Title: Quinolone-3-Diarylethers: A new class of drugs for a new era of malaria eradication

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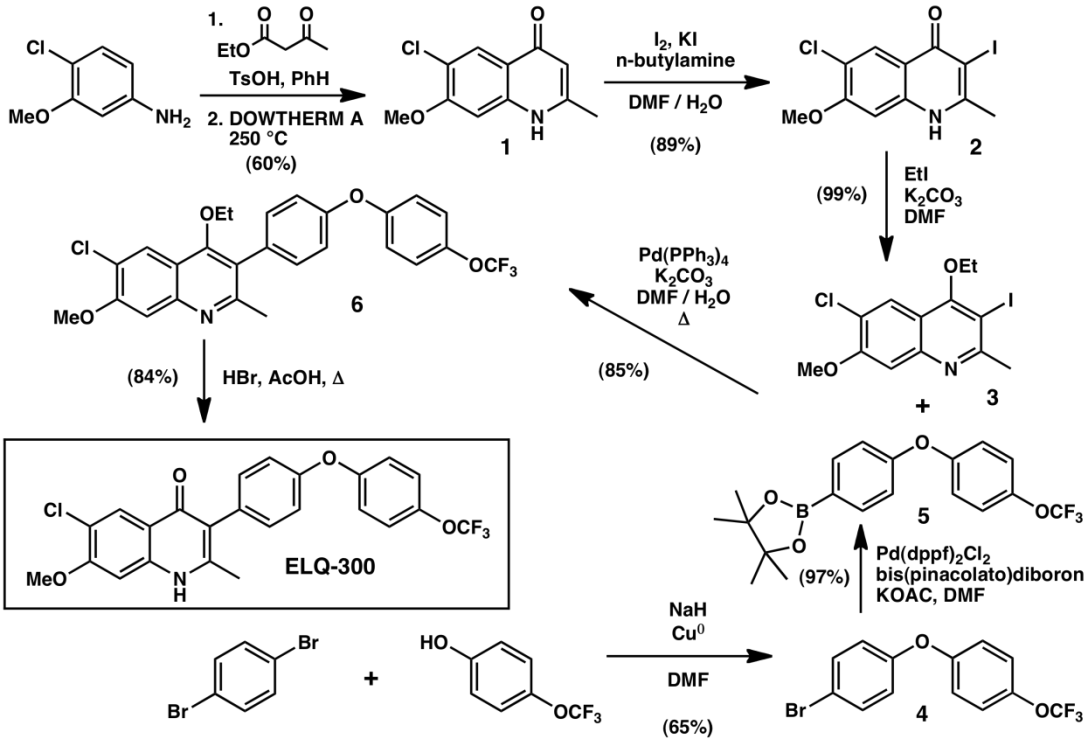
Materials and Methods

ELQ-300 synthesis



General. Anhydrous solvents and reagents were purchased from various fine chemical suppliers and were used without further purification. Inert atmosphere operations were conducted under argon in flame-dried glassware. ^{1}H NMR spectra were taken on a Bruker 400 MHz instrument. Data reported were calibrated to internal TMS (0.0 ppm) for all solvents and are reported as follows: chemical shift, multiplicity (bs, broad singlet; s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet), coupling constant and integration. High-resolution mass spectrometry (HRMS) using electrospray ionization was performed by the Portland State University BioAnalytical Mass Spectrometry Facility. The final compound was judged to be >99% pure by HPLC analysis using an HP1100 HPLC at 254 nm with Phenomenex Luna C8(2) reverse phase column (5 mm, 50 x 2 mm i.d) at 40°C and eluted with methanol/water with 0.5% TFA at 0.4 mL/min.

6-Chloro-7-methoxy-2-methylquinolin-4(1*H*)-one (1). A solution of 5-amino-2-chloroanisole (10.0 g, 63.5 mmol), ethyl acetoacetate (8.1 mL, 63.5 mmol) and catalytic *para*-toluene sulfonic acid (302 mg, 1.59 mmol) in 65 mL benzene was stirred 6 hours at reflux with a Dean-Stark trap. The reaction mixture was then concentrated *in vacuo,* and the resulting Schiff base added to 65 mL boiling (250°C) DOWTHERM A and stirred 20 minutes at 250°C. The reaction mixture was cooled to room temperature. The precipitate was triturated with ethyl acetate and filtered to give 6-chloro-7-methoxy-2-methylquinolin-4(1*H*)-one (8.57 g, 60% yield) as an off-white powder. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 11.54 (bs, 1H), 7.94 (s, 1H), 7.02 (s, 1H), 5.86 (s, 1H), 3.94 (s, 3H), 2.31 (s, 3H).

6-Chloro-3-iodo-7-methoxy-2-methylquinolin-4(1*H*)-one (2). To a stirred solution of 6-chloro-7-methoxy-2-methylquinolin-4(1*H*)-one (6.43 g, 28.7 mmol) and *n*-butylamine (28 mL, 287 mmol) in dimethylformamide (114 mL) was added iodine (7.30 g, 28.7 mmol) in a saturated solution of aqueous

potassium iodide (29 mL). The reaction mixture was stirred 12 hours at room temperature. Residual iodine was quenched with 0.1 M aqueous sodium thiosulfate, and the resulting solution was concentrated *in vacuo*. The residue was resuspended in water and filtered to give 6-chloro-3-iodo-7-methoxy-2-methylquinolin-4(1*H*)-one (8.93 g, 89% yield) as a light brown powder. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 11.65 (bs, 1H), 7.59 (s, 1H), 6.41 (s, 1H), 3.91 (s, 3H), 2.18 (s, 3H).

6-Chloro-4-ethoxy-3-iodo-7-methoxy-2-methylquinoline (3). To a stirred solution of 6-chloro-3-iodo-7-methoxy-2-methylquinolin-4(1*H*)-one (2.00 g, 5.72 mmol) in dimethylformamide (57 mL) was added potassium carbonate (1.58 g, 11.4 mmol) at room temperature. The resulting suspension was stirred 0.5 hours at 50°C. Ethyl iodide was added dropwise at room temperature, and the reaction mixture was stirred 8 hours at 50 °C. The solvent was removed *in vacuo*. The resulting residue was resuspended in ethyl acetate and water and filtered. The organic layer was extracted with brine, dried over magnesium sulfate and concentrated *in vacuo* to give 6-chloro-4-ethoxy-3-iodo-7-methoxy-2-methylquinoline (2.12 g, 99% yield) as a light brown solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (s, 1H), 7.40 (s, 1H), 4.19 (q, *J* = 7.1 Hz, 2H), 4.02 (s, 3H), 2.92 (s, 3H), 1.61 (t, *J* = 7.1 Hz, 3H).

1-Bromo-4-(4-(trifluoromethoxy)phenoxy)benzene (4). To a stirred solution of 4-trifluoromethoxy-phenol (89.0 g, 0.50 mol) in DMF (400 mL) cooled in an ice bath, was added sodium hydride 60% dispersion in mineral oil (24.0 g, 0.60 mol, 1.2 eq) portion wide over a period of 1 hour resulting in a thick viscous yellow solution. This was then added to a mixture of 1,4-dibromobenzene (235 g, 1.00 mol, 2.0 eq) and copper powder (16 g, 0.25 mol, 0.5 eq) in DMF (400 mL). The mixture was stirred in an oil bath at 160°C and the progression of the reaction was monitored by GC-MS. After 60 hours the mixture blackened and it was estimated that less than 5% of the starting 4-trifluoromethoxy-phenol remained in the reaction mixture. The mixture was allowed to cool to room temperature and then filtered through Celite and washed with 50-100 mL of DMF. Water (50 mL) was then added to the DMF filtrate. DMF was then removed by rotoevaporation under vacuum and the resulting mixture was again filtered through Celite (to remove the insoluble sodium bromide), washed with ethylacetate (100 mL) and rotoevaporated. The resulting black solution was then allowed to stand at room temperature for 48 hours. Black crystalline material was formed which was filtered, washed with hexane (50 mL) and rotoevaporated. The resulting black oil was then distilled under high vacuum. The middle fraction with a boiling point of 96-100°C at 85 millitorr corresponding to the desired diaryl ether 4 as a light yellow oil was collected yielding 100.7 g (60%). GC-MS: M+ 332, 100%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (d, *J* = 9.3 Hz, 2H), 7.19 (d, *J* = 8.9 Hz, 2H), 6.99 (d, *J* = 9.3 Hz, 2H), 6.89 (d, *J* = 8.9 Hz, 2H).

1-Bromo-4-(4-(trifluoromethoxy)phenoxy)benzene (4 via alternative method). Using a method adapted from Hart et al. (*39*), to a solution of 4-(trifluoromethoxy)phenylboronic acid (10.0 g, 48.6 mmol) and 4-bromophenol (4.20 g, 24.3 mmol) in dichloromethane (250 mL) over heat-activated 3 Å molecular sieves was added copper (II) acetate (4.41 g, 24.3 mmol), diisopropylethylamine (21 mL, 121 mmol) and pyridine (10 mL, 121 mmol). The reaction mixture was stirred 12 hours at room temperature under positive pressure of dry air and concentrated *in vacuo*. The resulting residue was resuspended in ethyl acetate and 0.5 M HCl. The organic layer was extracted with water and brine, dried over magnesium sulfate and concentrated *in vacuo*. Purification by silica gel chromatography (hexanes) provided 1-bromo-4-(4-(trifluoromethoxy) phenoxy) benzene (5.07 g, 63% yield) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (d, *J* = 9.3 Hz, 2H), 7.19 (d, *J* = 8.9 Hz, 2H), 6.99 (d, *J* = 9.3 Hz, 2H), 6.89 (d, *J* = 8.9 Hz, 2H).

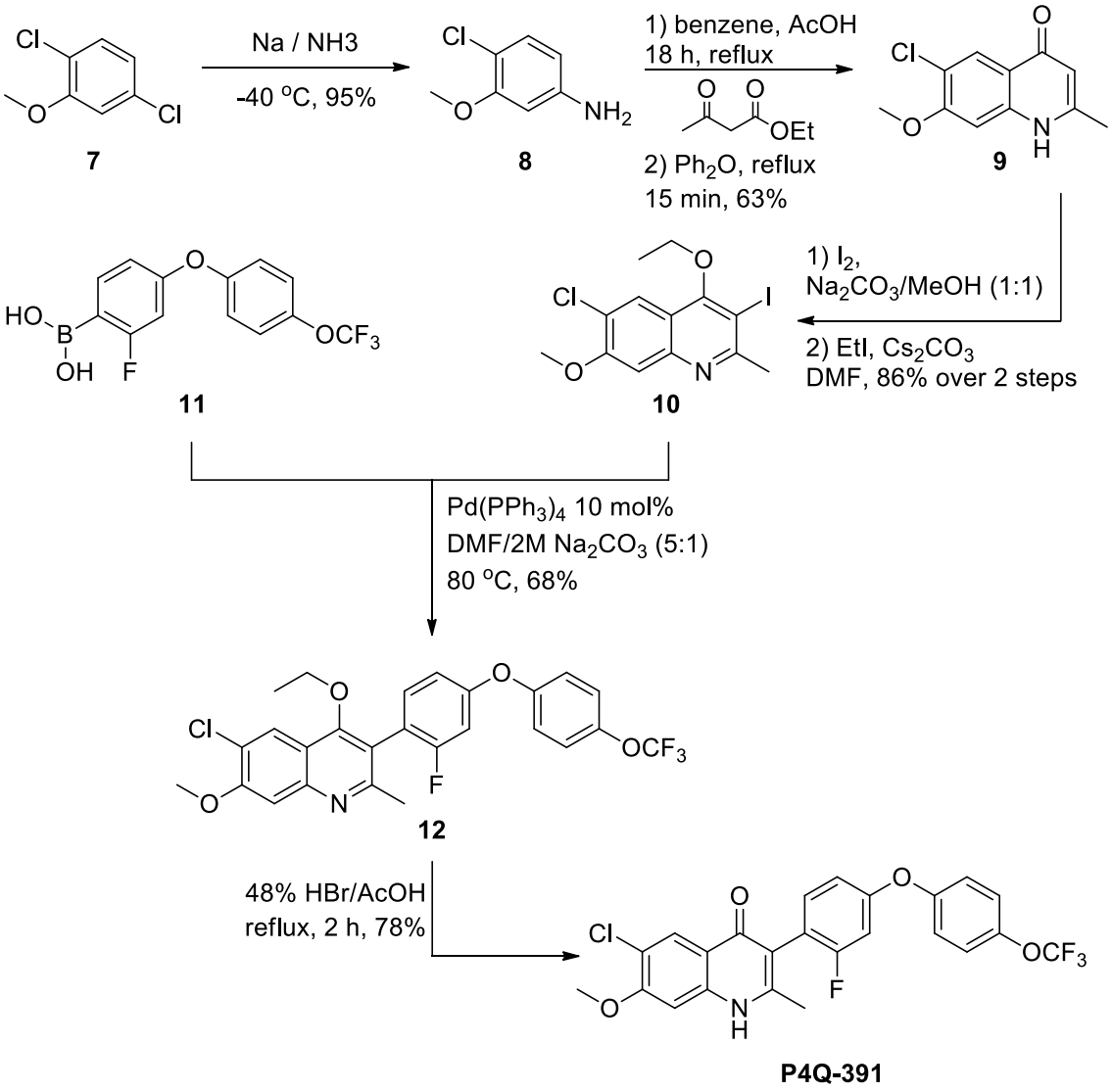
4,4,5,5-tetramethyl-2-(4-(4-(trifluoromethoxy)phenoxy)phenyl)-1,3,2-dioxaborolane (5). To a solution of 1-bromo-4-(4-(trifluoromethoxy)phenoxy)benzene (11.0 g, 33.0 mmol) in DMF (130 mL) over heat-activated 3 Å molecular sieves (13 g) was added 1,1'-bis(diphenylphosphino)ferrocene dichloropalladium (II) (1.21 g, 1.65 mmol), bis(pinacolato) diboron (9.22 g, 36.3 mmol) and potassium

acetate (9.72 g, 99.0 mmol). The reaction mixture was heated to 80 °C, stirred for 18 hours, cooled and filtered. The filtrate was concentrated *in vacuo*, resuspended in ethyl acetate and water and separated. The organic layer was extracted with brine and dried over magnesium sulfate. Purification by silica gel chromatography (ethyl acetate / hexanes) provided 4,4,5,5-tetramethyl-2-(4-(4-(trifluoromethoxy)phenoxy)phenyl)-1,3,2-dioxaborolane (11.5 g, 92% yield) as a light brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.9 Hz, 2H), 7.19 (d, *J* = 8.9 Hz, 2H), 6.97-7.04 (m, 2H), 1.35 (s, 12H).

6-chloro-4-ethoxy-7-methoxy-2-methyl-3-(4-(4-(trifluoromethoxy)phenoxy) phenyl) quinoline (6). To a solution of 6-chloro-4-ethoxy-3-iodo-7-methoxy-2-methylquinoline (1.26 g, 3.35 mmol), 4,4,5,5-tetramethyl-2-(4-(4-(trifluoromethoxy)phenoxy)phenyl)-1,3,2-dioxaborolane (1.91 g, 5.02 mmol) and palladium (0) tetrakis triphenylphosphine (194 mg, 0.168 mmol) in degassed dimethylformamide (17 mL) was added 6.7 mL of 2 N aqueous potassium carbonate solution. The reaction mixture was stirred 18 hours at 85 °C, filtered through celite and concentrated *in vacuo*. The resulting residue was resuspended in ethyl acetate and water and separated. The organic layer was extracted with brine, dried over magnesium sulfate and concentrated *in vacuo*. Purification by silica gel chromatography (ethyl acetate / dichloromethane) provided 6-chloro-4-ethoxy-7-methoxy-2-methyl-3-(4-(4-(trifluoromethoxy)phenoxy)-phenyl)quinoline (1.44 g, 85% yield) as a light brown solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (s, 1H), 7.43 (s, 1H), 7.31-7.36 (m, 2H), 7.22-7.26 (m, 2H), 7.07-7.14 (m, 4H), 4.04 (s, 3H), 3.71 (q, *J* = 7.1 Hz, 2H), 2.49 (s, 3H), 1.18 (t, *J* = 7.1 Hz, 3H).

6-chloro-7-methoxy-2-methyl-3-(4-(4-(trifluoromethoxy)phenoxy)phenyl) quinolin-4(1*H*)-one (ELQ-300). To a solution of 6-chloro-4-ethoxy-7-methoxy-2-methyl-3-(4-(4-(trifluoromethoxy)phenoxy)-phenyl)quinoline (1.16 g, 2.30 mmol) in acetic acid (10 mL) was added 50% aqueous hydrobromic acid solution (5 mL). The reaction mixture was stirred 24 hours at 90 °C, cooled, and neutralized with saturated KOH solution. The collected solid was triturated in ethyl acetate and recrystallized from DMF / methanol to give 6-chloro-7-methoxy-2-methyl-3-(4-(4-(trifluoromethoxy)phenoxy) phenyl) quinolin-4(1*H*)-one (923 mg, 84% yield) as a white powder, which was found to be >99% pure by HPLC analysis. ^{1}H NMR (400 MHz, d6-DMSO) δ 11.97 (s, 1H), 8.05 (s, 1H), 7.42 (d, *J* = 8.7 Hz, 2H), 7.29 (d, *J* = 8.3 Hz, 2H), 7.17 (d, *J* = 8.3 Hz, 2H), 7.12 (s, 1H), 7.08 (d, *J* = 8.7 Hz, 2H), 3.97 (s, 3H), 2.26 (s, 3H). ^{13}C NMR (100 MHz, d6-DMSO) δ 174.39 (q), 157.58 (q), 156.67 (q), 155.63 (q), 147.49 (q), 144.50 (q), 140.46 (q), 133.62 (CH), 132.36 (q), 127.06 (CH), 123.89 (CH), 122.27 (q), 120.80 (q), 120.66 (CH), 119.61 (q), 119.15 (CH), 118.87 (q), 100.30 (CH), 57.28 (CH_3), 19.82 (CH_3). HRMS (EI+) *m*/*z* for $C_{24}H_{17}ClF_3NO_4$: calculated 475.0798, found 475.0801.

P4Q-391 synthesis



General. All reagents and anhydrous solvents were obtained from Aldrich Chemical Co. and used without further purification unless otherwise noted. 2,5-Dichloroanisole was bought from Alfa Aesar. The identity of all compounds was verified via ^{1}H NMR, ^{13}C NMR, and HPLC/HRMS. The chemical purity of the titled compounds was determined using the following conditions: an Agilent 1100 series LC/MSD with a Eclipse XDB-C18 (4.6mm x 100 mm, 5 µm) reversed phase column; method: 10% (v/v) of acetonitrile (+0.05% TFA) in 90% (v/v) of H_2O (+0.05% TFA), ramped to 100% acetonitrile (+0.05% TFA) over 9 min, and holding at 100% acetonitrile for 4 min with a flow rate of 0.7 mL/min, UV detector, 254 nm. The purity of each compound was ≥95% in this analysis. NMR spectra were recorded at ambient temperature on a 400 or 500 MHz Varian NMR spectrometer in the solvent indicated. All ^{1}H NMR experiments are reported in parts per million (ppm) downfield of TMS and were measured relative to the signals for chloroform (7.26 ppm) and dimethylsulfoxide (2.50 ppm). All ^{13}C NMR spectra are reported in ppm relative to the signals for chloroform (77 ppm) and dimethylsulfoxide (39.5 ppm) with ^{1}H decoupled observation. Data for ^{1}H NMR were reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration and coupling constant (Hz), whereas ^{13}C-NMR analyses

were obtained at 101 MHz and reported in terms of chemical shift. NMR data was analyzed by using MestReNova Software ver. 5.3.2-4936. High resolution mass spectra (HRMS) were performed on an Agilent LC/MSD TOF system G3250AA. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F254 pre-coated plates (0.25 mm) from EMD Chemical Inc. and components were visualized by ultraviolet light (254 nm). Silicycle silica gel 230-400 (particle size 40-63 µm) mesh was used for all flash column chromatography.

4-Chloro-3-methoxyaniline (8). 900 mL of ammonia was condensed at -78 °C. 1.0 g of thinly shaven strips of sodium was added followed by 1.0 g of iron (III) nitrate nonahydrate. Upon disappearance of the deep blue color, additional 25 g of sodium was added. After 30 minutes of stirring at -78°C, 50 g of 2,5-dichloroanisole (**7**) was added dropwise as a solution in hexanes (70 mL) and the reaction was warmed to 45 °C for 2 hours. The ammonia was allowed to evaporate. The crude was then diluted in chloroform and 100 g of NH_4Cl was added slowly. The organic layer was washed with H_2O (X3) followed by brine (X1). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The resulting solid was used without further purification. Yield: 95%. ^{1}H NMR (400 MHz, DMSO) δ 6.98 (d, *J* = 8.4, 1H), 6.34 (d, *J* = 1.8, 1H), 6.16 (dd, *J* = 8.4, 1.9, 1H), 5.23 (s, 2H), 3.74 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 154.90, 149.16, 129.68, 107.02, 106.74, 98.66, 55.38. HRMS calc. for C_7H_8ClNO $[M+H]^+$: 157.03672, found: 157.03612

6-Chloro-7-methoxy-2-methylquinolin-4(1*H*)-one (9). To a 500 mL round bottom flask was added 4-chloro-3-methoxyaniline (**8**) (15 g, 95 mmol), 24 mL of ethyl acetoacetate, 7 mL AcOH, 160 mL of benzene. The contents were refluxed in a Dean-Stark trap with azeotropic removal of water. After 18 hours the reaction was cooled to room temperature and concentrated *in vacuo*. The resulting oil was added dropwise to refluxing biphenyl ether (160 mL) over 5 minutes and set to reflux with a Dean-Stark trap to remove ethanol. After 16 minutes, the reaction was cooled to room temperature. The resulting solid was diluted in ice cold hexanes and filtered. The resulting solid was washed with ice cold diethyl ether. Yield: 63%. ^{1}H NMR (400 MHz, DMSO) δ 11.53 (s, 1H), 7.94 (s, 1H), 6.99 (s, 1H), 5.85 (s, 1H), 3.92 (s, 3H), 2.30 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 175.91, 157.26, 150.24, 141.01, 126.28, 119.54, 118.51, 108.93, 100.29, 56.97, 20.07. HRMS calc. for $C_{11}H_{10}ClNO_2$ $[M+H]^+$: 224.04728, found: 224.04634.

6-Chloro-4-ethoxy-3-iodo-7-methoxy-2-methylquinoline (10). 6-Chloro-7-methoxy-2-methyl-quinolin-4(1*H*)-one (**9**) was dissolved in 450 mL of MeOH and 450 mL of Na_2CO_3. After 30 minutes of stirring, 51 g of iodine was added and the reaction was monitored by HPLC. Upon completion, the reaction mixture was poured onto water and ice. AcOH was then slowly added while maintaining stirring. The slurry was filtered and the resulting solid was dried in an oven over 24 hours. The intermediate 6-chloro-3-iodo-7-methoxy-2-methylquinolin-4(1*H*)-one was taken on to the alkylation step without any further purification. ^{1}H NMR (400 MHz, DMSO) δ 12.06 (s, 1H), 7.95 (s, 1H), 6.98 (s, 1H), 3.93 (s, 3H), 2.57 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 171.38, 156.81, 151.15, 139.12, 126.16, 118.77, 114.82, 99.29, 85.78, 56.40, 26.08.

A flame-dried flask was backfilled with argon (X2) and intermediate 6-chloro-3-iodo-7-methoxy-2-methylquinolin-4(1*H*)-one (51 g, 146 mmol) was dissolved in 2.8 L of DMF. Cs_2CO_3 (118 g, 363 mmol) and ethyl iodide (44 mL, 365 mmol) were added to this solution and the resulting mixture was stirred at room temperature for 12 hours. The reaction mixture was then poured onto ice and water and further diluted with chloroform. The organic layer was washed with water (X2) and brine (X2) and concentrated *in vacuo*. The resulting oil was purified via flash chromatography (hexanes:EtOAc=8:1) to obtain the product (**10**) in 90% yield as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.91 (s, 1H), 7.31 (s, 1H), 4.13 (q, *J* = 7.0 Hz, 2H), 3.98 (s, 3H), 2.86 (s, 3H), 1.56 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ

162.21, 162.14, 156.42, 148.87, 124.13, 122.45, 117.57, 107.97, 87.73, 70.88, 56.36, 30.53, 15.72. HRMS calc. for $C_{13}H_{14}ClINO_2$ $[M+H]^+$: 377.97577, found: 377.97581.

4-(4-(trifluoromethoxy)phenoxy)phenylboronic acid (11). The titled compound was prepared exactly as Yeates et al. described (*11*). In a flame-dried 25 mL schlenk tube backfilled with argon (X3), a solution of 4-bromo-3-fluorophenol (0.346 g, 2 mmol) in *N*-methylpyrrolidine (8 mL) was added 4-(trifluoromethoxy)iodobenzene (0.63 mL, 4 mmol), 2,2,6,6-tetramethylheptane-3,5-dione (0.09 mL, 0.44 mmol) and cesium carbonate (1.30 g, 4 mmol). The slurry was degassed by bubbling argon for 15 minutes and CuCl (0.099 g, 1 mmol) was then added. The reaction mixture was again degassed and then warmed to 100 ºC for 7 hours. After the reaction mixture was cooled to room temperature, Et_2O (75 mL) was added slowly. The resulting slurry was filtered and the solid washed with Et_2O (3 x 50 mL). The combined filtrates were washed with 2 M NaOH (100 mL), water (100 mL), 1 M aq HCl (100 mL), water (100 mL) and saturated brine (100 mL). Subsequently, the crude was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified via flash chromatography with 100% hexanes. This column was repeated three times combining the purest fractions to obtain pure material due to similarly eluting 4-(trifluoromethoxy)iodobenzene to afford 1-bromo-2-fluoro-4-(4-(trifluoro-methoxy)phenoxy)benzene (0.15 g, 45%) as a colorless liquid. To a solution of 1-bromo-2-fluoro-4-(4-(trifluoromethoxy)phenoxy)benzene (2.1 mmol, 0.7 g) and triisopropyl borate (2.7 mmol, 0.63 mL) in dry THF (15 mL) at -78°C was added dropwise 2.5 M BuLi (6.5 mL) in hexanes over a period of 5 minutes. The reaction was stirred for 3 hours at -78°C at which point 10 mL of 6M HCl is added and the solution is allowed to warm up to room temperature and stir overnight. The reaction mixture was diluted with EtOAc (150 mL) and water (150 mL). The organic layer was taken separately and rinsed with water (150 mL), followed by brine (150 mL) and then dried over Na_2SO_4. The EtOAc is then concentrated in vacuo to afford a waxy solid which was then treated with 2M NaOH (40 mL) and stirred for 15 minutes, diluted with water (300 mL) and stirred for an additional 20 minutes. The solution was then filtered and the filtrate washed with hexanes (3 x 100 mL). The aqueous layer was carefully acidified to pH 1 with 6 M HCl. The resulting white solid was filtered and dried on a high vacuum overnight to afford the titled compound in 67% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (t, *J* = 8.1 Hz, 1H), 7.22 (d, *J* = 9.0 Hz, 2H), 7.09 – 7.02 (m, 2H), 6.80 (dd, *J* = 8.3, 2.2 Hz, 1H), 6.63 (dd, *J* = 12.0, 2.2 Hz, 1H), 5.14 (d, *J* = 6.3 Hz, 2H).

6-Chloro-3-(2-fluoro-4-(4-(trifluoromethoxy)phenoxy)phenyl)-7-methoxy-2-methylquinolin-4(1H)-one (P4Q-391). A 1 L 2-neck flask was evacuated and back-filled with argon (X3). The flask was charged with 6-chloro-4-ethoxy-3-(2-fluoro-4-(4-(trifluoromethoxy)phenoxy)phenyl)-7-methoxy-2-methylquinoline (**10**) (4.0g, 10 mmol), $Pd(PPh_3)_4$ (1.21 g, 10 mol%), and 2-fluoro-4-(4-(trifluoromethoxy)-phenoxy)phenylboronic acid (**11**) (6g, 18 mmol). Next, 200 mL of DMF was added via cannula to the flask, followed by 40 mL of 2 M Na_2CO_3. The reaction was heated between 80-85°C for 2-3 hours, and monitored for completion via HPLC analysis. The reaction was quenched by pouring the contents on ice water and chloroform. The organic layer was washed with water (X2) and brine (X2). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The crude oil was purified via flash chromatography (hexanes/EtOAc=5:1). Compound **12** was isolated as an orange foam (3.32 g, 68%) and dried thoroughly under high vacuum. Compound **12** was dissolved in 3.3 mL of HBr and 3.3 mL of AcOH and transferred to a 25 mL round bottom flask and set to vigorous reflux until completion. This reaction was carefully monitored by HPLC and TLC to prevent over de-alkylation. Upon completion, the flask contents were poured onto ice and water and allowed to stand for 1 hour. Next, the solid was filtered and thoroughly dried in an oven before being recrystallized from EtOAc (X3) (2.45g, 78%). ^{1}H NMR (400 MHz, DMSO) δ 11.79 (s, 1H), 7.99 (d, *J* = 1.3 Hz, 1H), 7.44 (d, *J* = 8.9 Hz, 2H), 7.29 (t, *J* = 8.0 Hz, 1H), 7.23 (dd, *J* = 9.0, 1.2 Hz, 2H), 7.08 (s, 1H), 6.99 (d, *J* = 10.4 Hz, 1H), 6.90 (d, *J* = 10.1 Hz, 1H), 3.97 (s, 3H), 2.20 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.09, 160.46 (d, *J* = 246.6 Hz), 156.79, 156.66 (d, *J* = 246.6 Hz), 154.87, 147.54, 144.07, 139.67, 134.22 (d, *J* = 5.05 Hz), 126.03, 123.04, 120.43, 118.68 (d, *J* = 17.17

Hz), 118.27 (d, *J* = 16.16 Hz), 114.05, 113.93 (d, *J* = 3.03 Hz), 106.13 (d, *J* = 26.26 Hz), 99.51, 56.32, 18.24. ^{19}F NMR (376 MHz, $CDCl_3$) δ -52.56, -105.28. HRMS calc. for $C_{25}H_{19}ClF_4NO_3$ $(M+H)^+$: 494.07822, found: 494.07817.

In vitro antimalarial activity

SyBr Green

Parasite culture and drug sensitivity
Laboratory strains of *P. falciparum* were cultured in human erythrocytes by standard methods under a low oxygen atmosphere (5% O_2, 5% CO_2, 90% N_2) in an environmental chamber (*40*). The culture medium was RPMI-1640, supplemented with 25 mM HEPES buffer, 25 mg/L gentamicin sulfate, 45 mg/L hypoxanthine, 10 mM glucose, 2 mM glutamine, and 0.5% Albumax II (complete medium). The parasites were maintained in fresh human erythrocytes suspended at a 2% hematocrit in complete medium at 37°C. Stock cultures were sub-passaged every 3 to 4 days by transfer of infected red cells to a flask containing complete medium and uninfected erythrocytes.

SyBr Green assay
In vitro antimalarial activity of the 4(1*H*)-quinolone-3-diarylether derivatives was assessed by the SYBR Green I fluorescence-based method (the "MSF assay") described previously by us (*15*). Experiments were set up in triplicate in 96 well plates (Costar, Corning) with two-fold dilutions of each drug across the plate in a total volume of 100 microliters and at a final red blood cell concentration of 2% (v/v). The dilution series was initiated at a concentration of 1μM and the experiment was repeated beginning with a lower initial concentration for those compounds in which the IC_{50} value was below 10nM. Automated pipeting and dilution was carried out with the aid of a programmable Precision 2000 robotic station (BioTek, Winooski, VT). An initial parasitemia of 0.2% was attained by addition of normal uninfected red cells to a stock culture of asynchronous parasite infected red cells (PRBC). The plates were incubated for 72 hrs at 37°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. After this period the SYBR Green I dye-detergent mixture (100μl) was added and the plates were incubated at room temperature for one hour in the dark and then placed in a 96-well fluorescence plate reader (Spectramax Gemini-EM, Molecular Diagnostics) for analysis with excitation and emission wavelength bands centered at 497 and 520 nm, respectively. The fluorescence readings were plotted against the logarithm of the drug concentration and curve fitting by nonlinear regression analysis (GraphPad Prism software) yielded the drug concentration that produced 50% of the observed decline relative to the maximum readings in drug-free control wells (IC_{50}).

^{3}H-Hypoxanthine

The methods employed were essentially as described by Desjardins et al. (*16*) however the procedure was modified to a 72-hour incubation period.

Ex vivo (*P. falciparum* and *P. vivax* field isolates)

Compounds
Standard anti-malarial drugs chloroquine (CQ), amodiaquine (AQ) (Sigma-Aldrich, Australia), piperaquine (PIP), mefloquine (MFQ), and artesunate (AS) (WWARN QA/QC Reference Material Programme), and experimental compounds ELQ-300 (VA Medical Center, Portland, USA) and P4Q-391 (University of South Florida, Tampa, USA) were prepared as 1 mg/mL stock solutions in dimethyl sulfoxide (DMSO). Drug plates were pre-dosed by diluting the compounds in 50% methanol followed by lyophilisation and stored at 4°C.

Field location and sample collection

Plasmodium isolates were collected from patients attending malaria clinics in Timika (Papua, Indonesia), a region endemic for multidrug-resistant strains of *P. vivax* and *P. falciparum*. Patients with symptomatic malaria presenting to an outpatient facility were recruited into the study if singly infected with *P. falciparum* or *P. vivax*, with a parasitaemia of between 2,000 μl^{-1} and 80,000μl^{-1}, and a majority (>60%) of parasites at ring stage of development. Venous blood (5 mL) was collected by venepuncture and after removal of host white blood cells by using CF 11 cellulose, packed infected red blood cells (iRBCs) were used for the *ex vivo* drug susceptibility assay.

Ex vivo drug susceptibility assay

Drug susceptibility of *P. vivax* and *P. falciparum* isolates was measured using a protocol modified from the WHO microtest as described previously (*20, 21, 41*). Two hundred µl of a 2% haematocrit Blood Media Mixture (BMM), consisting of RPMI 1640 medium plus 10% AB+ human serum (*P. falciparum*) or McCoy's 5A medium plus 20% AB+ human serum (*P. vivax*) was added to each well of pre-dosed drug plates containing 11 serial concentrations (2-fold dilutions) of the anti-malarials (maximum concentration shown in brackets): Chloroquine/CQ (2991 nM), Amodiaquine/AQ (81 nM), Piperaquine/PIP (792 nM), Mefloquine/MFQ (338 nM), Artesunate/AS (25 nM), ELQ-300 (66 nM), and P4Q-391 (1042 nM). A candle jar was used to mature the parasites at 37°C for 30-56 hours. Incubation was stopped when >40% of ring stage parasites had reached mature schizont stage in the drug-free control well. Thick blood films made from each well were stained with 5% Giemsa solution for 30 minutes and examined microscopically. The number of schizonts per 200 asexual stage parasites was determined for each drug concentration and normalised to the control well. The dose-response data were analyzed using nonlinear regression analysis (WinNonLin 4.1, Pharsight Corporation) and the IC_{50} value derived using an inhibitory sigmoid E_{max} model. *Ex vivo* IC_{50} data were only used from predicted curves where the E_{max} and E_0 were within 15% of 100 and 1, respectively.

Quality control (QC) procedures

Drug plate quality was assured by running schizont maturation assays (two independent experiments) with the chloroquine-resistant strain K1 and the chloroquine-sensitive strain FC27. For microscopy slide reading quality control, two randomly selected drugs per isolate were read by a second microscopist. If the raw data derived by the two microscopists lead to a dramatic shift in the IC_{50} estimates of the selected drugs, the whole assay (i.e., all standard drugs and experimental compounds) was re-read by a second reader and the results compared. If necessary, discrepant results were resolved by a third reading by an expert microscopist.

Mechanistic studies

***Measurement of cytochrome bc_1 complex inhibition* (human, *P. yoelii* and *P. falciparum*).**

Isolation of HEK-293 derived mitochondria
Cell Culture/Harvesting/Mitochondrial Preparation
HEK-293 cells were grown in DMEM containing 10% FCS using standard methods. When cell monolayers were confluent, flasks were treated with trypsin and the detached cells were pelleted and washed twice in ice cold PBS. The washed cells were resuspended in PBS containing 1 mM PMSF, and processed three times with an ice cold dounce homegenizer. Large or insoluble matter was removed from the broken cells by centrifugation at 800 X G for 10 minutes. The pellet was discarded and the supernatant was centrifuged at 20,000 X G for 40 minutes. The pellet containing mitochondria was resuspended in a minimum of PBS, and made 30% (v/v) glycerol for storage at -80°C until needed for enzyme assays. For details concerning the measurement of cytochrome bc_1 complex inhibition, see below.

P. yoelii mitochondria enrichment
P. yoelii – Outbred female CF-1 mice were infected by injection of 10^6 parasitized red blood cells (RBCs) from an infected donor. When parasitemia reached ~30% (estimated by microscopy of Giemsa-stained blood smears), anesthetized mice were exsanguinated by cardiac puncture, and blood was collected in ACD anticoagulant tubes, pooled, kept on ice, and maintained at this temperature during processing. Leukocyte removal was achieved by published methods (*42*) and verified by microscopic examination of Giemsa-stained blood smears.

Mitochondrial fragments were prepared by a procedure modified from Krunkrai et al. (*43, 44*). Fresh blood was suspended in 125 mL of RPMI 1640 medium containing 0.075% saponin. The mixture was incubated for 20 minutes at 37°C. The parasites were harvested by centrifugation at 8,000 X G for 10 minutes. The intact parasites were then washed four times in phosphate buffered saline (PBS) containing 1 mM PMSF and then centrifuged at 8000 X G for 10 minutes. Once the parasites were harvested, they were frozen and stored until needed for preparation of mitochondrial fragments. The thawed parasites were disrupted by suspending them in "Lysis buffer", containing 75 mM sucrose, 225 mM mannitol, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM EDTA, 1 mM PMSF, 5 mM HEPES, at pH 7.4. The cells were then broken by homogenization in a glass homogenizer. Parasite debris was removed by centrifugation at 800 X G for 5 minutes. Mitochondrial particles were then harvested at 20,000 X G for 40 minutes.

The pellet was resuspended in a minimum of lysis buffer, and mixed to a final concentration of 30% glycerol. The aliquots of mitochondrial particles were frozen at -80°C until needed.

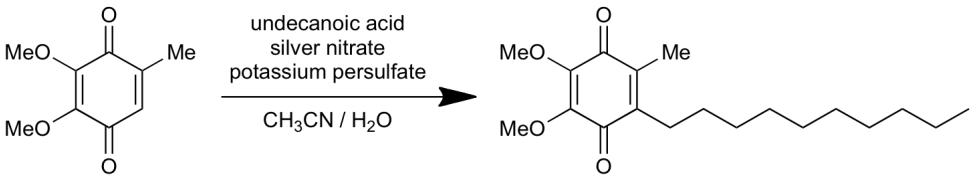
Measurement of cytochrome bc_1 complex inhibition
Mitochondrial material was diluted to a concentration that yielded suitable activity levels (usually 10^{-4}-10^{-3} absorbance units per second - see below), and dispersed in 2 mg/mL n-dodecyl β-D-maltoside. The mixture was allowed to incubate for 45 minutes on ice and then clarified by micro-centrifugation at 10,000 X G.

Enzymatic activity was measured in the following reaction buffer: 50 mM Tricine, 100 mM KCl, 4 mM KCN, 50 µM cyt *c* (horse heart, Sigma), 0.1 mg/mL n-dodecyl β-D-maltoside, 50 µM decylubiquinol (prepared freshly before each experiment by reduction of decylubiquinone with sodium borohydride followed by HCl quenching), pH = 8.0. Cytochrome *c* reductase measurements were made at 550-542 nm at 30°C. Measurements were initiated by the addition of decylubiquinol, and a baseline was

collected for approximately 20 seconds to account for the non-enzymatic reduction of cytochrome *c* by decylubiquinol. Once the baseline collection was complete, enzyme was added to the mixture, and the reaction was allowed to proceed. Once the kinetic trace had been collected, the baseline was subtracted from the initial rate of enzymatic activity. The activity of each kinetic trace is reported as the fraction of activity with respect to control uninhibited enzyme activity under identical conditions.

Synthesis of Decylubiquinone

During the course of our study we were unable to obtain decylubiquinone from commercial sources. As this reagent is required for the cytochrome bc_1 assays the compound had to be synthesized. Accordingly we devised a method for its synthesis and this is described below.



Synthetic Method:

2-Decyl-5,6-dimethoxy-3-methylcyclohexa-2,5-diene-1,4-dione. To a stirred suspension of 2,3-dimethoxy-5-methyl-p-benzoquinone (2.50 g, 13.7 mmol), undecanoic acid (2.55 g, 13.7 mmol) and silver nitrate (2.12 g, 12.5 mmol) in 280 mL 1:1 acetonitrile/water (0.05 M) at 75ºC was added 150 mL of an aqueous potassium persulfate solution (0.1 M). The reaction mixture was stirred 1 hour at 75ºC, cooled to room temperature and filtered. The filter cake was resuspended in ethyl acetate and water. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were washed with water, 1 M aqueous sodium bicarbonate and brine. The organic layer was dried with magnesium sulfate, filtered and concentrated *in vacuo*. Purification of the resulting residue by flash chromatography (ethyl acetate / hexanes) provided 2-decyl-5,6-dimethoxy-3-methylcyclo-hexa-2,5-diene-1,4-dione (2.33 g, 53% yield) as a red-orange oil (100% pure by GC-MS).

Inhibition of parasite respiration by ELQ-300

P. yoelii infected red cells were obtained from infected mice (CF-1, female) with local IACUC approval. The assay is based on the use of BDTM Oxygen Biosensor plates (*45*) that have an oxygen-sensitive dye embedded in a matrix at the bottom of each well. Oxygen quenches the fluorescence of the dye in a concentration dependent manner. Normal parasite respiration processes rapidly deplete the surrounding medium of oxygen thereby allowing the dye to fluoresce. Experiments were set up in a typical 96-well plate and the contents of each well were transferred to a biosensor plate to initiate the reactions, allowing 10 minutes after setup for equilibration and sedimentation of the assay components. ≈60 million parasitized red cells (PRBC) (final hematocrit = 12.5%) were incubated in phosphate buffered saline (pH 7.4, w/o Ca^{++} or Mg^{++}) in the presence or absence of ELQ-300 and in a total volume of 80µl. The set-up was conducted at 4°C and then the plates were transferred to a Gemini EM 96-well plate fluorometer pre-equilibrated to 37°C. The instrument was programmed to take readings (485nm excitation/630nm emission) at 2-minute intervals over a 2 hour period.

Cytochrome bc_1 (*P. falciparum*)
Ubiquinol-cytochrome c oxidoreductase (cytochrome bc_1) activity. Cytochrome *c* reductase activity was assayed by a modification of the method of Trumpower and Edwards (*46*). The assay was performed at 35°C in a stirred cuvette with a final volume of 1 mL containing various amounts of mitochondrial preparation (generally ~6-12mL), 100 mM 2,3-dimethoxy-5-methyl-6-decyl-1,4 benzohydro-quinone (QDH2) (5mL 20 mM), 100 mM horse heart cytochrome *c* (Sigma Aldrich), 0.1 mg/mL n-*dodecyl*-β-D-*maltoside*, 60 mM HEPES (pH 7.4), 10 mM sodium malonate, 1.0 mM EDTA, and 2.0 mM KCN. The reduction of cytochrome *c* was recorded with a modified SLM-AMINCO DW2C dual wavelength spectrophotometer (On-Line Instrument Systems, Inc., Bogart, GA, USA) in dual mode (550 nm – 541 nm). The short chain ubiquinol analog QDH2 was prepared by reducing QD in dimethyl sulfoxide with sodium borohydride and acidifying the mixture with concentrated HCl, and stored under argon in aliquots at -80°C.

Resistance propensity

Assessing Resistance Frequency for Quinolone Compounds
Strain used: *P. falciparum* Dd2
Viability control: 10 parasitized erythrocytes
Positive control: 10 nM atovaquone
Growth conditions: 5% hematocrit in RPMI with 20% inactivated human serum
Experimental approach: Seed counted parasites in triplicate flasks for test compounds; Change medium with indicated drug (at 10x EC_{50} experimentally determined in 20% serum containing medium using a 48 hour assay) every day for the first week, followed by twice a week medium change and once a week 1:2 split with new red cells provided to maintain 5% hematocrit. Thin smears made twice a week to assess emergence of parasites.

Fixed-ratio isobologram analysis of drug interactions between ELQ-300 and proguanil.

In vitro interaction of ELQ-300 and proguanil was assessed by isobolar analysis using fixed-ratio combinations, where drugs were diluted in fixed ratios of starting concentrations predetermined to generate well-defined concentration response curves(*25*). After determination of the intrinsic IC_{50}'s for selected drugs, stock solutions were prepared with each drug at concentrations such that the final concentration in our 96-well drug susceptibility assay after four or five two-fold dilutions approximated the IC_{50}. If we call these stock solutions drug A and drug B, then six final stock solutions were prepared from this initial stock: drug A alone, drug B alone, and volume-volume mixtures of drugs A and B in the following ratios: 4:1, 3:2, 2:3, and 1:4. Two-fold dilutions of each of the six final stock solutions were performed across a 96-well plate in quadruplicate. Initial data analysis yielded the intrinsic dose-response curve for each drug alone and four different fixed-ratio combination dose-response curves, with corresponding IC_{50}'s. The fractional inhibitory concentration (FIC) was then calculated by the following formula: FIC (A) = IC_{50} of drug A in combination/ IC_{50} of drug A alone; FIC (B) = IC_{50} of drug B in combination/ IC_{50} of drug B alone; FIC index = FIC (A) +FIC (B). The isobolograms were constructed by plotting a pair of FICs for the combination of ELQ and proguanil. An interpretation of a straight diagonal line (FIC index = 1) on the isobologram indicates an additive effect between the two drugs. A concave curve below the line (FIC index < 1) indicates synergy of the combination, while a convex curve above the line (FIC index > 1) indicates antagonism.

Parasite reduction ratio (PRR)
The methods used for investigation of ELQ-300 parasite-cidal kinetics, determination of PRR and the time required to kill 99.9% of parasites in vitro, i.e., Parasite Clearance Time (PCT), 99.9%, have been described previously by Sanz et al.(*24*).

In vivo antimalarial activity

Blood stage Thompson tests (*P. berghei*) (University of South Florida)

We used a "modified Thompson model"(*47*), in which infections were established by an intraperitoneal inoculation of 2 x 10^6 *P. berghei* (GFP)-infected red blood cells. By day 3, the first day of drug treatment, parasitemia typically reaches 1% (i.e. 1% of the RBC will be parasitized). Drugs were administered per os once-daily (qd) for 3 days. Atovaquone was included as a standard drug for comparison. Infected mice, drug treated on days 3-5 post parasite inoculum, were followed for 30 days total. Parasitemia was determined from blood collected from the tail vein on days 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30; parasitemia was quantified by flow cytometry and by microscopic examination of Giemsa-stained blood smears. We will also monitor whether (and when) recrudescence occurs. Endpoints for the efficacy analysis were the ED_{50} and ED_{90} (dose of drug that suppresses 50 and 90% of parasitemia on day 6, respectivcely), the no recrudescence dose (ie., no parasitemia following initial clearance of parasitemia), and percent cure at 30 days. This study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council for the National Academies. The protocol was approved by the University of South Florida Institutional Animal Care and Use Committee.

In vivo therapeutic efficacy against *P. yoelii* (GSK Tres Cantos, Spain)

The *in vivo* efficacy of ELQ-300 against *P. yoelii* was evaluated in a standard 4-day test with minor modifications (*48*). Aged matched female CD1 Swiss (Harlan) mice were infected i.v. with 6.4×10^6 *P. yoelii* 17XNL-infected erythrocytes and randomly distributed in groups of n=4. A range of doses of ELQ-300 solubilized in PEG-400 were administered to mice QD from one hour after infection (day 0) to day 3 by oral gavage. Parasitemia was measured in 2 µL blood samples taken at day 4 of the assay using flow cytometry using the nucleic acid dye YOYO-1 as described (*28*).

Blood stage 4-day suppression tests (*P. yoelii*) (Oregon Health & Science University)

In vivo efficacy against murine malaria

The in vivo activity of selected ELQ derivatives was assessed against the blood stages using a modified 4-day test (*47*). Mice (female, CF1, Charles River Labs) were infected intravenously with 2.5 to 5.0 x10^5 *P. yoelii* (Kenya strain, MR4 MRA-428) parasitized erythrocytes from a donor animal. Drug administration commenced the day after the animals were inoculated (Day 1). The test compounds were dissolved in PEG-400 and administered by oral gavage once daily for 4 successive days; chloroquine was used as a positive control. On the 5th day blood films were prepared and the extent of parasitemia was determined by microscopic examination of Giemsa stained smears. ED_{50} and ED_{90} values (mg/kg/day) were derived graphically from the dose required to reduce parasite burden by 50% and 90%, respectively, relative to drug-free controls. Animals remaining parasite free 30 days after the last drug dose were considered cured of their infection. As described the malaria infection in this model system is rapidly fulminate producing average parasitemias of ≈30% in untreated control animals by Day 5. The procedures involved, together with all matters relating to the care, handling and housing of the animals used in this study, were approved by the Portland VA Medical Center Institutional Animal Care and Use Committee.

In vivo therapeutic efficacy against P. falciparum

The *in vivo* efficacy of ELQ-300 against *P. falciparum* was evaluated in a 4-day test as previously described (*27, 49*). Briefly, a group of non-myelodepleted, age-matched, NOD-*scid IL-2Rγ^{null}* (Charles River, Gannat, France, under license from The Jackson Laboratory, Bar Harbor, ME) female mice (n= 11) having 72.1 ± 7 % human erythrocytes in peripheral blood were infected with 2 × 10^7 *P. falciparum* Pf3D7$^{0087/N9}$-infected erythrocytes suspended in 0.25 mL saline solution (day 0). A range of different doses of ELQ-300 was administered to mice QD from day 3 to day 6 in aqueous suspension (1%

hydroxypropyl cyclodextrin, 10% ethanol, 10% propylene glycol, 40% PEG-400, 39% PBS) by oral gavage at 10 mL/Kg.

Parasitemia in peripheral blood of humanized mice was measured by flow cytometry using the nucleic acid dye SYTO-16 and the anti-mouse erythrocyte phycoerythrine-conjugated Ter-119 mAb (Pharmingen, San Diego, CA) in serial 2 µL blood samples taken every 24 hours until assay completion as described (*50*).

The levels of ELQ-300 in blood upon oral administration were measured in all the mice of the efficacy study in order to estimate its efficacious exposure. Serial samples of peripheral blood (25 µl) were taken by tail puncture at 0.5, 1, 3, 6, 8, 10, 18 and 23 hours after the first administration of ELQ-300, mixed with 25 µl of water containing 0.1% saponin for erythrocyte lysis and immediately frozen on dry ice. The frozen samples were stored at -80ºC until analysis. Vehicle-treated mice (n=3) underwent the same blood-sampling regimen. Protein precipitation by liquid-liquid extraction was performed directly in a 96-well plate with filter system (MultiScreen Solvinert 0.45µm Hidrophobic PTFE; Millipore) by putting 10 µL of blood/saponin lysate sample per well, then adding 120 µL of suitable organic solvent as extractant, vortexing for 10 minutes and filtrating with the help of a vacuum system. The filtrates were stored frozen at -80ºC until analysis by LC/MS/MS method specifically devised for the compound. Concentration versus time data were analyzed by non-compartmental analysis (NCA) methods using WinNonlin® Professional Version 5.2 (Pharsight Corporation, Mountain View, CA). Additional statistical analysis of the data was performed with GraphPad Prism® Version 5.01 (GraphPad Software Inc, San Diego CA).

Efficacy is expressed as the daily exposure (AUC, $\mu g \cdot h \cdot mL^{-1} \cdot day^{-1}$) of ELQ-300 in whole blood necessary to reduce parasitemia at day 7 by 90 % with respect to vehicle-treated mice (AUC_{ED90}). The AUC_{ED90} is estimated by fitting a four parameter logistic equation for the $\log_{10}$ [parasitemia at day 7 for individual *i*] *versus* the $AUC_{0\text{-}23h}$ of ELQ-300 in blood for individual *i* using GraphPad Prism 5.01.

All the experiments were approved by the DDW Ethical Committee on Animal Research, performed at the DDW Laboratory Animal Science facilities accredited by AAALAC, and conducted according to European Union legislation and GlaxoSmithKline policy on the care and use of animals.

Liver stage antimalarial activity

***In vitro* liver stage activity (*P. berghei*)**

All mice used in these experiments were female Balb/c mice (average weight was approximately 18 g) obtained from Harlan (Frederick, MD). The transgenic *P. berghei* line 1052 cl1 (Pb1052 cl1) that expresses luciferase was used in the liver stage studies and was obtained from C.J. Janse at Leiden University. HepG2 cells (75,000 per well) were seeded into collagen coated, white 96-well plates for analysis with the Top Count microplate luminometer (Packard, Meriden, CT) as previously described (*51, 52*). Cells were maintained at 37°C in 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 1.0% penicillin-streptomycin (Sigma), and 1.0% L-glutamine. Mosquito salivary glands were dissected and 5,000 sporozoites were added per well. Plates were incubated at 37°C for 3 hours, and then washed three times with PBS. Serial dilutions of experimental compounds were prepared as previously described (*53*), added to parasite infected HepG2 cells in triplicate, and incubated at 37°C for 44 hours. Following the incubation, cells were washed once with PBS and then lysed with 10 µl of cell culture lysis reagent (Promega Luciferase Assay System Kit®; Promega, Madison, WI). Immediately after cell lysis, 100 µl of Luciferase Assay Substrate was added and then the parasite lysates were analyzed. This study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council for the National Academies. The protocol was approved by the University of South Florida Institutional Animal Care and Use Committee.

In vitro activity vs. large and small forms (*P. cynomolgi*)

These assays were conducted essentially as recently described in the published literature (*29*). 96-Well plates are seeded with freshly isolated or thawed cryopreserved stocks of primary rhesus monkey hepatocytes one or two days before infection with 5 x 10^4 freshly dissected *P. cynomolgi* sporozoites per well. After 2-3 hours of sporozoite invasion into the hepatocytes, culture medium is exchanged for culture medium containing appropriate compound dilutions. Initially, compounds are tested in duplicate in 3 dilutions: 0.1, 1 and 10 µM. Plates are incubated for 6 days with medium exchange (including compound dilutions) every other day. Plates are fixed in methanol and parasites are stained with rabbit anti-PcyHSP70 antiserum in the presence of DAPI to stain the nuclei. Plates are automatically counted in a high-content imager (Operetta®) and small parasites (hypnozoites) and large schizonts are recorded, as well as number of hepatocytes as a measure for cytotoxic effects of the compounds. Controls include uninfected hepatocytes, sporozoite infected wells without compound and infected wells with primaquine and atovaquone. When activity is recorded and reported, a more extensive dilution curve can be evaluated: a 7-point 3-fold dilution series from days 0-6, to evaluate IC_{50} on freshly invaded sporozoites and on established EEF.

Effects on gametocyte development and transmission-blocking activity

Late stage *P. falciparum* (IV-V) anti-gametocyte compound screen

The assay uses highly synchronous stage IV gametocytes induced from a transgenic NF-54-pfs16-GFP parasite strain. The gametocytes are harvested by magnetic isolation on day 7, rested overnight then added to the test compounds in 384 well imaging plates. The number of gametocytes is standardized to 20,000 parasites per well (*4, 33*).

Once compound and parasite are combined in the well, the plates are incubated for 72 hours under reduced oxygen tension (5% CO_2, 5% O_2, 80% N_2). After incubation, Mitotracker Red (MitoTracker Red CM-H2XRos) is added to the plates, which are then incubated for an hour. After this period the plates are incubated overnight as described above. After overnight incubation, the plates are evaluated on the OPERA™ confocal imaging system. Images for GFP and Mitotracker Red are acquired, overlayed and the number of elongated viable gametocytes per image determined using a script based on Acapella™ software developed for use with the OPERA™ imaging system. Using DMSO or Puromycin as controls, the percent inhibition is calculated for the compounds screened in dose response.

Principle of script used for image analysis

The average Mitotracker Red fluorescence intensity is determined for objects greater than a determined size. Objects which are more than three times longer than they are wide are identified using the GFP images and are representative of gametocytes. Objects which are elongated and have Mitotracker Red fluorescent intensity above the cut off limit are then identified as viable gametocytes.

In vitro activity against ookinetes and oocysts

P. berghei ookinete development assay

Whilst *P. falciparum* ookinetes cannot currently be produced *in vitro* in bulk, *P. berghei* readily forms ookinetes in culture. This assay (*34, 35*) reports on the ability of a drug to inhibit the transformation of mature gametocytes into ookinetes, encompassing gamete formation, fertilization, and zygote development into the mature ookinete. The assay uses a transgenic parasite expressing GFP under an ookinete-specfic promoter, and fluorescence detection in a plate reader in either 96 or 384 well formats. (Note that this transgenic parasite line contains a DHFR selection marker and so does not accurately

report on antifolate compounds.) Gametocyte-containing blood for the assay is obtained from donor mice by cardiac puncture before being immediately distributed to wells of the 96 well plate to a final hematocrit of ~4% in ookinete medium. Final assay volumes are: 200 µl (96 well). Final DMSO concentration does not exceed 0.5%. Plates are incubated in the dark at 19°C for 22 hours before being read in a plate reader. All drugs are screened at 10 µM in triplicate independent experiments with the blood of three different mice. As a negative control, DMSO alone is added in at least triplicate to each plate and the mean fluorescence reading of these wells corresponds to "100% ookinete production". As a positive control, 10µM cycloheximide (a protein synthesis inhibitor) is added in at least triplicate to each plate and the mean fluorescence reading of these wells corresponds to "0% ookinete production". All datapoints are evaluated with reference to these controls and are expressed as the % inhibition of ookinete production. As fluorescence is dependent on ookinete number, which in turn is dependent on initial mouse gametocytemia, which is variable, a plate must have a difference of at least 150 fluorescent units between positive and negative control to be acceptable. The typical experimental range is from 150-600 fluorescent units. Those drugs exhibiting >50% inhibition at 10µM are then subjected to a 10-datapoint dose response analysis to determine an IC_{50} value.

P. berghei transmission-blocking assay

This in vitro assay provides the essential *in vivo* confirmation of transmission-blocking activity detected in the higher throughput *in vitro* transmission assays (*P berghei* ookinete development assay).

Blood from GFP-expressing gametocyte-infected mice is aliquoted into an array of membrane feeders +/- drug, and each fed to batches of 75 *Anopheles stephensi* mosquitoes. 7 days later fluorescent oocysts are counted on the dissected midguts using semi-automated image-capture/analysis. DMSO is used as a negative control in this assay and serves as a 100% oocyst production control by which to compare all other data. Data is presented as % inhibition of oocyst production. Each experimental datapoint represents 30-50 dissected mosquitoes and each experiment is repeated 3 times independently with blood derived from a different pool of mice so that a mean and standard error can be calculated. Drugs are typically screened at 1 µM with a final DMSO concentration of no more than 0.5%. If a compound is prioritized, it may also be screened at three concentrations: 1 µM, 100 nM and 10nM to obtain an indication of potency. This assay does not use a positive control as the base line is no fluorescent oocysts detected.

***I**n vivo transmission blocking*

Donor mice were inoculated ip with *P. berghei*-infected erythrocytes from thawed cryopreserved blood. Following development of patent infections, blood from the donor mice was pooled and then 2 x 10^6 *P. berghei*-infected erythrocytes were inoculated into each experimental mouse (n=10). On day 4 following inoculation the parasitemia was ~3% in the experimental mice. Groups of mice (n=2) were then treated per os with P4Q-391, ELQ-300, primaquine, atovaquone, or vehicle control. One hour following drug treatment, each mouse was anesthetized and adult female *A. stephensi* mosquitoes were allowed to feed. Efficacy was assessed by determining the development of oocysts on the midgut of moquitoes.

P. falciparum transmission-blocking assay

This assay uses human-infective *P. falciparum* and can assess the ability of a drug to interrupt transmission anywhere from the mature gametocyte right through to the oocyst.

Mature gametocyte cultures are pooled and divided into 4 mL "minicultures" and exposed to the drugs of interest at 1 µM for 24 hours in culture. DMSO serves as a negative control, and final DMSO concentrations in cultures did not exceed 0.5%. After the 24 h incubation, the gametocytes are reconstituted in fresh blood and fed to *A. stephensi* mosquitoes. After a further 10 days, the mosquitoes are dissected and mudgut oocyst burden quantified microscopically by trained observers. All data is

compared to the DMSO control which represents 100% oocyst production. Data is expressed as % inhibition of oocyst production and experiments are repeated 3 times independently with different source cultures so that a mean and standard error can be calculated.

Standard Membrane Feeding Assay (*P. falciparum* strain 3D7)
Two *P. falciparum* 3D7 strain gametocyte cultures were started by passage from asexual cultures at 1% parasitemia/4% haematocrit staggered by two days. Culture medium was changed once daily whilst ensuring culture temperature remained at 37°C at all times. When the cultures reached Day 17 and Day 15 respectively, the maturity of the cultures was confirmed by sampling their ability to exflagellate when stimulated by temperature decrease and the presence of xanthurenic acid. The cultures were then pooled and centrifuged whilst maintaining a constant temperature of 37°C. The cell pellet was then resuspended in warmed whole blood to 50% haematocrit and divided into individual tubes containing either DMSO as a control, or different concentrations of ELQ-300. The parasite/blood mixes were then introduced into a warmed membrane feeding apparatus and offered to groups of ~75 *Anopheles stephensi* mosquitoes (SD500 strain) for 30 min. After feeding, mosquitoes were maintained at 26°C 80% relative humidity with a 12 hr light/dark cycle being fed on a fructose/p-aminobenzoic acid (PABA) solution replenished every 2-3 days. At 10 days post-feeding, mosquitoes were drowned in ethanol and midgets dissected. Midgut oocyst burdens were recorded only for those mosquitoes that had eggs in their ovaries, indicating that they had blood fed.

Physicochemical Properties and Pharmacokinetics

Physicochemical Properties

In silico physicochemical properties were obtained using ACD/Labs Release 9.0 software (Advanced Chemistry Development, Toronto, Canada) and ChemDraw (Cambridgesoft, Cambridge, MA, USA). Solubility was estimated using two methods:

First, PBS kinetic solubility (Apredica, Inc.): Serial dilutions of test agent are prepared in test agent at 100x the final concentration. Test agent solutions (50 mM stock) are diluted 100-fold into PBS in a 96-well plate and mixed. The absorbance of the PBS-containing plate is measured prior to adding the test agents to determine the background absorbance. After 45 min and 16 hr, the presence of precipitate is then detected by turbidity (absorbance at 540 nm). An absorbance value of greater than (mean + 3x standard deviation of the blank), after subtracting the pre-experiment background, is indicative of turbidity. For brightly colored compounds, a visual inspection of the plate is performed to verify the solubility limit determined by UV absorbance. The solubility limit is reported as the highest experimental concentration with no evidence of turbidity.

Second, an equilibrium / dissolution solubility method: Conducted by adding physiologically-relevant media (representative of the fasted (FaSSIF) and fed (FeSSIF) state intestinal fluids[1]) to solid material, briefly sonicating and incubating at 37ºC for 1 and 4 h. Samples were centrifuged and aliquots of the supernatant analysed by HPLC using a Waters 2695 system coupled to a Waters 486 UV detector (254 nm). The column was a Phenomenex Luna C8(2) column (5 µm, 50 x 2 mm i.d.) maintained at a column temperature of 40°C, and the mobile phase consisted of water, methanol, and 1% aqueous formic acid delivered at 0.4 mL/min under gradient conditions.

In vitro metabolism

Human, dog, rat and mouse liver microsomes (BD Gentest, Discovery Labware Inc., Woburn, Massachusetts) were suspended in 0.1 M phosphate buffer (pH 7.4) at a final protein concentration of 0.4 (P4Q-391) or 1 (ELQ-300) mg/mL and incubated with compounds (0.2 µM ELQ-300; 0.5 µM P4Q-391) at 37°C. An NADPH-regenerating system (1 mg/mL NADP, 1 mg/mL glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase) and $MgCl_2$ (0.67 mg/mL) was added to initiate the reactions, which were subsequently quenched with ice-cold acetonitrile at 4-5 time points over 60 (P4Q-391) or 180 (ELQ-300) min. Samples were also incubated in the absence of the NADPH-regenerating system to monitor for non-cytochrome P450-mediated metabolism in the microsomal matrix. Samples were centrifuged and the concentration of compound remaining in the supernatant was monitored by LC-MS. The first order rate constant for substrate depletion was determined by fitting the data to an exponential decay function and these values were used to calculate the *in vitro* intrinsic clearance value which was scaled to predict the *in vivo* intrinsic clearance value (CL_{int}, mL/min/kg) as previously described (*54*).

Inhibition of cytochrome P450 (CYP) enzymes was assessed in human liver microsomes using a substrate specific approach where the formation of metabolites specific to a particular CYP isoform was monitored. Microsomes were suspended in phosphate buffer and incubated at 37ºC in the presence of probe substrates; conditions for each pathway are shown below:

CYP450 enzyme	Probe Substrate	Substrate Conc. (µM)	Microsomal protein conc. (mg/mL)	Assay incubation duration (min)
CYP1A2	phenacetin	40	0.4	30
CYP2C9	tolbutamide	140	0.4	20
CYP2C19	S-mephenytoin	30	1.0	40
CYP2D6	dextromethorphan	3	0.4	10
CYP3A4/5	testosterone	50	0.4	5

Reactions were initiated by the addition of an NADPH-regenerating system as described above and were quenched at appropriate times using acetonitrile. Samples were centrifuged and concentrations of the metabolites assessed by LC/MS. The IC_{50} value for each compound or control inhibitor was the concentration at which there was a 50% reduction in the amount metabolite formed relative to the maximum metabolite formation in the absence of inhibitor.

Plasma and media binding

Pharmacokinetic analysis

Animal studies were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the study protocol was approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee.

Pharmacokinetic studies were conducted in non-fasted male Swiss outbred mice and overnight fasted male Sprague Dawley rats after IV and oral administration. Mice had unrestricted access to food and water throughout the experimental period, whereas rats had unrestricted access to water and access to food was re-instated 4 hours post-dose. For IV administration, compounds were formulated in either mouse plasma (for mice) or 20% v/v ethanol / 80% v/v propylene glycol (for rats) and for oral administration compounds were formulated in PEG400. IV doses were administered to mice by bolus injection of 50 µL into the tail vein and to rats via a 10 minute infusion of 0.5 mL into an indwelling jugular

cannula. For oral dosing, compounds were administered by gavage of 0.1 mL (mice) or 1 mL followed by 1 mL of water (rats). At specific time points, blood samples were collected from mice (maximum of two samples per mouse) via submandibular bleed (conscious sampling) or terminal cardiac puncture (under inhaled isofluorane anesthesia) and from rats via an in-dwelling carotid cannula. Collected samples were immediately transferred to heparinized tubes that were centrifuged and the plasma collected and stored at -20ºC until analysis (with two weeks).

Compound concentrations were quantitated in plasma samples by LC-MS using a Waters Micromass Premier triple quadrupole instrument. Chromatographic separation was conducted using a Waters Acquity UPLC with Supelco Ascentis Express RP Amide column (2.7 μm particle size, 50 x 2.1 mm i.d.) equipped with a Phenomenex Security Guard column with Synergy Polar packing material (both at 40°C). The mobile phase (0.4 mL/min) consisted of 0.05% formic acid in water and 0.05% formic acid in acetonitrile mixed using a linear gradient over 4 minutes. The injection volume was 5 µL and elution of analytes was confirmed by multiple-reaction monitoring (MRM) using diazepam as the internal standard. Plasma samples and calibration standards were prepared by protein precipitation with acetonitrile (3 parts acetonitrile to 1 part plasma), followed by centrifugation and analysis of the supernatant. Sample concentrations were determined by comparison to calibration standards prepared in blank plasma and assayed using the same conditions. The analytical lower limit of quantitation in plasma was typically 0.5-1 ng/mL and accuracy, precision and recovery were within acceptable limits.

Pharmacokinetic parameters were calculated using non-compartmental methods. The terminal elimination half-life ($t_{1/2}$), the area under the plasma concentration versus time profile from time zero to either the last sample time point ($AUC_{0\text{-}48}$) or extrapolated to infinity ($AUC_{0\text{-}inf}$), plasma clearance, and volume of distribution at steady-state were determined using WinNonlin software (version 5.2.1, Pharsight Corporation, Mountain View, CA). The maximum plasma concentration (C_{max}) and the time to reach the maximum concentration (T_{max}) were taken directly from the concentration versus time profiles. Oral bioavailability (%F) was calculated by comparing the average dose-normalized $AUC_{0\text{-}inf}$ after oral administration to the average dose-normalized $AUC_{0\text{-}inf}$ after IV administration.

Cytotoxicity assays

In vitro cytotoxicity (T-Cell Lymphocyte Proliferation Assay)

We assessed the cytotoxicity of 4(1*H*)-quinolone-3-diarylether derivatives (ELQ-271, ELQ-300, and P4Q-391) *in vitro* against murine splenic lymphocytes induced to proliferate and differentiate following concanavalin A stimulation. Briefly, $2x10^5$ splenic cells were incubated at 37°C in 200 µl of RPMI-1640 supplemented with 5% heat-inactivated fetal calf serum, antibiotics (penicillin and streptomycin), and the test drug (in concentrations ranging from 0 to 25 µM). Conanavalin A was added at 10 µg/mL. 2-fold drug dilutions were made starting at 25 µM. After 72 hours of incubation 20 µl of PrestoBlue™ Cell Viability Reagent (Invitrogen) was added and plates were incubated for another 60 minutes. This reagent consists of a resazurin-based dye that is reduced by cellular dehydrogenases to a fluorescent product (excitation 560nm/emission 590 nM). Cytotoxic IC_{50} values were calculated from the dose response curves using Prism 5 software and reflect the concentration of drug required to inhibit lymphocyte proliferation by 50 percent relative to drug-free controls. A primary goal of the drug testing studies is the determination of IC_{50} values for drug activity (vs. parasite infected red blood cells) and drug toxicity (vs. splenic lymphocytes). The ratio of the 2 IC_{50}'s, i.e., IC_{50} (vs. lymphocytes)/IC_{50} (vs. PRBC) is used to generate the in vitro selectivity index (SI) for selected quinolones.

In vitro Cytotoxicity Testing of ELQ-300 and P4Q-391 against human bone marrow progenitors

Introduction: It is now accepted that morphologically recognizable bone marrow precursor cells are derived from hematopoietic progenitor cells committed to a specific lineage of hematopoietic differentiation. When these progenitors are cultured in vitro under appropriate conditions, they give rise

to colonies of mature progeny. Thus, the granulocyte-macrophage progenitor cell (colony forming unit-granulocyte, macrophage; CFU-GM) forms colonies of granulocytes and or macrophages and burst forming units-erythroid (BFU-E) when cultured in semi-solid medium in the presence of human Steel Factor, IL-3, and erythropoietin. Pluripotent stem cells which give rise to committed stem cells such as the CFU-GM and BFU-E (as well as the multipotential stem cell, CFU-GEMM) undergo self-replication and differentiation into committed stem cells. Gauging the inhibitory effects of candidate antimalarial drugs on the formation of CFU-GEMM, CFU-GM and BFU-E colonies represents an excellent opportunity to assess comparative cytotoxicity against normal mammalian cell proliferation and differentiation and provides evidence of potential for bone marrow suppression. The inhibitory activity of ELQ-300 (alongside of atovaquone and P4Q-391) against murine CFU-GM, CFU-GEMM, and BFU-E formation in vitro was evaluated in vitro. The methods that were employed to set up this cytotoxicity experiment have been described previously (*55, 56*). The assessment of drug activity on colony type, size and morphology was determined by an individual with experience in hematology and hematopoiesis. In summation of our findings, the amount of drug needed to reduce colony formation by 50% (IC_{50}) for both drugs was above 10 μM. Taken together, ELQ-300 and P4Q-391 appear to be highly selective antimalarial compounds with an in vitro therapeutic index (IVTI) of over 1,000-fold.

Methods: Normal human bone marrow cells were cultured in the presence of quinolone derivatives ELQ-300 and P4Q-391 and the antimalarial atovaquone. For this experiment, a preparation of immature heparinized hematopoietic cells was obtained from the bone marrow of a mouse. The bone marrow cells were enriched for stem cells by density gradient centrifugation to produce “low density bone marrow cells” or LDBMC, which contain the mononuclear hematopoietic stem cells (*55*). These cells were suspended in semisolid medium, Methocult H4230 methylcellulose medium (Stem Cell Technologies, Vancouver, Canada) containing recombinant murine Stem Cell Factor (10 ng/mL, R&D Systems, Minneapolis, MN), murine interleukin-3 (10 ng/mL, R&D Systems), and human erythropoietin (2 U/mL, Amgen, Thousand Oaks, CA) as described previously(*56*). Conditions in which the cells were incubated in the absence or presence of 0–10 μM drug were set up in triplicate and allowed to incubate for 10 days after which colonies and bursts were counted as CFU-GEMM, CFU-GM, and BRU-E using a dissecting microscope.

ATP depletion assays in 2 different mammalian cell lines

Toxicity to host mitochondria and to host mitochondrial processes has been implicated in drug-induced idiosyncratic toxicity. Some of these drugs that have been withdrawn from the market due to organ toxicity, have been found to be mitochondrial toxicants. These compounds injure mitochondria by blocking the respiratory complexes of the electron transport chain, inhibiting or uncoupling oxidative phosphorylation, resulting in mitochondrial oxidative stress, or inhibition of DNA replication, transcription, or translation. Examples of such toxicants are diphenylamine containing NSAIDs such as Diclofenac (*57*).

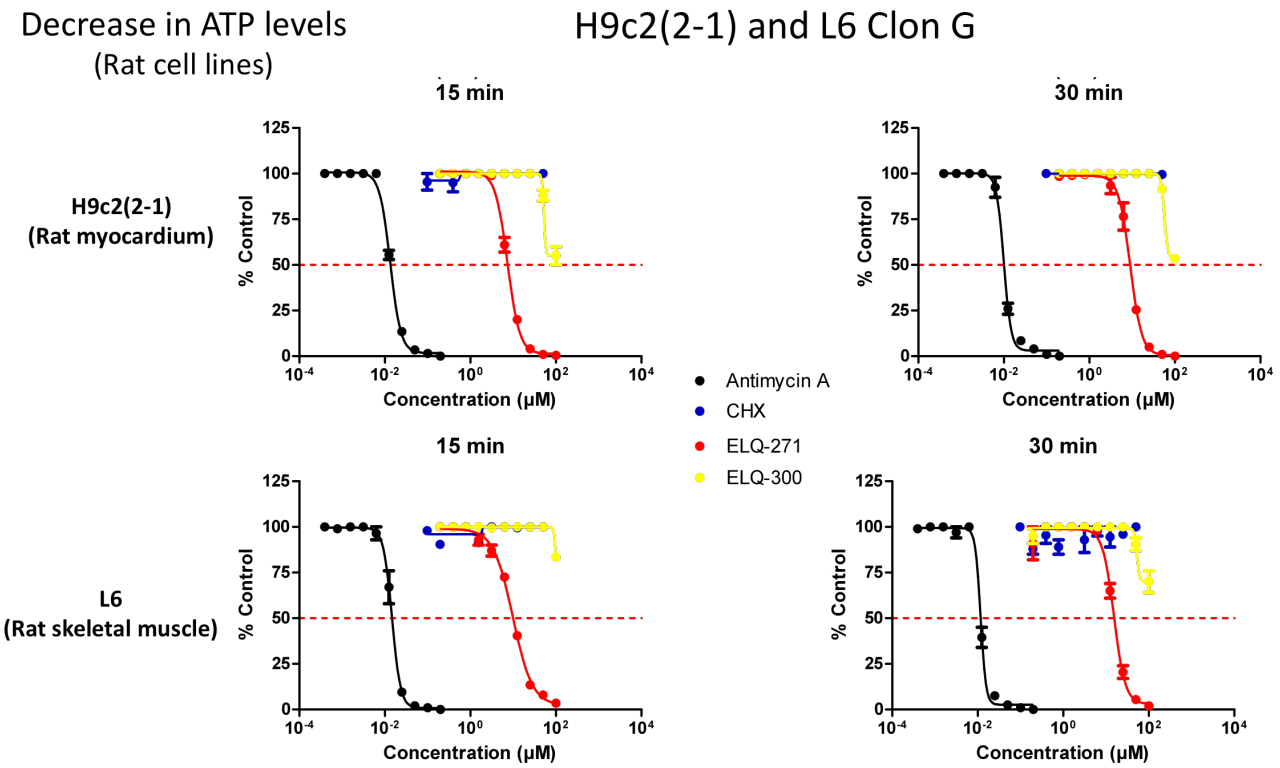
Toxicity testing of drug candidates is usually evaluated in transformed immortalized cell lines that have been adapted for exponential growth in a reduced-oxygen environment. Some of these cell lines were derived from tumors that adapted to survival in the host in the relative hypoxia of a rapidly growing tumor mass. The metabolism of these transformed cells is often anaerobic, by means of glycolysis, despite their having fully functional mitochondria and an adequate oxygen supply (this fairly describes the so-called “Crabtree and Warburg effect”). On the other hand, normal cells generate ATP for energy consumption by mitochondrial oxidative phosphorylation. The anaerobic metabolism of transformed cell lines makes them refractory to drugs that block the components of the mammalian electron transport chain. As a result scientists have devised culture conditions that force cell lines to rely on their mitochondria for ATP production (*58, 59*). The key to this clever technique is the replacement of glucose with galactose.

Scientists at the GSK Tres Cantos facility have adapted the growth of two cell lines, H9C2 (rat, myocardium) and L6 (rat, skeletal muscle), for growth *in vitro* with galactose as the sole source of carbon and energy. Intracellular ATP produced by the mitochondrial oxidative phosphorylation was detected and quantified by the standard luciferin-luciferase methodology.

The ability of ELQ-271 and ELQ-300 to deplete ATP levels in these two cell lines in galactose medium was evaluated with antimycin A and cycloheximide as positive and negative controls, respectively. As shown in Figure S1 while ELQ-271 caused a concentration dependent decline in ATP levels (indicating that it was inhibiting host electron transport processes), ELQ-300 did not have a measureable adverse effect on ATP levels in either cell line with an IC_{50} level above 100μM.

hERG and Ion Channel Inhibition

ELQ-300 was tested for inhibition of the hERG, hNaV1.5 and hKV1.5 cardiac ion channels using Ionworks patch clamp electrophysiology and CaV1.2 channels using a fluorescence Ca^{++} assay with Essen Bioscience. The electrophysiology assays employed repeated gating voltage-command protocols to determine 'use-dependent' and 'open-channel' block. Similarly, the Ca^{++} flux assay was conducted in a depolarised membrane potential format to explore 'open- and inactivated-channel' inhibition. Concentration-dependence was assessed by testing at 8-11 concentrations from a maximum concentration of 100 μM with 3-fold dilutions ('n'=2-3).



		Antimycin A	Cycloheximide	ELQ-271	ELQ-300
		IC50 (µM)			
H9c2 (2-1)Clon G	15min	0.013	>50	7.4	>100 (55%)*
	30min	0.010	>50	8.2	>100 (53%)*
L6 Clon G	15min	0.014	>50	10	>100 (85%)*
	30min	0.011	>50	15.4	>100 (70%)*

*: The value in brackets shows the percentage of control at the highest concentration tested.

Cell lines L6 and H9c2(2-1) were adapted to grow in galactose to avoid the Crabtree and Warburg effect. It was done in base on the article published by Marroquin et al. Tox. Sci. 97:539,2007, introducing some modifications.

Figure S1. Comparative effect of ELQ-271 and ELQ-300 on the intracellular level of ATP in two different mammalian cell lines.

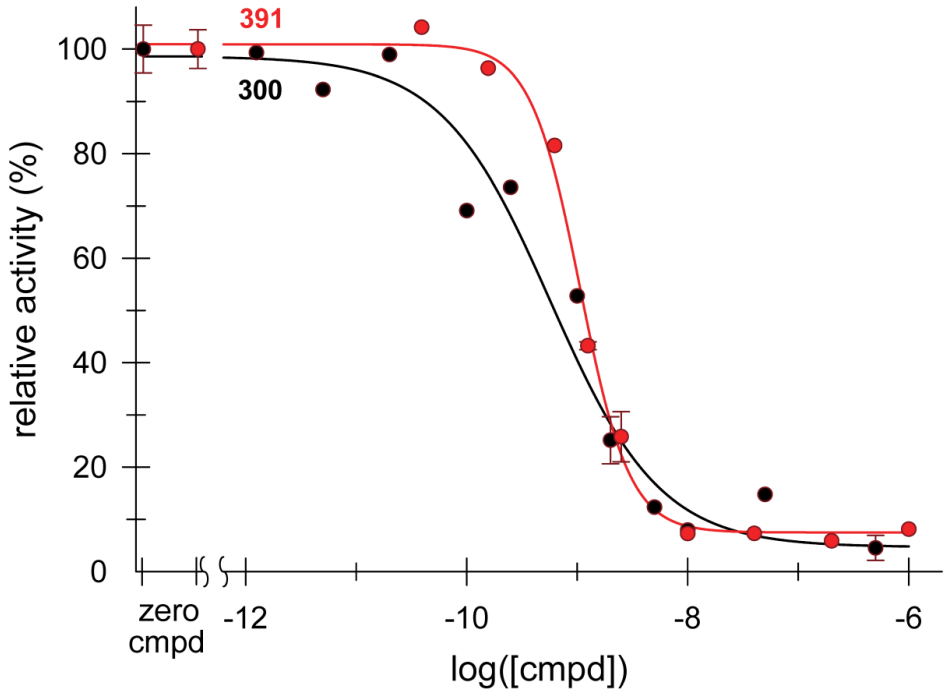
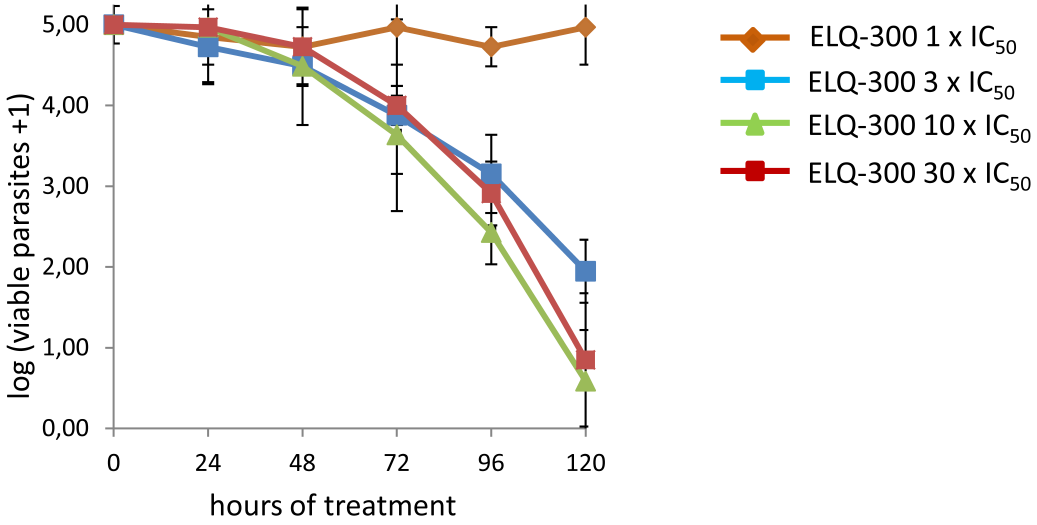


Figure S2. EC_{50} curves for ELQ-300 and P4Q-391 vs. *P. falciparum* cytochrome bc_1.

Panel A



Panel B

Compound	Dose	Lag Phase, h	Slope	R	Log PRR	$PCT_{99.9\%}$, h
ELQ-300	10x IC_{50}	48	-0.064	-0.993	3.1	100
Artemisinin	10x IC_{50}	0	-0.101	-0.92	>4.8*	<24
Atovaquone	10x IC_{50}	48	-0.060	-0.992	2.9	81
Pyrimethamine	10x IC_{50}	24	-0.072	-0.999	3.7	57
Chloroquine	10x IC_{50}	0	-0.094	-0.999	4.5	34

Figure S3. Parasite Reduction Ratio (PRR) of ELQ-300. Panel A. Number of viable parasites after ELQ-300 treatment for all the concentrations tested. Panel B. Table showing the PRR parameters for ELQ-300 and standard antimalarials.

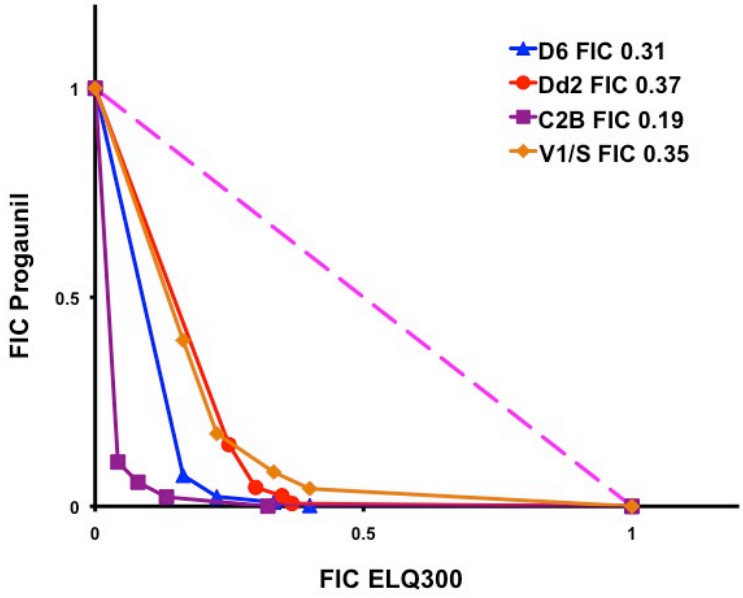


Figure S4. Isobolograms of the interactions of ELQ-300 with proguanil against 4 different strains of *P. falciparum* (D6, Dd2, TM90-C2B, and V1/S). The mean FIC indices were derived from three independent experiments. The *x*-axis represents the FICs of ELQ-300, and *y*-axis represents the FICs of proguanil. The diagonal line (FIC index = 1) indicates the hypothetical additive drug effect. A concave curve (FIC index < 1) below the diagonal line indicates synergy, while a convex curve (FIC index > 1) above the diagonal line indicates antagonism.

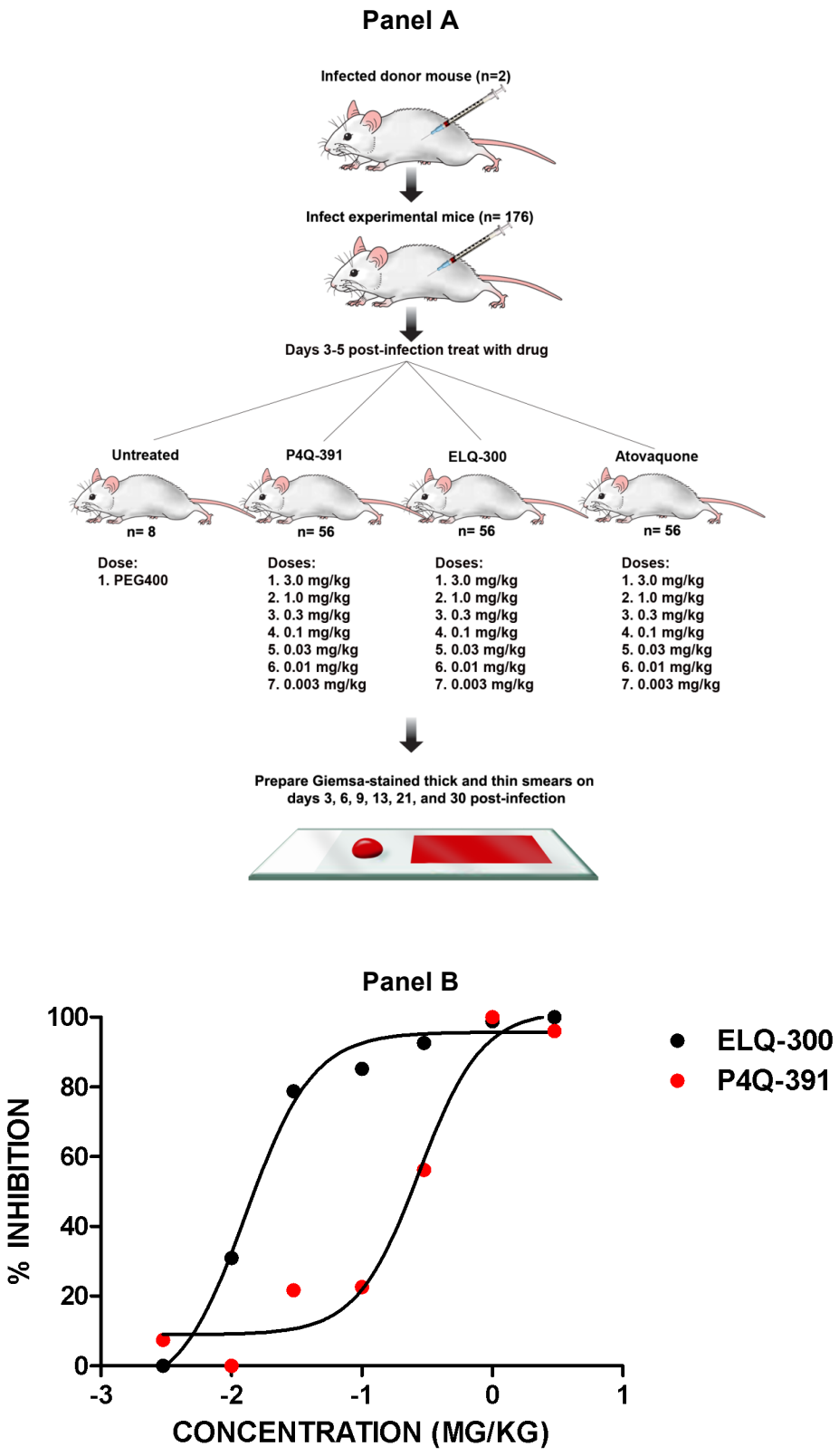


Figure S5. Schematic overview (Panel A) and results of Thompson test method for evaluation of candidate antimalarial drugs in mice (Panel B).

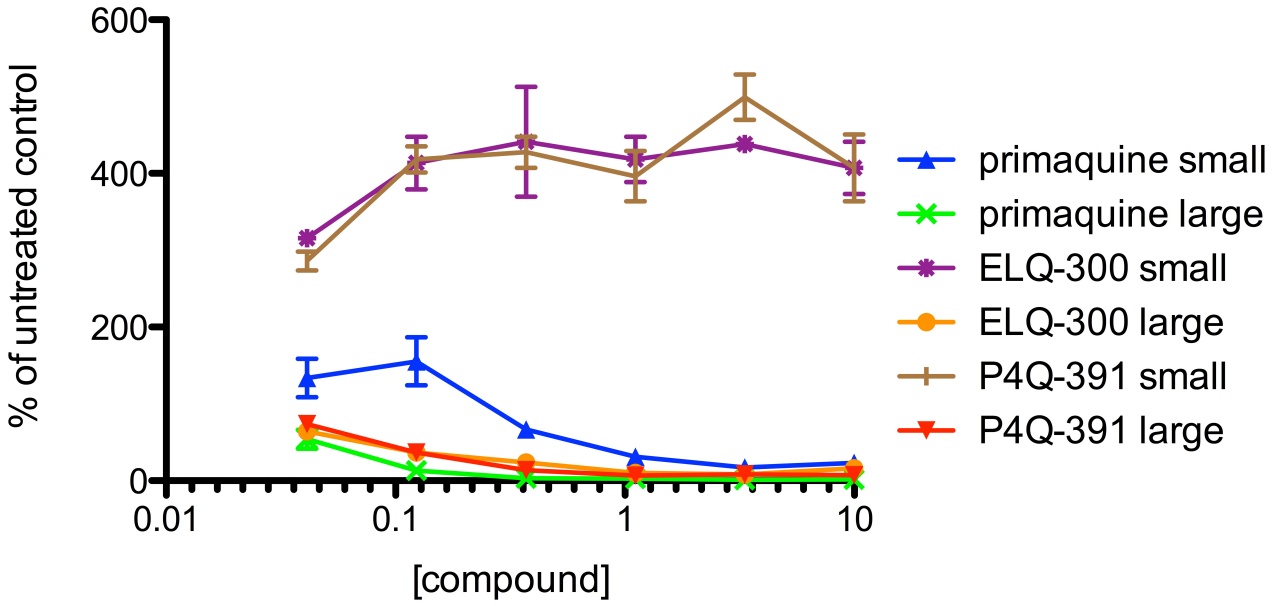


Figure S6. Effect of drugs on *P. cynomolgi* large and small hepatic forms *in vitro*. Concentration is in μM.

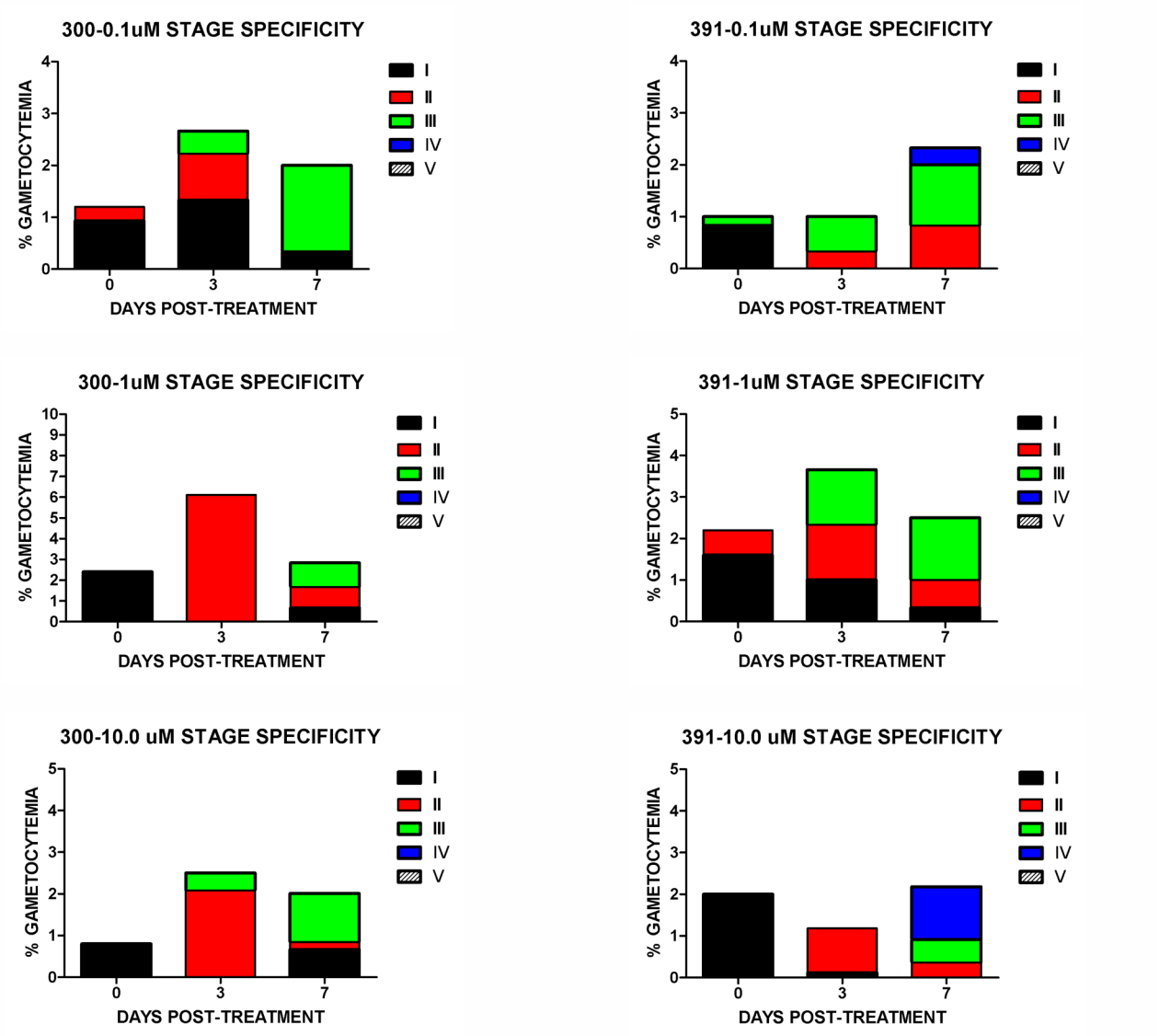


Figure S7. *In vitro* activity of ELQ-300 and P4Q-391 against development of early stage gametocytes (*P. falciparum*). Gametocytes of NF54 were exposed in vitro on days 7-9 of development. The percent of each stage of development was determined by microscopy on the first day of treatment (Day 0), day 3 and day 7 following initiation of treatment. Both ELQ-300 and P4Q-391 inhibited the development of gametocytes to fully mature Stage V gametocytes at all doses (0.1, 1.0, and 10 μM). By day 7 following treatment untreated cultures were mostly Stage V, whereas treated gametocytes were arrested mostly at Stage III. The increase in development of gametocytes to Stage IV in the 10 μM P4Q-391 group is likely due to limited solubility and the drug falling out of solution.

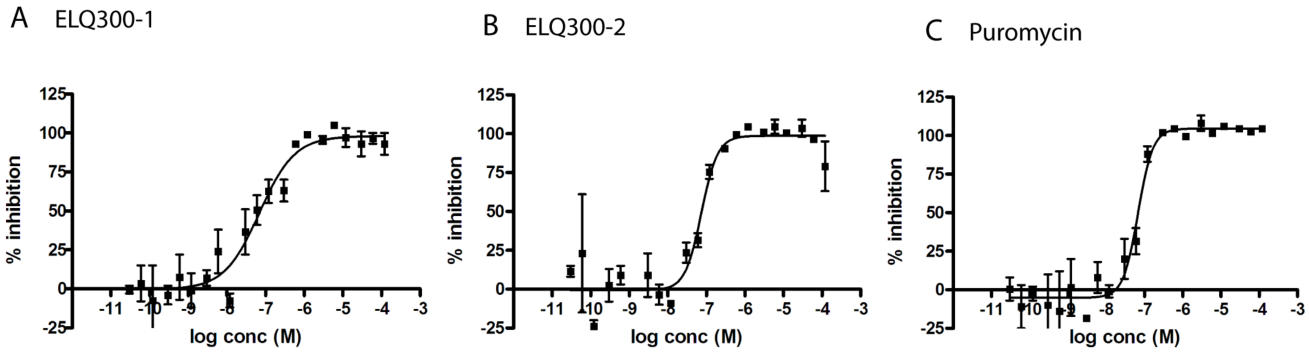


Figure S8. Comparative inhibitory activity of ELQ-300 (Panel A and B) and puromycin (control drug, Panel C) on the development of stage IV to stage V *P. falciparum in vitro*.

Panel A

		ELQ-300, nM		
	DMSO control	1000	100	10
	377	0	0	156
	43	0	0	97
	375	0	0	207
	181	0	0	93
	266	0	0	27
	211	0	0	15
	256	0	0	135
	245	0	0	0
	240	0	0	149
	291	0	0	106
	403	0	0	259
	302	0	0	0
	240	0	0	417
	224	0	0	128
	271	0	0	76
	152	0	0	433
	0	0	0	0
	137	0	0	0
	0	0	17	174
	0	0	0	259
	239	0	0	42
	47	0	0	29
	256	0	16	496
	145	0	0	22
	162	0	0	397
	144		0	346
	111		0	0
	412		0	0
	233		0	131
	209		0	109
	118		0	45
	18		0	121
	429		0	128
	76		0	0
	0		0	146
	107		0	98
	271		0	83
	41		0	84
	281		0	0
	0		0	173
	93		0	0
	396		0	81
	239		0	240
	0		0	142
	20		0	65
	93		32	382
	234		0	165
	416		0	240
	301		0	100
	332		0	184
	8		0	144
	58		0	289
	0		0	245
	257		0	445
	117		0	256
	496		0	380
				98
Mean	188.8	0	1.2	152.5
% inhib.		100	99.4	19.2

Panel B

	ELQ-300		
Conc (nM)	1000	100	10
% inhibition			
Expt 1	100.00	100.00	42.39
Expt 2	100.00	100.00	62.75
Expt 3	100.00	99.39	19.24
Mean	100.00	99.80	41.46
SD	0	0.35494	21.77218
SEM	0	0.204924	12.57017

Panel C

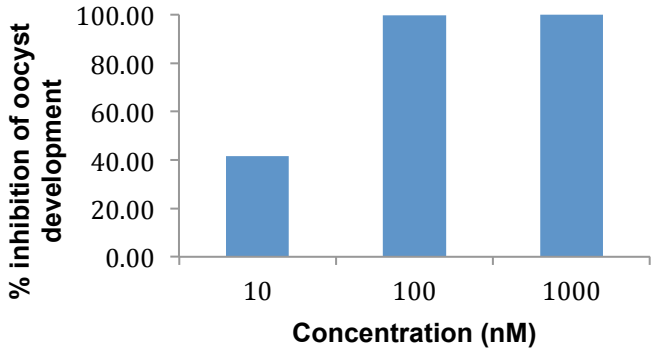


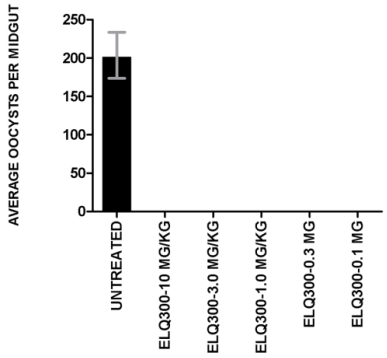
Figure S9. Effect of ELQ-300 on the formation of *P. berghei* oocysts in mosquitoes after feeding on a blood meal containing various concentrations of the drug. The data from a single exemplary experiment is presented below (Panel A). Summary data from three independent studies is provided in Panel B and Panel C.

Expt 1		ELQ-300		
	DMSO	10nM	100nM	1000nM
	1	0	0	0
	3	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	12	0	0	0
	8	0	0	0
	3	0	0	0
	6	0	0	0
	1	0	0	0
	7	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	4	0	0	0
	0	0	0	0
	11	0	0	0
	1	0	0	0
	4	0	0	0
	1	0	0	0
	3	0	0	0
	4	0	0	0
	0	0	0	0
	4	0	0	0
	5	0	0	0
	3	0	0	0
	3	0	0	0
	0	0	0	0
	4	0	0	0
	7	0	0	0
	0	0	0	0
	14	0	0	0
	1	0	0	0
	1	0	0	0
	10	0	0	0
	1	0	0	0
	8		0	0
	5		0	0
	3		0	0
	4		0	0
			0	0
				0
				0
				0
Mean	3.55	0	0	0
Inhibition (%)		100	100	100
Prevalence (%)	75	0	0	0

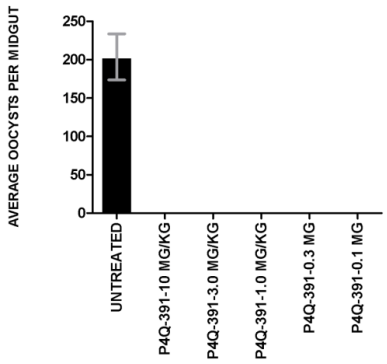
Expt 2		ELQ-300		
	DMSO	10nM	100nM	1000nM
	6	0	0	0
	4	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	4	0	0	0
	0	0	0	0
	5	0	0	0
	5	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	5	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	4	5	0	0
	0	0	0	0
	0	0	0	0
	1	0	0	0
	2	0	0	0
	3	0	0	0
	0	0	0	0
	7	0	0	0
	9	0	0	0
	1	0	0	0
	4	0	0	0
	2	0	0	0
	10	0	0	0
	6	0	0	0
	13	0	0	0
	0	0	0	0
	2	0	0	0
	0	0	0	0
	1	0	0	0
	0	0	0	0
	2	0	0	
	6	0	0	
	1		0	
	4		0	
	0		0	
Mean	2.52	0.10	0	0
Inhibition (%)		95.87	100	100
Prevalence	51.92	2.08	0	0

Figure S10. Effect of ELQ-300 on *P. falciparum* oocyst development in blood-fed mosquitoes as assessed in a Standard Membrane Feeding Assay (SMFA). The methods employed for this SMFA are described in the methods section of the supplementary materials.

A


AVERAGE OOCYSTS PER MIDGUT
250
200
150
100
50
0
UNTREATED
ELQ300-10 MG/KG
ELQ300-3.0 MG/KG
ELQ300-1.0 MG/KG
ELQ300-0.3 MG
ELQ300-0.1 MG

B


AVERAGE OOCYSTS PER MIDGUT
250
200
150
100
50
0
UNTREATED
P4Q-391-10 MG/KG
P4Q-391-3.0 MG/KG
P4Q-391-1.0 MG/KG
P4Q-391-0.3 MG
P4Q-391-0.1 MG

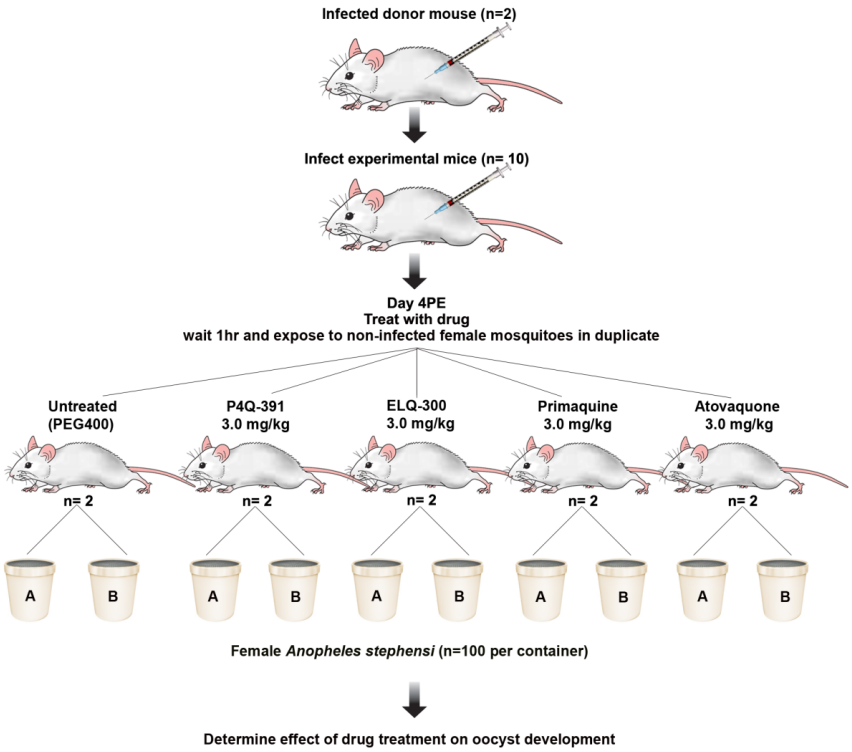


Figure S11. Schematic overview and results of *in vivo* transmission blocking activity of ELQ-300 (Panel A) and P4Q-391 (Panel B) in mice infected with *P. berghei*.

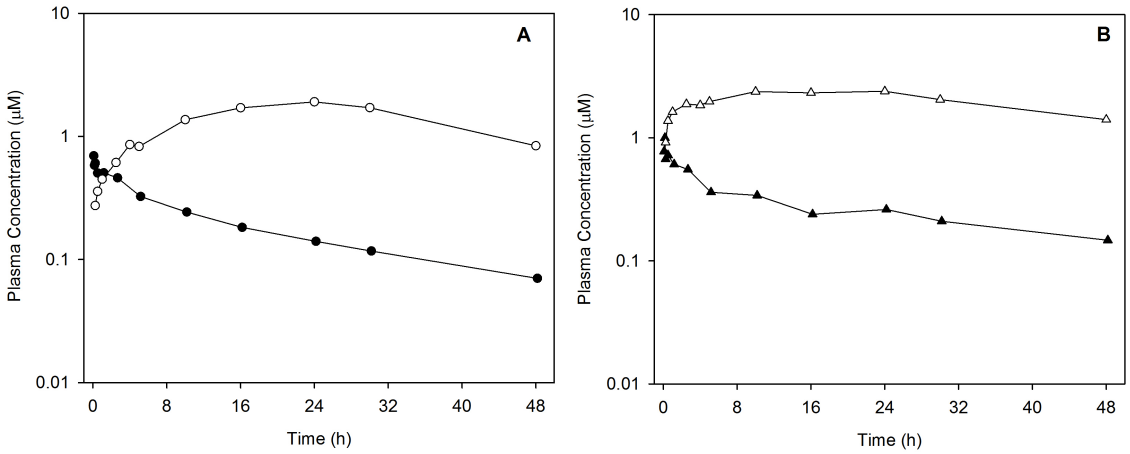


Figure S12. Plasma concentration versus time profiles for ELQ-300 (Panel A) and P4Q-391 (Panel B) following IV (filled, 0.15 mg/kg) and oral (open, 10 mg/kg) administration to fasted male Sprague Dawley rats.

Drug	D6	W2	Dd2	TM90-C2B	D10yDHOD
ELQ-300	2.2	1.8	2.5	1.7	>2,500
P4Q-391	7.7	7.6	7.7	20.4	>2,500
Atovaquone	0.1	0.3	0.1	>2,500	>2,500
Chloroquine	7.8	126	153	92.7	82

Table S1. Antiplasmodial IC_{50} values (nM)[a] (SyBr Green method) for standard isolates and clones of *Plasmodium falciparum.* [a]IC_{50} values were determined by the 72 hour SyBr Green fluorescence based method described previously by Smilkstein et al. in 2004 (*15*).

Pf Strain	ATQ	ELQ-300	P4Q-391	GSK932121A	Chloroquine
W2	0.3	2.27	7.45	3.39	215
TM90-C2B	> 170	2.84	23.5	56.8	63
PL08-009	0.17	1.32	3.18	2.57	59.9
PL08-025	2.18	13.6	32.3	15.5	262
ARC08-0063	0.17	1.52	4.44	2.37	38.3
A6	> 170	> 210	> 200	> 235	21.5
3D7 luc	0.36	4.79	12	6.45	21.9
D6	0.31	3.06	11.27	3.66	22.7
Dd2 EndR1	5.13	3.24	5.02	8.89	63.1
Dd2 EndR2	2.1	1.36	7.13	5.95	69.1
Dd2 EndR3	8.26	5.99	11.3	14.8	106
D10 attb yDHOD	> 170	> 210	> 200	>235	21.4
TM91-C235	0.56	4.34	12	6.25	123

Table S2. Antiplasmodial IC_{50} values (nM)[b] (^{3}H-hypoxanthine incorporation method) for isolates and clones of *Plasmodium falciparum*. [b]IC_{50} values were determined by monitoring for the incorporation of ^{3}H-hypoxanthine into parasite nucleic acids as described previously (*16*). The PL and ARC series are isolates from Cambodia (2008). The DD2 End isolates were selected for resistance to endochin in vitro (Vaidya, unpublished observations).

Compound	Mouse Macrophage (J774) Cell line	Human (HEPG2) CC_{50}, nM	Murine conA-stimulated lymphocyte proliferation	Murine bone marrow derived CFU-GM colony formation	Murine bone marrow derived BFU-E colony formation	Murine bone marrow derived CFU-GEMM colony formation
ELQ-300	>10μM	>10μM	>10μM	>10μM	>10μM	>10μM
P4Q-391	>10μM	NT	NT	>10μM	>10μM	>10μM

Table S3. Cytotoxicity evaluation of ELQ-300 vs. mammalian cells in culture. NT = not tested.

Compound	IC_{50} vs. human* cytochrome bc_1 (μM)
ELQ-271	1.99
ELQ-300	>10
P4Q-391	>10
ATQ	0.41

Table S4. Inhibition of Human cytochrome bc_1 by selected 4-(1*H*)-Quinolones. *Human cytochrome bc_1 complex was derived from HEK-293 cells as described below.

Treatment	Initial Parasite Count	Days to Parasite Emergence
Experiment 1		
None (Control)	10	18
10 nM atovaquone	10^8	30
10 nM atovaquone	10^8	30
150 nM ELQ-300	10^8	--
150 nM ELQ-300	10^8	--
150 nM ELQ-300	10^8	--
Experiment 2		
None (Control)	10	16
10 nM atovaquone	10^8	16
10 nM atovaquone	10^8	16
150 nM ELQ-300	10^8	--
150 nM ELQ-300	10^8	--
150 nM ELQ-300	10^8	--
Experiment 3		
None (Control)	10	13
10 nM atovaquone	10^8	--
10 nM atovaquone	10^8	--
300 nM P4Q-391*	10^8	--
300 nM P4Q-391*	10^8	--
300 nM P4Q-391*	10^8	--

Table S5. Comparison of the propensity for resistance in *P. falciparum* (Dd2 strain) to ELQ-300, P4Q-391 and atovaquone. *P4Q-391 was added to the medium after filtration.

Compound	***P. berghei*** (USF) **Thompson Test**[a]			***P. yoelii*** (GSK Tres Cantos) **4-day Peters Test**[b]			***P. yoelii*** (OHSU) **4-day Test**[c]		
	ED_{50}, mg/kg/d	ED_{90}, mg/kg/d	NRD, mg/kg/d	ED_{50}, mg/kg/d	ED_{90}, mg/kg/d	NRD, mg/kg/d	ED_{50}, mg/kg/d	ED_{90}, mg/kg/d	NRD, mg/kg/d
ELQ-300	0.016	0.04	1.0	0.1	0.15	1.0	0.02	0.04	0.3
P4Q-391	0.27	0.92	1.0	NT	NT	NT	0.04	0.1	1.0
ATQ	0.006	0.052	ND	0.02	0.04	30	NT	NT	NT
CQ	NT	NT	NT	2.05	NT	NT	1.1	1.8	>64

Table S6. In vivo efficacy of ELQ-300 and P4Q-391 in three different murine models of malaria infection. [a, b, c] Complete details of in vivo models are presented in supplemental information. NRD = non-recrudescence dose, ATQ = atovaquone, CQ = chloroquine. NT = not tested.

Compound	EC_{50} (nM)	EC_{90} (nM)
ELQ-300	1.02	4.18
P4Q-391	5.11	10.7
ATQ	2.98	3.88

Table S7. In vitro liver stage activity: *P. berghei* in HepG2.

Compound	Activity against	IC_{50} (μM)
Primaquine	Developing EEF	0.03
Primaquine	Small forms	0.36
ELQ-300	Developing EEF	0.07
ELQ-300	Small forms	ND
P4Q-391	Developing EEF	0.09
P4Q-391	Small forms	ND

Table S8. IC_{50} values of the compounds tested against *P. cynomolgi* developing liver stages (large forms) and small forms (presumably hypnozoites).

Property	ELQ-300	P4Q-391
Molecular weight	475.84	493.83
Rotatable bonds	6	6
H-bond acceptors	5	5
H-bond donors	1	1
Polar surface area ($Å^2$)	56.8	56.8
ClogP	5.66	5.81
logD	4.2	4.2
Kinetic solubility at pH 7.4 (μM), 24 h	16	8
Solubility in FaSSIF media (pH 6.5) at 4 h (μM)	0.3	<1
Solubility in FeSSIF media (pH 5.0) media at 4 h (μM)	1.7	<1
Melting point (ºC)	308-310 (dec)	~300 (dec)
Plasma protein binding (%)	99.9	99.9
Albumax media binding (%)	99.8	99.8

Table S9. Physicochemical properties and binding characteristics for ELQ-300 and P4Q-391.

CYP Isoform	IC_{50} (μM)	
	ELQ-300	P4Q-391[a]
CYP1A2	>20	>5
CYP2C9	6	1.6
CYP2C19	>20	>5
CYP2D6	15	>5
CYP3A4/5	>20	>5

Table S10. Cytochrome P450 inhibition by ELQ-300 and P4Q-391. [a] solubility was limited to 5 μM in the assay media.

	Mice		Rats	
	ELQ-300	P4Q-391	ELQ-300	P4Q-391
IV dose (mg/kg)	0.1	0.1	0.15	0.15
IV CL (mL/min/kg)	0.77	0.61	0.45	0.3
IV Vss (L/kg)	1.2	1.1	0.9	0.7
IV Half life (h)	15	23	24	32
Oral dose (mg/kg)	0.3	0.3	0.3 7.0	10
Oral C_{max} (μM)	0.45	0.54	0.24 2.2	2.6
Oral T_{max} (h)	7.5	7.5	7.0 20	15
Oral F (%)	~100	~100	38 16	14

Table S11. Pharmacokinetic properties of ELQ-300 and P4Q-391 in mice and rats.

Compound	hERG pIC_{50}	$K_v1.5$ pIC_{50}	$Na_V1.5$ pIC_{50}	$Ca_V1.2$ pIC_{50}
ELQ-300	<4.5	<4.0	<4.0	<4.0

Table S12. Summary of the blocking actions of ELQ-300 on hERG, KV1.5, NaV1.5 and CaV1.2 channels.

In vitro recombinant protein binding assay	Species	Mean % of Control (10 µM ELQ-300)
Acetylcholinesterase	Human	103.7
Adenosine 1 receptor	Human	106.9
Adenosine 2A receptor	Human	159.6
Adenosine 3 receptor	Human	79.2
Adenylyl cyclase (activator effect)	Rat	1.6
Adrenergic Alpha 1	Rat	111.0
Adrenergic Alpha 2	Rat	112.8
Adrenergic Beta 1	Human	108.8
Adrenergic Beta 2	Human	100.8
AMPA	Rat	91.1
Angiotensin II AT1 receptor	Human	104.5
Angiotensin II AT2 receptor	Human	93.7
ATPase (Na^+/K^+)	Porcine	100.8
Benzodiazepine receptor	Human	111.8
Bradykinin 1 receptor	Human	99.5
Bradykinin 2 receptor	Human	90.4
Ca^{2+} channel (L, dihydropyridine)	Rat	93.1
Ca^{2+} channel (L, diltiazem site)	Rat	109.4
Ca^{2+} channel (L, verapamil site)	Rat	87.1
Cannabinoid 1 receptor	Human	81.2
Cannabinoid 2 receptor	Human	110.1
Catechol-O-methyl-transferase	Porcine	106
CENP-E	Human	105.7
Cholecystokinin 1 receptor	Human	73.9
Cholecystokinin 2 receptor	Human	108.2
Choline transporter (CHT1)	Human	112.7
Cl^- channel (GABA gated)	Rat	90.7
COX (Cyclooxyenases) 1	Human	81.8
CRF1 receptor	Human	127.7
Dopamine D1 receptor	Human	108.3
Dopamine D2S receptor	Human	87.5
Dopamine D3 receptor	Human	89.0
Dopamine D4.4 receptor	Human	99.5
Dopamine Transporter	Human	86.1
Eg5	Human	92
Endothelin A receptor	Human	113.4
Endothelin B receptor	Human	102.9
GABA receptor	Rat	77.7
GABA transaminase	Rat	100.1
GABA transporter	Rat	117.7
Glutamate channel PCP	Rat	94.2
Guanylyl cyclase (activator effect)	Human	0.7
HDAC3	Human	89.7

HDAC4	Human	111.5
HDAC6	Human	100.1
HDAC11	Human	92.1
Histamine H1 receptor	Human	87.2
Histamine H2 receptor	Human	116.8
Histamine H3 receptor	Human	107.0
Imidazoline I1 receptor	Rat	107.9
Imidazoline I1 receptor	Rat	97.7
K^+ channel KATP	Rat	94.6
K^+ channel KV	Rat	116.0
K^+ channel SKCa	Rat	107.6
Kainate	Rat	99.6
Leukotrienes BLT1 (LTB4) receptor	Human	96.1
Leukotrienes CysLT1 (LTD4) receptor	Human	96.8
Melanocortin MC4 receptor	Human	103.9
Melatonin MT1 (ML1A) receptor	Human	88.9
Monoamine Oxydase A	Human	103.0
Monoamine Oxydase B	Human	78.9
Muscarinic M receptor	Rat	91.4
Neurokinin NK1 receptor	Human	108.3
Neurokinin NK2 receptor	Human	93.9
Neurokinin NK3 receptor	Human	91.8
NeuropeptideY Y receptor	Rat	86.6
Nicotinic (CNS) receptor	Human	102.3
NMDA channel site recept.	Rat	95.5
Norepinephrine Transporter	Human	99.7
Opioid receptor	Rat	75.3
Opioid NOP (ORL1) receptor	Human	97.8
Phosphatase 1B	Human	92.2
Phosphatase CDC25A	Human	109.5
Phosphodiesterase PDE1B	Human	105.2
Phosphodiesterase PDE2A1	Human	101.1
Phosphodiesterase PDE3A	Human	103.2
Phosphodiesterase PDE4D2	Human	100.3
Phosphodiesterase PDE5	Human	94.4
PKCalpha	Human	100.8
PPARgamma receptor	Human	92.2
Prostanoid EP2 receptor	Human	89.6
Prostanoid IP (PGI2) receptor	Human	111.0
Purinergic P2X receptor	Rat	89.8
Purinergic P2Y receptor	Rat	106.3
Serotonin 5-HT receptor	Rat	104.7
Serotonin 5-HT transporter	Human	87.3
Sigma receptor	Human	104.3
Steroid nuclear GR receptor	Human	114.7

Steroid nuclear ER receptor	Human	99.9
Steroid nuclear PR receptor	Human	155
Steroid nuclear AR receptor	Human	96.2
Thyrotropin TRH1	Human	120.9
Vasopressin V1a receptor	Human	88.1
Vasopressin V2 receptor	Human	104.5
Tyrosine hydroxylase	Rat	86.3
Sirtuin 1	Human	99.3

Table S13. Inhibition by ELQ-300 of selected off-target receptors, ion channels and biogenic amine transporters (% of no-inhibitor control values at 10 μM ELQ-300).