Supporting Information

Discovery of a Series of 2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)acetamides as Novel Molecular Switches that Modulate Modes of Kv7.2 (KCNQ2) Channel Pharmacology: Identification of (S)-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (ML252) as a Potent, Brain Penetrant Kv7.2 Channel Inhibitor

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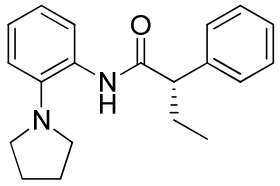
Experimental Section

General. All NMR spectra were recorded on a 400 MHz AMX Bruker NMR spectrometer. ^{1}H chemical shifts are reported in δ values in ppm downfield with the deuterated solvent as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constant (Hz). Low resolution mass spectra were obtained on an Agilent 1200 series 6130 mass spectrometer with electrospray ionization. High resolution mass spectra (HRMS) were recorded on a Waters Q-TOF API-US plus Acquity system with electrospray ionization. Analytical thin layer chromatography was performed on EM Reagent 0.25 mm silica gel 60-F plates. Analytical HPLC was performed on an Agilent 1200 series with UV detection at 214 nm and 254 nm along with ELSD detection. LC/MS: Method 1 = (J-Sphere80-C18, 3.0 x 50 mm, 4.1 min gradient, 5%[0.05%TFA/CH_3CN]:95%[0.05%TFA/H_2O] to 100%[0.05%TFA/CH_3CN]; Method 2 = (Phenomenex-C18, 2.1 X 30 mm, 2 min gradient, 7%[0.1%TFA/CH_3CN]:93%[0.1%TFA/H_2O] to 100%[0.1%TFA/CH_3CN]; Method 3 = (Phenomenex-C18, 2.1 X 30 mm, 1 min gradient, 7%[0.1%TFA/CH_3CN]:93%[0.1%TFA/H_2O] to 95%[0.1%TFA/CH_3CN]. Preparative purification was performed on a custom HP1100 purification system with collection triggered by mass detection. Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification. For chiral SFC separation: Compounds were separated on a Thar Investigator SFC system using either a 4.6 x 250 mm 5 um Chiral Technologies CHIRALPAK IB column or a 4.6 x 250 mm 5 um Regis Technologies RegisPack column. Gradient conditions were 5% to 50% IPA (0.1% DEA) in CO2 over 6.3 minutes, then held at 50% IPA (0.1% DEA) in CO2 for 1 minute. Flow rate was 3.5 mL/min with a back pressure of 100 bar.



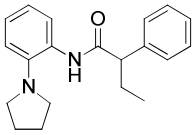
(*S*)-2-Phenyl-*N*-(2-(pyrrolidin-1-yl)phenyl)butanamide ((*S*)-5):

General Procedure: To a stirred solution of (*S*)-(–)-2-phenylbutyric acid (50 mg, 0.304 mmol), HATU (116 mg, 0.304 mmol) in DMF (1.5 mL) was added ethyldiisopropylamine (79 mL, 0.456 mmol) followed by 2-(1-pyrrolidinyl)aniline (49 mg, 0.304 mmol) and the reaction mixture was stirred at room temperature overnight. The reaction was diluted with water (1.5 mL) and extracted with EtOAc (2× 2mL). The organic extracts were combined, concentrated and the residue was purified by RP prepHPLC eluting with 10 to 90% CH_3CN/H_2O (0.1% TFA) to give the product as the TFA salt which was dissolved in methylene chloride (5 mL), washed with aqueous saturated sodium bicarbonate solution, dried ($MgSO_4$), filtered and concentrated. The free base was dissolved in methylene chloride (2 mL) and treated with 4M HCl in dioxane (0.2 mL). After 20 min, the reaction mixture was concentrated to give the product as a solid (60 mg, 57%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.10 (br s, 1H), 7.52–7.41 (m, 3H), 7.41 – 7.30 (m, 3H), 7.31–7.10 (m, 3H), 3.76 (t, J = 7.5 Hz, 1H), 3.72–3.42 (m, 1H), 3.84 (br s, 4H), 2.20–2.02 (m,

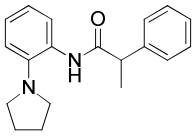
1H), 1.88 (br s, 4H), 1.81–1.66 (m, 1H), 0.89 (t, J = 7.3 Hz, 3H). LCMS: R_T = 0.66 min., >98% @ 215 nm and 254 nm, MS (ESI^+) m/z = 309.3 $[M+H]^+$. HRMS (TOF, ES^+), calc'd for $C_{20}H_{25}N_2O$ $[M + H]^+$: 309.1967, found 309.1969. Chiral HPLC: 94.2% (*S*)-isomer; R_T = 3.97 min. 5.75% (*R*)-isomer; R_T = 4.29 min. 88.4% ee.

All compounds were prepared following the general procedure outlined above in library format and isolated/purified via mass-directed HPLC system. Purity of all final compounds was determined by HPLC analysis is >95%. All compounds were isolated as their TFA salts, unless otherwise noted. All compounds were isolated as solids.



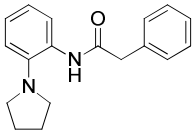
2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (5): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-phenylbutanoic acid yielded **5**.

LCMS: R_T = 0.73 min., >98% @215 and 254 nm, MS (ESI^+) m/z 309.3 $[M + H]^+$.



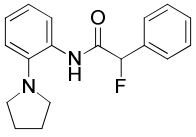
2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)propanamide (6): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-phenylpropanoic acid yielded **6**.

^1H NMR: (400 MHz, $CDCl_3$) δ (ppm): 9.66 (br s, 1H), 7.90 (d, J = 8.0 Hz, 1H), 7.46 (d, J = 7.30 Hz, 2H), 7.36 (t, J = 6.6 Hz, 3H), 7.27 (m, 2H), 7.21 (m, 2H), 3.92 (q, J = 6.9 Hz, 1H), 3.20 (m, 4H), 1.98 (m, 4H), 1.62 (d, J = 7.1 Hz, 3H). LCMS: R_T = 0.60 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 295.2 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{19}H_{23}N_2O$ $[M + H]^+$: 295.1810, found 295.1811.



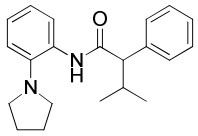
2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (7): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-phenylacetic acid yielded **7**.

LCMS: R_T = 0.56 min, 92.5% @ 215 nm and >98% ELSD, MS (ESI^+) m/z = 281.2 $[M + H]^+$.



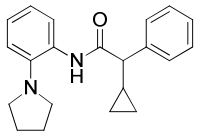
2-fluoro-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (8): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-fluoro-2-phenylacetic acid yielded **8**.

LCMS: R_T = 0.60 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 299.2 $[M + H]^+$.



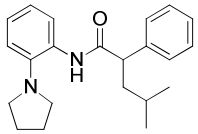
3-methyl-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (9): Coupling of 2-(pyrrolidin-1-yl)aniline and 3-methyl-2-phenylbutanoic acid yielded **9**.

LCMS: R_T = 0.72 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 323.2 $[M + H]^+$.



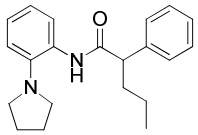
2-cyclopropyl-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (10): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-cyclopropyl-2-phenylacetic acid yielded **10**.

LCMS: R_T = 0.69 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 321.3 $[M + H]^+$.



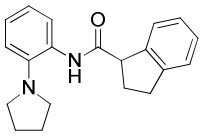
4-methyl-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)pentanamide (11): Coupling of 2-(pyrrolidin-1-yl)aniline and 4-methyl-2-phenylpentanoic acid yielded **11**.

LCMS: R_T = 0.78 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 337.3 $[M + H]^+$.



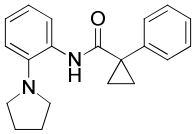
2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)pentanamide (12): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-phenylpentanoic acid yielded **12**.

LCMS: R_T = 0.72 min., >98% @ 214 nm and ELSD, MS (ESI^+) m/z =323.3 $[M + H]^+$.



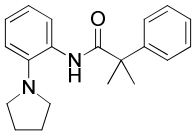
N-(2-(pyrrolidin-1-yl)phenyl)-2,3-dihydro-1H-indene-1-carboxamide (13): Coupling of 2-(pyrrolidin-1-yl)aniline and 2,3-dihydro-1*H*-indene-1-carboxylic acid yielded **13**.

LCMS: R_T = 0.59 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 307.2 $[M + H]^+$.



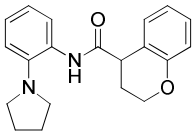
1-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)cyclopropanecarboxamide (14): Coupling of 2-(pyrrolidin-1-yl)aniline and 1-phenylcyclopropanecarboxylic acid yielded **14**.

LCMS: R_T = 0.75 min., 92.5% @ 215 nm and >98% ELSD, MS (ESI^+) m/z = 307.2 $[M + H]^+$.



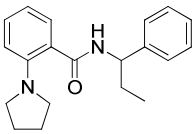
2-methyl-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)propanamide (15): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-methyl-2-phenylpropanoic acid yielded **15**.

LCMS: R_T = 0.72 min., 92.5% @ 215 nm and >98% ELSD, MS (ESI^+) m/z = 309.3 $[M + H]^+$.



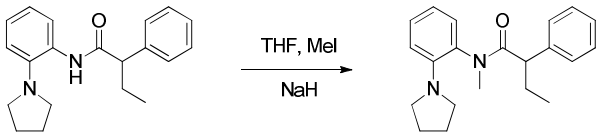
N-(2-(pyrrolidin-1-yl)phenyl)chroman-4-carboxamide (16): Coupling of 2-(pyrrolidin-1-yl)aniline and chroman-4-carboxylic acid yielded **16**.

LCMS: R_T = 0.61 min., >98% @ 214 nm and ELSD, MS (ESI^+) m/z =323.2 $[M + H]^+$.



N-(1-phenylpropyl)-2-(pyrrolidin-1-yl)benzamide (17): Coupling of 2-(pyrrolidin-1-yl)benzoic acid and 1-phenylpropan-1-amine yielded **17**.

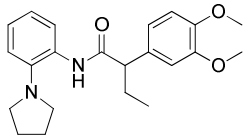
LCMS: R_T = 0.66 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 309.3 $[M + H]^+$.



N-methyl-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (18): To a solution of 2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)butanamide, **6**, (64 mg; 0.21 mmol) in THF (2 mL) was added NaH (11 mg; 0.27 mmol) followed by MeI (14.2 μL; 0.23 mmol). After 2 h, the reaction mixture was quenched with water and extracted with EtOAc. The organic extracts were

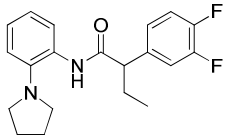
combined, concentrated and the residue was purified by RP prepHPLC eluting with 10 to 90% CH_3CN/H_2O (0.1% TFA) to give the product, **18**, 36.2 mg (0.11 mmol; 53%).

^{1}H NMR: (400 MHz, $CDCl_3$) δ (ppm): 7.19 (m, 10H), 7.06 (dd, *J* = 1.6, 7.7 Hz, 1H), 6.86 (m, 3H), 6.78 (t, *J* = 7.6 Hz, 1H), 6.71 (d, 8.12 Hz, 1H), 6.57 (t, *J* = 7.6 Hz, 1H), 6.42 (dd, *J* = 1.4, 7.6 Hz, 1H), 3.39 (m, 4H), 3.28 (s, 3H), 3.23 (m, 2H), 3.21 (s, 3H), 2.88 (m, 4H), 1.99 (m, 6H), 1.68 (m, 2H), 1.53 (m, 2H), 1.35 (m, 2H), 0.77 (dt, *J* = 7.4 Hz). LCMS: R_T = 0.84 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 323.1 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{18}H_{20}ClN_2O$ $[M + H]^+$: 315.1264, found 315.1266.



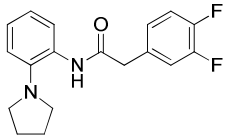
2-(3,4-dimethoxyphenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (19): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3,4-dimethoxyphenyl)butanoic acid yielded **19**.

LCMS: R_T = 0.65 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 369.3 $[M + H]^+$.



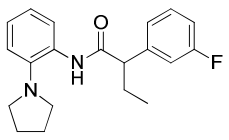
2-(3,4-difluorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (20): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3,4-difluorophenyl)butanoic acid yielded **20**.

^{1}H NMR: (400 MHz, $CDCl_3$) δ (ppm): 8.29 (br s, 1H), 8.28 (d, *J* = 7.3 Hz, 1H), 7.10 (m, 7H), 3.42 (t, *J* = 7.6 Hz, 1H), 2.80 (m, 4H), 2.31 (sep, *J* = 7.0 Hz, 1H), 1.8 (m, 5H), 0.97 (t, *J* = 7.4 Hz). LCMS: R_T = 0.69 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 345.3 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{20}H_{23}F_2N_2O$ $[M + H]^+$: 345.1778, found 345.1779.



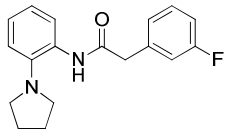
2-(3,4-difluorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (21): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3,4-difluorophenyl)acetic acid yielded **21**.

LCMS: R_T = 0.60 min., >98% @ 215 nm and 254 nm, MW (ESI^+) m/z = 317.2 $[M + H]^+$.



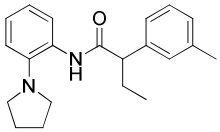
2-(3-fluorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (22): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3-fluorophenyl)butanoic acid yielded **22**.

LCMS: R_T = 0.68 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 327.1 $[M + H]^+$.



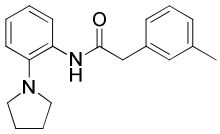
2-(3-fluorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (23): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3-fluorophenyl)acetic acid yielded **23**.

LCMS: R_T = 0.58 min., >98% @ 215 nm and 254 nm, MS (ESI^+) m/z = 299.2 $[M + H]^+$.



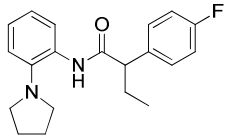
N-(2-(pyrrolidin-1-yl)phenyl)-2-(m-tolyl)butanamide (24): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(*m*-tolyl)butanoic acid yielded **24**.

LCMS: R_T = 0.71 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 323.1 $[M + H]^+$.



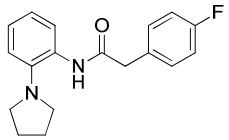
N-(2-(pyrrolidin-1-yl)phenyl)-2-(m-tolyl)acetamide (25): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(m-tolyl)acetic acid yielded **25**.

LCMS: R_T = 0.62 min., >98% @ 215 nm and 254 nm, MS (ESI^+) m/z = 295.4 $[M + H]^+$.



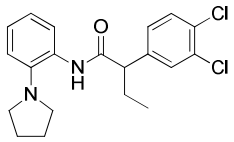
2-(4-fluorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (26): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(4-fluorophenyl)butanoic acid yielded **26**.

^{1}H NMR: (400 MHz, $CDCl_3$) δ (ppm): 8.35 (br s, 1H), 8.25 (d, *J* = 7.9 Hz, 1H), 7.36 (dd, *J* = 5.5, 8.3 Hz, 2H), 7.08 (m, 5H), 3.47 (t, *J* = 8.0 Hz, 1H), 2.79 (m, 4H), 2.33 (sep, *J* = 7.2 Hz, 1H), 1.95-1.74 (m, 5H), 0.96 (t, *J* = 7.3 Hz, 3H). LCMS: R_T = 0.68 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 327.1 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{20}H_{24}FN_2O$ $[M + H]^+$: 327.1873, found 327.1871.



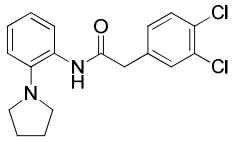
2-(4-fluorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (27): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(4-fluorophenyl)acetic acid yielded **27**.

LCMS: R_T = 0.55 min., >95% @ 215 nm and 254 nm, MS (ESI^+) m/z = 439.2 $[M + H]^+$.



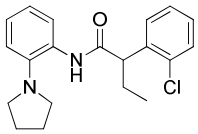
2-(3,4-dichlorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (28): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3,4-dichlorophenyl)butanoic acid yielded **28**.

^1H NMR: (400 MHz, $CDCl_3$) δ (ppm): 8.27 (m, 2H), 7.51 (d, *J* = 1.6 Hz, 1H), 7.46 (d, *J* = 8.3 Hz, 1H), 7.25 (dd, *J* = 1.9, 8.3 Hz, 1H), 7.1-7.0 (m, 3H), 3.42 (t, *J* = 7.4 Hz, 1H), 2.79 (m, 4H), 2.33 (sep, *J* = 7.1 Hz, 1H), 1.9-1.7 (m, 5H). LCMS: R_T = 0.78 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 376.9 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{20}H_{23}Cl_2N_2O$ $[M + H]^+$: 377.1187, found 377.1185.



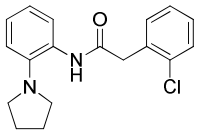
2-(3,4-dichlorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (29): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3,4-dichlorophenyl)acetic acid yielded **29**.

LCMS: R_T = 0.69 min., >95% @ 215 nm and 254 nm, MS (ESI^+) m/z = 349.1 $[M + H]^+$.



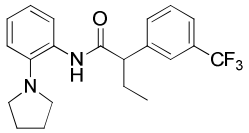
2-(2-chlorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (30): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(2-chlorophenyl)butanoic acid yielded **30**.

LCMS: R_T = 0.76 min., >98% @ 215 nm and 254 nm, MS (ESI^+) m/z = 343.3 $[M + H]^+$.



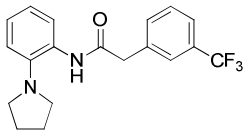
2-(2-chlorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (31): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(2-chlorophenyl)acetic acid yielded **31**.

LCMS: R_T = 0.61 min., >98% @ 215 nm and 254 nm, MS (ESI^+) m/z = 315.1 $[M + H]^+$.



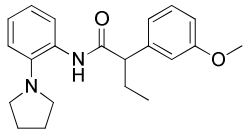
N-(2-(pyrrolidin-1-yl)phenyl)-2-(3-(trifluoromethyl)phenyl)butanamide (32): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3-(trifluoromethyl)phenyl)butanoic acid yielded **32**.

LCMS: R_T = 0.76 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 377.2 $[M + H]^+$.



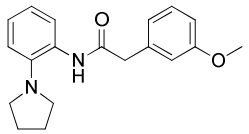
N-(2-(pyrrolidin-1-yl)phenyl)-2-(3-(trifluoromethyl)phenyl)acetamide (33): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3-(trifluoromethyl)phenyl)acetic acid yielded **33**.

LCMS: R_T = 0.67 min., >95% @ 215 nm and 254 nm, MS (ESI^+) m/z = 349.2 $[M + H]^+$.



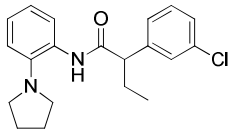
2-(3-methoxyphenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (34): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3-methoxyphenyl)butanoic acid yielded **34**.

LCMS: R_T = 0.66 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 339.30 $[M + H]^+$.



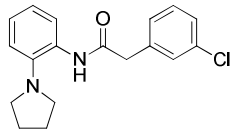
2-(3-methoxyphenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (35): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3-methoxyphenyl)acetic acid yielded **35**.

LCMS: R_T = 0.57 min., >95% @ 215 nm and 254 nm, MS (ESI^+) m/z = 311.2 $[M + H]^+$.



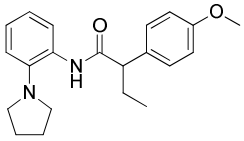
2-(3-chlorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (36): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3-chlorophenyl)butanoic acid yielded **36**.

LCMS: R_T = 0.72 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 343.0 $[M + H]^+$.



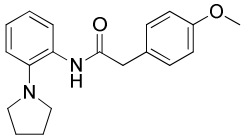
2-(3-chlorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (37): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(3-chlorophenyl)acetic acid yielded **37**.

^{1}H NMR: (400 MHz, $CDCl_3$) δ (ppm): 8.32 (m, 2H), 7.36 (m, 3H), 7.27 (m, 2H), 7.09 (m, 2H), 7.03 (m, 1H), 3.78 (s, 2H), 2.75 (m, 4H), 1.75 (m, 4H). LCMS: R_T = 0.63 min., >98% @215 nm and 254 nm, MS (ESI^+) m/z = 315.1 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{18}H_{20}ClN2O$ $[M + H]^+$: 315.1264, found 315.1266.



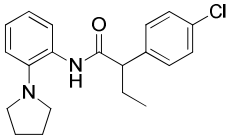
2-(4-methoxyphenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (38): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(4-methoxyphenyl)butanoic acid yielded **38**.

LCMS: R_T = 0.66 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 339.0 $[M + H]^+$.



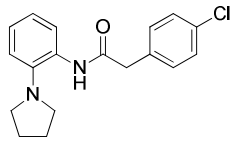
2-(4-methoxyphenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (39): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(4-methoxyphenyl)acetic acid yielded **39**.

LCMS: R_T = 0.56 min., >98% @215 nm and 254 nm, MS (ESI^+) m/z = 311.3 $[M + H]^+$.



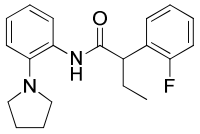
2-(4-chlorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (40): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(4-chlorophenyl)butanoic acid yielded **40**.

^{1}H NMR: (400 MHz, $CDCl_3$) δ (ppm): 8.29 (br s, 1H), 8.26 (d, J = 8.0 Hz, 1H), 7.35 (app q, J = 8.5 Hz, 4H), 7.09 (m, 2H), 7.03 (m, 1H), 3.46 (m, 1H), 2.77 (m, 4H), 2.34 (sep, J = 7.1 Hz, 1H), 1.96-1.75 (m, 5H), 0.96 (t, J = 7.5 Hz). LCMS: R_T = 0.72 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 343.2 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{20}H_{24}ClN_2O$ $[M + H]^+$: 343.1577, found 343.1580.



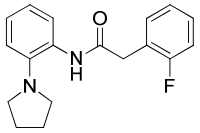
2-(4-chlorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (41): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(4-chlorophenyl)acetic acid yielded **41**.

LCMS: R_T = 0.63 min., >98% @215 nm and 254 nm, MS (ESI^+) m/z = 315.1 $[M + H]^+$.



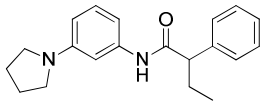
2-(2-fluorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)butanamide (42): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(2-fluorophenyl)butanoic acid yielded **42**.

LCMS: R_T = 0.66 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 372.2 $[M + H]^+$.



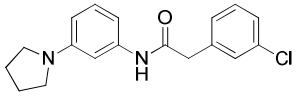
2-(2-fluorophenyl)-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (43): Coupling of 2-(pyrrolidin-1-yl)aniline and 2-(2-fluorophenyl)acetic acid yielded **43**.

LCMS: R_T = 0.60 min., >98% @ 215 nm and 254 nm, MS (ESI^+) m/z = 299.2 $[M + H]^+$.



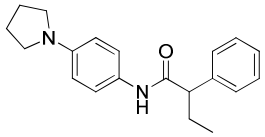
2-phenyl-N-(3-(pyrrolidin-1-yl)phenyl)butanamide (44): Coupling of 3-(pyrrolidin-1-yl)aniline and 2-phenylbutanoic acid yielded **44**.

LCMS: R_T = 0.71 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 309.3 $[M + H]^+$.



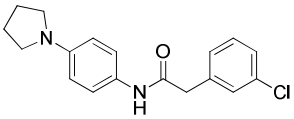
2-(3-chlorophenyl)-N-(3-(pyrrolidin-1-yl)phenyl)acetamide (45): Coupling of 3-(pyrrolidin-1-yl)aniline and 2-(3-chlorophenyl)acetic acid yielded **45**.

^1H NMR: (400 MHz, $CDCl_3$) δ (ppm): 7.37 (s, 1H), 7.33 (m, 2H), 7.26 (m, 2H), 7.14 (t, *J* = 8.5 Hz, 1H), 7.0 (br s, 1H), 6.93 (br s, 1H), 6.58 (d, *J* = 7.5 Hz, 1H), 6.36 (d, *J* = 7.5 Hz, 1H), 3.71 (s, 2H), 3.29 (m, 4H), 2.01 (m, 4H). LCMS: R_T = 0.68 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 315.0 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{18}H_{20}ClN_2O$ $[M + H]^+$: 315.1264, found 315.1266.



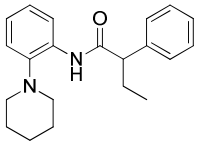
2-phenyl-N-(4-(pyrrolidin-1-yl)phenyl)butanamide (46): Coupling of 4-(pyrrolidin-1-yl)aniline and 2-phenylbutanoic acid yielded **46**.

^{1}H NMR: (400 MHz, $CDCl_3$) δ (ppm): 7.61 (br s, 1H), 7.45 (d, *J* = 8.5 Hz, 2H), 7.38 (m, 4H), 7.31 (m, 1H), 6.99 (d, *J* = 8.9 Hz, 2H), 3.48 (m, 5H), 2.27 (m, 1H), 2.15 (m, 4H), 1.88 (m, 1H), 0.94 (t, *J* = 7.6 Hz, 3H). LCMS: R_T = 0.62 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 309.2 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{20}H_{25}N_2O$ $[M + H]^+$: 309.1967, found 309.1967.



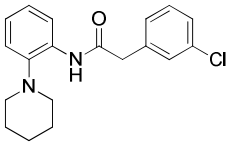
2-(3-chlorophenyl)-N-(4-(pyrrolidin-1-yl)phenyl)acetamide (47): Coupling of 4-(pyrrolidin-1-yl)aniline and 2-(3-chlorophenyl)acetic acid yielded **47**.

LCMS: R_T = 0.61 min., 96.6% @ 215 nm and >98% ELSD, MS (ESI^+) m/z = 315.0 $[M + H]^+$.



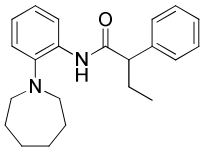
2-phenyl-N-(2-(piperidin-1-yl)phenyl)butanamide (48): Coupling of 2-(piperidin-1-yl)aniline and 2-phenylbutanoic acid yielded **48**.

LCMS: R_T = 0.74 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 323.2 $[M + H]^+$.



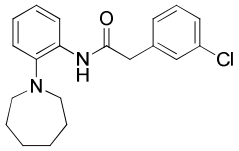
2-(3-chlorophenyl)-N-(2-(piperidin-1-yl)phenyl)acetamide (49): Coupling of 2-(piperidin-1-yl)aniline and 2-(3-chlorophenyl)acetic acid yielded **49**.

LCMS: R_T = 0.70 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 329.0 $[M + H]^+$.



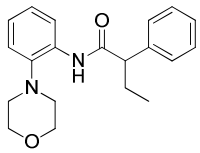
N-(2-(azepan-1-yl)phenyl)-2-phenylbutanamide (50): Coupling of 2-(azepan-1-yl)aniline and 2-phenylbutanoic acid yielded **50**.

LCMS: R_T = 0.89 min., >95% @ 215 nm, 254 nm and ELSD, MS (ESI^+) m/z = 351.1 $[M + H]^+$.



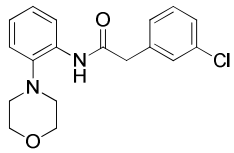
N-(2-(azepan-1-yl)phenyl)-2-(3-chlorophenyl)acetamide (51): Coupling of 2-(azepan-1-yl)aniline and 2-(3-chlorophenyl)acetic acid yielded **51**.

LCMS: R_T = 0.81 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 357.0 $[M + H]^+$.



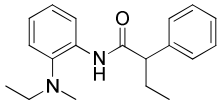
N-(2-(morpholin-4-yl)phenyl)-2-phenylbutanamide (52): Coupling of 2-morpholinoaniline and 2-phenylbutanoic acid yielded **52**.

LCMS: R_T = 0.84 min., >95% @ 215 nm, 254 nm and ELSD, MS (ESI^+) m/z = 325.2 $[M + H]^+$.



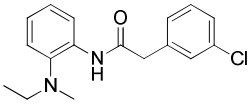
2-(3-chlorophenyl)-N-(2-(morpholin-4-yl)phenyl)acetamide (53): Coupling of 2-morpholinoaniline and 2-(3-chlorophenyl)acetic acid yielded **53**.

LCMS: R_T = 0.78 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 331.0 $[M + H]^+$.



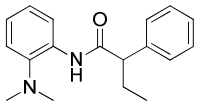
N-(2-(ethyl(methyl)amino)phenyl)-2-phenylbutanamide (54): Coupling of N^1-ethyl-N^1-methylbenzene-1,2-diamine and 2-phenylbutanoic acid yielded **54**.

LCMS: R_T = 0.66 min., >95% @ 215 nm, 254 nm and ELSD, MS (ESI^+) m/z = 297.1 $[M + H]^+$.



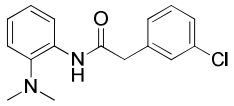
2-(3-chlorophenyl)-N-(2-(ethyl(methyl)amino)phenyl)acetamide (55): Coupling of N^1-ethyl-N^1-methylbenzene-1,2-diamine and 2-(3-chlorophenyl)acetic acid yielded **55**.

LCMS: R_T = 0.59 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 303.0 $[M + H]^+$.



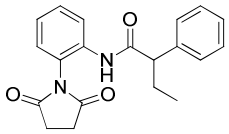
N-(2-(dimethylamino)phenyl)-2-phenylbutanamide (56): Coupling of N^1,N^1-dimethylbenzene-1,2-diamine and 2-phenylbutanoic acid yielded **56**.

LCMS: R_T = 0.63 min., >98% @ 215 and 254 nm, MS (ESI^+) m/z = 283.2 $[M + H]^+$.



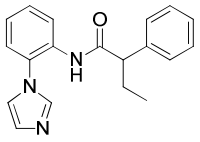
2-(3-chlorophenyl)-N-(2-(dimethylamino)phenyl)acetamide (57): Coupling of N^1,N^1-dimethylbenzene-1,2-diamine and 2-(3-chlorophenyl)acetic acid yielded **57**.

LCMS: R_T = 0.57 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 289.0 $[M + H]^+$.



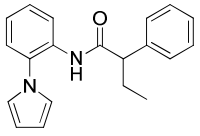
N-(2-(pyrrolidin-1-yl-2,5-dione)phenyl)-2-phenylbutanamide (58): Coupling of 1-(2-aminophenyl)pyrrolidine-2,5-dione and 2-phenylbutanoic acid yielded **58** (Free base).

LCMS: R_T = 0.67 min., >95% @ 215 nm, 254 nm and ELSD, MS (ESI^+) m/z = 337.1 $[M + H]^+$.



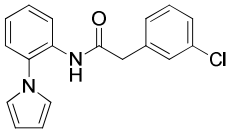
N-(2-(1H-imidazol-1-yl)phenyl)-2-phenylbutanamide (59): Coupling of 2-(1H-imidazol-1-yl)aniline and 2-phenylbutanoic acid yielded **59**.

LCMS: R_T = 0.69 min., >98% @ 215 and 254 nm, MS (ESI^+) m/z = 306.2 $[M + H]^+$.



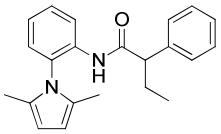
N-(2-(1H-pyrrol-1-yl)phenyl)-2-phenylbutanamide (60): Coupling of 2-(1H-pyrrol-1-yl)aniline and 2-phenylbutanoic acid yielded **60**.

LCMS: R_T = 0.87 min., >98% @ 215 and 254 nm, MS (ESI^+) m/z = 305.2 $[M + H]^+$.



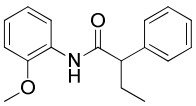
N-(2-(1H-pyrrol-1-yl)phenyl)-2-(3-chlorophenyl)acetamide (61): Coupling of 2-(1H-pyrrol-1-yl)aniline and 2-(3-chlorophenyl)acetic acid yielded **61**.

LCMS: R_T = 0.82 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 254.1 $[M + H]^+$.



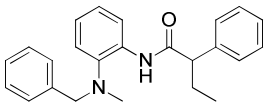
N-(2-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-2-phenylbutanamide (62): Coupling of 2-(2,5-dimethyl-1H-pyrrol-1-yl)aniline and 2-phenylbutanoic acid yielded **62**.

LCMS: R_T = 0.94 min., >98% @ 215 and 254 nm, MS (ESI^+) m/z = 333.2 $[M + H]^+$.



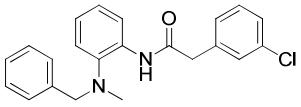
N-(2-methoxyphenyl)-2-phenylbutanamide (63): Coupling of 2-methoxyaniline and 2-phenylbutanoic acid yielded **63** (Free base).

LCMS: R_T = 0.81 min., >98% @ 215 and 254 nm, MS (ESI^+) m/z = 270.2 $[M + H]^+$.



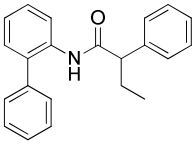
N-(2-(benzyl(methyl)amino)phenyl)-2-phenylbutanamide (64): Coupling of N^1-benzyl-N^1-methylbenzene-1,2-diamine and 2-phenylbutanoic acid yielded **64**.

LCMS: R_T = 0.91 min., >98% @ 215 and 254 nm, MS (ESI^+) m/z = 359.2 $[M + H]^+$.



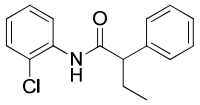
N-(2-(benzyl(methyl)amino)phenyl)-2-(3-chlorophenyl)acetamide (65): Coupling of N^1-benzyl-N^1-methylbenzene-1,2-diamine and 2-(3-chlorophenyl)acetic acid yielded **65**.

LCMS: R_T = 0.85 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 365.0 $[M + H]^+$.



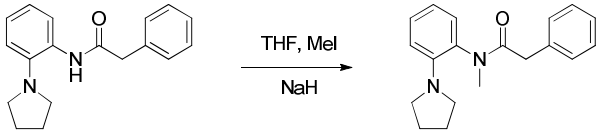
N-([1,1'-biphenyl]-2-yl)-2-phenylbutanamide (5): Coupling of [1,1'-biphenyl]-2-amine and 2-phenylbutanoic acid yielded **4** (Free base).

LCMS: R_T = 0.88 min., 92.5% @ 215 nm and >98% ELSD, MS (ESI^+) m/z = 316.2 $[M + H]^+$.



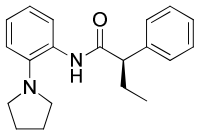
N-(2-chlorophenyl)-2-phenylbutanamide (66): Coupling of 2-chloroaniline and 2-phenylbutanoic acid yielded **66** (Free base).

LCMS: R_T = 0.86 min., >95% @ 215 nm, 254 nm and ELSD, MS (ESI^+) m/z = 274.1 $[M + H]^+$.



N-methyl-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)acetamide (67): To a solution of 2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)acetamide, **7**, (52 mg; 0.19 mmol) in THF (2 mL) was added NaH (10 mg; 0.24 mmol) followed by MeI (14 μL; 0.22 mmol). After 2 h, the reaction mixture was quenched with water and extracted with EtOAc. The organic extracts were combined, concentrated and the residue was purified by RP prepHPLC eluting with 10 to 90% CH_3CN/H_2O (0.1% TFA) to give the product, **67**, 35.2 mg (0.12 mmol; 63%).

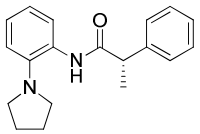
LCMS: R_T = 0.72 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 295.1 $[M + H]^+$.



(*R*)-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)butanamide ((*R*)-5): Coupling of 2-(pyrrolidin-1-yl)aniline and (*R*)-2-phenylbutanoic acid yielded **(*R*)-5**.

LCMS: R_T = 0.66 min., >98% @ 215 nm and 254 nm, MS (ESI^+) m/z = 309.3 $[M+H]^+$.

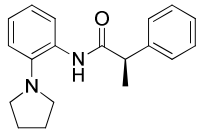
Chiral HPLC: 6.9% (*S*)-isomer; R_T = 4.4 min. 93.1% (*R*)-isomer; R_T = 4.97 min. 86% ee.



(*S*)-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)propanamide ((*S*)-6): Coupling of 2-(pyrrolidin-1-yl)aniline and (*S*)-2-phenylpropanoic acid yielded **(*S*)-6**.

^1H NMR: (400 MHz, $CDCl_3$) δ (ppm): 8.33 (d, *J* = 8.5 Hz, 1H), 8.22 (br s, 1H), 7.40 (m, 4H), 7.33 (m, 1H), 7.08 (m, 2H), 7.00 (m, 1H), 3.8 (q, *J* = 7.4 Hz, 1H), 2.67 (m, 4H), 1.70 (m, 4H), 1.66 (d, *J* = 7.4 Hz, 3H). LCMS: R_T = 0.60 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z =

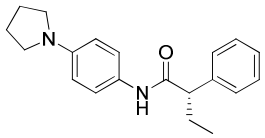
295.1 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{19}H_{23}N_2O$ $[M + H]^+$: 295.1810, found 295.1808. Chiral HPLC: 84.7% (*S*)-isomer; R_T = 4.2 min. 15.3% (*R*)-isomer; R_T = 4.54 min. 70% ee.



(*R*)-2-phenyl-N-(2-(pyrrolidin-1-yl)phenyl)propanamide ((*R*)-6): Coupling of 2-(pyrrolidin-1-yl)aniline and (*R*)-2-phenylpropanoic acid yielded **(*R*)-6**.

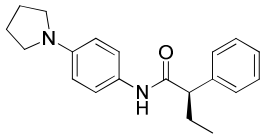
LCMS: R_T = 0.60 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 295.1 $[M + H]^+$.

Chiral HPLC: 11.8% (*S*)-isomer; R_T = 4.2 min. 88.2% (*R*)-isomer; R_T = 4.53 min. 77% ee.



(*S*)-2-phenyl-N-(4-(pyrrolidin-1-yl)phenyl)butanamide ((*S*)-46): Coupling of 4-(pyrrolidin-1-yl)aniline and (*S*)-2-phenylbutanoic acid yielded **(*S*)-46**.

^1H NMR: (400 MHz, $CDCl_3$) δ (ppm): 7.46-7.23 (m, 7H), 6.80 (d, *J* = 8.4 Hz, 2H), 3.39 (m, 5H), 2.28 (sep, *J* = 6.9 Hz, 1H), 2.09 (m, 4H), 1.88 (sep, *J* = 7.2 Hz, 1H), 0.94 (t, *J* = 7.2 Hz). LCMS: R_T = 0.63 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 309.1 $[M + H]^+$. HRMS (TOF, ES^+), calc'd for $C_{20}H_{25}N_2O$ $[M + H]^+$: 309.1967, found 309.1966. Chiral HPLC: 97.0% (*S*)-isomer; R_T = 6.24 min. 3.0% (*R*)-isomer; R_T = 6.58 min. 94% ee.



(*R*)-2-phenyl-N-(4-(pyrrolidin-1-yl)phenyl)butanamide ((*R*)-46): Coupling of 4-(pyrrolidin-1-yl)aniline and (*R*)-2-phenylbutanoic acid yielded **(*R*)-46**.

LCMS: R_T = 0.63 min., >98% @ 215 nm and ELSD, MS (ESI^+) m/z = 309.1 $[M + H]^+$.

Chiral HPLC: 3.4% (*S*)-isomer; R_T = 6.27 min. 96.6% (*R*)-isomer; R_T = 6.58 min. 93% ee.

In Vitro Pharmacology

Cell culture

Stable lines expressing KCNQ2 were generated by standard protocols using pcDNA3.1(+) and cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 medium(DMEM/F12) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Gemini, Calabasas, CA), 2 mM L-Glutamine (Invitrogen, Carlsbad, CA) and 500 μg/mL G418 (Invitrogen, Carlsbad, CA). Cells have been maintained and passaged when reaching 80% confluency.

Thallium-based fluorescence assay

Activity of potassium channels was monitored by the influx of a surrogate ion for potassium, thallium (Tl+). Thallium influx was detected through the use of a thallium-sensitive fluorescent dye, FluxOR^{TM} (Invitrogen, Carlsbad, CA). Cells were plated using a Multidrop (Thermo scientific, Hudson, New Hampshire) at 6,500 cells/well into BD Biocoat poly-D-Lysine coated 384-well plates and incubated overnight (16-20 hr) at 37°C and 5% CO2. The thallium-based fluorescence assay protocol was adapted from the manufacturer's recommended protocol. Briefly, medium was removed; cells were loaded with 1x FluxOR dye solution, 25 μl/well, for 90 min at room temperature in the dark; the 1x FluxOR solution was replaced by assay buffer (Hanks Balanced Salt Solution containing 5.8 mM potassium; Catalog # 14065, Invitrogen), 20 μl/well; test compounds at final concentration 10 μM from a 5 mM stock supplied by the NIH Molecular Libraries Small Molecule Repository (BioFocus DPI, San Francisco, CA) or controls in assay buffer were then added to cells, 4 μl /well; 20 minutes later, cell plates were loaded to Hamamatsu FDSS 6000 kinetic imaging plate reader (Hamamatsu Photonics, Hamamatsu city, Japan); after establishing fluorescence baseline by 1 Hz scanning for 10 seconds, the KCNQ2 channels were activated by addition of 6 μl/well stimulus buffer containing 12.5 mM K_2SO4 and 12.5 mM Tl_2SO4 to the assay buffer giving a final potassium concentration of 15.8 mM and final Tl+ concentration of 5 mM; fluorescence measurement was continued at 1 Hz for another 110 seconds. To evaluate the robustness of the HTS thallium-based fluorescence assays, ZTZ240 at 10 μM was applied as positive activator control, while assay buffer was employed as negative control. Both the positive and negative controls are prepared with 0.2% (v/v) DMSO, corresponding to the test concentration 10 μM. The fluorescence ratio, F(max-min)/F0 (ΔF/F0), was calculated for each well using the entire 120 second detection window and then normalized to the positive and negative control wells. Hit selection was based on the B scores of test compounds calculated from the fluorescence ratios. If the B score of the test compound was more than 3 times the standard deviation (SD) of the B scores of ratios of the library compounds, and the B score of initial fluorescence intensity is within 2 times the standard deviation of the B scores of the library compounds, the compound is designated as an activator of KCNQ2 channels. Otherwise, it is designated as inactive.

Automated electrophysiology assay

KCNQ2 activity was examined in an electrophysiological assay using the population patch clamp mode on the Ionworks Quattro (MDC, Sunnyvale, CA), an automated patch clamp instrument. The CHO cells stably expressing KCNQ2 channels were freshly dislodged from flasks and dispensed into a 384-well population patch clamp (PPC) plate. The cell plating density

was 7,000 cells/well suspended in the extracellular solution, composed of (in mM): 137 NaCl, 4 KCl, 1 MgCl2, 1.8 CaCl2, 10 HEPES, and 10 glucose, pH 7.4 adjusted with NaOH.

After dispensing, seal resistance of cells was measured for each well and cells were perforated by incubation with 50μg/ml amphotericin B (Sigma, St. Louis, MO), which was dissolved in the internal solution composed of (in mM): 40 KCl, 100 K-Gluconate, 1 MgCl2, 5 HEPES, 2 CaCl2, pH 7.2 adjusted with KOH. Activity of KCNQ2 was then measured with the recording protocol as followings. Leak currents were linear subtracted extrapolating the current elicited by a 100-ms step to -100 mV from a holding potential of -90 mV. During the voltage pulse protocol, cells were held at -90 mV, followed by by 2,000 ms depolarizing step from -90 mV to -10 mV, and then back to -90mV for 2000 ms. The currents were measured at the end of the depolarization pulse before and after the application of compounds for 3 min. Only cells with a current amplitude more than 100 pA at -10 mV and a seal resistance >30 MΩ were included in the data analysis.

Compound effects were assessed by the percentage changes in the KCNQ2 steady state currents, which were calculated by dividing the difference between pre- and post-compound KCNQ2 currents by the respective pre-compound currents in the same well. When constructing conductance-voltage curves, conductance values were calculated by dividing the steady state outward currents measured during the voltage steps by the driving force (step voltage minus the calculated potassium reversal potential).

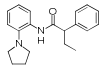
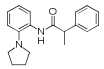
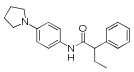
The KCNQ2 protocol was also used for KCNQ2/KCNQ3 recording. But for KCNQ1 and KCNQ4, the cells were depolarized to +40mV from the holding potential -70 mV. Currents were measured at the step current at +40 mV. And for KCNQ1/KCNE1, cells were stimulated by 3,000 ms depolarizing step from -70 mV to +40 mV, followed by hyperpolarization to -20 mV for 500 ms. Currents were measured at the steady state of +40mV voltage step.

No corrections for liquid junction potentials (estimated as -20 mV by comparing the KCNQ2 reversal potential with the calculated Nernst potential for potassium) were applied. The current signal was sampled at 0.625 kHz.

Statistics

The raw data of thallium flux assay were analyzed and exported from manufacturer's software package provided by Hamamatsu Photonics (Hamamatsu, Japan). Automated patch clamp data were analyzed in IonWorks 2.0.4.4 (Moledular Devices Corp., Sunnyvale, CA) and then exported for further analysis by Excel (Microsoft, CA) and Origin 6.

Table 1. SAR evaluation of enantiospecific isomers of lead KCNQ2 inhibitor compounds.

Entry	R	KCNQ2 IC_{50} (μM)[a] (*S*)	(R)
5		0.069 ± 0.006	0.944 ± 0.046
6		0.59 ± 0.10	5.13 ± 7.68
46		0.11 ± 0.02	0.36 ± 0.34

[a] IC_{50}'s were generated from 8-point concentration response curves with 3-fold dilutions starting from the maximal concentration (30μM) with quadruplicate determinations in the automated patch clamp assay . In cases in which a saturating response was not obtained at the highest tested concentration, (% at 30μM) is listed. IC_{50} values are expressed as IC_{50} ± SD, using estimated standard deviations provided by the fitting software (Origin 6.0).

Table 2. Selectivity profile of (*S*)-5, other key analogs and previously identified KCNQ2 channel inhibitors against a panel of KCNQ channels.

Cmpd	KCNQ2	IC_{50} (μM) KCNQ1	KCNQ1/E1	KCNQ2/3	KCNQ4
(*S*)-5	0.07 ± 0.01	2.92 ± 3.90	8.12 ± 1.47	0.12 ± 0.02	0.20 ± 0.06
(*S*)-6	0.59 ± 0.10	>30			
(*S*)-48	0.11 ± 0.02	>30			
20	0.17 ± 0.02	2.58			
22	0.36 ± 0.03	17.65			
24	0.33 ± 0.05	>30			
26	0.13 ± 0.03	6.80			
28	0.47 ± 0.08	9.96			
32	0.39 ± 0.14	>30			
34	0.12 ± 0.03	22.06			
36	0.24 ± 0.04	11.17			
40	0.28 ± 0.03	>30			
42	0.44 ± 0.09	>30			
1	0.06 ± 0.01 (0.71 ± 0.07)[a]	0.40 ± 0.10	1.60 ± 0.17	0.31 ± 0.11	0.38 ± 0.15
2	0.71 ± 0.23 (4.8 ± 0.6)[a]	0.71 ± 0.28	2.08 ± 0.78	0.92 ± 0.14	1.68 ± 0.31
3	9.42 ± 1.12 (1.07)[a]	0.06 ± 0.02	0.13 ± 0.12	>30	19.29 ± 11.3

[a] IC_{50}'s from published data were taken from[6,11]

In vitro PK Analysis:

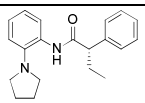
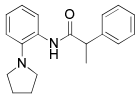
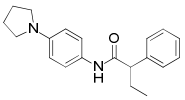
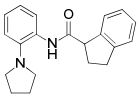
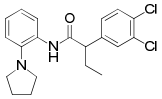
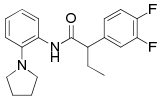
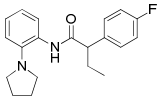
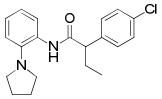
Microsomal stability: The metabolic stability of each compound was investigated in rat hepatic microsomes (BD Biosciences, Billerica, MA) using substrate depletion methodology (% parent compound remaining). In separate 96-well plates for each time point, a mixture of 0.1M potassium phosphate-buffered (pH 7.4), 1μM test compound, 0.5 mg/mL microsomes, and 1mM NADPH (t=3, 7, 15, 25, or 45min) or buffer (t=0) were continually incubated at 37°C under ambient oxygenation. At the respective time, each plate's reaction was precipitated by the addition of 2 volumes of ice-cold acetonitrile containing glyburide as an internal standard (50 ng/mL). The plates were centrifuged at 3000 rpm (4°C) for 10 min. The resulting supernatants were transferred and diluted 1:1 (supernatant: water) into new 96-well plates in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate. The in vitro half-life ($t_{1/2}$, min, Eq. 1), intrinsic clearance (*CLint*, mL/min/kg, Eq. 2) and subsequent predicted hepatic clearance (*CLhep*, mL/min/kg, Eq. 3) was determined employing the following equations:

1) $t_{1/2}$ = Ln(2) / k ; where k represents the slope from linear regression analysis (% test compound remaining)

2) *CLint* = (0.693 / $t_{1/2}$) (rxn volume / mg of microsomes) (45 mg microsomes / gram of liver) (20[a] gm of liver / kg body weight); [a]scale-up factors of 20 (human) and 45 (rat)

3) $$CLhep = \frac{Q \cdot CL\,\text{int}}{Q + CL\,\text{int}}$$

Table 3. Intrinsic clearance values for selected analogs.

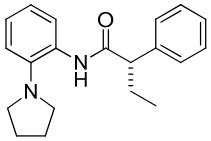
Entry		Human		Rat	
		CL_{INT}	CL_{HEP}	CL_{INT}	CL_{HEP}
(*S*)-5		668	20.4	1720	67.3
6		204.2	19.0	1563.5	67.0
46		333.2	19.8	3574.3	68.7
13		770.6	20.4	3709.3	68.7
28		1168.8	20.6	3137.8	68.5
20		572.6	20.3	1626.3	67.1
26		709.6	20.4	1561.9	67.0
40		668.4	20.4	1937.3	67.6

In vivo PK:

IP PBL: Compound was formulated as 10% Tween 80 in sterile water at the concentration of 3 mg/mL and administered i.p. to male Sprague-Dawley rats weighing 250 g (Harlan, Indianapolis, IN) at the dose of 10 mg/kg. The rat blood (cardiac puncture) and brain were collected at 1 hour. Animals were euthanized and decapitated, and the brains were removed, thoroughly washed in cold phosphate-buffered saline, and immediately frozen on dry ice. Plasma was separated by centrifugation and stored at -80°C until analysis. On the day of analysis, frozen whole-rat brains were weighed and diluted with 1:3 (w/w) parts of 70:30 isopropanol:water. The mixture was then subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products) followed by centrifugation. The sample extraction of plasma (20 µl) and brain homogenate (20 µl) was performed by a method based on protein precipitation using 120µL acetonitrile containing an internal standard (50 ng/mL carbamazepine). Extracts were centrifuged at 4000 x g for 5 min. The supernatants of plasma and brain homogenate extracts were then diluted 1:1 with water.

Liquid chromatography/mass spectrometry analysis: In vivo samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 3.0 x 30 mm, 3 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 10% B after a 0.2 min hold and was linearly increased to 90% B over 0.8 min; held at 90% B for 0.5 min and returned to 10% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500°C and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage) (see Table 1). All data were analyzed using AB Sciex Analyst 1.5.1 software.

Table 4. Ricerca profiling.

Cmpd	Structure	Primary Biochemical Assay	Species	% inhibition @ 10 μM
(*S*)-5, ML252		Melatonin, MT_1	Human	61%