Electronic Supplementary Information

Primaquine-based ionic liquids as a novel class of antimalarial hits

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CONTENTS:

1. Detailed synthetic procedures and analytical/spectral data for target compounds

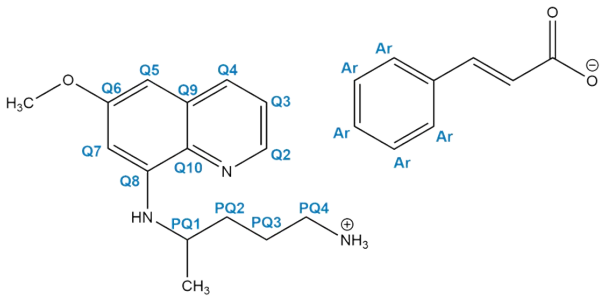
Chemicals and instrumentation. Primaquine biphosphate (purity 97.5 – 102.5 by titration with NaOH, according to manufacturer) and triethylamine (purity ≥ 99.5 % by GC, according to manufacturer) were from Sigma–Aldrich. Cinnamic acids with minimal 98% purity, according to manufacturer, were all from Acros Organics. All solvents were of analytical grade, and purchased to VWR International. NMR analyses were carried out on a Brucker Avance III 400 MHz spectrometer, and samples were prepared in $(CD_3)_2SO$ with tetramethylsilane (TMS) as an internal reference. Chemical shifts are reported downfield in parts per million (ppm) and multiplicity of proton signals is indicated as s (singlet), d (doublet), dd (double doublet), t (triplet), m (unresolved multiplet). High resolution mass spectra (HRMS) were obtained on an *LTQ Orbitrap XL* / *LTQ Tune Plus 2.5.5* spectrometer, and processed with the *2.1.0 Xcalibur* software (Thermo Scientific).

General procedure for the synthesis of the ionic liquids. Primaquine was first obtained as a free base, by addition of triethylamine (0.25 mL) to a suspension of primaquine biphosphate (0.1298 g; 0.285 mol) in dichloromethane (4 mL). The mixture was stirred for 30 minutes in ice and in the dark, after which the organic layer was washed with water (3×10 mL), dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. Free primaquine base was quantitatively obtained as a yellow oil. This procedure was repeated as many times as needed.

Free primaquine was next dissolved in methanol, and titrated with an equimolar amount of the relevant organic acid, upon dropwise addition of a methanolic solution of the acid. The neutralization reaction proceeded in the dark at room temperature for 30 minutes, after which methanol was removed by evaporation at reduced pressure and the target ILs obtained as chromatographically homogeneous yellow-orange viscous liquids. Amounts of starting materials used to produce each one of the ILs synthesized are presented on the table below.

Primaquine quantity	**Cinnamic acid derivatives**		**Abbreviation of IL produced**
	Anion abbreviation	**Quantity**	
0.051 g; 0.20 mmol	[Cinm]	0.030 g; 0.20 mmol;	[PQ][Cinm]
0.069 g; 0.27 mmol	[MeCinm]	0.044 g; 0.27	[PQ][MeCinm]
0.051 g; 0.20 mmol	[OHCinm]	0.033 g; 0.20 mmol	[PQ][OHCinm]
0.058 g; 0.22 mmol	[MeOCinm]	0.037 g; 0.21 mmol	[PQ][MeOCinm]
0.065 g; 0.25 mmol	[iPropCinm]	0.038 g; 0.20 mmol	[PQ][iPropCinm]
0.051 g; 0.20 mmol	[ClCinm]	0.036 g; 0.20 mmol	[PQ][ClCinm]

MS and NMR analysis of the products provided confirmation of the expected chemical structures, as shown by detailed spectral data given below. Assignment of NMR peaks is provided according to the following labels:



[PQ][Cinm] Yellow oil (0.18 mmol, 77 mg, 95%); δ_H (400.13 MHz, DMSO-d_6) 8.53 (dd, 1H, *J* = 4.2, 1.6 Hz, Q2), 8.07 (dd, 1H, *J* = 8.3, 1.6 Hz, Q4), 7.56 (dd, 2H, *J* = 8.1, 1.4 Hz, Ar*H*), 7.42 (dd, 1H, *J* = 8.2, 4.2 Hz, Q3), 7.39 - 7.29 (m, 4H, Ar*H*, -C*H*=CH-), 6.48 (dd, 2H, *J* = 9.2, 6.7 Hz, Q7, -CH=C*H*-), 6.28 (d, 1H, *J* = 2.5 Hz, Q5), 6.14 (d, 1H, *J* = 8.8 Hz, N*H*-), 3.82 (s, 3H, -OCH_3), 3.69 – 3.59 (m, 1H, PQ1), 2.74 (t, 2H, *J* = 6.2 Hz, PQ4), 1.75-1.53 (m, 4H, PQ2 e PQ3), 1.21 (d, 3H, *J* = 6.3 Hz, -CH_3); δ_C (100 MHz, DMSO-d_6) 169.6 (-*C*OO), 159.0 (Q6), 146.8 (-CH=CH-) 144.6 (Q), 144.2 (Q), 139.5 (Q), 135.5 (Q), 134.8 (ArC), 134.5 (ArC), 129.6 (Q), 129.0 (ArC), 128.7 (ArC), 127.5 (ArC), 124.9 (-CH=CH-), 122.1 (ArC), 96.2 (Q), 91.7 (Q), 55.0 (-OCH_3), 47.0 (PQ), 33.0 (PQ),27.2 (PQ) 25.3 (PQ), 20.2 (-CH_3); **(EI$^+$)** *m/z* calcd for $C_{15}H_{22}N_3O^+$: 260.1757, found 260.1751; **(EI$^-$)** *m/z* calcd for $C_9H_7O_2^-$: 147.0452, found 147.0456.

[PQ][MeCinm] Yellow oil (0.24 mmol; 105 mg, 92%); δ_H (400.13 MHz, DMSO-d_6) 8.53 (dd, 1H, *J* = 4.2, 1.6 Hz, Q2), 8.07 (dd, 1H, *J* = 8.3, 1.5 Hz, Q4), 7.49-7.40 (m, 3H, ArH, Q3), 7.31 (d, 1H, *J* = 15.9 Hz, -C*H*=CH-), 7.18 (d, 2H, *J* = 7.9 Hz, ArH), 6.44 (m, 2H, -CH=C*H*-, Q7), 6.27 (d, 1H, *J* = 2.5 Hz, Q5), 6.14 (d, 1H, *J* = 8.7 Hz, -N*H*-), 3.82 (s, 3H, -OCH_3), 3.68-3.60 (m, 1H, PQ1), 2.71 (t, 2H, *J* = 6.2 Hz, PQ4), 2.30 (s, 3H, Ar-CH_3), 1.74-1.52 (m, 4H, PQ2, PQ3), 1.21 (d, 3H, *J* = 6.3 Hz, -CH_3); δ_C (100 MHz, DMSO-d_6) 169.7 (-*C*OO), 159.4 (Q), 145.18 (-*C*H=CH-), 144.7 (Q), 135.3 (Q), 135.1 (Ar*C*), 135.0 (Ar*C*), 130.1 (Q), 129.8 (Ar*C*), 128.0 (Ar*C*), 122.6 (Ar*C*), 96.6 (Q), 92.2 (Q), 55.5 (-OCH_3), 47.5 (PQ), 33.6 (PQ), 21.4 (Ar*C*), 20.7 (-*C*H_3); **(EI$^+$)** *m/z* calcd for $C_{15}H_{22}N_3O^+$: 260.1757, found 260.1751; **(EI$^-$)** *m/z* calcd for $C_{10}H_9O_2^-$ 161.0608, found 161.0610.

[PQ][OHCinm] Yellow oil (0.18mmol; 77mg, 91%); δ_H (400.13 MHz, DMSO-d_6) 8.53 (dd, 1H, *J* = 4.2, 1.6 Hz, Q2), 8.07 (dd, 1H, *J* = 8.3, 1.6 Hz, Q4), 7.45 – 7.38 (m, 3H, Ar*H*, Q3), 7.31 (d, 1H, *J* = 15.9 Hz, -C*H*=CH-), 6.79-6.75 (m, 2H, -CH=C*H*-, Q7), 6.47 (d, 1H, *J* = 2.5 Hz, Q5), 6.26 (dd, 2H, *J* = 10.8, 9.2 Hz, Ar*H*), 6.13 (d, 1H, *J* = 8.8 Hz, -N*H*-), 3.82 (s, 3H, -OCH_3), 3.67-3.59 (m, 1H, PQ1), 2.71 (t, 2H, *J* = 6.7 Hz, PQ4), 1.72-1.52 (m, 4H, PQ2, PQ3), 1.21 (d, 3H, *J* = 6.3 Hz, -CH_3); δ_C (100 MHz, DMSO-d_6) 169.8 (-*C*OO), 159.6 (Q), 159.5 (Ar*C*), 145.1 (-*C*H=CH-), 144.7 (Q), 141.5 (Q), 135.3 (Q), 135.0 (Ar*C*), 130.1 (Q), 129.8 (-CH=*C*H-), 126.4 (Ar*C*), 122.6 (Ar*C*), 120.9 (Q), 116.2 (Ar*C*), 96.6 (Q), 92.2 (Q), 55.6 (PQ), 47.5 (PQ), 33.6 (PQ), 26.3 (PQ), 20.7 (-CH_3) ppm. **(EI$^+$)** *m/z* calcd for $C_{15}H_{22}N_3O^+$: 260,1757, found 260,1750. **(EI$^-$)** *m/z* calcd for $C_9H_7O_3^-$ 163.0401, found 163.0409.

[PQ][MeOCinm] Yellow oil (0.19mmol; 79mg, 89%) δ_H (400.13 MHz, DMSO-d_6) 8.53 (dd, 1H, *J* = 4.2, 1.6 Hz, Q2), 8.07 (dd, 1H, *J* = 8.3, 1.6 Hz, Q4), 7.53 (d, 2H, *J* = 8.7 Hz, Ar*H*), 7.42 (dd, 1H, *J* = 8.2, 4.2 Hz, Q3), 7.34 (d, 1H, *J* = 15.9 Hz, -C*H*=CH-), 6.93 (d, 2H, *J* = 8.8 Hz, Ar*H*), 6.47 (d, 1H, *J* = 2.4 Hz, Q7), 6.33 (d, 1H, *J* = 15.9 Hz, -

CH=C*H*), 6.27 (d, 1H, *J* = 2.4 Hz, Q5), 6.13 (d, 1H, *J* = 8.7 Hz, -NH-), 3.82 (s, 3 H, -OCH_3), 3.77 (s, 3 H, -OCH_3), 3.68-3.57 (m, 1H, PQ1), 2.71 (t, 2H, *J* = 6.7 Hz, PQ4), 1.74-1.52 (m, 4H, PQ2 e PQ3), 1.21 (d, 3H, *J* = 6.3 Hz, -CH_3); δ_C (100 MHz, DMSO-d_6) 169.4 (-*C*OO), 160.2 (Q), 159.0 (-*C*H=CH-), 144.6 (Q), 144.2 (Q), 140.1 (Q), 134.8 (Q), 134.5 (Q), 129.6 (-CH=*C*H-), 129.2 (Ar*C*), 127.8 (Ar*C*), 122.1 (Q), 114.2 (Ar*C*), 96.2 (Q), 91.7 (Q), 55.2 (-OCH_3), 55.0 (-OCH_3), 48.6 (PQ), 47.0 (PQ), 25.5 (PQ), 20.2 (-*C*H_3); **(EI$^+$)** *m/z* calcd for $C_{15}H_{22}N_3O^+$: 260,1757, found 260,1752. **(EI$^-$)** *m/z* calcd for $^-$ $C_{10}H_9O_3^-$: 177.0557, found 177.0570.

[PQ][iPropCinm] Yellow oil (0.20mmol; 84mg, 99%) δ_H (400.13 MHz, DMSO-d_6) 8.53 (dd, 1H, *J* = 4.1, 1.5 Hz, Q2), 8.08 (dd, 1H, *J* = 8.3, 1.4 Hz, Q4), 7.52 (d, 2H, *J* = 8.2 Hz, Ar*H*), 7.45-7.36 (m, 2H, Q3, -*CH*=CH-), 7.25 (d, 2H, *J* = 8.2 Hz, Ar*H*), 6.45 (m, 2H, Q7, -*C*H=C*H*-), 6.28 (d, 1H, *J* = 2.3 Hz, Q5), 6.14 (d, 1H, *J* = 8.8 Hz, -N*H*-), 3.82 (s, 3H, -OCH_3), 3.64 (m, 1H , PQ1), 2.89 (m, 1H,-C*H*-), 2.71 (t, 2H, *J* = 6.7 Hz, PQ4), 1.76-1.46 (m, 4H, PQ2 e PQ3), 1.20 (m, 9H, -CH_3); δ_C (100 MHz, DMSO-d_6) 169.4 (-*C*OO), 160.2 (Q), 159.0 (Q), 158.7 (Q), 145.18 (-*C*H=CH-), 144.6 (Q), 135.3 (Q), 135.1 (Ar*C*), 135.0 (Ar*C*), 130.1 (Q), 129.8 (Ar*C*), 126.7 (Ar*C*), 127.8 (Ar*C*), 122.6 (Ar*C*), 96.6 (Q), 92.2 (Q), 54.8 (PQ), 47.5 (PQ), 33.6 (PQ.), 33.3 (*C*H), 23.7 (-*C*H_3), 20.3 (-*C*H_3); **(EI$^+$)** *m/z* calcd for $C_{15}H_{22}N_3O^+$: 260,1757, found 260,1756. **(EI$^-$)** *m/z* calcd for $C_{12}H_{13}O_2^-$ 189.0921, found 189.0927.

[PQ][ClCinm] Yellow oil (0.19 mmol; 82mg, 93%) δ_H (400.13 MHz, DMSO-d_6) 8,53 (dd, 1H *J* = 4.2, 1.6 Hz, Q2), 8.07 (dd, 1H, *J* = 8.3, 1.5 Hz, Q4), 7.62 (d, 2H, *J* = 8.5 Hz, Ar*H*), 7.42 (m, 3H, Q3, Ar*H*), 7.35 (d, 1H, *J* = 16.0 Hz, -C*H*=CH-), 6,52-6,48 (m, 2H, 1H, *J* = 15.9 Hz, -CH=C*H,* Q7), 6.28 (d, 1H, *J* = 2.4 Hz, Q5), 6.14 (d, 1H, *J* = 8.8 Hz, -N*H*-), 3.82 (s, 3H, -OCH_3), 3.64 (m, 1H, PQ1), 2.76 (t, 2H, *J* = 6.5 Hz, PQ4), 1.75-1.54 (m, 4H, PQ2, PQ3), 1.18 (d, 3H, 6.4 Hz, –CH_3); δ_C (100 MHz, DMSO-d_6) 168.9 (-COO), 159.0 (Q), 144.6 (-*C*H=CH-), 144.2 (Q), 138.6 (Q), 134.5 (Q), 134.3 (Ar*C*), 133.5 (ArC), 129.6 (-CH=*C*H-), 129.3 (Ar*C*), 128.7 (Ar*C*), 122.1 (Q), 96.2 (Q), 91.7 (Q), 55.0 (-OCH_3), 46.9 (PQ), 33.0 (PQ), 24.9 (PQ), 20.2 (-*C*H_3); **(EI$^+$)** *m/z* calcd for $C_{15}H_{22}N_3O^+$: 260.1757, found 260.1755. **(EI$^-$)** *m/z* calcd for $C_9H_6ClO_2^-$: 181.0062, found 181.0081.

2. Assays of *in vitro* activity against *Plasmodium* liver stages

Inhibition of liver stage infection by test compounds was assessed by measuring the luminescence intensity of lysates of Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* line, *Pb*GFP-Luc_{con}, as previously described.[27] Huh-7 cells, a human hepatoma cell line, were cultured in 1640 RPMI medium supplemented with 10% v/v fetal calf serum, 1% v/v non-essential amino acids, 1% v/v penicillin/streptomycin, 1% v/v glutamine and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH 7, and maintained at 37 °C with 5% CO_2. For infection assays, Huh-7 cells (1.2×10^4 per well) were seeded in 96-well plates the day before drug treatment and infection. Medium in the cells was replaced by medium containing the appropriate concentration of each compound approximately 1 h prior to infection with sporozoites freshly obtained through disruption of salivary glands of infected female *Anopheles stephensi* mosquitoes. Sporozoite addition was followed by centrifugation at 1700 g for 5 min. The effect of the compounds on the viability of Huh-7 cells was assessed 46 hours after sporozoite addition by the AlamarBlue assay (Invitrogen, UK), using the manufacturer's protocol. Parasite load was assessed at 48 h of infection by luminescence measurement of cell lysates, following addition of the luciferin substrate.

3. Assays of *in vitro* activity against *Plasmodium* asexual blood stages

Laboratory-adapted *P. falciparum* Dd2 (chloroquine-resistant) and 3D7 (chloroquine-susceptible) strains were continuously cultured as previously described by us (Faísca Phillips AM et al., 2015). Briefly, parasites were cultivated on human erythrocytes suspended in RPMI 1640 medium supplemented with 25 mM HEPES, 6.8 mM hypoxanthine and 10% AlbuMAX II, at pH 7.2. Cultures were maintained at 37ºC under an atmosphere of 5% O_2, 3-5% CO_2, and N_2 and synchronized by sorbitol treatment prior to the assays. Staging and parasitaemia were determined by light microscopy of Giemsa-stained thin blood smears.

The antimalarial activities of the test compounds were determined using the SYBR Green I assay as previously described (Faísca Phillips AM et al, 2015). Briefly, early ring stage parasites were tested in triplicate in a 96-well plate and incubated with drugs for 48 h (37ºC, 5% CO_2), parasite growth was assessed with SYBR Green I. Each compound was tested using 12 concentrations serially diluted (1/3), ranging from 0 to 10 μM. Fluorescence intensity was measured with a multi-mode microplate reader (Dynex Triad) with excitation and emission wavelengths of 485 nm and 535 nm, respectively, and analyzed by nonlinear regression using a GraphPad Prism 5 demo version.

Reference:

Faísca Phillips AM, Nogueira F, Murtinheira F, Barros MT. Synthesis and antimalarial evaluation of prodrugs of novel fosmidomycin analogues. Bioorg Med Chem Lett. 2015;25(10):2112-6.

4. Assays of *in vitro* activity against *Plasmodium* gametocytes

***P. falciparum* culture and gametocyte induction.** The transgenic *P. falciparum* 3D7 strain 3D7elo1-pfs16-CBG99 expressing the *Pyrophorus plagiophthalamus* CBG99 luciferase under a gametocyte specific promoter was used in all the experiments. Parasites were cultured and gametocytes obtained as previously described.[28] Late-stage gametocytes were exposed to compounds at day 11 after *N*-acetylglucosamine (NAG) addition. Gametocytes stages were counted in Giemsa stained smears and the percentage of stage V gametocytes was higher than 80%.

Drug susceptibility assay. Compouds were prepared by serial dilution, in 96-well plates, in complete medium. Primaquine was used as reference drug. Plates were incubated for 72 h at 37°C under 1 % O2, 5 % CO2, 94 % N2 atmosphere. Luciferase activity was taken as measure of gametocytes viability, as previously described.[28] Briefly, drug-treated gametocyte samples at 2% haematocrit were transferred to 96-well black microplates and D-luciferin (1 mM in citrate buffer 0.1 M, pH 5.5) was added at a 1:1 volume ratio. Luminescence measurements were performed after 10 min with 500 ms integration time using a Sinergy 4 (Biotek) microplate reader. The IC_{50} was extrapolated from the non-linear regression analysis of the concentration–response curve.