

SUPPORTING MATERIAL FOR:
Identification of Quinolizidinone Carboxylic Acids as CNS Penetrant,
Selective M1 Allosteric Receptor Modulators

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General: All commercially available chemicals and solvents were used without further purification. Automated flash chromatography was performed on an ISCO CombiFlash with peak detection at 254 nm. Reverse phase purification was accomplished using a Gilson 215 liquid handler equipped with a YMC Pack Pro C18 column (150 x 20 mm I.D., S-5 μ m). Peak collection was triggered by UV detection at 214 or 254 nm. ¹H (400MHz) NMR spectra were recorded on a Varian VXR 400 spectrometer unless otherwise noted. The chemical shifts are reported in δ (ppm) using the δ 0.00 signal of Me₄Si as an internal standard. High-resolution MS data were obtained on a Bruker Daltonics FTICR/MS. High-resolution mass spectral analysis was performed on a Bruker-daltonics BioApex 3T mass spectrometer. HPLC spectra were recorded on a Hewlett-Packard 1100 with a CombiScreen Pro C-18 column. The purity of compounds was assessed to be >95% by analytical HPLC: i) system 1: linear gradient over 10 min of CH₃CN/0.1% TFA and H₂O/0.1% TFA 10:90 to 95:5 and 2 min at 95:5; flow rate 1.0 mL/min, detection at 215 and 254 nm (YMC-Pack Pro C18, 50 x 4.6 mm column). ii) linear gradient over 3.5 min of CH₃CN/0.1% TFA and H₂O/0.1% TFA 5:95 to 95:5; flow rate 1.5 mL/min, detection at 215 nm (YMC-Pack Pro C18, 50 x 4.6 mm column).

All animal studies described herein were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee.

General Methods for the Preparation of Compounds Illustrated in Schemes 1 and 2:

Ethyl 1-[hydroxyl(phenyl)methyl]-4-oxo-4*H*-quinolizine-3-carboxylate (6a). Ethyl 1-formyl-4-oxo-4*H*-quinolizine-3-carboxylate (**5**) was prepared by the procedure described by Komatsu, et. al. (Komatsu, H.; Iwasawa, N.; Citterio, D.; Suzuki, Y.; Kubota, T.; Tokuno, K.; Kitamura, Y.; Oka, K.; Suzuki, K. Design and synthesis of highly sensitive and selective fluorescein-derived magnesium fluorescent probes and application to intracellular 3D Mg²⁺ imaging. *J. Am. Chem. Soc.* **2004**, *126*(50), 16353-16360).

To a solution of ethyl 1-formyl-4-oxo-4*H*-quinolizine-3-carboxylate (0.400 mg, 1.63 mmol) in 30 mL 1:1 THF:CH₂Cl₂ at -20 °C was added phenylmagnesium bromide (0.979 mL, 1.96 mmol, 2M in THF) dropwise. The reaction was allowed to warm to room temperature. After 30 minutes, saturated aqueous ammonium chloride was added, and the mixture was filtered and concentrated to provide crude ethyl 1-[hydroxy(phenyl)methyl]-4-oxo-4*H*-quinolizine-3-carboxylate as a yellow solid (MS *m/e* 324.1 (M+H)⁺) which was carried on directly to **9a**.

1-[Hydroxy(phenyl)methyl]-4-oxo-4*H*-quinolizine-3-carboxylic acid (9a). To a solution of the ethyl 1-[hydroxy(phenyl)methyl]-4-oxo-4*H*-quinolizine-3-carboxylate **6a** (115 mg, 0.356 mmol) in 4 mL 1:2:1 DMSO:MeOH:H₂O was added sodium hydroxide (0.100 g, 2.50 mmol). The reaction was heated to 60 °C for 1 h, cooled to room temperature, and subjected to purification via reverse phase HPLC to provide 1-[hydroxy(phenyl)methyl]-4-oxo-4*H*-quinolizine-3-carboxylic acid: MS *m/e* 296.2 (M+H)⁺; ¹H NMR(CD₃OD) δ 9.49 (d, *J* = 7.1 Hz, 1H), 8.40 (s, 1H), 8.07 (d, *J* = 9.1 Hz, 1H), 7.99-7.94 (m, 1H), 7.67-7.53 (m, 3H), 7.38-7.28 (m, 2H), 7.22 (d, *J* = 8.8 Hz, 1H), 6.21 (bd, 1H).

1-Benzoyl-4-oxo-4*H*-quinolizine-3-carboxylic acid (9b). To a solution of 1-[hydroxy(phenyl)methyl]-4-oxo-4*H*-quinolizine-3-carboxylic acid (0.142 g, 0.508 mmol) in 3 mL CH₃CN was added 1-hydroxy-1,2-benziodoxol-3(1*H*)-one 1-oxide (0.075 g, 0.254 mmol). The mixture was heated to 75 °C for 3 h, cooled to room temperature, and concentrated *in vacuo*. The resultant residue was subjected to purification via reverse

phase HPLC to provide 1-benzoyl-4-oxo-4*H*-quinolizine-3-carboxylic acid (0.015 g, 20%): MS *m/e* 294.2 (M+H)⁺; ¹H NMR (400 MHz, d₆-DMSO) δ 9.50 (d, *J* = 7.3 Hz, 1H), 8.80 (d, 2H), 8.42 (s, 1H), 8.23 (t, 1H), 8.09-8.05 (m, 1H), 7.85-7.47 (m, 6H).

1-(4-Chlorobenzyl)-4-oxo-4*H*-quinolizine-3-carboxylic acid (9d). To a solution of 1-[(4-chlorophenyl)(hydroxy)methyl]-4-oxo-4*H*-quinolizine-3-carboxylic acid (0.553 mg, 1.677 mmol) in 5 mL of CH₂Cl₂ was added triethylsilane (0.54 mL, 3.4 mmol) and trifluoroacetic acid (0.65 mL, 8.4 mmol). After 1.5 h, 10 mL of water was added, and the reaction was extracted with CH₂Cl₂, dried over sodium sulfate, filtered, and concentrated in vacuo. The resultant residue was subjected to purification via reverse phase HPLC to provide 1-(4-chlorobenzyl)-4-oxo-4*H*-quinolizine-3-carboxylic acid: MS *m/e* 313.96 (M+H)⁺; ¹H NMR(CD₃OD) δ 9.49 (d, *J* = 7.1 Hz, 1H), 8.40 (s, 1H), 8.07 (d, *J* = 9.1 Hz, 1H), 7.99-7.94 (m, 1H), 7.62-7.58 (m, 1H), 7.32 (d, *J* = 8.5 Hz, 2H), 7.22 (d, *J* = 8.8 Hz, 1H), 4.32 (s, 2H).

1-(Biphenyl-4-ylmethyl)-4-oxo-4*H*-quinolizine-3-carboxylic acid (9e). To a solution of 1-(4-chlorobenzyl)-4-oxo-4*H*-quinolizine-3-carboxylic acid (0.060 g, 0.19 mmol) in 2 mL of THF under N_{2(g)} was added phenylboronic acid (0.035 g, 0.29 mmol), 1 *N* cesium carbonate (0.38 mL, 0.38 mmol), and bis (tri-*tert*-butylphosphine) palladium(0). The reaction was heated to 100 °C for 15 h, cooled to room temperature, and acidified with 6 *N* HCl to pH ~3. The mixture was then filtered and subjected to purification via reverse phase HPLC to provide 1-(biphenyl-4-ylmethyl)-4-oxo-4*H*-quinolizine-3-carboxylic acid: MS *m/e* 356.0 (M+H)⁺; ¹H NMR(400 MHz, d₆-DMSO) δ 9.40 (d, *J* = 7.2 Hz, 1H), 8.41 (s, 1H), 8.23 (d, *J* = 9.0 Hz, 1H), 8.09-8.05 (m, 1H), 7.69 (d, *J* = 6.9 Hz, 1H), 7.63-7.58 (m, 4H), 7.46-7.42 (m, 2H), 7.35-7.33 (m, 3H), 4.39 (s, 2H).

Compounds **9e** and **9f** were made via this procedure substituting 2- or 3-pyridyl boronic acid for phenyl boronic acid.

4-Oxo-1-(piperidin-1-yl-methyl)-4H-quinolizine-3-carboxylic acid (10a). In a 2-5 mL EmrysTM process vial, a mixture of 1-formyl-4-oxo-4H-quinolizine-3-carboxylic acid (51 mg, 0.24 mmol), piperidine (24 mg, 0.28 mmol), glacial acetic acid (0.081 mL, 1.4 mmol) and 1.2 mL of dichloroethane was stirred vigorously. To the stirring mixture was added resin-bound MP-cyanoborohydride (0.27 g, 0.56 mmol). The mixture was heated via Emrys OptimizerTM microwave to 120 °C for 20 minutes. The mixture was then filtered and concentrated *in vacuo*. The residue was subjected to purification via reverse phase HPLC to provide 4-oxo-1-(piperidin-1-ylmethyl)-4H-quinolizine-3-carboxylic acid: MS *m/e* 287.25 (M+H)⁺; ¹H NMR(400 MHz, CD₃OD) δ 9.55 (d, *J* = 7.3 Hz, 1H), 8.70 (s, 1H), 8.39 (d, *J* = 8.8 Hz, 1H), 8.19-8.16 (m, 1H), 7.73-7.70 (m, 1H), 4.68 (s, 2H), 3.56 (d, *J* = 11.7 Hz, 2H), 3.12-3.07 (m, 2H), 1.94 (d, *J* = 13.7 Hz, 2H), 1.84 (d, *J* = 12.7 Hz, 1H), 1.74 (d, *J* = 13.7 Hz, 2H), 1.53 (d, *J* = 12.4 Hz, 1H).

All compounds in Table 2 were prepared via the procedure above. Additionally, compound **10i** (1-([4-(4-cyanophenyl)piperazin-1-yl]methyl)-4-oxo-4H-quinolizine-3-carboxylic acid) was also made as follows:

A mixture of the ethyl 1-formyl-4-oxo-4H-quinolizine-3-carboxylate (0.891 g, 3.63 mmol), 4-piperazin-1-ylbenzonitrile (0.952 g, 5.09 mmol), glacial acetic acid (1.25 mL, 21.8 mmol) and 18.2 mL of dichloroethane was stirred vigorously. To the stirring mixture was added sodium triacetoxymethylborohydride (1.08 g, 5.09 mmol), which was stirred for 18 hours. The mixture was quenched with a 10% sodium carbonate solution, extracted with dichloromethane (1 x 40 mL), and partitioned between water and dichloromethane. The organic layer was dried over sodium sulfate, filtered, concentrated, and purified by silica gel chromatography eluting with EtOAc to yield ethyl 1-([4-(4-cyanophenyl)piperazin-1-yl]methyl)-4-oxo-4H-quinolizine-3-carboxylate.

The above carboxylate (178 mg, 0.43 mmol) was dissolved in a 2:1 THF:EtOH solution (4.3 mL), cooled to 0 °C, treated with 2.5 *N* NaOH (0.19 mL, 0.47 mmol), and warmed to ambient temperature for 16 hours. The mixture was filtered, concentrated, and the crude material purified by reverse phase HPLC to afford the title compound as

the trifluoroacetate salt that gave a proton NMR spectrum consistent with theory and a high resolution mass spectrum (ES+) m/z of 389.1628 calculated for $M+H^+$ [$C_{22}H_{20}N_4O_3$: 389.1608]: 1H NMR (500 MHz, CD_3OD) δ 9.57 (d, J = 6.8 Hz, 1H), 8.69 (s, 1H), 8.43 (d, J = 9.0 Hz, 1H), 8.17 (t, J = 7.8 Hz, 1H), 7.72 (t, J = 7.0 Hz, 1H), 7.58 (d, J = 8.8 Hz, 1H), 7.08 (d, J = 8.8 Hz, 1H), 4.65 (bs, 2H), 3.34-3.44 (m, 6H). ^{13}C NMR (400 MHz, d_6 -DMSO) δ 166.19, 160.13, 152.58, 146.34, 144.03, 137.43, 134.10, 130.20, 123.94, 120.35, 120.18, 115.34, 104.66, 100.23, 54.87, 50.53, 44.64.

Fluorometric Imaging Plate Reader (FLIPR). CHO-NFAT cells expressing human mAChRs or rhesus, dog, mouse, or ratM1 (in CHO-K1, ATCC) were plated (25,000 cells/well) in clear-bottomed polyd-lysine (PDL)-coated 384-well plates in growth medium using Labsystems Multidrop. Receptor expression levels for the human lines were measured by radioligand binding and were M1 (0.70 ± 0.03), M2 (3.22 ± 0.08), M3 (0.53 ± 0.04), M4 (1.61 ± 0.09), and M5 (0.80 ± 0.07) pmol/mg protein ($n = 3$). Cells were grown overnight $37^\circ C/6\%$ CO_2 , washed 3 X 100 μL assay buffer (Hanks' balanced salt solution/20 mM HEPES/2.5 mM probenecid/ 0.1%BSA) then incubated with 1 μM Fluo-4 a.m. (Invitrogen) 1hr at $37^\circ C/6\%CO_2$. Extracellular dye was removed and Ca^{2+} was measured with a FLIPR384 fluorometric imaging plate reader (Molecular Devices) during incubation with compound 4 min followed by 4 min with the EC15 of ACh or titrated ACh for fold potentiation assay.

Fluorometric Imaging Plate Reader (FLIPR): CHONFAT cells expressing M1, M2, M3, M4, M5, rhesus M1, dog M1, mouse M1 and rat M1 (in CHOK1 from ATCC) receptors were plated (25,000 cells per well) in clear-bottomed, poly-d-lysine-coated 384-well plates in growth medium by using a Labsystems (Chicago) Multidrop. The plated cells were grown overnight at $37^\circ C$ in the presence of 6% CO_2 . The next day, the cells were washed with $3 \times 100 \mu l$ assay buffer (Hanks' balanced salt solution containing 20 mM Hepes, 2.5 mM probenecid, and 0.1% BSA). The cells were incubated with 1 μM Fluo-4AM (Molecular Probes) for 1 hr at $37^\circ C$ and 6% CO_2 . The extracellular dye was removed by washing as described above. Ca^{2+} flux was measured by using a FLIPR384 fluorometric imaging plate reader (Molecular Devices). Compounds were serially diluted

in 100% DMSO and then diluted in assay buffer to a 3X stock at 2% DMSO. This stock was then applied to the cells for a final DMSO concentration of 0.67%. For potency determination, the cells were pre-incubated with various concentrations of compound for 4 min and then stimulated for 4 min with an EC15 concentration of agonist (i.e., ACh) for potentiation measurements.