Supporting Information

Squaramides with cytotoxic activity against human gastric carcinoma against HGC-27.

Synthesis, biological evaluation and mechanism study as novel anticancer agents

Mireia Quintana,¹a Juan V. Alegre-Requena,¹a,b Eugenia Marqués-López,b Raquel P. Herrera,b,* Gemma Triolaª*

ªBiomedicinal Chemistry Department, Institute of Advanced Chemistry of Catalonia (IQAC), CSIC, E-08034, Barcelona, Spain; e-mail: gemma.triola@iqac.csic.es

bLaboratorio de Organocatálisis Asimétrica, Departamento de Química Orgánica, Instituto de Síntesis Química y Catálisis Homogénea (ISQCH), CSIC-Universidad de Zaragoza, E-50009 Zaragoza, Spain; e-mail: raquelph@unizar.es

Supp Fig. 1 Dose-dependent curves obtained with an ATP-based assay or a MTT assay show the difference of cell viability at given concentrations, thus supporting the need of a 8 µM concentration of 34 to correlate a decrease in cell viability with the effects on cell death induction.
Supp Fig. 2 Detection of intracellular ROS levels in HGC-27 cells exposed to increased concentrations of H₂O₂ and 34.
Synthesis of squaramates 5-8 and 11-14

To a mixture of 3,4-dimethoxy-3-cyclobutene-1,2-dione (40) (0.2 mmol) in MeOH (0.75-1.5 ml), amine 41a-h (0.2 mmol) was added at room temperature. After the corresponding reaction time, the product was purified either by filtration, washing with MeOH at –25 °C, or by column chromatography.

3-Ethyl-4-(2-methoxy-3,4-dioxocyclobut-1-enylamino)benzonitrile (5)

Following the general procedure, using 0.75 ml MeOH and after 5 days, 5 was obtained by filtration as a yellow solid in 60% yield. M.p. 173–175 °C. $^1$H-NMR (400 MHz, DMSO-$d_6$) δ 10.54 (br s, 1H), 7.75–7.63 (m, 2H), 7.30 (d, $J = 8.2$ Hz, 1H), 4.33 (s, 3H), 2.73 (q, $J = 7.5$ Hz, 2H), 1.14 (t, $J = 7.5$ Hz, 3H). $^{13}$C-NMR-APT (100 MHz, DMSO-$d_6$) δ 188.0 (1C), 184.9 (1C), 179.3 (1C), 170.0 (1C), 139.3 (1C), 137.6 (1C), 132.5 (1C), 130.2 (1C), 124.8 (1C), 118.7 (1C), 108.0 (1C), 60.5 (1C), 23.0 (1C), 13.5 (1C). IR (KBr film) (cm$^{-1}$) ν 3222, 2924, 2854, 1811, 1703, 1584,
3-((2,4-Dimethoxyphenyl)amino)-4-methoxycyclobut-3-ene-1,2-dione (6)

Following the general procedure, using 0.75 ml MeOH and after 1 h, 6 was obtained by filtration as a pale yellow solid in 64% yield. M.p. 186–189 °C. $^{1}H$-NMR (300 MHz, DMSO-$d_6$) δ 10.13 (br s, 1H), 7.09 (d, $J = 8.6$ Hz, 1H), 6.63 (d, $J = 2.6$ Hz, 1H), 6.50 (dd, $J = 8.6$, 2.6 Hz, 1H), 4.26 (s, 3H), 3.78 (s, 3H), 3.77 (s, 3H). $^{13}C$-NMR-APT (75 MHz, DMSO-$d_6$) δ 183.7 (1C), 178.0 (1C), 170.9 (1C), 158.6 (1C), 153.5 (1C), 125.5 (1C), 118.8 (2C), 104.2 (1C), 99.0 (1C), 60.0 (1C), 55.6 (1C), 55.3 (1C). IR (KBr film) (cm$^{-1}$) ν 3299, 3250, 2923, 2853, 1804, 1708, 1598, 1459, 1419, 1366, 1205, 1048, 928, 865, 801, 454. HRMS (ESI+) calcd C$_{14}$H$_{12}$N$_2$NaO$_3$ 279.0746; found 279.0726 [M + Na].

3-((2,4-Dichloro-5-isopropoxyphenyl)amino)-4-methoxycyclobut-3-ene-1,2-dione (7)

Following the general procedure, using 0.75 ml MeOH after 61 h, 7 was obtained by filtration as a yellow solid in 50% yield. M.p. 161–164 °C. $^{1}H$ NMR (300 MHz, DMSO-$d_6$) δ 10.65 (s, 1H), 7.61 (s, 1H), 7.23 (s, 1H), 4.67 (q, $J = 6.1$ Hz, 1H), 4.30 (s, 3H), 1.29 (d, $J = 6.1$ Hz, 6H). $^{13}C$-NMR-APT (75 MHz, DMSO-$d_6$) δ 187.9 (1C), 184.9 (1C), 178.9 (1C), 170.0 (1C), 151.9 (1C), 133.9 (1C), 129.7 (1C), 120.0 (1C), 118.0 (1C), 112.7 (1C), 71.8 (1C), 60.4 (1C), 21.6 (2C). IR (KBr film) (cm$^{-1}$) ν 3268, 2923, 2853, 1801, 1710, 1608, 1583, 1503, 1448, 1405, 1389, 1249, 1088, 879, 442. HRMS (ESI+) calcd C$_{14}$H$_{13}$Cl$_2$NaNO$_4$ 352.0119; found 352.0118 [M + Na].

3-Methoxy-4-((3,4,5-trifluorophenyl)amino)cyclobut-3-ene-1,2-dione (8)

Following the general procedure, using 0.75 ml MeOH after 44 h, 8 was obtained by filtration as a pale yellow solid in 68% yield. M.p. 212–213 °C. $^{1}H$-NMR (300 MHz, DMSO-$d_6$) δ 10.94 (s, 1H), 7.29 (d, $J = 9.6$, 6.1 Hz, 2H), 4.40 (s, 3H). $^{13}C$-NMR-APT (100 MHz, DMSO-$d_6$) δ 187.4 (1C),
184.1 (1C), 179.5 (1C), 168.9 (1C), 150.4 (dd, $J = 245.2, 9.9, 5.0$ Hz, 2C), 135.5 (dt, $J = 190.0$ Hz, 13.7 Hz, 1C), 134.1 (1C), 104.0 (d, $J = 24.4$ Hz, 2C), 60.8 (1C). IR (KBr film) (cm$^{-1}$) $\nu$ 3238, 3069, 2924, 2853, 1816, 1636, 1595, 1532, 1457, 1362, 1249, 1213, 1111, 1048, 983, 929, 852, 796, 748, 717, 609, 418. HRMS (ESI+) calcd C$_{11}$H$_6$F$_3$NNaO$_3$ 280.0197; found 280.0178 [M + Na].

3-(((1R,2S)-1-Hydroxy-2,3-dihydro-1H-inden-2-yl)amino)-4-methoxycyclobut-3-ene-1,2-dione (11)

Following the general procedure, using 0.75 ml MeOH after 42 h, 11 was obtained by column chromatography (SiO$_2$, using Hex:AcOEt 9:1 to Hex:AcOEt 4:6) as a pale yellow solid in 93% yield. M.p. 86–89 °C. $[\alpha]_{D}^{26} = +107.1$ (c 0.12, DMSO). $^1$H-NMR (300 MHz, DMSO-$d_6$, 50 °C) $\delta$ 8.59 (br s, 1H), 7.35–7.15 (m, 4H), 5.45 (br s, 0.5H), 5.18 (s, 1H), 4.97 (br s, 0.5H), 4.53–4.45 (m, 1H), 4.32 (s, 3H), 3.06 (dd, $J = 15.8, 5.9$ Hz, 1H), 2.91 (dd, $J = 16.0, 3.1$ Hz, 1H). $^{13}$C-NMR-APT (100 MHz, DMSO-$d_6$) $\delta$ 189.8 (1C), 182.9 (1C), 174.4 (1C), 172.7 (1C), 140.9 (1C), 140.7 (1C), 126.5 (2C), 124.6 (2C), 72.3 (1C), 60.9 (1C), 59.8 (1C), 38.8 (1C). IR (KBr film) (cm$^{-1}$) $\nu$ 3337, 2924, 2853, 1805, 1593, 1461, 1377, 1336, 1049, 933, 740. HRMS (ESI+) calcd C$_{14}$H$_{13}$NNaO$_4$ 282,0742; found 282.0728 [M + Na].

(S)-3-Methoxy-4-(((1-(naphthalen-1-yl)ethyl)amino)cyclobut-3-ene-1,2-dione (12)

Following the general procedure, using 0.75 ml MeOH after 95 h, 12 was obtained by column chromatography (SiO$_2$, using Hex:AcOEt 9:1 to Hex:AcOEt 5:5) as a pale yellow solid in 95% yield. M.p. 115–117 °C. $[\alpha]_{D}^{26} = +97.6$ (c 0.12, DMSO). $^1$H-NMR (300 MHz, DMSO-$d_6$, 50 °C) $\delta$ 9.38 (br s, 0.5H), 9.15 (br s, 0.5H), 8.10 (br s, 1H), 7.96 (d, $J = 8.0$ Hz, 1H), 7.86 (d, $J = 8.1$ Hz, 1H), 7.66–7.49 (m, 4H), 6.11 (br s, 0.5H), 5.65 (br s, 0.5H), 4.30 (s, 1.5H), 4.14 (s, 1.5H), 1.64 (d, $J = 6.8$ Hz, 3H). $^{13}$C-NMR-APT for the major conformer (100 MHz, DMSO-$d_6$) $\delta$ 189.3 (1C), 182.5 (1C), 177.7 (1C), 171.5 (1C), 139.0 (1C), 133.3 (1C), 129.5 (1C), 128.7 (1C), 127.7 (1C), 126.4 (1C), 125.7 (1C), 125.4 (1C), 122.7 (1C), 122.5 (1C), 60.0 (1C), 50.1 (1C), 22.7
IR (KBr film) (cm$^{-1}$) ν 3186, 2923, 2853, 1804, 1693, 1620, 1531, 1462, 1376, 1306, 1074, 939, 781. HRMS (ESI+) calcd C$_{17}$H$_{15}$NNaO$_3$ 304.0944; found 304.0938 [M + Na].

$N$-((1$R$,2$R$)-2-((2-Methoxy-3,4-dioxocyclobut-1-en-1-yl)amino)cyclohexyl)-4-methylbenzenesulfonamide (13)

Following the general procedure, using 1.5 ml MeOH after 96 h, 13 was obtained by filtration as a white solid in 74% yield.

M.p. 187–189 °C. $[\alpha]_D^{27}$ = +95.2 (c 0.76, MeOH). $^1$H-NMR (400 MHz, CDCl$_3$) δ 7.72 (d, $J$ = 7.3 Hz, 2H), 7.25 (d, $J$ = 7.3 Hz, 2H), 7.12 (d, $J$ = 6.2 Hz, 0.5H), 6.14 (d, $J$ = 7.6 Hz, 0.5H), 5.87 (d, $J$ = 6.6 Hz, 0.5H), 5.56 (d, $J$ = 8.4 Hz, 0.5H), 4.37 (s, 1.7H), 4.32 (s, 1.3H), 3.76-3.55 (m, 0.5H), 3.35-3.00 (m, 1.5H), 2.40 (s, 3H), 2.17-0.92 (m, 8H). $^{13}$C-NMR-APT for the major conformer (100 MHz, CDCl$_3$) δ 190.1 (1C), 182.3 (1C), 177.3 (1C), 171.2 (1C), 143.6 (1C), 138.4 (1C), 129.7 (2C), 126.6 (1C), 126.5 (1C), 60.7 (1C), 58.1 (1C), 57.0 (1C), 33.2 (2C), 24.0 (2C), 21.5 (1C). IR (KBr film) (cm$^{-1}$) ν 3364, 2923, 2853, 1794, 1713, 1603, 1485, 1458, 1431, 1381, 1326, 1170, 1077, 1048, 933, 762, 697, 431. HRMS (ESI+) calcd C$_{18}$H$_{22}$NNaO$_3$S 401.1147; found 401.1140 [M + Na].

3-Methoxy-4-(methyl(phenyl)amino)cyclobut-3-ene-1,2-dione (14)

Following the general procedure, using 0.75 ml MeOH after 44 h, 14 was obtained by filtration as a pale yellow solid in 32% yield.

M.p. 155–158 °C. $^1$H-NMR (300 MHz, DMSO-$d_6$) δ 7.55-7.20 (m, 5H), 4.27 (s, 3H), 3.63 (s, 3H). $^{13}$C-NMR-APT (75 MHz, DMSO-$d_6$) δ 183.7 (1C), 177.9 (1C), 170.3 (1C), 141.4 (1C), 128.7 (2C), 126.4 (1C), 123.1 (2C), 60.4 (1C), 38.7 (1C). IR (KBr film) (cm$^{-1}$) 3070, 2923, 2853, 1793, 1713, 1603, 1485, 1458, 1431, 1381, 1326, 1170, 1077, 1048, 933, 762, 697, 431. HRMS (ESI+) calcd C$_{18}$H$_{17}$NNaO$_3$ 240.0637; found 240.0623 [M + Na].
$^1$H-NMR AND $^{13}$C-NMR-APT SPECTRA OF SQUARAMATES 5-8 AND 11-14

3-Ethyl-4-(2-methoxy-3,4-dioxocyclobut-1-enlamino)benzonitrile (5)
3-((2,4-Dimethoxyphenyl)amino)-4-methoxycyclobut-3-ene-1,2-dione (6)
3-((2,4-Dichloro-5-isopropoxyphenyl)amino)-4-methoxycyclobut-3-ene-1,2-dione (7)
3-Methoxy-4-((3,4,5-trifluorophenyl)amino)cyclobut-3-ene-1,2-dione (8)
3-(((1R,2S)-1-Hydroxy-2,3-dihydro-1H-inden-2-yl)amino)-4-methoxycyclobut-3-ene-1,2-dione (11)
(S)-3-Methoxy-4-((1-(naphthalen-1-yl)ethyl)amino)cyclobut-3-ene-1,2-dione (12)
\(N-((1R,2R)-2-((2\text{-Methoxy}-3,4\text{-dioxocyclobut-1-en-1-yl}amino)cyclohexyl)-4\text{-methylbenzenesulfonamide (13)}}\)
3-Methoxy-4-(methyl(phenyl)amino)cyclobut-3-ene-1,2-dione (14)