Squaric acid: a valuable scaffold for developing antimalarials?

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General Chemistry Methods:

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected. The infrared spectra were collected on a Nicolet Impact 400 FTIR infrared spectrophotometer. Low resolution mass spectra (MS) were performed in LCLEM, Faculdade de Farmácia, Universidade de Lisboa. High resolution mass spectra (HRMS) were performed in Unidade de Espectrometria de Massas, Santiago de Compostela. Merck Silica Gel 60 F254 plates were used as analytical TLC; flash column chromatography was performed on Merck Silica Gel (200-400 mesh). 1H and 13C NMR spectra were recorded on a Bruker 400 Ultra-Shield. Proton nuclear magnetic resonance spectra were recorded at 400 MHz. Carbon nuclear magnetic resonance spectra were recorded at 100 MHz. 1H and 13C chemical shifts are expressed in δ (ppm) referenced to the solvent used and the proton coupling constants J in hertz (Hz). Spectra were assigned using appropriate COSY, DEPT and HMQC sequences.

General preparation of squaric derivatives 4a-b, 6i-m

A solution of the suitable amine (1 eq.) in dry methanol (2 ml for 0.22 mmol of amine) was stirred for 5 min at room temperature before addition of 3,4-dimethoxy-3-cyclobutene-1,2-dione (1 eq). The mixture was stirred for 12 h at reflux. The methanol was evaporated and the obtained residue was recrystallized in methanol/ethyl acetate.

**Compound 4a:** Obtained as a white solid (40 mg, 67%); Rf=0.3 (EtOAc/hexane 1:1); mp: 259-261ºC; δH (DMSO-d6) 10.60 (s, 1H), 7.17 (s br, 2H), 6.89 (d, J = 12 Hz, 2H), 4.35 (s, 3H), 3.09 (t, J = 8 Hz, 4H), 1.60 (m, 4H), 1.52 (m, 2H) ppm; δC (DMSO-d6) 149.22 (Cq), 130.71 (Cq), 119.99 (CH), 117.17 (CH), 116.61 (2 CH), 60.81 (OCH3), 50.19 (2CH2), 25.67 (2CH2), 24.29 (CH2) ppm; IR (KBr): ν = 3267, 2932, 1791, 1695 cm-1; HRMS-ESI-TOF: m/z [M+H]+ calcd for C16H19N2O3: 287.1396, found: 287.1390.

**Compound 4b:** Obtained as a yellow solid (41 mg, 76%); Rf=0.6 (EtOAc/hexane 1:1); mp: 245-247ºC; δH (DMSO-d6) 10.72 (s, 1H, NH), 7.35 (d, J =8 Hz, 2H), 7.26 (s br, 2H), 4.37 (s, 3H), 1.27 (s, 9H) ppm; δC (DMSO-d6) 181.57 (CO), 165.36 (Cq), 145.71 (Cq), 135.98 (Cq), 125.75 (2 CH), 119.34 (CH), 118.23 (CH), 60.45 (OCH3), 34.06 (C(CH3)3), 31.09 (C(CH3)3) ppm; IR (KBr): ν = 3254, 2945, 1797, 1708, 1568 cm -1; HRMS-ESI-TOF: m/z [M+H]+ calcd for C15H18NO3: 260.1287, found: 260.1281.

**Compound 6i:** Obtained as a yellow solid (25 mg, 43%); Rf=0.2 (EtOAc/MeOH 9:1); mp: 212-214ºC; δH (DMSO-d6) 7.47 (s, 2H, NH), 3.50 (s br, 4H), 2.24 (t, J =8 Hz, 4H), 2.12 (s, 12H), 1.65 (t, J = 8 Hz, 4H) ppm; δC (DMSO-d6) 182.32 (CO), 167.79 (Cq), 55.98 (C(H2N(CH3)2), 45.05 (N(C(CH3)2), 41.57 (NCH2), 28.67 (NHCH2C3H2) ppm; IR (KBr): ν = 3190, 2945, 1797, 1708, 1568 cm -1; HRMS-ESI-TOF: m/z [M+H]+ calcd for C14H27N4O2: 283.2134, found: 283.2129.

**Compound 6j:** Obtained as a yellow solid (28 mg, 40%); Rf=0.0 (EtOAc/MeOH 9:1); mp: 244-246ºC; δH (DMSO-d6) 7.34 (s, NH, 1H), 3.50 (s br, 2H), 2.43 (m, 6H), 1.63 (m, 2H), 0.93 (t, J =6 Hz, 4H) ppm; δC (DMSO-d6) 182.46 (CO), 167.85 (Cq), 49.21 (C(CH2N(CH2CH3)2), 46.18 (N(CH2CH3)2), 41.79 (NHC3H7), 28.33 (NHCH2C3H2), 11.62 (N(CH2CH3)2) ppm; IR (KBr): ν = 3222, 3141, 1650, 1544, 1403 cm -1; MS (ESI, CP 4.0 kV, SP 10V, 5 eV): m/z 339 [M+H]+.

**Compound 6l:** Obtained as a yellow solid (25 mg, 43%); Rf=0.15 (EtOAc/MeOH 9:1); mp: 235-237ºC; δH (DMSO-d6) 8.49 (s, 2H, NH’s), 4.79 (m, 2H), 3.29 (m, 8H), 1.88 (m, 4H), 1.20 (d, 12H, J =7 Hz) ppm; δC (DMSO-d6) 181.06 (CO), 168.30 (Cq), 49.66 (C(CH2CH3)2), 45.93 (CH2NH), 45.63 (NHCH2), 29.86 (NH2CH2CH3), 20.07 (CH(CH3)2) ppm; IR (KBr): ν = 3164, 2958, 1797, 1670 cm -1; MS (ESI, CP 3.0 kV, SP 30V): m/z 339 [M+H]+.

**Compound 6k:** Obtained as a yellow solid (25 mg, 39%); Rf=0.1 (EtOAc/MeOH 9:1); mp: 235-237ºC; δH (DMSO-d6) 7.04 (s, 2H, NH’s), 4.79 (m, 2H), 3.29 (m, 8H), 1.88 (m, 4H), 1.20 (d, 12H, J =7 Hz) ppm; δC (DMSO-d6) 181.06 (CO), 168.30 (Cq), 49.66 (C(CH2CH3)2), 45.93 (CH2NH), 45.63 (NHCH2), 29.86 (NH2CH2CH3), 20.07 (CH(CH3)2) ppm; IR (KBr): ν = 3164, 2958, 1797, 1670 cm -1; MS (ESI, CP 4.0 kV, SP 30V): m/z 339 [M+H]+.
General preparation of squaric derivatives 4c-f, 6h, 6n-o and 6q
A mixture of the suitable amine (1 eq.) and 3,4-dimethoxy-3-cyclobutene-1,2-dione (1 eq.) in dry methanol (2 ml for 0.22 mmol of amine) was stirred for 12 h at room temperature. The methanol was evaporated and the obtained residue was recrystallized in methanol/ethyl acetate.

**Compound 6m**: Obtained as a white solid (31 mg, 37%); $R_f$=0.15 (EtOAc/MeOH 9:1); mp: 257-259°C; $\delta_{\text{H}}$ (DMSO-d$_6$) 7.20 (s br, 2H, NH$_2$), 4.02 (m, 2H), 2.41 (q, $J$=1.8 Hz, 8H), 2.34 (t, $J$=1.8 Hz, 4H), 1.44 (m, 8H), 1.18 (d, $J$=1.8 Hz, 6H), 0.92 (t, $J$=1.8 Hz, 12H) ppm; $\delta_{\text{C}}$ (DMSO-d$_6$) 181.79 (CO), 167.19 (Cq), 51.89 (CH$_2$), 49.56 (CH), 46.24 (N(CH$_2$CH$_2$)$_2$), 35.08 (CH$_3$), 23.18 (CH$_2$), 22.30 (CH$_3$), 11.83 (N(CH$_2$CH$_2$)$_2$) ppm; IR (KBr): $\tilde{\nu}$ = 3434, 3254, 1791, 1670 cm$^{-1}$; MS (ESI, CP 3.0 kV, SP 30V): m/z 395 [M+H]$^+$. 

**Compound 6n**: Obtained as a white solid (31 mg, 37%); $R_f$=0.15 (EtOAc/MeOH 9:1); mp: 257-259°C; $\delta_{\text{H}}$ (DMSO-d$_6$) 7.20 (s br, 2H, NH$_2$), 4.02 (m, 2H), 2.41 (q, $J$=1.8 Hz, 8H), 2.34 (t, $J$=1.8 Hz, 4H), 1.44 (m, 8H), 1.18 (d, $J$=1.8 Hz, 6H), 0.92 (t, $J$=1.8 Hz, 12H) ppm; $\delta_{\text{C}}$ (DMSO-d$_6$) 181.79 (CO), 167.19 (Cq), 51.89 (CH$_2$), 49.56 (CH), 46.24 (N(CH$_2$CH$_2$)$_2$), 35.08 (CH$_3$), 23.18 (CH$_2$), 22.30 (CH$_3$), 11.83 (N(CH$_2$CH$_2$)$_2$) ppm; IR (KBr): $\tilde{\nu}$ = 3434, 3254, 1791, 1670 cm$^{-1}$; MS (ESI, CP 3.0 kV, SP 30V): m/z 395 [M+H]$^+$. 

**Compound 6o**: Obtained as a white solid (61 mg, 44%); $R_f$=0.25 (AcOEt/hexane 1:1); mp: 246-248°C; $\delta_{\text{H}}$ (DMSO-d$_6$) 7.73 (s, 2H, NH), 7.30 (m, 10H), 5.20 (m, 2H), 1.52 (d, $J$=4 Hz, 6H) ppm; $\delta_{\text{C}}$ (DMSO-d$_6$) 182.92 (CO), 167.33 (Cq), 129.13 (CH), 127.86 (Cq), 126.47 (CH), 53.14 (NH$_2$CH), 23.46 (CH$_2$) ppm; IR (KBr): $\tilde{\nu}$ = 3151, 2971, 1791, 1644 cm$^{-1}$; MS (ESI, CP 3.0 kV, SP 30V): m/z 321 [M+H]$^+$. 

**Compound 6p**: Obtained as a white solid (61 mg, 44%); $R_f$=0.25 (AcOEt/hexane 1:1); mp: 246-248°C; $\delta_{\text{H}}$ (DMSO-d$_6$) 7.73 (s, 2H, NH), 7.30 (m, 10H), 5.20 (m, 2H), 1.52 (d, $J$=4 Hz, 6H) ppm; $\delta_{\text{C}}$ (DMSO-d$_6$) 182.92 (CO), 167.33 (Cq), 129.13 (CH), 127.86 (Cq), 126.47 (CH), 53.14 (NH$_2$CH), 23.46 (CH$_2$) ppm; IR (KBr): $\tilde{\nu}$ = 3151, 2971, 1791, 1644 cm$^{-1}$; MS (ESI, CP 3.0 kV, SP 30V): m/z 321 [M+H]$^+$. 

**Compound 6q**: Obtained as a white solid (61 mg, 44%); $R_f$=0.25 (AcOEt/hexane 1:1); mp: 246-248°C; $\delta_{\text{H}}$ (DMSO-d$_6$) 7.73 (s, 2H, NH), 7.30 (m, 10H), 5.20 (m, 2H), 1.52 (d, $J$=4 Hz, 6H) ppm; $\delta_{\text{C}}$ (DMSO-d$_6$) 182.92 (CO), 167.33 (Cq), 129.13 (CH), 127.86 (Cq), 126.47 (CH), 53.14 (NH$_2$CH), 23.46 (CH$_2$) ppm; IR (KBr): $\tilde{\nu}$ = 3151, 2971, 1791, 1644 cm$^{-1}$; MS (ESI, CP 3.0 kV, SP 30V): m/z 321 [M+H]$^+$. 

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**Compound 6q:** Obtained as a yellow solid (41 mg, 43%); Rf=0.25 (AcOEt/hexane 1:1); mp: 216-218°C; δH (DMSO-d6) 7.35 (s br, 2H, NH), 3.76 (s br, 2H), 1.89 (m, 4H), 1.68 (m, 4H), 1.55 (m, 2H), 1.25 (m, 10H) ppm; δC (DMSO-d6) 182.31 (CO), 167.42 (Cq), 52.44 (NH(CH3)), 34.17 (CH2), 25.24 (CH2), 24.47 (CH2) ppm; IR (KBr): ṽ= 3151, 2920, 1797, 1644, 1561 cm⁻¹; HRMS-ESI-TOF: m/z [M+H]+ calcd for C16H25N2O2: 277.1916, found: 277.1911.

**General preparation of squaric derivatives 6g and 6p**

A mixture of the suitable amine (2 eq.) and 3,4-dimethoxy-3-cyclobutene-1,2-dione (1 eq.) in dry methanol (2 ml for 0.22 mmol of amine) was stirred for 12 h at room temperature. The methanol was evaporated and the obtained residue was recrystallized in methanol/ethyl acetate.

**Compound 6g:** Obtained as a white solid (149 mg, 83%); Rf=0.15 (AcOEt/MeOH 9:1); mp: 239-241°C; δH (DMSO-d6) 7.54, (s br, 2H, NH), 3.62 (d, 4H, J=5 Hz), 2.41 (t, 4H, J=5 Hz), 2.19 (s, 12H) ppm; δC (DMSO-d6) 182.88 (CO), 167.97 (Cq), 59.66 (CH2), 45.43 (N(CH3)2), 41.32 (NHCH2) ppm; IR (KBr): ṽ= 3112, 2765, 1797, 1542 cm⁻¹; MS (ESI, CP 3.0 kV, SP 30V): m/z [M]+.

**Compound 6p:** Obtained as a yellow solid (82 mg, 72%); Rf=0.2 (EtOAc/hexane 1:1); mp: 228-230°C; δH (DMSO-d6) 9.89 (s, 2H, NH), 7.29 (m, 4H), 6.98 (dd, J=8.0 Hz, J=1.2 Hz, 2H), 6.67 (dd, J=8.0 Hz, J=1.2 Hz, 2H), 3.78 (s, 6H) ppm; δC (DMSO-d6) 181.57 (CO), 165.61 (Cq), 160.22 (Cq), 139.74 (CH), 130.33 (CH), 110.60 (CH), 109.09 (Cq), 104.20 (CH), 55.17 (OCH3) ppm; IR (KBr): ṽ= 3151, 2965, 1691, 1540 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]+ calcd for C18H16N2NaO4: 347.1008, found: 347.1002.

**General preparation of squaric derivatives 5a-b**

A mixture of the suitable amine (1 eq.) and 3,4-Dibutoxy-3-cyclobutene-1,2-dione (1 eq.) in dry methanol (2 ml for 0.22 mmol of amine) was stirred for 12 h at room temperature. The methanol was evaporated and the obtained residue was recrystallized in methanol/ethyl acetate.

**Compound 5a:** Obtained as a yellow solid (79 mg, 55%); Rf=0.5 (AcOEt/hexane 1:1); mp: 261-263°C; δH (DMSO-d6) 10.63 (s br, 1H, NH), 7.22 (s br, 2H), 6.97 (s br, 2H), 4.71 (t, J=8 Hz, 2H), 3.12 (m, 4H), 1.73 (m, 2H), 1.63 (m, 4H), 1.53 (m, 2H), 0.91 (t, J=8 Hz, 3H) ppm; δC (DMSO-d6) 181.41 (CO), 177.27 (Cq), 149.18 (Cq), 129.56 (Cq), 121.42 (2 CH), 116.55 (2 CH), 73.22 (OCCH3), 50.22 (2 CH2), 31.90 (OCH2CH3), 25.67 (2 CH2), 24.29 (CH2), 18.60 (CH2CH3), 13.95 (CH3) ppm; IR (KBr): ṽ= 3254, 3100, 1791, 1689, 1593 cm⁻¹; HRMS-ESI-TOF: m/z [M+H]+ calcd for C19H25N2O3: 329.1865, found: 329.1860.

**Compound 5b:** Obtained as a light green solid (36 mg, 52%); Rf=0.25 (AcOEt/hexane 1:1); mp: 94-96°C; δH (DMSO-d6) 10.72 (s, 1H, NH), 7.40 (d, J=8 Hz, 2H), 7.35 (d, J=8 Hz, 2H), 4.73 (t, J=8 Hz, 2H), 1.76 (m, 2H), 1.41 (m, 2H), 0.93 (t, J=8 Hz, 3H) ppm; δC (DMSO-d6) 181.36 (CO), 165.38 (Cq), 145.69 (Cq), 136.01 (Cq), 126.01 (CH), 125.67 (CH), 119.45 (CH), 118.26 (CH), 72.93 (OCH2CH2), 31.90 (OCH2CH2), 31.08 (C(CH3)3), 18.09 (C(CH3)), 13.45 (CH3) ppm; IR (KBr): ṽ= 3178, 2960, 2870, 1805, 1712, 1602, 1579, 1267, 1188, 1116, 1068 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]+ calcd for C18H23NNaO3: 324.1576, found: 324.1570.

**Preparation of squaric derivative 6a**

A mixture of the suitable amine (2 eq.) and 3,4-Dibutoxy-3-cyclobutene-1,2-dione (1 eq.) in dry methanol (2 ml for 0.22 mmol of amine) was stirred for 12 h at reflux. The methanol was evaporated and the obtained residue was recrystallized in methanol/ethyl acetate. The product was obtained as a grey solid (70 mg, 73%); Rf=0.25 (AcOEt/MeOH 9:1); mp: >300°C; δH (DMSO-d6) 10.72 (s, 1H, NH), 7.40 (d, J=8 Hz, 2H), 7.35 (d, J=8 Hz, 2H), 4.73 (t, J=8 Hz, 2H), 1.76 (m, 2H), 1.41 (m, 2H), 0.93 (t, J=8 Hz, 3H) ppm; δC (DMSO-d6) 181.36 (CO), 165.38 (Cq), 145.69 (Cq), 136.01 (Cq), 126.01 (CH), 125.67 (CH), 119.45 (CH), 118.26 (CH), 72.93 (OCH2CH2), 34.04 (C(CH3)3), 31.39 (OCH2CH2), 31.08 (C(CH3)3), 18.09 (C(CH3)), 13.45 (CH3) ppm; IR (KBr): ṽ= 3178, 2960, 2870, 1805, 1712, 1602, 1579, 1267, 1188, 1116, 1068 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]+ calcd for C19H25N4NaO2: 453.2266, found: 453.2261.
General preparation of squaric derivatives 6b and 6e

A mixture of the suitable amine (1 eq.) and 3,4-Dibutoxy-3-cyclobutene-1,2-dione (1 eq.) in dry methanol (2 ml for 0.22 mmol of amine) was stirred for 12 h at reflux. The methanol was evaporated and the obtained residue was recrystallized in methanol/ethyl acetate.

**Compound 6b**: Obtained as a white solid (76 mg, 46%); \( R_f = 0.3 \) (EtOAc/hexane 1:1); mp: > 330 °C, \( \delta_H \) (DMSO-d6) 9.83 (s, 2H, NH), 7.41 (s, 8H), 1.28 (s, 18H) ppm; \( \delta_C \) (DMSO-d6) 181.85 (CO), 165.83 (Cq), 146.19 (Cq), 136.46 (Cq), 126.53 (CH), 118.72 (CH), 34.52 (C(CH3)3), 31.63 (C(C(H3)3) ppm; IR (KBr): \( \tilde{\nu} = 3177, 2958, 2367, 1785, 1542 \) cm\(^{-1}\); HRMS-ESI-TOF: \([M+H]^+\) calcd for C\(_{24}\)H\(_{29}\)N\(_2\)O\(_2\): 377.2229, found: 377.2224.

**Preparation of squaric derivative 6f**

A solution of the suitable amine (1 eq.) in dry methanol (2 ml for 0.22 mmol of amine) and TEA (1 eq.) was stirred for 5 min at room temperature before addition of 3,4-Dibutoxy-3-cyclobutene-1,2-dione (1 eq). The mixture was stirred for 12 h at room temperature. The methanol was evaporated and the obtained residue was recrystallized in methanol/ethyl acetate. The product was obtained as a white solid (47 mg, 47%); \( R_f = 0.2 \) (EtOAc/hexane 1:1); mp: 270-272ºC; \( \delta_H \) (DMSO-d6) 7.50 (d, 2H, NH, \( J = 6.4 \) Hz), 7.21 (m, 10H), 5.03 (s, 2H), 4.15 (s, 2H), 3.44 (m, 4H), 2.88 (dd, 2H, \( J = 6.8 \), \( J = 7.2 \) Hz), 2.75 (dd, 2H, \( J = 7.2 \), \( J = 7.6 \) Hz ppm; \( \delta_C \) (DMSO-d6) 182.24 (CO), 167.63 (Cq), 138.38 (Cq), 129.23 (CH), 128.26 (CH), 126.23 (CH), 62.51 (C\(_{H2}\)OH), 56.91 (C\(_{HNH}\)), 38.18 (C\(_{H2}\)Ph) ppm; IR (KBr): \( \tilde{\nu} = 3202, 2932, 1791, 1644, 1574 \) cm\(^{-1}\); HRMS-ESI-TOF: \([M+H]^+\) calcd for C\(_{22}\)H\(_{25}\)N\(_2\)O\(_4\): 381.1814, found: 381.1809.

**General preparation of squaric derivatives 8b and 8f**

A solution of the suitable amine (1 eq.) in dry methanol (2 ml for 0.22 mmol of amine) and TEA (1 eq.) was stirred for 5 min at room temperature before addition of MeOSQLeuazaPheCOVSPh\(^1\) (1 eq). The mixture was stirred for 12 h at reflux. The methanol was evaporated and the obtained residue was purified by column chromatography.

**Compound 8b**: Obtained as a white powder (48 mg, 42%); \( R_f = 0.46 \) (AcOEt/Hex 3:2); mp: 117-118ºC; \( \delta_H \) (CDCl3) 7.92 (d, \( J = 8 \) Hz, 2H), 7.84 (d, \( J = 16 \) Hz, 1H), 7.57 (m, 3H), 7.57 (m, 3H), 5.67 (s, 1H), 4.94 (t, \( J = 8 \) Hz, 1H), 4.24 (s, 1H), 3.94 (d, \( J = 8 \) Hz, 1H), 1.76 (t, \( J = 8 \) Hz, 1H), 1.66 (m, 2H), 1.42 (s, 9H), 1.04 (s, 6H) ppm; \( \delta_C \) (CDCl3) 184.81 (C=O), 184.25 (C=O), 170.91 (Cq), 169.65 (C=O), 168.62 (Cq), 165.36 (C=O), 147.95 (Cq), 140.97 (Cq), 139.62 (CH=CHSO2Ph), 138.27 (CH), 136.25 (CH), 133.09 (CH), 129.14 (CH=CHSO2Ph), 128.81 (CH), 128.47 (CH), 128.21 (CH), 127.64 (CH), 126.23 (CH), 122.33 (CH), 58.35 (NHC\(_{HLeu}\)), 53.21 (NCH\(_2\)Ph), 40.47 (C\(_{CH2(CH2)3}\)), 35.44 (C\(_{CH2(CH2)3}\)), 31.47 (C\(_{CH2(CH2)3}\)), 25.42 (C\(_{CH2(CH2)3}\)), 23.18 (C=O) ppm; IR (NaCl): \( \tilde{\nu} = 3254, 3241, 3087, 2945, 1797, 1714, 1612, 1568, 1517, 1446, 1389, 1076 \) cm\(^{-1}\); HRMS-ESI-TOF: \([M+Na]^+\) calcd for C\(_{36}\)H\(_{30}\)N\(_4\)O\(_6\)S\(_2\): 679.2566, found: 679.2559.

**Compound 8f**: Obtained as a white powder (53 mg, 47%); \( R_f = 0.43 \) (AcOEt/Hex 3:2); mp: 111-112ºC; \( \delta_H \) (CDCl3) 8.99 (s, 1H), 7.96-7.91 (m, 3H), 7.55 (m, 3H), 7.20-6.97 (m, 11H), 5.17 (s, 1H), 4.64 (t, \( J = 4 \) Hz, 1H), 4.09 (m, 3H), 3.81 (m, 1H), 3.70 (m, 2H), 2.96 (m, 1H), 2.66 (m, 1H), 2.23 (m, 1H), 2.08 (t, \( J = 8 \) Hz, 1H), 1.49 (t, \( J = 8 \) Hz, 1H), 1.21 (s, 1H), 1.12 (d, \( J = 8 \) Hz, 6H) ppm; \( \delta_C \) (CDCl3) 184.81(2C=O), 171.83 (Cq), 170.91 (Cq), 170.91 (Cq), 168.62 (Cq), 165.36 (C=O), 147.95 (Cq), 140.97 (Cq), 139.62 (CH=CHSO2Ph), 138.27 (CH), 136.25 (CH), 133.09 (CH), 129.14 (CH=CHSO2Ph), 128.81 (CH), 128.47 (CH), 128.21 (CH), 127.64 (CH), 126.23 (CH), 122.33 (CH), 58.35 (NHC\(_{HLeu}\)), 53.21 (NCH\(_2\)Ph), 40.47 (C\(_{CH2(CH2)3}\)), 35.44 (C\(_{CH2(CH2)3}\)), 31.47 (C\(_{CH2(CH2)3}\)), 25.42 (C\(_{CH2(CH2)3}\)), 23.18 (C\(_{CH2(CH2)3}\)) ppm; IR (NaCl): \( \tilde{\nu} = 3254, 3241, 3087, 2945, 1797, 1714, 1612, 1568, 1517, 1446, 1389, 1076 \) cm\(^{-1}\); HRMS-ESI-TOF: \([M+Na]^+\) calcd for C\(_{36}\)H\(_{30}\)N\(_4\)O\(_6\)SNa: 679.2566, found: 679.2559.
(CH₂OH), 60.81 (CH(CH₂OH)(CH₂Ph)), 58.35 (NHCH₂Leu), 53.21 (NCH₂Ph), 40.47 (CH₂CH(CH₃)₂), 38.56 (CH₂Ph), 25.42 (C(CH₃)₂), 23.14 (CH₂CH(C(CH₃)₃), 61.46 (OCH₃), 54.25 (CH₃)₃), 73.09 (CH₂), 50.58 (NHC(CH₃)₂) ppm; IR (NaCl): ν = 3409, 2976, 1691, 1536, 1458, 1398, 1293, 1259, 1085 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]⁺ calcd for C₁₉H₂₃N₂O₄Na: 468.2135, found: 468.2134.

General preparation of aza squaric derivatives 11a and 13a
To a solution of t-butyl carbazate (1 eq.) in methanol was added the suitable squaric acid ester (1 eq.). The reaction mixture was stirred overnight at r.t.. The solvent was then removed under reduced pressure. The obtained residue was purified by column chromatography affording the corresponding product as a white solid. To a solution of the Boc protected hydrazone 10 (1 eq.) in DCM obtained in the previous step was added TFA (4 eq.). After 2h the solvent was removed under reduced pressure affording quantitatively a pale yellow oil.

To a solution of the BocLeuOH (1 eq.) in a mixture of THF:DMF (1:1) was added CDI (1 eq.) and the mixture stirred at r.t. for 30 minutes. After this time, the suitable hydrazine 10 was added in THF:DMF (1:1) and the mixture was stirred overnight at r.t.. The reaction mixture was then diluted with water and extracted with AcOEt and the organic layers combined, dried with anhydrous Na₂SO₄, filtered and concentrated to dryness. The obtained residue was purified by column chromatography affording the corresponding product as a white solid.

**Compound 11a:** Obtained as a white solid (58 mg, 68%); Rf = 0.41 (AcOEt/Hex 3:2); mp: 111-112°C; δH (CDCl₃) 8.32 (s, 1H), 5.74 (s, 1H), 5.35 (s, 1H), 4.82 (t, J = 4 Hz, 1H), 3.93 (s, 3H), 1.80 (m, 2H), 1.51 (s, 9H), 1.00 (m, 1H), 0.94 (s, 6H) ppm; δC (CDCl₃) 190.10 (C=O), 178.94 (C=O), 173.66 (C=O), 172.61 (Cq), 158.31 (C=O), 80.65 (Cq), 61.45 (OCH₃), 50.58 (NHCH₂Leu), 40.43 (CH₂CH(CH₂)₂), 28.41 (C(CH₃)₂), 23.14 (CH₂CH(C(CH₃)₃), 61.46 (OCH₃), 54.25 (CH₂Ph), 51.09 (NHCH₂Leu), 40.44 (CH₂CH(CH₂)₂), 28.41 (C(CH₃)₂), 23.14 (CH₂CH(C(CH₃)₃), 73.09 (CH₂), 50.58 (NHCH₂Leu), 40.43 (CH₂CH(CH₂)₂), 31.14 (CH₃), 28.41 (C(CH₃)₂), 25.38 (CH₂CH(CH₂)₂), 23.14 (CH₂CH(C(CH₃)₃), 19.94 (CH₃), 14.01 (CH₃) ppm; IR (NaCl): ν = 3285, 2928, 2873, 1681, 1613, 1443, 1375, 1300, 1211, 1177 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]⁺ calcd for C₁₉H₂₃N₃O₆Na: 378.1641, found: 378.1632.

**Compound 13a:** Obtained as a white solid (73 mg, 78%); Rf = 0.42 (AcOEt/Hex 3:2); mp: 119-120°C; δH (CDCl₃) 7.55 (s, 1H), 4.88 (s, 1H), 4.62 (t, J = 4 Hz, 1H), 4.45 (m, 3H), 1.98 (m, 1H), 1.78 (m, 2H), 1.70 (m, 1H), 1.67 (m, 2H), 1.38 (s, 9H), 1.01 (s, 6H) ppm; δC (CDCl₃) 190.17 (C=O), 180.27 (Cq), 178.45 (C=O), 170.87 (C=O), 158.51 (Cq), 156.31 (C=O), 80.65 (C(CH₃)₂), 73.09 (CH₂), 50.58 (NHCH₂Leu), 40.43 (CH₂CH(CH₂)₂), 31.14 (CH₃), 28.41 (C(CH₃)₂), 25.38 (CH₂CH(CH₂)₂), 23.14 (CH₂CH(C(CH₃)₃), 19.94 (CH₃), 14.01 (CH₃) ppm; IR (NaCl): ν = 3151, 2971, 2932, 1791, 1644, 1561, 1459, 1210, 1153, 1095 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]⁺ calcd for C₁₉H₂₃N₃O₆Na: 420.2111, found: 420.2095.

General preparation of aza squaric derivatives 11b-c, 11f-h, 13b and 13e-g
To a solution of the suitable amino acid hydrazide (1 eq.) in methanol was added the suitable squaric acid ester (1 eq.). The reaction mixture was stirred overnight at r.t.. The solvent was then removed under reduced pressure. The obtained residue was purified by column chromatography affording the corresponding product as a white solid.

**Compound 11b:** Obtained as a white solid (42 mg, 42%); Rf = 0.48 (AcOEt/Hex 3:2); mp: 117-118°C; δH (CDCl₃) 7.49 (d, J = 8 Hz, 2H), 7.27 (t, J = 8 Hz, 2H), 7.19 (m, 1H), 6.38 (s, 1H), 5.58 (s, 1H), 5.08 (s, 1H), 4.85 (t, J = 4 Hz, 1H), 4.73 (s, 1H), 3.70 (s, 3H), 1.80 (dd, J = 8 Hz, J = 4 Hz, 1H), 1.70 (dd, J = 8 Hz, J = 4 Hz, 1H), 1.58 (s, 10H), 1.01 (d, J = 8 Hz, 6H) ppm; δC (CDCl₃) 190.17 (C=O), 180.27 (Cq), 178.45 (C=O), 170.87 (C=O), 158.51 (Cq), 158.31 (C=O), 136.31 (Cq), 128.76 (CH), 128.40 (CH), 127.59 (CH), 80.65 (C(CH₃)₂), 61.46 (OCH₃), 54.25 (CH₂Ph), 51.09 (NHCH₂Leu), 40.44 (CH₂CH(CH₂)₂), 28.41 (C(CH₃)₂), 25.39 (CH₂CH(CH₂)₂), 23.14 (CH₂CH(C(CH₃)₃), 21.73 (CH₂CH(CH₂)₂), 19.94 (CH₃), 14.01 (CH₃) ppm; IR (NaCl): ν = 3357, 2971, 2868, 1797, 1695, 1606, 1574, 1459, 1370, 1146, 1051 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]⁺ calcd for C₁₉H₂₃N₃O₆Na: 468.2111, found: 468.2099.

**Compound 11c:** Obtained as a white solid (47 mg, 47%); Rf = 0.47 (AcOEt/Hex 3:2); mp: 129-130°C; δH (CDCl₃) 7.51 (s, 1H), 7.29-7.14 (m, 5H), 5.15 (s, 1H), 4.82 (t, J = 4 Hz, 1H), 4.38 (t, J = 8 Hz, 1H), 3.92 (s, 3H), 3.80 (t, J = 8 Hz, 1H), 2.91 (t, J = 8 Hz, 2H), 1.88 (dd, J = 8 Hz, J = 4 Hz, 1H), 1.80 (dd, J = 8 Hz, J = 4 Hz, 1H), 1.51 (s, 9H), 1.17
(m, 1H), 0.98 (d, J = 8 Hz, 6H) ppm; δc (CDCl3) 190.17 (C=O), 183.73 (Cq), 178.45 (C=O), 170.87 (C=O), 163.36 (Cq), 160.36 (Cq), 154.73 (C=O), 138.64 (Cq), 137.48 (Cq), 137.19 (CH), 129.19 (CH), 128.31 (CH), 128.17 (CH), 126.36 (CH), 66.68 (OCH2Ph), 61.45 (OCH3), 51.50 (NCH3), 46.38 (NHCH2CO), 51.42 (CH2Ph) ppm; IR (NaCl): v = 3292, 2958, 1797, 1651, 1587, 1465, 1229, 1165 cm−1; HRMS-ESI-TOF: m/z [M+Na]+ caleed for C32H23N3O6Na: 544.1572, found: 544.1562.

Compound 11f: Obtained as a white solid (91 mg, 72%); Rf = 0.36 (AcEt/Hex 3:2); mp: 104-105°C; δh (CDCl3) 7.34 (s, 1H), 7.27-7.14 (m, 10H), 5.37 (s, 2H), 5.28 (s, 1H), 4.26-4.21 (m, 2H), 4.10 (s, 1H), 3.85 (m, 4H), 2.89 (t, J = 8 Hz, 2H) ppm; δc (CDCl3) 190.17 (C=O), 183.73 (Cq), 178.45 (C=O), 171.16 (C=O), 160.36 (Cq), 154.73 (C=O), 138.64 (Cq), 137.48 (Cq), 137.19 (CH), 129.19 (CH), 128.31 (CH), 128.17 (CH), 126.36 (CH), 66.68 (OCH2Ph), 61.45 (OCH3), 51.50 (NCH3), 46.38 (NHCH2CO), 51.42 (CH2Ph) ppm; IR (NaCl): v = 3292, 2958, 1670, 1580, 1427, 1331, 1229, 1114 cm−1; HRMS-ESI-TOF: m/z [M+Na]+ caleed for C32H23N3O6Na: 546.1485, found: 460.1478.

Compound 11h: Obtained as a white solid (87 mg, 79%); Rf = 0.41 (AcEt/Hex 3:2); mp: 96-97°C; δh (CDCl3) 7.25-7.05 (m, 13H), 6.99 (d, J = 8 Hz, 2H), 6.46 (s, 1H), 5.47 (m, 1H), 5.36 (s, 2H), 4.36 (t, J = 4 Hz, 1H), 4.22 (s, 1H), 3.98 (t, J = 4 Hz, 1H), 3.77 (s, 3H), 3.02 (dd, J = 12 Hz, J = 4 Hz, 1H), 2.86-2.80 (m, 3H) ppm; δc (CDCl3) 190.17 (C=O), 183.73 (Cq), 178.45 (C=O), 163.36 (Cq), 154.73 (C=O), 138.64 (Cq), 137.48 (Cq), 137.19 (CH), 129.38 (CH), 129.19 (CH), 129.06 (CH), 128.31 (CH), 128.17 (CH), 126.36 (CH), 66.68 (OCH2Ph), 61.45 (OCH3), 56.18 (NCH3), 51.50 (NCH3), 38.09 (CH2Ph), 31.42 (CH2Ph) ppm; IR (NaCl): v = 3421, 3318, 3074, 2958, 1714, 1682, 1510, 1134 cm−1; HRMS-ESI-TOF: m/z [M+Na]+ caleed for C30H29N3O6Na: 550.1954, found: 550.1941.

Compound 13b: Obtained as a white solid (85 mg, 82%); Rf = 0.46 (AcEt/Hex 3:2); mp: 132-133°C; δh (CDCl3) 7.29-7.14 (m, 6H), 5.34 (s, 1H), 4.97 (t, J = 4 Hz, 1H), 4.45 (t, J = 8 Hz, 2H), 3.41 (t, J = 4 Hz, 1H), 3.84 (t, J = 8 Hz, 1H), 2.92 (t, J = 8 Hz, 1H), 1.83-1.77 (m, 4H), 1.61 (m, 1H), 1.52 (sl, 1H), 1.02 (d, J = 8 Hz, 9H) ppm; δc (CDCl3) 190.17 (C=O), 185.03 (Cq), 180.09 (C=O), 170.87 (C=O), 163.32 (Cq), 158.31 (C=O), 138.64 (Cq), 129.19 (CH), 129.06 (CH), 126.36 (CH), 80.65 (C(CH3)3), 73.09 (CH3), 51.50 (NCH3), 51.09 (NHCH2Leu), 40.43 (CH2CH(CH3)2), 31.42 (CH2Ph), 31.14 (C(CH3)3), 28.41 (CH2CH(CH3)2), 25.38 (CH2CH(CH3)2), 23.14 (CH2CH(CH3)2), 19.94 (CH3), 14.01 (CH3) ppm; IR (NaCl): v = 3426, 3166, 2932, 2860, 1735, 1675, 1621, 1464, 1379, 1253, 1217, 1048 cm−1; HRMS-ESI-TOF: m/z [M+Na]+ caleed for C27H30N3O6Na: 524.2737, found: 524.2732.

Compound 13c: Obtained as a white solid (97 mg, 83%); Rf = 0.43 (AcEt/Hex 3:2); mp: 87-88°C; δh (CDCl3) 10.21 (s, 1H), 7.26-7.08 (m, 10H), 5.33 (s, 2H), 4.95 (t, J = 8 Hz, 1H), 4.35 (t, J = 8 Hz, 1H), 4.26 (t, J = 8 Hz, 2H), 4.11 (s, 1H), 3.90 (t, J = 4 Hz, 1H), 2.88 (t, J = 8 Hz, 2H), 1.83 (m, 1H), 1.75 (m, 3H), 1.64 (m, 1H), 1.44 (m, 2H), 1.07-0.99 (m, 9H) ppm; δc (CDCl3) 190.17 (C=O), 185.03 (Cq), 180.09 (C=O), 170.87 (C=O), 164.32 (Cq), 159.04 (C=O), 138.64 (Cq), 137.08 (Cq), 129.19 (CH), 129.06 (CH), 128.31 (CH), 128.17 (CH), 126.36 (CH), 73.09 (CH2), 66.68 (OCH2Ph), 51.50 (NCH3), 51.09 (NHCH2Leu), 40.43 (CH2CH(CH3)2), 31.42 (CH2Ph), 31.14 (CH3), 25.38 (CH2CH(CH3)2), 23.14 (CH2CH(CH3)2), 19.94 (CH3), 14.01 (CH3) ppm; IR (NaCl): v = 3426, 3340, 3244, 3051, 2969, 1722, 1647, 1538, 1416, 1265, 1150, 1088 cm−1; HRMS-ESI-TOF: m/z [M+Na]+ caleed for C39H38N3O6Na: 558.2580, found: 558.2571.
**General preparation of Muaza squaric derivatives 11d-e and 13c-d**

To a solution of the suitable Boc azapeptide squaric acid derivative (1 eq.) in DCM was added TFA (4 eq.). After 2 h the solvent was removed under reduced pressure affording quantitatively a pale yellow oil. The residue was dissolved in DCM and Et₂N (2 eq.) was added followed by the MuCOCl (1 eq.) under N₂. The reaction mixture was stirred at r.t. for 2 h. The solvent was then removed under reduced pressure and the obtained residue was purified by column chromatography affording the corresponding product as a white solid.

**Compound 11d:** Obtained as a white solid (43 mg, 71%); Rf = 0.37 (AcOEt/Hex 1:1); mp: 88-89°C; δ (CDCl₃) 5.88 (s, 1H), 5.01 (t, J = 8 Hz, 1H), 4.43 (s, 1H), 3.89 (s, 3H), 3.81 (m, 6H), 3.57 (t, J = 4 Hz, 2H), 1.85 (t, J = 8 Hz, 1H), 1.58 (m, 2H), 1.02 (sl, 6H) ppm; δ (CDCl₃) 190.10 (C=O), 178.94 (C=O), 173.66 (C=O), 172.61 (Cq), 163.32 (Cq), 157.71 (C=O), 155.09 (C=O), 138.64 (Cq), 137.68 (Cq), 137.08 (Cq), 129.19 (CH), 129.05 (CH), 128.31 (CH), 127.15 (CH), 126.36 (CH), 66.68 (CH₂CH₂), 56.18 (CH₂), 51.50 (NCH₂), 38.09 (CH₂), 31.42 (CH₂), 19.94 (CH₃), 14.01 (CH₃) ppm; IR (NaCl): v = 3305, 2971, 1727, 1657, 1523, 1153, 1031 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]⁺ calcd for C₁₉H₁₆N₃O₆Na: 592.2424, found: 592.2411.

**Compound 11e:** Obtained as a white solid (46 mg, 74%); Rf = 0.37 (AcOEt/Hex 1:1); mp: 89-90°C; δ (CDCl₃) 7.28-7.15 (m, 6H), 5.66 (s, 1H), 5.30 (t, J = 4 Hz, 1H), 4.44-4.35 (m, 2H), 3.93 (s, 3H), 3.81 (m, 6H), 3.61 (t, J = 8 Hz, 2H), 2.83 (t, J = 8 Hz, 2H), 1.66 (t, J = 4 Hz, 1H), 1.50 (t, J = 8 Hz, 1H), 1.20 (m, 1H), 0.91 (d, J = 8 Hz, 6H) ppm; δ (CDCl₃) 190.17 (C=O), 183.73 (Cq), 178.45 (C=O), 170.87 (C=O), 165.15 (Cq), 157.43 (C=O), 138.64 (Cq), 137.68 (Cq), 137.08 (Cq), 129.19 (CH), 129.05 (CH), 128.31 (CH), 127.15 (CH), 126.36 (CH), 66.68 (OCH₂Ph), 56.18 (NCH₂CO), 51.50 (NCH₂), 38.09 (CH₂), 31.42 (CH₂), 19.94 (CH₃), 14.01 (CH₃) ppm; IR (NaCl): v = 3305, 3010, 2932, 1791, 1644, 1597, 1465, 1537, 1038 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]⁺ calcd for C₂₀H₂₁N₅O₇Na: 593.1594, found: 593.1579.

**Compound 13c:** Obtained as a white solid (46 mg, 76%); Rf = 0.35 (AcOEt/Hex 1:1); mp: 89-90°C; δ (CDCl₃) 5.92 (s, 1H), 5.00 (t, J = 4 Hz, 1H), 4.67 (s, 1H), 4.37 (t, J = 4 Hz, 2H), 3.88 (t, J = 8 Hz, 2H), 3.79 (t, J = 8 Hz, 2H), 3.63 (t, J = 4 Hz, 2H), 2.01 (t, J = 8 Hz, 1H), 1.85 (m, 1H), 1.73 (m, 2H), 1.49 (m, 3H), 0.99 (m, 10H) ppm; δ (CDCl₃) 190.10 (C=O), 180.01 (C=O), 174.62 (Cq), 173.66 (C=O), 166.93 (Cq), 157.71 (C=O), 137.68 (Cq), 137.08 (Cq), 129.19 (CH), 129.05 (CH), 128.31 (CH), 127.15 (CH), 126.36 (CH), 51.33 (NCH₂CO), 51.50 (NCH₂), 40.43 (CH₂CH₂), 28.85 (CH₂CH₂), 23.15 (CH₂CH₂), 19.94 (CH₃), 14.01 (CH₃) ppm; IR (NaCl): v = 3447, 1702, 1651, 1504, 1465, 1287, 1197, 1140, 1095 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]⁺ calcd for C₁₉H₁₉N₄O₇Na: 433.2063, found: 433.2056.
3.86-3.77 (m, 7H), 3.60 (t, J = 4 Hz, 2H), 2.93 (t, J = 8 Hz, 2H), 1.88-1.78 (m, 4H), 1.60 (m, 1H), 1.47 (m, 1H), 1.03-0.99 (m, 9H) ppm; δC (CDCl3) 190.17 (C=O), 185.03 (Cq), 180.09 (C=O), 170.87 (C=O), 164.32 (Cq), 157.71 (C=O), 138.64 (Cq), 129.19 (CH), 129.06 (CH), 126.36 (CH), 73.09 (CH3), 66.09 (CH2), 51.98 (NHCsLeu), 51.50 (NCH3), 46.58 (CH2), 40.43 (CH2CH(CH3)2), 31.42 (CH2Ph), 31.14 (CH3), 25.38 (CH2CH(CH3)2), 23.14 (CH2CH(CH3)2), 19.94 (CH3), 14.01 (CH3) ppm; IR (NaCl): ν = 3302, 3066, 2953, 1721, 1642, 1518, 1450, 1394, 1230, 1118, 1028 cm⁻¹; HRMS-ESI-TOF: m/z [M+Na]+ calcd for C27H38N4O6Na: 537.2689, found: 537.2673.
Pharmacology Methods

Falcipain-2 assay
Falcipain-2 was assayed at 25 °C using 25 µM Z-Leu-Arg-AMC as substrate in 100 mM sodium acetate pH 6.0, 5 mM DTT, 0.75% DMSO. One microliter of serial dilutions of each compound dissolved in DMSO (highest concentration was 50 µM diluted 1:5 in 8 steps resulting in the lowest concentration used 0.65 nM) was diluted into 100 µL of assay buffer. Enzyme (1nM) was added, the mixture was incubated for 10 min, the reaction was initiated by addition of 50 µL of assay buffer containing the substrate, and fluorescence was read immediately in a Fluoroskan Ascent microplate spectrofluorometer. IC₅₀ values were determined by plotting percentage inhibition relative to controls without inhibitor using GraphPad Prism 4 (GraphPad Software).

Papain assay
Assays were carried out in 200 µL assay buffer (10 mM PBS, pH 7.4, 5 mM DTT) containing 20 µL of papain activated in assay buffer at 5 µg/mL, and 5 µL of each concentration of tested inhibitors. Reactions were initiated by the addition of 30 µM fluorogenic substrate (Z-Leu-Leu-Arg-AMC, from Bachem, Germany) and activity was monitored (excitation 355 nm; emission 460 nm) for 30 min, at 37°C on a Fluorescence Microplate Reader Tecan infinite M200 (Tecan, Switzerland). The Km of this substrate of papain was previously determined to be 0.4 µM (data not shown). For all assays, saturated substrate concentrations were used in order to obtain linear fluorescence curves. Inhibitors stock solutions were prepared in DMSO, and serial dilutions were made in DMSO. Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (trans-Epoxysucciny-L-leucyl-amido(4-guanidino) butane E64, Calbiochem, Germany). The IC₅₀ values were determined by non-linear regression analysis based on the log of inhibitors concentrations versus the percentage of activity using GraphPad PRISM software. Assays were performed in triplicate.

In vitro Antiplasmodial Activity in human red blood cells
Human red blood cells infected with ~1% parasitemia of ring stage P. falciparum synchronized with 5% sorbitol were incubated with tested compounds in 96 well plates at 37°C for 48 h in RPMI-1640 medium, supplemented with 25 mM HEPES pH 7.4, 10% heat inactivated human serum (or 0.5% Albumax, 2% human serum), and 100 µM hypoxanthine under an atmosphere of 3% O₂, 5% CO₂, 91% N₂. After 48 h the cells were fixed in 2% HCHO in PBS, transferred into PBS with 100 mM NH₄Cl, 0.1% Triton X-100, 1 nM YOYO-1, and then analyzed in a flow cytometer (FACSort, Beckton Dickinson; EX 488 nm, EM 520 nm.) IC₅₀s were calculated using GraphPad PRISM software.

In Vitro Cytotoxicity
The cytotoxicity was assessed using general cell viability endpoint MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). MTT is a yellow, water-soluble tetrazolium dye that is converted by mitochondrial dehydrogenases in viable cells to a water-insoluble, purple formazan. The day before experiment cells obtained from American Type Culture Collection, (ATCC, Rockville, Md.), (NIH 3T3 - mouse embryonic fibroblast cell line (ATCC CRL-1658) or HEK 293T - human embryonic kidney epithelial cell line, (ATCC CRL-11268)) are seeded in 96 well tissue culture plates, in RPMI 1640 culture medium supplemented with 10% Fetal serum bovine, 100 units of penicillin G (sodium salt) and 100µg of streptomycin sulfate and 2mM L-glutamine, at a concentration that allow cells to grow exponentially during the time of the assay. Compounds to be tested are diluted in dimethylsulfoxide (DMSO) and then serially diluted in the culture medium. Compounds at different concentrations and DMSO are then added to the cells. Each compound concentration was tested in triplicate in a single experiment which was repeated at
least 3 times. Cells are incubated at 37°C in humidified 5% CO2 atmosphere. After 48 hours, cell media containing DMSO (for control cells) or tested compound solution (for test cells) was removed and replaced with fresh medium. The MTT dye solution was added to each well (5mg/mL in 10mM phosphate buffer solution at pH 7.4). After 3h of incubation the complete media was removed and the intracellular formazan crystals were solubilised and extracted with DMSO. After 15 min at room temperature the absorbance measured at 570 nm in microplate reader (Infinite M200, Tecan, Austria).

The percentage of cell viability was determined for each concentration of tested compound as described previously. The concentration of a compound reflecting a 50% inhibition of cell viability (i.e. IC50) was determined from the concentration-response curve. This was done by applying non-linear regression procedure to the concentration-response data using GraphPad PRISM software.
Supplementary References


