Supporting Information

Discovery of MK-5046, a Potent, Selective Bombesin Receptor Subtype-3 Agonist for the Treatment of Obesity

[*a]Iyassu K. Sebhat, [a]Christopher Franklin, [a]Michael Lo, [a]David Chen, [a]James P. Jewell, [b]Randy Miller, [b]Qingmei Pang, [c]Oksana Palyha, [c]Yanqing Kan, [c]Theresa M. Kelly, [c]Xiao-Ming Guan, [c]Donald J. Marsh, [d]Joseph M. Metzger, [b]Kathryn Lyons, [b]Jasminka Dragovic, [e]Peter R. Guzzo, [e]Alan J. Henderson, [c]Marc L. Reitman, [a]Ravi P. Nargund, [a]Matthew J. Wyvratt and [a]Linus S. Lin

Departments of [a]Medicinal Chemistry, [b]Drug Metabolism [c]Metabolic Diseases (Obesity) and [d]Pharmacology, Merck Research Laboratories, Rahway, NJ 07065. [e]AMRI, 26 Corporate Circle, Albany, NY12212, USA.

** To whom correspondence should be addressed. Phone 732-594-0168. E-mail: iyassu_sebhat@merck.com*

Contents:

Experimental Section

General Procedures

The LC/MS analyses were preformed using a MICROMASS ZMD mass spectrometer coupled to an AGILENT 1100 Series HPLC utilizing a YMC ODS-A 4.6 x 50 mm column eluting at 2.5 mL/min with a solvent gradient of 10 to 95% B over 4.5 min, followed by 0.5 min at 95% B: solvent A = 0.06% TFA in water; solvent B = 0.05% TFA in acetonitrile. ^{1}H-NMR spectra were obtained on a 500 MHz VARIAN Spectrometer in $CDCl_3$ or CD_3OD as indicated and chemical shifts were reported as δ. using the solvent peak as reference and coupling constants were reported in hertz (Hz). All reported compounds were ≥95% pure, as evidenced by analytical HPLC traces under different conditions, high resolution mass spectra, and high resolution NMR spectra.

Chiral resolution of racemic products was effected via HPLC utilizing a Chiralpak AD-H column eluting with 15% isopropanol/heptane to afford two individual enantiomers (E1 and E2). In all cases compounds described and characterized are for E1, the isolated, faster eluting enantiomers.

For metabolism assays, concentrations of test compounds were determined using an LC-MS system consisting of a Perkin-Elmer HPLC system and a Sciex 4000 QTRAP mass spectrometer. The Perkin-Elmer HPLC system (Perkin-Elmer Life and Analytical Sciences, Boston, MA) was equipped with two Series 200 micro pumps and a Perkin-Elmer Series 200 autosampler. Mass spectrometric detection of the analytes was accomplished using a Turbo Ionspray interface operated in the positive ionization mode. Analyte response was measured by multiple reaction monitoring (MRM) of transitions unique to each compound.

Data were acquired and processed using the Sciex Analyst 1.4 software. Calibration was achieved by plotting the peak area ratios of analyte to IS as a function of the nominal concentrations of the standard samples. A line of best fit was generated from the curve points by powerfit regression with a weighting factor of 1. The equation of this line was used to calculate the concentrations in all samples.

4-(2,2-dimethylpropyl)-*N,N*-dimethyl-1*H*-imidazole-1-sulfonamide (5).

Step A: To a cooled (0°C) solution of 3,3-dimethylbutyraldehyde (32.7 g, 0.33mol) in THF (500 mL) was added TosMIC (51.2g) followed by t-BuOK (1.5g) and the reaction was warmed to ambient temperature and stirred for an additional 2 h. The mixture was concentrated *in vacuo*, redissolved in ammonia/MeOH (500 mL) and heated in a steal tube at 100°C for 16 h. The crude reaction mixture was concentrated *in vacuo*, and the residue was purified by silica gel chromatography using acetone as eluent to give 5-(2,2-dimethylpropyl)-1*H*-imidazole as a dark oil which was used directly to the next step.

Step B: To a solution of 5-(2,2-dimethylpropyl)-1*H*-imidazole, dimethylsulfamoyl chloride (25 mL), triethylamine (45 mL) in methylene chloride (300 mL) was added DMAP (0.8 g). The reaction mixture was heated at reflux for 18 h. The solvent was evaporated and the residue was purified by silica gel chromatography to give the title compound as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 8.01 (1 H, s),

7.23 (1 H, s), 2.85 (6 H, s), 2.46 (2 H, s), 0.93 (11 H, s); LCMS: m/z = 246.1 [M+H]+.

2-[(4-bromophenyl)acetyl]-4-(2,2-dimethylpropyl)-*N,N*-dimethyl-1*H*-imidazole-1-sulfonamide (6).

To a solution of compound **5** at -78℃ (2.45 g, 10 mmol) in THF (40 mL) was added n-BuLi (5 mL, 12.5 mmol). The temperature was raised to –10℃ over a period of 1 h, and 4-bromophenyl acetic acid methyl ester (2.2 g, 10 mmol) in THF (5 mL) was added. The reaction mixture was stirred overnight at ambient temperature, then poured into ice-water, extracted with ethyl acetate and dried (magnesium sulfate). After filtration, the solvent was concentrated and the residue was used in the next step without further purification.

2-[4-(2,2-dimethylpropyl)-1*H*-imidazol-2-yl]-1-[4-(5-fluoropyridin-2-yl) phenyl]propan-2-ol (7).

Step A: Palladium tetrakis(triphenylphosphine) (107 mg, 0.09 mmol) was added to a degassed, ambient temperature solution of 2-[(4-bromophenyl)acetyl]-4-(2,2-dimethylpropyl)-*N*,*N*-dimethyl-1*H*-imidazole-1-sulfonamide (compound **6**) (410 mg, 0.9 mmol), 2-bromo-5-fluoropyridine (163 mg, 0.9 mmol) and hexamethylditin (304 mg, 0.9 mmol) in 1,4-dioxane (20 mL). After stirring at reflux for 18 h, the reaction mixture was diluted with water and extracted with ethyl acetate and methylene chloride. The combined organic extracts were dried (magnesium sulfate) and concentrated *in vacuo*. Chromatography over silica eluting with 0-50% ethyl acetate/hexane afforded 4-(2,2-dimethylbutyl)-2-{[4-(5-fluoropyridin-2-yl)phenyl]acetyl}-*N*,*N*-dimethyl-1*H*-imidazole-1-sulfonamide (177 mg, 0.39 mmol, 43%).

Step B: Methylmagnesium bromide (3 M in diethyl ether) (6.1 mL, 18.4 mmol) was added to a -78°C solution of 4-(2,2-dimethylbutyl)-2-{[4-(5-fluoropyridin-2-yl)phenyl]acetyl}-*N*,*N*-dimethyl-1*H*-imidazole-1-sulfonamide (4.22 g, 9.21 mmol) in tetrahydrofuran (50 mL). After warming slowly to ambient temperature, the reaction was quenched with saturated aqueous ammonium chloride and extracted with methylene chloride. The combined organic extracts were dried (magnesium sulfate) and concentrated *in vacuo*. Chromatography over silica eluting with 0-60% ethyl acetate/hexane afforded 4-(2,2-dimethylpropyl)-2-{2-[4-(5-fluoropyridin-2-yl)phenyl]-1-hydroxy-1-methylethyl}-*N*,*N*-dimethyl-1*H*-imidazole-1-sulfonamide (2.1 g, 53%).

Step C: Hydrogen chloride (4 M in 1,4-dioxane) (6 mL, 24 mmol) was added to a solution of 4-(2,2-dimethylpropyl)-2-{2-[4-(5-fluoropyridin-2-yl)phenyl]-1-hydroxy- 1-methylethyl}-*N*,*N* -dimethyl-1*H* -imidazole-1-sulfonamide (1.5 g, 3.27 mmol) in methanol (20 mL). After stirring at 70°C for 1 h, volatiles were removed. The residue was partitioned between methanol/ethyl acetate and 10% aqueous sodium hydroxide. The aqueous phase was extracted with ethyl acetate. The combined organic extracts were dried (magnesium sulfate) and concentrated *in vacuo*. Chromatography over silica eluting with 0-100% acetone/methylene chloride afforded the title compound (1.1 g, 3.0 mmol, 92%). ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 8.48 (d, J=2.8Hz, 1H), 7.82 (dd, J=5,8.3Hz, 1H), 7.74 (d, J=8.3Hz, 2H), 7.64 (dt, J=2.8,8.3Hz, 1H), 7.08 (d, J=8.3Hz, 2H), 6.70 (s, 1H), 3.17 (s, 2H), 2.43 (s, 2H), 1.65 (s, 3H), 0.86 (s, 9H); LCMS: m/z = 368.2 [M+H]+.

1-[4-(5-fluoropyridin-2-yl)phenyl]ethanone (9).

Step A: Palladium tetrakis(triphenylphosphine) (1.38 g, 1.2 mmol) was added to a degassed, ambient temperature solution of 2-bromo-5-fluoropyridine (5 g, 28.4 mmol) and (4-{[methoxy(methyl)amino]carbonyl}phenyl)boronic acid (5 g, 23.9 mmol) and potassium carbonate (4.3 g, 31.1 mmol) in toluene/methanol (9.5:1) (105 mL). After stirring at 90°C for 1.5 h, the reaction mixture was concentrated in vacuo. The residue was partitioned between ethyl acetate and water. The organic phase was washed with brine, dried (magnesium sulfate) and concentrated *in vacuo*. Chromatography over silica eluting with 0-80% ethyl acetate/hexane afforded 4-(5-fluoropyridin-2-yl)-*N*-methoxy-*N*-methylbenzamide (4.5 g, 17.4 mmol, 73%).

Step B: Methylmagnesium bromide (3 M in tetrahydrofuran) (2.1 mL, 6.2 mmol) was added to a -78°C solution of 4-(5-fluoropyridin-2-yl)-*N*-methoxy-*N*-methylbenzamide (800 mg, 3.1 mmol) in tetrahydrofuran (20 mL). After stirring at 0°C for 30 min, the reaction mixture was quenched with methanol and saturated aqueous ammonium chloride. The organic layer was dried (magnesium sulfate) and concentrated *in vacuo*. Chromatography over silica eluting with 0-15% ethyl acetate/hexane afforded the title compound (580 mg, 2.7 mmol, 87%). ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 8.56 (1 H, d, J = 2.87 Hz), 8.12-8.06 (4 H, m), 8.00 (1 H, dd, J = 8.82, 4.32 Hz), 7.82 (1 H, d, J = 8.17 Hz), 7.70 (1 H, td, J = 8.57, 2.94 Hz), 2.64 (3 H, s); LCMS: m/z = 216.1 [M+H]+.

1-[4-(1H-pyrazol-1-yl)phenyl]ethanone (11a).

4-Bromoacetophenone (4.0 g, 20 mmol) was added to a stirred, ambient temperature mixture of pyrazole (1.4 g, 21.1 mmol), potassium carbonate (5.8 g, 42.1 mmol), rac-trans-*N*,*N*'-dimethylcyclohexane-1,2-diamine (0.9 g, 6.3 mmol) and copper (I) iodide (320 mg, 1.7 mmol) in toluene (15 mL) and the mixture was stirred at reflux for 18 h. The reaction mixture was cooled, filtered and concentrated *in vacuo*. Chromatography over silica eluting with 5-30% ethyl acetate/hexane afforded the title compound as an off-white solid (2.3 g, 12.3 mmol, 61%) ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 8.56 (1 H, d, J = 2.87 Hz), 8.12-8.06 (6 H, m), 8.00 (1 H, dd, J = 8.82, 4.32 Hz), 7.82 (1 H, d, J = 8.17 Hz), 7.70 (1 H, td, J = 8.57, 2.94 Hz), 2.64 (5 H, s); LCMS: m/z = 187.2 [M+H]+.

2,2,2-trifluoro-1-[4-(1H-pyrazol-1-yl)phenyl]ethanone (11b).

Compound **11b** was prepared via a similar procedure to compound **11a**. ^{1}H NMR (500 MHz, CD_3OD) δ ppm:

8.25 (1 H, dd, J = 2.5, 7.5Hz), 7.81-7.73 (5 H, m), 6.54-6.53 (1 H, m); LCMS: m/z = 241.1 [M+H]+.

4-(2,2-dimethylpropyl)-2-methyl-1-trityl-1H-imidazole (13).

Step A: 4,4-Dimethyl-1-pentene (59 g, 0.6 mol) was added to a 10°C suspension of nitrosonium tetrafluoroborate (66 g, 0.56 mmol) in acetonitrile (600 mL) After stirring vigorously at ambient temperature for 30 min, acetonitrile was removed *in vacuo* to afford 4-(2,2-dimethylpropyl)-2-methyl-1*H*-imidazol-1-ol which was used in the subsequent step without further purification.

Step B: Titanium (III) chloride (30% weight solution in 2N HCl) (600 mL, 1.5 mol) was added to an ambient temperature solution of 4-(2,2-dimethylpropyl)-2-methyl-1*H*-imidazol-1-ol (ca. 0.56 mmol) in methanol (1200 mL). After stirring at ambient temperature for 3 days, the reaction mixture was basified with saturated aqueous sodium bicarbonate until the black solution turned white. The reaction mixture was extracted with ether. The combined organic extracts were washed with brine, dried (magnesium sulfate), filtered and concentrated *in vacuo*. Chromatography over silica eluting with 10-20% ethyl acetate/hexane afforded 4-(2,2-dimethylpropyl)-2-methyl-1*H*-imidazole.

Step C: Triethylamine (70 mL, 0.5 mol) followed by trityl chloride (90 g, 0.32 mol) were added to an ambient temperature solution of 4-(2,2-dimethylpropyl)-2-methyl-1*H*-imidazole (49 g, 0.32 mmol) in methylene chloride (1 L). After stirring at ambient temperature for 30 min, the reaction mixture was washed with water and brine, dried (magnesium sulfate) and concentrated *in vacuo*. Chromatography over silica eluting with 10-20% ethyl acetate/hexane afforded the title compound. ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 7.37-7.34 (11 H, m), 7.13-7.10 (7 H, m), 6.43 (1 H, s), 2.32 (2 H, s), 1.57 (3 H, s), 0.87 (10 H, s); LCMS: m/z = 395.3 [M+H]+.

1-[4-(2,2-dimethylpropyl)-1H-imidazol-2-yl]-2-[4-(5-fluoropyridin-2-yl) phenyl]propan-2-ol (14).

Compound **14** was prepared via a similar procedure to compound **22**. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 8.52 (d, J=2.7Hz, 1H), 7.90 (d, J=8.2Hz, 2H), 7.69 (dd, J=4.3,8.7Hz, 1H), 7.48-7.42 (m, 3H), 6.62 (s, 1H), 3.22 (dd, J=15.1, 51.1Hz, 2H), 2.39 (s, 2H), 1.68 (s, 3H), 0.86 (s, 9H); LCMS: m/z = 368.2 [M+H]+.

1-[4-(2,2-dimethylpropyl)-1H-imidazol-2-yl]-2-[4-(1H-pyrazol-1-yl)phenyl]propan-2-ol (15).

Compound **15** was prepared via a similar procedure to compound **22**. ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 8.14 (1 H, d, J = 2.62 Hz), 7.69 (1 H, s), 7.61 (2 H, d, J = 8.22 Hz), 7.50 (2 H, d, J = 8.36 Hz), 6.55 (1 H, s), 6.50 (1 H, s), 3.16-3.04 (2 H, m), 2.34 (2 H, s), 1.59 (3 H, s), 0.81 (9 H, s);LCMS: m/z = 339.2 [M+H]+.

4-iodo-2-methyl-1-trityl-1H-imidazole (19).

Step A: Sodium carbonate (25.8 g, 243.6 mmol) followed by iodine (46.4 g, 182.7 mmol) were added to an ambient temperature solution of 2-methyl-1*H*-imidazole (5 g, 60.9 mmol) in 1,4-dioxane/water (1:1) (500 mL). After stirring in the dark at ambient temperature overnight, the reaction mixture was concentrated to about half its original volume, diluted with ethyl acetate and washed with saturated aqueous sodium thiosulfate and brine, dried (sodium sulfate) and concentrated *in vacuo* to afford 4,5-diiodo-2-methyl-1*H*-imidazole which was used in the subsequent step without further purification.

Step B: A solution of 4,5-diiodo-2-methyl-1*H*-imidazole (ca. 20 g, 59.9 mmol) and sodium sulfite (22.6 g, 179 mmol) in ethanol (400 mL) and water (400 mL) was heated at 100°C for 18 h. The reaction mixture was concentrated to half its original volume and partitioned between ethyl acetate and water. The organic phase was washed with brine, dried (sodium sulfate) and concentrated *in vacuo* to afford 4-iodo-2-methyl-1*H*-imidazole which was used in the subsequent step without further purification.

Step C: Triethylamine (13.4 mL, 96.2 mmol) followed by trityl chloride (20.1 g, 72.1 mmol) were added to an ambient temperature solution of 4-iodo-2-methyl-1*H*-imidazole (ca. 10 g, 48.1 mmol) in methylene chloride (90 mL). After stirring at ambient temperature for 2 days, the reaction mixture was diluted with methylene chloride and washed with water and brine, dried (sodium sulfate) and concentrated *in vacuo*. Chromatography over silica eluting with 0-15% acetone/methylene chloride afforded the title compound. ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 7.39 (6 H, dd, J = 5.49, 1.89 Hz), 7.28-7.18 (4 H, m), 7.13-7.11 (6 H, m), 6.76 (1 H, s), 1.59 (3 H, s); LCMS: m/z = 451.0 [M+H]+.

2-methyl-4-{[1-(trifluoromethyl)cyclopropyl]methyl}-1-trityl-1H-imidazole (20).

Step A: *N*,*O*-Dimethylhydroxylamine hydrochloride (7.17 g, 73.5 mmol) was added to an ambient temperature solution of 1-trifluoromethylcyclopropane-1-carboxylic acid (10.3 g, 66.8 mmol), EDC (15.4 g, 80.2 mmol), hydroxybenzotriazole hydrate (12.28 g, 80.2 mmol) and *N*-methylmorpholine (36.7 mL, 33.8 mmol) in methylene chloride (50 mL) at ambient temperature. After stirring at ambient temperature overnight, the reaction mixture was poured into ethyl acetate and washed successively with 2 M hydrochloric acid, saturated aqueous sodium bicarbonate and brine, dried (sodium sulfate) and concentrated to afford *N*-methoxy-*N*-methyl-1-(trifluoromethyl)cyclopropanecarboxamide which was used in the subsequent step without further purification.

Step B: Ethylmagnesium bromide (3 M solution in diethyl ether) (633 mL, 1.9 mol) was added over 1 h to a 5°C solution of 4-iodo-2-methyl-1-trityl-1*H*-imidazole (compound **19**) (855 g, 1.9 mol) in methylene chloride (8 L). After stirring at 5°C for 30 min, the reaction mixture was allowed to warm to ca. 12°C and a solution of *N*-methoxy-*N*-methyl-1-

(trifluoromethyl)cyclopropanecarboxamide (355 g, 1.8 mol) in methylene chloride (2 L) was added over 1 h. After stirring at ambient temperature for 18 h, the reaction mixture was poured into saturated aqueous ammonium chloride. The organic phase was washed with saturated aqueous sodium bicarbonate, dried (magnesium sulfate) and concentrated *in vacuo*. The residue was dissolved in a minimal volume of ether and precipitated with heptane. After stirring in heptane (ca. 3 L) for 1 h, solid was collected by filtration, washing with heptane to afford (2-methyl-1-trityl-1*H*-imidazol-4-yl)[1-(trifluoromethyl)cyclopropyl]methanone.

Step C: Hydrazine hydrate (30 mL) was added to a solution of (2-methyl-1-trityl-1*H*-imidazol-4-yl)[1-(trifluoromethyl)cyclopropyl]methanone (15.78 g, 34.2 mmol) and powdered potassium hydroxide (9.6 g, 171 mmol) in ethylene glycol (200 mL). After stirring at 120°C for 20 min, the reaction mixture was heated at 180°C for 2 h. After cooling to ambient temperature, water was added and the mixture was extracted twice with ethyl acetate. The combined organic extracts were washed with brine, dried (sodium sulfate) and concentrated *in vacuo*. Chromatography over silica eluting with 0-60% ethyl acetate/hexane afforded the title compound. ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 7.37 (9 H, d, J = 5.62 Hz), 7.12-7.09 (6 H, m), 6.57 (1 H, s), 2.86 (2 H, s), 1.56 (3 H, s), 0.86 (2 H, t, J = 6.17 Hz), 0.65 (2 H, s); LCMS: m/z = 447.2 [M+H]+.

2-[4-(1H-pyrazol-1-yl)phenyl]-1-(4-{[1-(trifluoromethyl)cyclopropyl]methyl}-1H-imidazol-2-yl)propan-2-ol (21).

Compound **21** was prepared via a similar procedure to compound **22**. ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 8.18 (1 H, s), 7.71-7.67 (3 H, m), 7.52 (2 H, d, J = 9 Hz), 7.18 (1 H, s), 6.51 (1 H, s), 3.32 (2 H, s), 3.00 (1 H, d, J=16 Hz), 2.94 (1 H, d, J=16 Hz), 1.70 (2 H, s), 0.98-0.89 (2 h, m), 0.71-0.62 (2 H, m); LCMS: m/z = 391.1 [M+H]+.

(2*S*)-1,1,1-trifluoro-2-[4-(1H-pyrazol-1-yl)phenyl]-3-(4-{[1-(trifluoromethyl)cyclopropyl]methyl}-1H-imidazol-2-yl)propan-2-ol (22).

n-BuLi (1.6 M in hexanes) (21 mL, 33.6 mmol) was added dropwise to a stirred, degassed, -78 °C solution of compound **20** (10 g, 22.4 mmol) in tetrahydrofuran (150 mL) and the mixture was stirred at -78 °C for 15 min. The mixture was added dropwise (via cannula) over 40 min to compound **11b** (8.07 g, 33.6 mmol) in degassed tetrahydrofuran (100 mL) and the resulting reaction mixture was stirred at -78 °C for 2 h. The reaction was quenched with saturated aqueous ammonium chloride and extracted with ethyl acetate. The organic phase was separated, washed with saturated brine, dried (sodium sulfate), filtered and concentrated *in vacuo* to give a crude residue. HCl (4 M in dioxane) (101 ml, 403 mmol) was added to a stirred, ambient temperature mixture of the crude residue in methanol (100 mL) and the mixture was stirred at 70 °C for 45 min. The volatiles were removed, and the resulting residue was partitioned between 2M aqueous sodium hydroxide and ethyl acetate. The organic phase was separated, washed with saturated brine, dried (sodium sulfate), filtered and concentrated *in vacuo* to give the crude product. Chromatography of the crude product over silica eluting with 20-80% EtOAc/hexane afforded the title compound as an off-white solid (3.05 g, 6.34 mmol, 28%) 242318-6. ^{1}H NMR (500 MHz, CD_3OD) δ ppm: 8.24 (d, J=2.6Hz, 1H), 7.87 (d, J=7.3Hz, 2H), 7.79 (d, J=8.9Hz, 2H), 7.73 (d, J=1.8Hz, 1H), 7.64 (d, J=8.7Hz, 2H), 7.20 (s, 1H), 6.53 (dd, J=2,2Hz, 1H) 3.73 (d, J=2Hz, 2H), 3.0 (d, J=16.3Hz, 1H), 2.92 (d, J=16Hz, 1H), 0.93 (m, 2H), 0.67 (d, J=9.8Hz, 1H), 0.62 (d, J=9.9Hz, 1H); LCMS: m/z = 445.2 [M+H]+.

Biological Evaluation

Binding and Functional Assays. Receptor binding and functional assessment were carried out as described.[1] Receptor binding was performed using membranes from CHO or HEK293 cells over-expressing the indicated receptor. For human BRS-3, NMBR, and GRPR binding, 30 pM [^{125}I]-[D-Tyr6,β-Ala^{11},Phe^{13},Nle^{14}]-Bombesin(6-14) (PerkinElmer, Boston, USA) was used, whereas for rat and mouse BRS-3 binding, 660 pM [^{3}H]Bag-3 was used. The Ki values are calculated from the IC_{50} values using the equation of Cheng and Prusoff (1973). For functional assays, agonist-induced mobilization of intracellular Ca^{2+} was measured in HEK293AEQ cells over-expressing BRS-3, using an aequorin bioluminescence assay. The %Act represents the maximum activations of tested compounds relative to that of dY-peptide (for human, dog and rhesus receptors) or a reference small molecule BRS-3 agonist (for mouse and rat receptors).

Pharmacokinetic assays. The blood samples were collected at various time points into lithium heparin tubes and centrifuged. The plasma samples were kept at -70°C until analysis. The plasma samples were extracted by protein precipitation and analyzed by LC/MS/MS.

Metabolic Stability. Test compounds (1 μM) were incubated with rat liver microsomes (0.2 mg protein/mL) in the presence of NADPH (1 mM) in 0.1 M phosphate buffer (pH 7.5). Incubations were carried out at 37ºC, and the reactions were quenched with an equal volume of acetonitrile containing internal standard at 0, 2, 5, 10, 15, 30 and 60 min, vortexed and centrifuged. Supernatants were transferred to 96-well plates, and 10 μL aliquots were analyzed using LC-MS. The percent (%) parent compound remaining in the sample was determined from the % of the peak area ratios of the parent compound to the internal standard at each time point compared to that at the t=0 time point. The parent compound disappearance data was used to fit a monoexponential decay function [$C(t) = Co*e^{(-Ke*t)}$]. The $t_{1/2}$ of parent disappearance was calculated using parameter Ke (first-order rate constant; $t_{1/2} = -0.693/ke$).

Protein Binding. Plasma protein and microsomal binding were carried out in a Harvard apparatus 96-well Equilibrium Dialyzer (Model 74-2330). Dulbecco's phosphate buffered saline (200 μL) was added to the buffer compartments and capped. Frozen human plasma (200 μL) or rat liver microsomes (0.2 mg/ml) was added to the protein compartments, spiked with 2 μL of 0.1 mM solution of test compounds in ethanol, and capped. The final concentration of compounds was 1 μM. The Dialyzer plate was secured into a rotator inside a Boekel Scientific Hybridization Oven (Model 230401). After rotating for ~24 hr at 37°C, 150 and 50 μL were removed from buffer and protein compartments, respectively, and 50 μL of control human plasma or rat liver microsomes was added to the buffer samples to maintain uniform matrix. A standard curve of test compounds (0.01 to 1 μM) was prepared with control human plasma or rat liver microsomes. Extraction was carried out with Water Oasis™ HLB extraction plate (10 mg). Samples were applied onto extraction plates containing 1 mL of 4 M urea, 50 μL of internal standard, washed once with water, followed by eluting with water:acetonitrile (10:90, by volume). Aliquots (10 μL) were analyzed using LC-MS/MS. The fraction of drug bound to proteins is calculated as follows: Fraction bound (%) = (B)/(P) x 100%, where B is the concentration in the buffer compartment and P is the concentration in the plasma or microsomal compartment.

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