M,* 2µ*M,* 0.4µ*M,* 80n*M,* 16n*M,* and 3.2nM. Cultures were maintained at 37^{o}C for 2 days after which the

parasites were washed and fixed with 1% formaldehyde in PBS. After 2 days parasitemia was measured by flow cytometry using the DNA stain YOYO-1 as a marker of cell survival.

In vitro* Antiplasmodial assays: 3D7 and K1 strains of *Plasmodium falciparum

The 3D7 clone is known to be sensitive to all antimalarials whereas the K1 which originates from Thailand is resistant to CQ and pyrimethamine, but sensitive to mefloquine. The cultures are naturally, asynchronous (65-75% ring stage) and are maintained in continuous log phase growth in RPMI 1640 supplemented with 5% washed human A+ erythrocytes , 25mM HEPES, 32nM $NaHCO_3$ and AlbuMAXII (lipid rich bovine serum albumin) (GIBCO, Grand Island, NY) (CM). All cultures and assays were conducted at 37^{o}C under an atmosphere of 5% CO_2 and 5% O_2 with the balance N_2. This assay was performed in various stages i.e. the primary and secondary screens.

Stock solutions were prepared in 100% DMSO at 20mg/ml and further dilutions were done using complete medium RPMI 1640 supplemented with 15nM cold hypoxanthine and AlbuMAXII. Assays were performed in sterile 96-well microtitre plates; each plate containing 100µl of parasite culture (0.5% parasitemia, 2.5% hematocrit). Each drug was tested in triplicate and parasite growth compared to the control and blank (uninfected erythrocytes) wells. After 24h incubation at 37^{o}C, 3.7 Bq of [^{3}H] hypoxathine was added to each well. Cultures were incubated for a further 24h before harvesting them onto glass fibre filter mats. Radioactivity was counted using a Wallac Microbeta 1450 scintillation counter. The results were recorded as counts per minute (CPM) per well at each drug concentration, control and blank. Percentage inhibition was calculated from comparison

to blank and control wells, and IC_{50} values were calculated using Microsoft XLFit line fitting software (IDBS, UK).

(a) Primary Screen

The primary screen used the 3D7 strain. The test compounds are tested at 4 concentrations (30, 10, 3, 1, 0.3, and 0.1µg/ml). Compounds that did not affect parasite growth at 10µg/ml are considered inactive, between 10 and 11µg/ml are designated as partially active and if <1µg/ml the compound was classified as active and was further evaluated by 3-fold serial dilutions in a repeat test.

(b) Secondary Screen

In this screen both 3D7 and K1 were used. The test drug was diluted 3-fold over at 12 different concentrations with appropriate starting concentrations based on the primary screen. The IC_{50} was determined by sigmoidal dose response analysis using Microsoft XLFit (IDBS, UK). For each assay, the $IC_{50,}$ and IC_{90} values for each parasite line were determined against CQ and other standard compounds appropriate for the assay.

Mammalian cell toxicity Assay

KB cells is a cell line derived from a human carcinoma of the nasopharynx, typically is used as an assay for antineoplastic agents. The KB cells were maintained as monolayers in RPMI 1640 + 10% HIFC. All cultures and assays were conducted at $37^{o}C$ under an atmosphere of 5%CO_2/ 95% air mixture. The Assay takes up to 5days.

(a) Day 1:

KB cells are harvested, counted and washed in serum free medium (2000rpm, 10 minutes. 4^{o}C), and resuspended in fresh medium (RPMI 1640 + 10% HIFC) at a concentration of 4 x 10^4/ml. 100µl is added to wells on a 96- well plate (4 x 10^3/well). The plate was incubated overnight at 37^{o}C under an atmosphere of 5%CO_2/ 95% air mixture to allow cells to adhere.

(b) Day 2:

Test compounds were prepared in 100% DMSO 20mg/ml and diluted to a starting concentration of 600µg/ml (2 x top concentration) with RPMI + 10% HIFC. The control wells had no drug. A 10-fold serial dilution was performed across the plate ie 300, 30, 3, etc. The plate was then incubated for 72h at atmosphere of 5%CO_2/ 95% air mixture.

Podophyllotoxin was used as the reference drug.

(c) Day 5:

Each well was assessed by microscopic observation. 20µl Alamar Blue was added to each well then the plates were incubated for 2-4h before reading (Gemini, EX/EM 530/580, cut-off 550nm) IC_{50} values were calculated using sigmoidal regression analysis (MS XLFit)

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