SUPPORTING MATERIAL FOR:

Identification of Amides as Carboxylic Acid Surrogates for Quinolizidinone Based M_1 Positive Allosteric 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 μm). Peak collection was triggered by UV detection at 214 or 254 nm. ^{1}H (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 Me_4Si 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 chromatograms on final compounds 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 CH_3CN/0.1% TFA and H_2O/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 CH_3CN/0.1% TFA and H_2O/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 Scheme 1:

The following compounds were synthesized as described in previously published literature: sodium 1-((4-cyano-4-(pyridin-2-yl)piperidin-1-yl)methyl)-4-oxo-4H-quinolizine-3-carboxylate[1,2] and 1-formyl-4-oxo-4H-quinolizine-3-carboxylic acid.

1-((4-Cyano-4-(pyridin-2-yl)piperidin-1-yl)methyl)-4-oxo-N-(tetrahydro-2H-pyran-4-yl)-4H-quinolizine-3-carboxamide (4o). A mixture of sodium 1-((4-cyano-4-(pyridin-2-yl)piperidin-1-yl)methyl)-4-oxo-4H-quinolizine-3-carboxylate (368 mg, 0.90 mmol) and 4-aminotetrahydropyran (109 mg, 1.07 mmol) in DMF (9.0 mL) was flushed with nitrogen and then charged with BOP (555 mg, 1.26 mmol). The reaction was titrated in triethylamine (TEA) (0.28 mL, 1.97 mmol) until pH ~8. After 1 hour, the reaction was diluted with DCM and washed three times with water. The organic layer was dried over sodium sulfate, filtered, concentrated and purified by isco column chromatography eluting with 0-3% MeOH in dichloromethane (DCM) to afford a yellow solid (338 mg, 79%). HRMS (ES+) m/z of 472.2354 calculated for $M+H^+$ [$C_{27}H_{29}N_5O_3$: 472.2343]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.69 (m, 2H), 2.06 (t, J = 13.9 Hz, 4H), 2.30 (m, 2H), 2.60 (t, J = 12.2 Hz, 2H), 3.01 (m, 2H), 3.59 (t, J = 11.2 Hz, 2H), 3.82 (s, 2H), 3.98 – 4.03 (m, 2H), 4.27 (m, 1H), 7.25 (m, 1H), 7.32 (t, J = 7.1 Hz, 1H), 7.60 (d, J = 8.0

Hz, 1H), 7.67 – 7.76 (m, 2H), 8.20 (d, *J* = 9.0 Hz, 1H), 8.59 (d, *J* = 4.0 Hz, 1H), 8.66 (s, 1H), 9.41 (d, *J* = 7.3 Hz, 1H), 9.79 (d, *J* = 7.6 Hz, 1H).

_N_-((1S,2S)-2-hydroxycyclohexyl)-1-((4-(isoquinolin-3-yl)piperazin-1-yl)methyl)-4-oxo-4_H_-quinolizine-3-carboxamide (5c).

A mixture of 1-formyl-4-oxo-4H-quinolizine-3-carboxylic acid (2.44 g, 11.2 mmol) in DMF (112 mL) was flushed with nitrogen and then charged with TEA (1.57 mL, 11.2 mmol). To the rxn mixture was added (1S,2S)-(-)-2-aminocyclohexanol (1.55 g, 13.5 mmol) and then BOP (6.96 g, 15.7 mmol). After 30 minutes, the reaction was diluted with DCM and washed three times with water. The organic layer was dried over sodium sulfate, filtered and reduced in volume of DCM. The resulting slurry was filtered via Buchner funnel, washing with DCM. The mother liquor was reduced in volume to a slurry, which was filtered via Buchner funnel. The solid was washed with additional DCM. The solids were combined and dried in vacuo to afford a yellow solid (1.77 g, 50%). HRMS (ES+) m/z of 315.1345 calculated for $M+H^+$ [$C_{17}H_{18}N_2O_4$: 315.1339]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 – 1.50 (m, 4H), 1.78 (d, *J* = 11.7 Hz, 2H), 2.11 (m, 2H), 3.53 (m, 1H), 3.93 (m, 2H), 7.55 (m, 1H), 8.05 (m, 1H), 9.13 (s, 1H), 9.34 (m, 1H), 9.51 (d, *J* = 7.3 Hz, 1H), 9.63 (d, *J* = 9.1 Hz, 1H), 10.00 (s, 1H).

In a microwave vial, the 1-formyl-*N*-((1S,2S)-2-hydroxycyclohexyl)-4-oxo-4H-quinolizine-3-carboxamide (100 mg, 0.32 mmol), 3-piperazin-1-ylisoquinoline (81 mg, 0.38 mmol), solid phase MP-cyanoborohydride resin® (40 mg) and glacial acetic acid (109 μL, 1.91 mmol) were taken up in dichloroethane (1.59 mL). The mixture was heated to 120 °C in a microwave for 20 minutes. Upon completion, the reaction was filtered through a Buchner funnel washing with

DCM 3x 10 mL, concentrated and purified by Shimadzu® reverse phase HPLC eluting with 15 - 35% acetonitrile in water with 0.05% trifluoroacetic acid (TFA). The fractions were combined, reduced in volume of acetonitrile in vacuo, neutralized with a saturated solution of sodium bicarbonate (50 mL) and extracted with DCM 2x 50 mL. The organic layers were combined, dried over sodium sulfate, filtered and concentrated to afford **5c** a yellow solid (130 mg, 79%). HRMS (ES+) m/z of 512.2664 calculated for $M+H^+$ [$C_{30}H_{33}N_5O_3$: 512.2656]. ^1H NMR (400 MHz, $CDCl_3$) δ 1.28 – 1.55 (m, 4H), 1.77 (m, 2H), 2.11 (m, 2H), 2.67 (t, *J* = 4.9 Hz, 4H), 3.55 (m, 5H), 3.79 (s, 2H), 3.89 (m, 1H), 4.41 (s, 1H), 6.74 (s, 1H), 7.26 (t, *J* = 7.4 Hz, 1H), 7.32 (t, *J* = 7.0 Hz, 1H), 7.49 (t, *J* = 7.5 Hz, 1H), 7.57 (d, *J* = 8.3 Hz, 1H), 7.71 (t, *J* = 7.9 Hz, 1H), 7.77 (d, *J* = 8.1 Hz, 1H), 8.30 (d, *J* = 9.0 Hz, 1H), 8.64 (s, 1H), 8.93 (s, 1H), 9.42 (d, *J* = 7.3 Hz, 1H), 9.90 (d, *J* = 6.6 Hz, 1H).

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°C in the presence of 6% CO2. The next day, the cells were washed with 3 × 100 μl 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 h at 37 °C and 6% CO2. The extracellular dye was removed by washing as described above. Ca2+ 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 EC_{20} concentration of agonist (i.e., ACh) for potentiation measurements.

Fold potentiation assay. CHO_{NFAT} cells expressing human mAChR 1 receptor 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°C in the presence of 6% CO_2. The next day, the cells were washed with 3 × 100 µl 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 h at 37 °C and 6% CO_2. The extracellular dye was removed by washing as described above. Ca^{2+} flux was measured by using a $FLIPR_{384}$ fluorometric imaging plate reader (Molecular Devices). Compounds were dissolved 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 the fold potentiation measurements, the cells were pre-incubated with various concentrations of compound for 4 min and then stimulated for 4 min with an serially diluted agonist (i.e., ACh).

1. Kuduk, S.D.; Chang, R.K. Quinolizidinone M_1 receptor positive allosteric modulators. WO 2009051715, 2009.

2. Chang, R..K.; Di Marco, C.N.; Pitts, D.R.; Greshock, T.J.; Kuduk, S.D. Preparation of 4-heteroaryl-4-cyanopiperidines via S_NAr substitution reactions. *Tetrahedron Lett.* **2009**, *50*, 6303-6306.