Supporting Information for

Discovery of 2-Pyridylpyrimidines as the First Orally Bioavailable GPR39 Agonists

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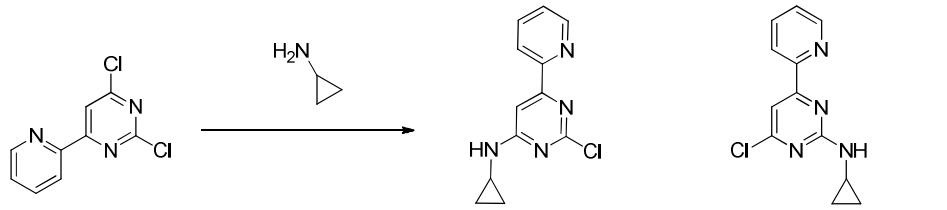
Experimental Procedures **Page**

Analytical methods:
NMR spectra were recorded on a Bruker AV400 (Avance 400 MHz) instrument. Analytical HPLC UV purity was assessed at both 254 and 214 nm using an Agilent 1100 HPLC system using the following method: 1.0 mL/min flow rate with the gradient from 5% to 95% acetonitrile in 10 min, 0.1% formic acid used as the modifier additive in the aqueous phase on a 3.0 x 100mm 3µm C18 column. LC/ESI-MS data were recorded using a Waters LCT Premier mass spectrometer with dual electrospray ionization source and Agilent 1100 liquid chromatograph. The resolution of the MS system was approximately 12 000 (fwhm definition). HPLC separation was performed at 1.0 mL/min flow rate with a gradient from 10% to 95% in 2.5 min. Ammonium formate (10 mM) was used as the modifier additive in the aqueous phase. Sulfadimethoxine (Sigma, protonated molecule *m*/*z* 311.0814) was used as a reference and acquired through the LockSpray channel every third scan. The mass accuracy of the system has been found to be <5 ppm.

Synthesis and characterization of compound 1 and 2

Preparation of N-(((1r,4r)-4-(((4-(Cyclopropylamino)-6-(pyridin-2-yl)pyrimidin-2-yl)amino)methyl)cyclohexyl)methyl)-3-fluorobenzenesulfonamide (1)



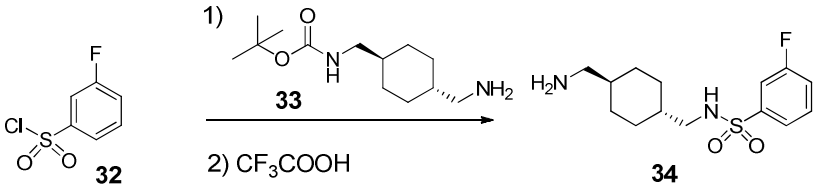
30 **31a** (major product) **31b** (minor product)

Step 1: 2-Chloro-N-cyclopropyl-6-(pyridin-2-yl)pyrimidin-4-amine (**31a**, major product) and 4-chloro-N-cyclopropyl-6-(pyridin-2-yl)pyrimidin-2-amine (**31b**, minor product)

In a 200 mL round bottom flask equipped with a stir bar, 2,4-dichloro-6-(pyridin-2-yl)pyrimidine (**30**, 2.04 g, 9.02 mmol) was dissolved in 30 mL of CH_3CN, and cooled down to 0 °C. Cyclopropylamine (1.90 mL, 27.1 mmol) taken up in 10 mL of CH_3CN was added dropwise and the final solution was allowed to warm to rt. After stirring for 2h, two regio-isomeric species were observed by LC-MS analysis. The solvent was evaporated and diluted with CH_2Cl_2 (100 mL), washed with 1N HCl (100 mL), and brine (100 mL). The organic fractions were dried over $MgSO_4$ and concentrated. The isomers were separated by flash chromatography using a 120 g column running a gradient from 20-100 % EtOAc in heptanes to afford the desired compound as the major product (1.34 g, 59%) and the regioisomer as the minor product (0.23g, 10%). Regioisomers were assigned with 2D-NMR studies.

31a: ^{1}H NMR (400 MHz, DMSO-d6) δ 8.69 (s, 1H), 8.41 - 8.10 (m, 2H), 7.94 (t, J = 7.5 Hz, 1H), 7.50 (dd, J = 45.0, 37.5 Hz, 2H), 2.89 - 2.57 (m, 1H), 1.01 - 0.67 (m, 2H), 0.63 - 0.39 (m, 2H).

31a: m/z (HRMS-ESI) meas./calc. ($C_{12}H_{12}ClN_4$) 247.0756/247.0750 (MH^+).

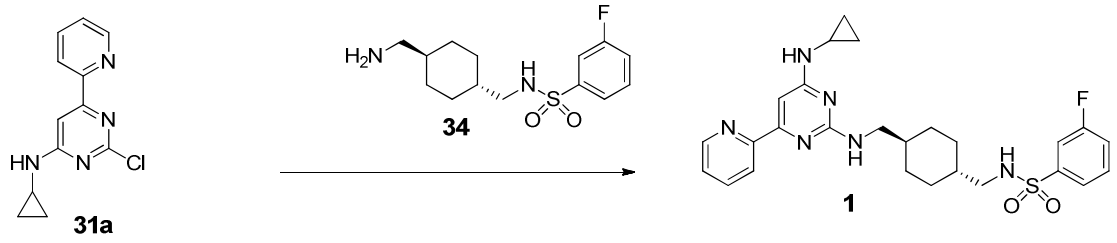


Step 2: N-(((1r,4r)-4-(Aminomethyl)cyclohexyl)methyl)-3-fluorobenzenesulfonamide (**34**), trifluoro acetate salt**.**

In a 100 mL round-bottomed flask equipped with a stir bar, *trans*-4-(boc-aminomethyl) cyclohexanemethanamide (**33**, 3.8 g, 15.68 mmol) was charged and taken up in CH_2Cl_2 (78 mL). While stirring, NEt_3 (6.56 mL, 47 mmol) was added followed by 3-fluorobenzene-1-sulfonyl chloride (**32**, 3.05 g, 15.68 mmol). The reaction was stirred overnight at rt upon which 1M HCl (50 mL) was added and extracted with CH_2Cl_2 (2 x 30 mL). The organic fraction was washed with water and brine (50 mL ea) and dried over $MgSO_4$ and concentrated *in vacuo*. Chromatographic purification was performed on a Biotage SP1 equipped with a 40g Isco Redisep-*RF* column (9:1 CH_2Cl_2: MeOH). Product containing fractions were combined and evaporated to afford a residue (1.74 g, 4.27 mmol, 91 %). (6.2 g, 15.5 mmol) was deprotected by dissolving in CH_2Cl_2 (77 mL) and treatment with CF_3COOH (12 mL, 155 mmol). After the solution was stirred for 2 h at rt the solvent was evaporated *in vacuo* and the residue was dried under high vacuum overnight to afford the title compound (6.33 g, 99%).

34: ^{1}H NMR (400 MHz, methanol-d_4) δ 7.63 - 7.69 (m, 1 H), 7.52 - 7.63 (m, 2 H), 7.33 - 7.42 (m, 1 H), 2.77 (d, J = 7.07 Hz, 2 H), 2.72 (d, J = 6.57 Hz, 2 H), 1.83 (m, 4 H), 1.49 - 1.67 (m, 1 H), 1.31 - 1.49 (m, 1 H), 0.84 - 1.10 (m, 4 H).

34: m/z (HRMS-ESI): meas./calc. ($C_{14}H_{22}N_2O_2FS$) 301.1377/301.1386 (MH^+).



Step 3: N-(((1r,4r)-4-(((4-(Cyclopropylamino)-6-(pyridin-2-yl))pyridin-2-yl)amino)methyl) cyclohexyl)methyl)-3-fluorobenzenesulfonamiden (**1**)

In a 5 mL microwave vial equipped with a stir bar, 2-chloro-N-cyclopropyl-6-(pyridin-2-yl)pyrimidin-4-amine (**31a**, 173.4 mg, 0.70 mmol) and N-(((1r,4r)-4-(aminomethyl)cyclohexyl)methyl)-3-fluorobenzenesulfonamide, trifluoroacetate salt (**34**, 874 mg, 2.11 mmol) were added, followed by NMP (1.4 mL) and DIPEA (1.2 mL). The vial was

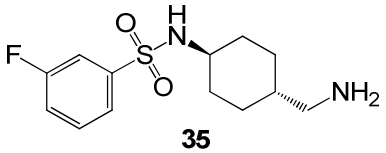
sealed and heated in the microwave reactor for 1.5 h at 180°C. The reaction was diluted with CH_2Cl_2 and then washed with 1N HCl, brine and H_2O. The combined organics were dried over $MgSO_4$, filtered and concentrated. Purification was performed with reverse phase preparative HPLC using a gradient of 10-98 % CH_3CN in 0.1 % n-propanol /H_2O over 10 min with a flow rate of 25 mL/min. Fractions were lyophilized to afford a white powder (158.5 mg, 44.2%)

1: ^{1}H NMR (400 MHz, methanol-d_4) δ 8.74 (d, J = 4.55 Hz, 1 H), 8.14 (br s, 1 H), 7.97 - 8.05 (m, 1 H), 7.63 - 7.69 (m, 1 H), 7.53 - 7.63 (m, 3 H), 7.36 (td, J = 8.59, 2.53 Hz, 1 H) 6.70 (br s, 1 H), 0.67 (br s, 2 H), 3.38 (br s, 1 H), 2.66 - 2.75 (m, 4 H), 1.70 - 1.93 (m, 4 H), 1.61 (br s, 1 H), 1.33 - 1.48 (m, 1 H), 0.81 - 1.10 (m, 6 H),

1: m/z (HRMS-ESI): meas./calc. ($C_{26}H_{32}FN_6O_2S$) 511.2270/511.2291 (MH^+)

Preparation of N-((1r,4r)-4-(((2-(cyclopropylamino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)cyclohexyl)-3-fluorobenzenesulfonamide (2)

Step 1: ((1r,4r)-4-(Aminomethyl)cyclohexyl)-3-fluorobenzenesulfonamide (**35**)



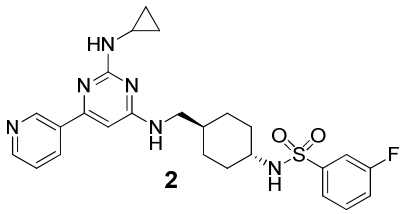
35

To a screw cap vial, tert-butyl *trans*-4-aminocyclohexylmethylcarbamate (2.11 g, 9.24 mmol) was dissolved in THF (15 mL). The vial was charged with 3-fluorobenzenesulfonyl chloride (1.23 mL, 9.24 mmol) and diisopropylethyl amine (2.42 mL, 13.86 mmol) and then stirred for 90 min at room temperature. The reaction mixture was acidified with 1 M HCl and diluted with dichloromethane. The organic phase was collected and concentrated to afford the crude product. The crude product was dissolved in 4 M HCl in dioxane (8 mL) and the solution was stirred for 1 h at room temperature. A precipitate formed and the solids were collected by vacuum filtration to obtain the title compound as a white solid (2.91 g, 94%).

35: ^{1}H NMR (400 MHz, methanol-d_4) δ 0.92 - 1.11 (m, 2 H) 1.17 - 1.33 (m, 2 H) 1.53 (td, J = 7.45, 4.29 Hz, 1 H) 1.71 - 1.88 (m, 4 H) 2.74 (d, J = 7.07 Hz, 2 H) 2.96 - 3.10 (m, 1 H) 7.32 - 7.42 (m, 1 H) 7.54 - 7.63 (m, 2 H) 7.67 - 7.73 (m, 1 H)

35: m/z (MS-ESI) meas./calc.($C_{13}H_{20}FN_2O_2S$): 287.2/287.1 (MH^+).

Step 2: N-((1r,4r)-4-(((2-(Cyclopropylamino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)cyclohexyl)-3-fluorobenzenesulfonamide (**2**)



2

In a 5 mL microwave vial equipped with a stir bar, 4-chloro-N-cyclopropyl-6-(pyridin-2-yl)pyrimidin-2-amine (**31b**, 125 mg, 0.51 mmol) and tert-butyl *trans*-4-aminocyclohexylmethylcarbamate (779 mg, 1.95 mmol) were added, followed by NMP (2.5 mL) and DIPEA (1.1 ml). The vial was sealed and heated in the microwave reactor for 1.5 h at 180 ^{o}C. The reaction was diluted with CH_2Cl_2 and then washed with 1N HCl, brine and H_2O. The combined organics were dried over $MgSO_4$, filtered and concentrated. Purification was performed with reverse phase preparative HPLC using a gradient of 10-98 % CH_3CN in 0.1 % trifluoroacetic acid /H_2O over 10 min with a flow rate of 25 mL/min. Fractions were lyophilized to afford the product (217 mg, 64 %).

2: ^{1}H NMR (400 MHz, methanol-d4) δ 8.58 (dd, J = 5.0, 1.6 Hz, 1H), 8.18 (d, J = 8.0 Hz, 1H), 7.90 (td, J = 7.8, 1.8 Hz, 1H), 7.72 - 7.66 (m, 1H), 7.62 - 7.56 (m, 2H), 7.46 - 7.41 (m, 1H), 7.36 (td, J = 8.4, 2.6 Hz, 1H), 6.61 (s, 1H), 3.20 (d, J = 6.7 Hz, 2H), 3.02 (ddt, J = 11.3, 7.6, 3.8 Hz, 1H), 2.77 (tt, J = 7.1, 3.7 Hz, 1H), 1.84 - 1.73 (m, 4H), 1.63 - 1.52 (m, 1H), 1.27 - 1.15 (m, 2H), 0.99 (qd, J = 14.0, 13.5, 3.5 Hz, 2H), 0.73 (td, J = 6.8, 4.7 Hz, 2H), 0.55 - 0.49 (m, 2H).

2: m/z (HRMS-ESI) meas./calc.($C_{25}H_{30}FN_6O_2S$): 497.2155/497.2135 (MH^+).

The UV purity of **2** at wavelength 214 and 254 nm was 98.2% and 97.7%, respectively (sample was dissolved in MeOH).

Synthesis and characterization of compounds of Table 1

Preparation of N-(3-chloro-4-(((2-(methylamino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)phenyl)methanesulfonamide (3)

Step 1: N-(3-Chloro-4-cyanophenyl)methanesulfonamide (**26**)

To a round-bottomed flask, 4-amino-2-chlorobenzonitrile (**25**) (500 mg, 3.28 mmol) was dissolved in pyridine (2 mL). The solution was cooled to 0 ^{o}C and mesyl chloride (0.5 mL, 6.55 mmol) was added. The reaction was warmed to room temperature and stirred for 15 min. The reaction mixture was concentrated and the crude product was purified by column chromatography (SiO_2, heptanes/EtOAc) to afford product **26** (610 mg, 80%).

26: ^{1}H NMR (400 MHz, methanol-d_4) δ 7.73 (d, J = 8.59 Hz, 1 H), 7.43 (d, J = 2.53 Hz, 1 H), 7.28 (dd, J = 8.59, 2.53 Hz, 1 H), 3.10 (s, 3 H).

26: m/z (LCMS-ESI): 229.2.

Step 2: N-(4-(Aminomethyl)-3-chlorophenyl)methanesulfonamide (**27**)

To a round-bottomed flask, lithium aluminium hydride (470 mg, 13 mmol) was dissolved in THF (15 mL). The solution was cooled to 0 ^{o}C and N-(3-chloro-4-cyanophenyl)methanesulfonamide (**26**) (600 mg, 2.6 mmol) was added dropwise. The reaction mixture was refluxed for 16 h. The reaction mixture was cooled with 0 ^{o}C and 1 M NaOH (5 mL) was added dropwise. The resulting suspension was filtered and the filtrate was concentrated to afford the crude product. The crude material was purified by column chromatography (SiO_2, dichloromethane/MeOH) to afford the title compound **27** (420 mg, 68%).

27: ^{1}H NMR (400 MHz, methanol-d_4) δ 3.14 (s, 3 H) 3.97 (s, 2 H) 7.74 (d, J = 8.08 Hz, 1 H) 7.88 (dd, J = 8.08, 1.52 Hz, 1 H) 7.96 (d, J = 2.02 Hz, 1 H).

27: m/z (LCMS-ESI): 233.3.

Step 3: N-(3-Chloro-4-(((2-chloro-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)phenyl)methanesulfonamide (**28**)

Into a round-bottom flask containing 2,4-dichloro-6-(pyridin-2-yl)pyrimidine (1.75 g, 7.74 mmol, synthesis described in Harden, D.B.; Mokroaz, M.J.; Strekowski, L. *J. Org. Chem.* **1988**, *53*, 4137-4140) in acetonitrile (15 ml) was added N-(4-(aminomethyl)-3-chlorophenyl) methanesulfonamide (**27**) (2.362 g, 10.06 mmol). The reaction was heated at 60 °C. After LCMS showed complete conversion, the material was concentrated onto silica gel. The crude product was purified using column chromotography using a RediSep 40g GOLD ISCO column eluting with a 15% to 100% EtOAc:heptanes gradient over 20 minutes. This method separated the two regioisomers with the undesired regioisomer eluting at 50% and the desired regioisomer eluting at 60%. The desired clean product fractions were pooled and concentrated. Some desired product eluted with the undesired regioisomer. Those fractions that contained a mixture were combined and repurified using the same method, which provided clean separation. The clean product fractions were then pooled with the product obtained from the first purification to yield in total 2.3 g (70%) of the desired product **28**.

28: ^{1}H NMR (400 MHz, methanol-d_4) δ 2.97 (s, 3 H) 4.67 (br. s., 2 H) 7.16 (d, *J*=8.59 Hz, 1 H) 7.28 - 7.39 (m, 2 H) 7.40 - 7.51 (m, 2 H) 7.93 (t, 1 H) 8.24 (d, *J*=8.08 Hz, 1 H) 8.63 (d, *J*=5.05 Hz, 1 H).

28: m/z (LCMS-ESI): 422.3.

Step 4: N-(3-Chloro-4-(((2-chloro-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)phenyl)methanesulfonamide (**3**)

In a microwave vial was added N-(3-chloro-4-(((2-chloro-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)phenyl)methanesulfonamide (**28**) (2.3 g, 5.42 mmol) to a solution of methylamine (0.56g, 5.42 mmol) in ethanol (20 mL). The yellow solution was heated in a microwave at 130 °C for 30 min. LCMS showed complete conversion to the desired product. The material was concentrated to remove excess amine. The crude product was then concentrated onto silica gel and purified by column chromotography using a 20% to 100% EtOAc: heptanes gradient. A RediSep 40g GOLD ISCO column was used. The desired product eluted at 100% EtOAc. The product fractions were pooled and concentrated. The product was dried under vacuum overnight to remove any remaining solvent to 1.97g (87%) of the product **3**.

3: ^{1}H NMR (400 MHz, methanol-d_4) δ 2.94 (d, *J*=8.59 Hz, 6 H), 4.66 (s, 2 H), 6.67 (s, 1 H), 7.13 (dd, *J*=8.08, 2.02 Hz, 1 H), 7.32 (d, *J*=2.02 Hz, 1 H), 7.38 - 7.51 (m, 2 H), 7.90 (td, *J*=7.83, 1.52 Hz, 1 H), 8.18 (d, *J*=7.58 Hz, 1 H), 8.58 (d, *J*=4.04 Hz, 1 H).

3: m/z (HMRS-ESI) meas./calc.($C_{18}H_{20}ClN_6O_2S$): 419.1068/419.1057 (MH^+).

The UV purity of **3** at wavelength 214 and 254 nm was 100% and 100%, respectively (sample was dissolved in MeOH).

Preparation of N^2-methyl-N^4-(4-(methylsulfonyl)benzyl)-6-(pyridin-2-yl)pyrimidine-2,4-diamine (20)

Step 1: 2-Chloro-N-(4-(methylsulfonyl)benzyl)-6-(pyridin-2-yl)pyrimidin-4-amine (**36**)

Into a screw top vial was added 2,4-dichloro-6-(pyridin-2-yl)pyrimidine (2.0 g, 8.85 mmol, Harden, D.B.; Mokroaz, M.J.; Strekowski, L. *J. Org. Chem.* **1988**, *53*, 4137-4140), (4-(methylsulfonyl)phenyl)methanamine (2.35 g, 10.62 mmol) and Huenig's base (4.64 ml, 26.5 mmol) in acetonitrile (16 ml). The orange-yellow solution was heated at 60 °C for 2 hr at which point LCMS showed complete consumption of the starting material and the formation of the two regioisomers. The crude material was concentrated onto silica gel and purified by column chromotography using a 0% to 75% gradient (EtOAc:heptanes). The undesired regioisomer eluted first at 40% EtOAc and the desired product eluted at 60% EtOAc to yield 2.82 g (85%) of the title compound.

36: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.06 (s, 3 H), 4.80 (d, *J*=5.56 Hz, 2 H), 5.58 (br. s., 1 H), 7.34 - 7.45 (m, 2 H), 7.55 (d, *J*=8.59 Hz, 2 H), 7.84 (td, *J*=7.71, 1.77 Hz, 1 H), 7.94 (d, *J*=8.08 Hz, 2 H), 8.38 (d, *J*=7.58 Hz, 1 H), 8.64 (dd, *J*=4.04, 1.01 Hz, 1 H).

36: m/z (LCMS-ESI): 375.2.

Step 2: N^2-Methyl-N^4-(4-(methylsulfonyl)benzyl)-6-(pyridin-2-yl)pyrimidine-2,4-diamine (**20**)

To a microwave vial was added 2-chloro-N-(4-(methylsulfonyl)benzyl)-6-(pyridin-2-yl)pyrimidin-4-amine (500 mg, 1.334 mmol) and methanamine in water (4.0 ml, 8.0 mmol) in a 1:1 mixture of acetonitrile (2 ml) and methanol (2 ml). The clear solution was heated in a microwave at 165 °C for 1 hr. LCMS shows complete conversion of the starting material with no visible side products. The crude material was purified by column chromatography using a 0% to 10% methanol:dichloromethane gradient. The product eluted at 4% MeOH as a sharp peak. The product fractions were collected, concentrated, dissolved in water and acetonitrile and freeze dried over the weekend. There still seemed to be a large amount of residual amine as the product was sticky. The product was then dissolved in acetonitrile and MeOH and purified by neutral phase HPLC (3% n-propanol) using a 15%-->95% gradient over 8 minutes. The product eluted as the sole peak at 90% MeCN. Lyophilization provided product **20** in 398 mg (86%) as a white powder.

20: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.03 (d, J = 5.05 Hz, 3 H), 3.07 (s, 3 H), 4.80 (d, J = 6.06 Hz, 2 H), 6.88 (s, 1 H), 7.36 (dd, J = 6.82, 4.80 Hz, 1 H), 7.57 (d, J = 8.08 Hz, 2 H), 7.79 - 7.87 (m, 1 H), 7.93 (d, J = 8.59 Hz, 2 H), 8.39 (d, J = 7.58 Hz, 1 H), 8.66 (d, J = 4.04 Hz, 1 H).

20: m/z (HMRS-ESI) meas./calc.($C_{18}H_{20}N_5O_2S$): 370.1340/370.1338 (MH^+).

The UV purity of **20** at wavelength 214 and 254 nm was 100% and 100%, respectively (sample was dissolved in MeOH).

Compounds **21**, **22** and **24** were prepared in a similar fashion and are characterized below:

N-(4-(((2-(Methylamino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)phenyl)-methanesulfonamide (21)

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.01 (s, 3 H), 3.05 (s, 3 H), 4.63 (d, J = 6.06 Hz, 2 H), 6.43 (br. s., 1 H), 6.84 (s, 1 H), 7.19 (d, J = 8.59 Hz, 2 H), 7.35 (d, J = 8.59 Hz, 3 H), 7.80 (t, J = 8.59 Hz, 1 H), 8.34 (d, J = 8.08 Hz, 1 H), 8.64 (d, J = 5.05 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{18}H_{21}N_6O_2S$): 385.1447/385.1447 (MH^+).

The UV purity of **21** at wavelength 214 and 254 nm was 95.8% and 95.4% respectively (sample was dissolved in MeOH).

N-(4-(((2-(Methylamino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)phenyl)acetamide (22)

^{1}H NMR (400 MHz, methanol-d_4) δ 2.10 (s, 3 H), 2.95 (s, 3 H), 4.57 (s, 2 H), 6.63 (s, 1 H), 7.32 (m, *J*=8.59 Hz, 2 H), 7.42 (ddd, *J*=7.58, 4.80, 1.26 Hz, 1 H), 7.45 - 7.53 (m, 2 H), 7.89 (td, *J*=7.71, 1.77 Hz, 1 H), 8.17 (d, *J*=7.58 Hz, 1 H), 8.53 - 8.62 (m, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{19}H_{21}N_6O$): 349.1777/349.1777 (MH^+).

The UV purity of **22** at wavelength 214 and 254 nm was 98.7% and 99.2% respectively (sample was dissolved in MeOH).

N-(3-Fluoro-4-(((2-(methylamino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)phenyl) acetamide (24)

^{1}H NMR (400 MHz, methanol-d_4) δ 2.10 (s, 3 H), 2.97 (s, 3 H), 4.63 (s, 2 H), 6.65 (s, 1 H), 7.17 (dd, *J*=8.08, 1.52 Hz, 1 H), 7.36 (t, *J*=8.59 Hz, 1 H), 7.45 (dd, *J*=7.07, 5.05 Hz, 1 H), 7.53 (dd, *J*=12.38, 1.77 Hz, 1 H), 7.91 (td, *J*=7.83, 1.52 Hz, 1 H), 8.15 (d, *J*=7.58 Hz, 1 H), 8.60 (d, *J*=4.04 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{19}H_{20}FN_6O$): 367.1686/367.1683 (MH^+).

The UV purity of **24** at wavelength 214 and 254 nm was 97.9% and 97.4% respectively (sample was dissolved in MeOH).

Compounds **4-7**, **9-16**, **18**, **19**, **23** and **29** were prepared similarly to example **3** and were obtained in UV purities ≥95% as measured at 214 nm and 254 nm wavelength.

N4-(Cyclopropylmethyl)-N2-methyl-6-(pyridin-2-yl)pyrimidine-2,4-diamine (4)

^{1}H NMR (400 MHz, methanol-d_4) δ 0.28 - 0.42 (m, 2 H), 0.56 - 0.67 (m, 2 H), 1.07 - 1.23 (m, 1 H), 3.06 (s, 3 H), 3.44 (d, *J*=7.07 Hz, 2 H), 6.73 (s, 1 H), 7.61 (ddd, *J*=7.33, 4.80, 1.01 Hz, 1 H), 7.97 - 8.16 (m, 2 H), 8.77 (dd, *J*=6.06, 1.52 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{14}H_{18}N_5$): 256.1588/256.1562 (MH^+).

N4-(Cyclopropylmethyl)-N2-neopentyl-6-(pyridin-2-yl)pyrimidine-2,4-diamine (5)

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.26 (d, 6.06 Hz, 2 H), 0.55 (dd, 8.08, 1.01 Hz, 2 H), 0.99 (s, 9 H), 1.03 - 1.17 (m, 1 H), 3.24 (t, J = 6.32 Hz, 2 H), 3.32 (d, *J* = 6.57 Hz, 2 H), 4.86 (br s, 1 H), 4.92 (br s, 1 H), 6.74 (s, 1 H), 7.29 - 7.35 (m, 1 H), 7.78 (td, *J* = 7.71, 1.77 Hz, 1 H), 8.29 (d, *J* = 7.58 Hz, 1 H), 8.64 (dt, *J* = 5.56, 1.01 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{18}H_{26}N_5$): 312.2191/312.2188 (MH^+).

N4-(Cyclopropylmethyl)-N2-(2-methoxyethyl)-6-(pyridin-2-yl)pyrimidine-2,4-diamine (6)

^{1}H NMR (400 MHz, methanol-d_4) δ 0.28 (br s, 2 H), 0.54 (br s, 2 H), 1.12 (br s, 1 H), 3.28 (br s, 2 H), 3.35 (d, *J* = 15.66 Hz, 2 H), 3.62 (br s, 5 H), 6.66 (br s, 1 H), 7.47 (br s, 1 H), 7.92 (br s, 1 H), 8.17 (br s, 1 H), 8.62 (br s, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{16}H_{22}N_5O$): 300.1826 /300.1824 (MH^+).

N4-(Cyclopropylmethyl)-6-(pyridin-2-yl)-N2-(4-(trifluoromethoxy)benzyl)pyrimidine-2,4-diamine (7)

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.26 (q, J = 5.05 Hz, 2 H), 0.49 - 0.62 (m, 2 H), 0.98 - 1.15 (m, 1 H), 3.25 (dd, J = 6.57, 5.56 Hz, 2 H), 4.63 - 4.78 (m, 2 H), 4.97 (br s, 1 H), 5.37 (br s, 1 H), 6.85 (s, 1 H), 7.19 (d, J = 8.08 Hz, 2 H), 7.30 - 7.37 (m, 1 H), 7.41 - 7.51 (m, 2 H), 7.80 (td, J = 7.83, 2.02 Hz, 1 H), 8.29 (d, J = 7.58 Hz, 1 H), 8.67 (dd, J = 4.04, 1.01 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{21}H_{21}F_3N_5O$): 416.1694 /416.1698 (MH^+).

N4-(Cyclopropylmethyl)-N2,N2-dimethyl-6-(pyridin-2-yl)pyrimidine-2,4-diamine (9)

^{1}H NMR (400 MHz, methanol-d_4) δ 0.22 - 0.34 (m, 2 H), 0.46 - 0.58 (m, 2 H), 1.03 - 1.22 (m, 1 H), 3.19 (s, 6 H), 3.27 (d, *J* = 7.07 Hz, 2 H), 6.61 (s, 1 H), 7.43 (ddd, *J* = 7.45, 4.67, 1.01 Hz, 1 H), 7.90 (td, *J* = 7.71, 1.77 Hz, 1 H), 8.29 (d, *J* = 8.08 Hz, 1 H), 8.53 - 8.63 (m, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{15}H_{20}N_5$): 270.1709/270.1719.

N2-Methyl-N4-neopentyl-6-(pyridin-2-yl)pyrimidine-2,4-diamine (10)

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 9 H), 3.04 (d, J = 5.05 Hz, 3 H), 3.22 (br s, 2 H), 4.85 (br s, 2 H), 6.80 (s, 1 H), 7.32 (dd, J = 7.33, 4.80 Hz, 1 H), 7.74 - 7.85 (m, 1 H), 8.33 (d, J = 8.08 Hz, 1 H), 8.65 (d, J = 4.55 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{15}H_{22}N_5$): 272.1876/272.1875.

N4-(Cyclohexylmethyl)-N2-methyl-6-(pyridin-2-yl)pyrimidine-2,4-diamine (11)

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (qd, J = 11.87, 2.78 Hz, 2 H), 1.06 - 1.23 (m, 3 H), 1.58 - 1.82 (m, 6 H), 2.96 (d, J = 5.05 Hz, 3 H), 3.19 (br s, 2 H), 4.96 (br s, 2 H), 6.71 (s, 1 H), 7.26 (ddd, J = 7.45, 4.93, 1.26 Hz, 1 H), 7.74 (td, J = 7.83, 2.02 Hz, 1 H), 8.29 (d, J = 8.08 Hz, 1 H), 8.55 - 8.63 (m, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{17}H_{24}N_5$): 298.2039/298.2032.

N4-(4-Chlorobenzyl)-N2-methyl-6-(pyridin-2-yl)pyrimidine-2,4-diamine (12)

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.95 (d, *J* = 5.05 Hz, 3 H), 4.55 (d, *J* = 5.56 Hz, 2 H), 5.11 (br s, 2 H), 6.75 (s, 1 H), 7.14 - 7.31 (m, 5 H), 7.73 (td, *J* = 7.71, 1.77 Hz, 1 H), 8.28 (d, *J* = 7.58 Hz, 1 H), 8.52 - 8.60 (m, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{17}H_{17}ClN_5$): 326.1185/326.1172.

N2-Methyl-6-(pyridin-2-yl)-N4-(pyridin-4-ylmethyl)pyrimidine-2,4-diamine (13)

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.93 - 3.05 (m, 3 H), 4.69 (d, *J* = 6.57 Hz, 2 H), 4.88 (br s, 1 H), 5.15 (br s, 1 H), 6.84 (s, 1 H), 7.27 - 7.29 (m, 2 H), 7.32 (ddd, *J* = 7.58, 4.55, 1.01 Hz, 1 H), 7.80 (td, *J* = 7.71, 1.77 Hz, 1 H), 8.32 (d, *J* = 7.58 Hz, 1 H), 8.48 - 8.59 (m, 2 H), 8.63 (dd, *J* = 4.04, 1.01 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{16}H_{17}N_6$): 293.1516/293.1515.

N-Methyl-N-(4-((2-(methylamino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)phenyl)acetamide (14)

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.83 (s, 3 H), 3.01 (d, J = 5.05 Hz, 3 H), 3.19 (s, 3 H), 4.99 (br s, 1 H), 6.64 (br s, 1 H), 7.04 (s, 1 H), 7.08 (d, J = 8.59 Hz, 2 H), 7.23 - 7.32 (m, 1 H), 7.52 (d, J = 8.08 Hz, 2 H), 7.73 (td, J = 7.71, 1.77 Hz, 1 H), 8.28 (d, J = 8.08 Hz, 1 H), 8.52 - 8.62 (m, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{19}H_{21}N_6O$): 349.1787/349.1777.

N4-(2,4-Dichlorobenzyl)-N2-methyl-6-(pyridin-2-yl)pyrimidine-2,4-diamine (15)

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.94 (d, J = 5.05 Hz, 3 H), 4.61 (d, J = 6.06 Hz, 2 H), 4.83 (br s, 1 H), 5.08 (br s, 1 H), 6.74 (s, 1 H), 7.10 (dd, J = 8.59, 2.02 Hz, 1 H), 7.19 - 7.25 (m, 1 H), 7.25 - 7.33 (m, 2 H), 7.70 (td, J = 7.71, 1.77 Hz, 1 H), 8.23 (d, J = 8.08 Hz, 1 H), 8.48 - 8.65 (m, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{17}H_{16}Cl_2N_5$): 360.0799/360.0783.

N4-(4-Chlorobenzyl)-6-(pyridin-2-yl)-N2-(2,2,2-trifluoroethyl)pyrimidine-2,4-diamine (16)

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.91 - 4.20 (m, 2 H), 4.54 (d, J = 5.56 Hz, 2 H), 5.24 (br s, 2 H), 6.86 (s, 1 H), 7.12 - 7.30 (m, 5 H), 7.73 (td, J = 7.71, 1.77 Hz, 1 H), 8.22 (d, J = 8.08 Hz, 1 H), 8.56 (dd, J = 5.56, 1.01 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{18}H_{16}ClF_3N_5$): 394.1055/394.1046.

N4-(1-(4-Chlorophenyl)cyclobutyl)-N2-methyl-6-(pyridin-2-yl)pyrimidine-2,4-diamine (18)

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.83 - 1.99 (m, 1 H), 1.99 - 2.16 (m, 1 H), 2.35 - 2.50 (m, 2 H), 2.50 - 2.68 (m, 2 H), 2.84 (d, *J* = 4.04 Hz, 3 H), 5.47 (br s, 1 H), 6.45 (br s, 1 H), 7.14 - 7.23 (m, 4 H), 7.36 (d, *J* = 8.59 Hz, 2 H), 7.66 (t, *J* = 7.58 Hz, 1 H), 8.14 (d, *J* = 7.58 Hz, 1 H), 8.51 (d, *J* = 4.04 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{20}H_{21}ClN_5$): 366.1489/366.1485.

N2-Methyl-6-(pyridin-2-yl)-N4-(4-(trifluoromethoxy)benzyl)pyrimidine-2,4-diamine (19)

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.94 (d, J = 5.05 Hz, 3 H), 4.56 (d, J = 6.06 Hz, 2 H), 4.90 (br s, 1 H), 5.04 (br s, 1 H), 6.76 (s, 1 H), 7.09 (d, J = 8.08 Hz, 2 H), 7.23 (ddd, J = 7.58, 4.80, 1.26 Hz, 1 H), 7.29 (d, J = 8.59 Hz, 2 H), 7.70 (td, J = 7.83, 1.52 Hz, 1 H), 8.24 (d, J = 8.08 Hz, 1 H), 8.55 (dt, J = 5.56, 1.01 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{18}H_{17}F_3N_5O$): 376.1388/376.1385.

N-Methyl-4-(((2-(methylamino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)benzamide (23)

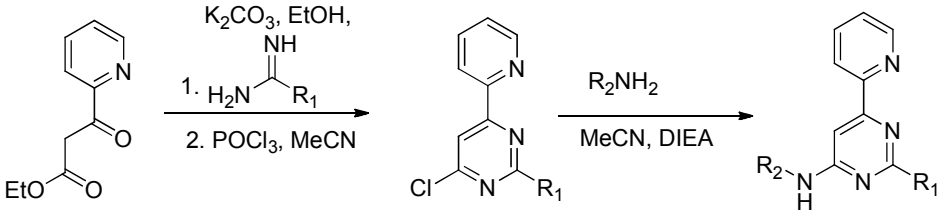
^{1}H NMR (400 MHz, methanol-d_4) δ 2.92 (s, 3 H), 2.90 (s, 3 H), 4.67 (s, 2 H), 6.65 (s, 1 H), 7.36 - 7.51 (m, 3 H), 7.70 - 7.80 (m, 2 H), 7.89 (td, *J* = 7.83, 2.02 Hz, 1 H), 8.17 (d, *J* = 8.08 Hz, 1 H), 8.57 (dt, *J* = 4.04, 1.01 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{19}H_{21}N_6O$): 349.1789/349.1777.

N-(((1r,4r)-4-(((2-((Cyclopropylmethyl)amino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)cyclohexyl)methyl)-4-fluorobenzenesulfonamide (29)

m/z (HMRS-ESI) meas./calc.($C_{27}H_{34}FN_6O_2S$): 525.2442/ 525.2448.

Alternatively, compounds of Table 1 can be prepared by the following scheme:



General method:

Step 1: To a round-bottomed flask, guanidine or amidine (2 eq.) was dissolved in ethanol (~0.4M). The reaction vessel was charged with potassium carbonate (3 eq.) followed by ethyl picolinoylacetate (1 eq.) as a solution in ethanol (~0.3 M). The reaction was heated at 80 ^{o}C overnight. The reaction mixture was concentrated and the residue was diluted with water and acidified to pH 1 with 5 N HCl. Water was removed, the crude product was concentrated and purified by silica gel chromatography using methanol/dichloromethane as eluent to yied the desired product.

Step 2: In a glass vial, the 6-(2-pyridyl)pyrimidin-4-ol (1 eq.) was suspended in acetonitrile (2 M) and phosphorus oxychloride (3 eq.) was added dropwise. The reaction was heated at 80 ^{o}C for 2 h. The reaction mixture was cooled to ambient temperature and added dropwise to ice (exothermic reaction). The quenched reaction mixture was basified with 2 M NaOH until pH 12. The aqueous layer was washed with dichloromethane, the organic layer was dried ($MgSO_4$), filtered, and concentrated to afford a coloured solid. The curde product was purified by silica gel chromatography unsing ethyl acetate/heptanes as eluent. The desired product was afforded as a white solid.

Step 3: To a microwave vial, 4-chloro-6-(2-pyridyl)pyrimidine (1 eq.) and amine (2 eq) were dissolved or suspended in acetonitrile (~0.04 M). The vial was charged with diisopropyl ethyl amine (1.1 eq) and the reaction was heated for 30-60 min at 160-180 °C under microwave radiation. The reaction mixture was then concentrated and the crude material was purified by reverse phase HPLC utilizing acetonitrile in water or flash chromatography on silica gel using methanol in dichloromethane as eluents.

N-(Cyclopropylmethyl)-6-(pyridin-2-yl)pyrimidin-4-amine (8)

Step 1: 2-Chloro-N-(cyclopropylmethyl)-6-(pyridin-2-yl)pyrimidin-4-amine
Into a 100 mL round bottom flask was added 2,4-dichloro-6-(pyridin-2-yl)pyrimidine (1.0 g, 4.42 mmol, Harden, D.B.; Mokroaz, M.J.; Strekowski, L. *J. Org. Chem.* **1988**, *53*, 4137-4140) dissolved in acetonitrile (25 ml). The clear solution was cooled to 0 °C in an ice bath and methylcyclopropylamine (1.15 ml, 13.27 mmol) was added. The solution was allowed to stir for 3 hr. LCMS showed complete product conversion with the expected ratio of 3:1 for both regioisomers. The solution was concentrated and then purified by silica column chromotography using a 0% to 60% ethyl acetate: heptanes gradient. The desired product eluted at ~ 25% ethyl

acetate and the undesired product eluted at ~40% ethyl acetate. Fractions containing the desired product were pooled and concentrated to yield 810 mg (70%) of the title compound.

Step 2: 2-Chloro-N-(cyclopropylmethyl)-6-(pyridin-2-yl)pyrimidin-4-amine (100 mg, 0.384 mmol) was dissolved in ethanol (5 mL) and 10% Pd/C (200 mg) was added to the hydrogenation flask. The flask was filled with hydrogen at 40 psi and the reaction was conducted for 16h. LCMS indicated partial conversion to the dechlorinated product. The mixture was filtered, concentrated and the crude product purified by reverse phase HPLC using a water/acetonitrile gradient to yield 13 mg (15%) of **8** as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.16 - 0.28 (m, 2 H), 0.47 - 0.58 (m, 2 H), 0.96 - 1.11 (m, 1 H), 3.21 (br. s., 2 H), 7.29 (ddd, *J*=7.45, 4.67, 1.01 Hz, 1 H), 7.36 (s, 1 H), 7.76 (td, *J*=7.83, 2.02 Hz, 1 H), 8.31 (d, *J*=8.08 Hz, 1 H), 8.52 - 8.65 (m, 2 H).

m/z (HMRS-ESI) meas./calc.($C_{13}H_{15}N_4$): 227.1201/227.1297 (MH^+).

The UV purity of **8** at wavelength 254 nm was 98.7%.

N-(4-Chlorobenzyl)-2-methyl-6-(pyridin-2-yl)pyrimidin-4-amine (17)

The compound was prepared following the above general method.

^{1}H NMR (400 MHz, methanol-d_4) δ 2.66 (s, 3 H), 4.76 (br. s., 2 H), 7.23 (br. s., 1 H), 7.33 - 7.44 (m, 4 H), 7.53 - 7.67 (m, 1 H), 8.03 (t, *J*=7.33 Hz, 1 H), 8.18 (br. s., 1 H), 8.77 (d, *J*=4.04 Hz, 1 H).

m/z (HMRS-ESI) meas./calc.($C_{17}H_{16}ClN_4$): 311.1055/311.1063 (MH^+).

The UV purity of **17** at wavelength 214 and 254 nm was 94.5% and 100% respectively (sample was dissolved in MeOH).

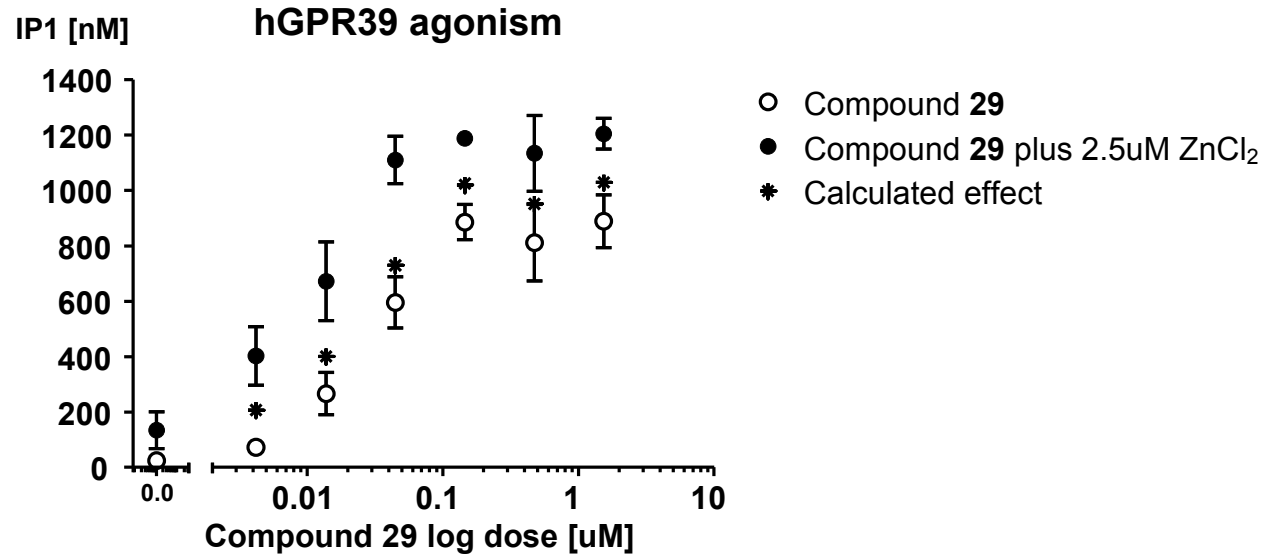
IP1 assay

HEK293 cell lines expressing GPR39 (mGPR39/HEK or hGPR39/HEK) or parental HEK293 cells were assayed for IP1 production using the IP1 kit from CisBio. Cells were cultured in media containing 1:1 F-12 Ham's: DMEM, 10% FBS, and geneticin (G418, 400 μg/mL). For assaying with compounds, cells were plated overnight in full media in 384 well plates at a density of 10,000 cells/well (mGPR39/HEK and hGPR39/HEK) or 15000 cells/well (HEK293 parental). Test compounds of interest were prepared for the assay by serial dilution in DMSO, followed by an intermediary dilution in assay buffer such that final [DMSO] was equal to 0.5%. Media was then aspirated from the cells and exchanged for stimulation buffer containing LiCl as provided in the CisBio IP1 assay kit. The compound mixture was added to assay plates and incubated for 60 minutes at 37 ^{o}C. IP1 d2 and the anti-IP1 Tb cryptate labeled antibody were then added as recommended by the manufacturer and IP1 production was measured by determining the signal ratio at 665 nm (IP1d2): 590 nm (anti-IP1 Tb Cryptate). The data were viewed using the signal ratio or converted to [IP1] by comparison with an IP1 standard. EC_{50} values for both the signal ratio and the corresponding IP1 concentration were defined as the inflection point of the logistic curve, as determined by non-linear regression of the fluorescent signal ratio vs $\log_{10}$(concentration) of test agonist using the R statistical software package. Each datapoint was acquired in triplicate. Where no calls could be made by the program, an estimated EC_{50} for a given agonist was reported. EC_{50} values falling outside the range tested were listed as "greater than" the highest dose..

IP1 assay with/without Zn^{2+}

Effect of GPR39 agonist N-(((1r,4r)-4-(((2-((cyclopropylmethyl)amino)-6-(pyridin-2-yl)pyrimidin-4-yl)amino)methyl)cyclohexyl)methyl)-4-fluorobenzenesulfonamide (**29)** combined with sub-EC_{50} dose of zinc chloride results in generation of IP1 greater than the amount produced by either substance alone. IP1 production measured in HEK293 cells expressing human GPR39 after 60 min stimulation utilizing IP1 terbium HTRF assay (see Figure). Open circle (o) represents effect of compound **29** alone, with x-axis zero point demonstrating baseline IP1 levels. Closed circle (•) represents combination of 2.5 μM $ZnCl_2$ to the compound **29** dose curve. The asterisk (∗) represents a calculated additive effect of $ZnCl_2$ to compound **29** dose curve based on the resulting IP1 generation by $ZnCl_2$ in the absence of compound **29**. IP1 levels above the asterisk indicate a synergistic effect between $ZnCl_2$ and compound **29**. EC_{50} values for $ZnCl_2$ and compound **29** on human GPR39 are 40 μM and 0.07 μM respectively, with an average ≥ 5 replicates. EC_{50} values were determined by the inflection point of the logistic curve as determined by non-linear regression of IP1 concentration vs $\log_{10}$(concentration) of test agonist, within the linear range of the assay.

Figure



cAMP assay

HEK293 cell lines expressing GPR39 (mGPR39/HEK or hGPR39/HEK) or parental HEK293 cells were assayed for cAMP production using the cAMP kit from CisBio. Cells were cultured as described above and plated for compound treatment at 20,000 cells/well in a 384 well plate in serum-free media. After an overnight incubation, compounds of interest were prepared for the assay by serial dilution in DMSO, followed by an intermediary dilution in assay buffer (Hanks' Balanced Salt Solution (HBSS), 10 mM HEPES, 3 mM IBMX, pH 7.4) containing IBMX such that final [DMSO] during the assay was equal to 0.7% and final [IBMX] =1 mM. The compound mixture was added to assay plates and incubated for 60 minutes at 37 ^{o}C. cAMP-d2 and the anti-cAMP Tb cryptate-labeled antibody were diluted 1:20 and cAMP production was measured by determining the signal satio at 665 nm (cAMP-d2): 590 nm (anti-cAMP Tb cryptate). The data were viewed using the signal ratio or converted to [cAMP] by comparison with a [cAMP] standard. EC_{50} values for both the signal ratio and the corresponding cAMP concentration were defined as the inflection point of the logistic curve, as determined by non-linear regression of the fluorescent signal ratio vs $\log_{10}$(concentration) of test agonist using the R statistical software package. Each datapoint was acquired in triplicate. Where no calls could be made by the program, an estimated EC_{50} for agiven agonist was reported. EC_{50} values falling outside the range tested were listed as "less than" or "greater than" the lowest or highest dose respectively.

GLP-1 secretion assay

Summary

STC-1 cells seeded in 96-well plates were treated with low glucose (5 mM) overnight and then starved for 2 hr in Earl's Balanced Salt Solution (EBSS) on the day of the assay. Compounds were prepared for the assay by serial dilution in DMSO and the cells were treated with compounds for 2 hr. The media was then collected and the secreted GLP-1 was detected in the media by a fluorescent bead based ELISA using the Luminex technology.

Cell Culture

SCT-1 cells (obtained from Dean Norgate at Novartis Horsham Research Center/England) were seeded at a density of approximately 4 million cells per 150 cm^2 flask (Corning cat# 430825in culture medium (DMEM (Gibco Cat# 11965) supplemented with 15% horse serum + 3% FBS + 1% Penicillin/Streptomycin/Glutamine). Cells were maintained at 37^0C and 5% CO_2 with media changed every two days and were passaged or harvested by treating with 0.25% trypsin for 5 minutes at 37^0C when the cells reached about 80% confluency. Cells were recovered by centrifugation. (1000RPM for 1 minute.)

GLP-1 secretion assay in STC-1 cells

STC-1 cells were plated at 35,000cells/well into poly-D-lysine-coated 96-well plates (BD bioscience, Cat#356461) and then maintained in a TC incubator for 24 hours. The medium was changed to low glucose (5.5 mM) DMEM (Gibco Cat# 11054) the evening before the assay. On the day of the assay, cells were washed twice with 100 µl per well of EBSS buffer (Earle's Balanced salt Solution: 100 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$-$7H_2O$, 1.65 mM NaH_2PO_4-$2H_2O$, 26 mM $NaHCO_3$, 10 mM HEPES, 1.6 mM $CaCl_2$, 0.1%BSA, 10 mM HEPES) and incubated in 100 µl of the same buffer for 2 hours as a glucose starvation step. During starvation, the positive control PMA (paramethoxyamphetamine) or test compounds were serially diluted at a ratio of 1:3 in DMSO. One and one-half (1.5) µl of compound solution for each concentration was added to 500 µl of assay buffer (EBSS buffer plus 5 mM glucose). After 2 hours of starvation, cells were washed again twice with EBSS buffer and 80 µl of assay buffer with or without compound was added to each well and incubated for 2 hours in a cell culture incubator. 60 µl of cell supernatant was transferred to tubes and sent to -80^0C refrigerator of BARS (Biological Assay Require System) for active GLP-1 detection with luminescence.

Data analysis for GLP-1 secretion assay in STC-1 cells

Results were calculated from the luminescence signal measurements expressed in relative light strength units (R). The amount of secreted GLP-1 in pM was extrapolated from the linear range of the GLP-1 standard curve per plate.

The activity of the test sample was expressed as relative fold increase of GLP-1 compared with DMSO control

Fold increase of GLP-1 = R_{sample}/average of triplicate R_{DMSO}

Where R_{sample} is the concentration of GLP-1 (pM) after incubation with the test compound and R_{DMSO} is the GLP-1 concentration in the DMSO control. The EC50 values were calculated with nonlinear regression using Graphpad Prism.

Cytokine assay

Summary

INS-1E cells (a gift from Prof. C.B. Wolheim, University Medical Center, Geneva, Switzerland) were cultured in 96-well plates. Compounds were prepared for the assay by serial dilution in DMSO. The cells were first treated with the compound or Exendin (positive control) for 5 hr and then with a mixture of cytokines containing 0.4 ng/ml IL-1β, 1 ng/ml IFNγ and 1 ng/ml TNFα for 24 hr. After 24 hr, Caspase Glo® reagent was added to each well and the caspase3/7 activity was measured following manufacturer's (Promega) instructions.

Cell Culture

INS-1E cells were routinely cultured in 15cm diameter culture dishes with RPMI containing 5% FBS at 37$_o$ C, 5% CO_2 and 95% humidity. Cells between passages 60 and 85 were used in all experiments. Subculturing was performed by washing cells once with 1x PBS at RT followed by incubating with 3 ml trypsin at 37$_o$C until all cells have detached. Trypsin was neutralized with 10 ml culture media, cells were resuspended and counted with the Coulter cell counter. INS-1E cells were seeded in a 96-well PDL plate at 40,000 cells in 100µl culture media per well 3 days prior treatment.

Assay method

On day 0, INS-1E cells were plated at 40,000 cells per well in a 96-well PDL plate in 100 µl RPMI growth medium supplemented with 5% FBS. The various cell treatments were initiated on day 3. DMSO was added in RPMI growth medium with 5% FBS 24 hr before cytokine challenge. Exendin-4 was added 5 hr before cytokine challenge in RPMI growth medium. On day 4, cytokines were added in RPMI containing 1% FBS at a final concentration of 0.4 ng/ml IL1-β, 1 ng/ml IFNγ and 1 ng/ml TNFα. Cells were incubated for 24 hr in 100 µl volume per well. Reagents such as Exendin 4 or DMSO were added during cytokine challenge. After incubation with cytokines, 100 µl Caspase Glo reagent was added to each well. The plate was put on a plate shaker for 5 seconds to mix reagents gently and assure proper cell lysis. The plate was incubated for 30 min at RT. The bottom of the plate was taped with a foil plate sealer. Caspase activity was measured at the Wallac Victor 3 plate reader from Perkin Elmer. Data was analyzed with Excel and GraphPad Prism software.

Off-target pharmacology

Compounds **21**, **22** and **3** were tested against a broad panel of enzymes (kinases and proteases), transporters, nuclear hormone receptors and GPCRs to demonstrate that the compounds act as selective GPR39 agonists. Data is reported in micromolar units (K_ds for binding asssays, IC_{50}s and EC_{50}s for functional assays).

Assay/compound	**21**	**22**	**3**
Serotonin 5HT2A receptor, human	>30		3.7
Serotonin 5HT2C receptor, human	>30	>30	28
Adenosin 1 receptor, human	>30	>30	>30
Adenosin 2A receptor, human	>30	>30	>30
Adenosin 3 receptor, human	>30	>30	30

Alpha 1A receptor, human			15
Adrenergic alpha 2B receptor, human	>30	23	>30
Adrenergic alpha 2C receptor, human	1.9	17	3.3
Adrenergic beta 1 receptor, human	>30	>30	>30
Adrenergic beta 2 receptor, human	>30		>30
Angiotensin II AT1 receptor, human	>30	>30	>30
Bradykinin 2 receptor, human	>30	>30	>30
Cholecystokinin A receptor, human	>30	>30	>30
Cholecystokinin B receptor, human	>30	>30	>30
Dopamine 2 receptor, human	17	16	12
Dopamine 3 receptor, human	>30	>30	>30
Endothelin A receptor, human	>30	>30	>30
Ghrelin receptor, human	**>30**	**>30**	**>30**
Histamine H1 receptor, human	>30	>30	>30
Histamine H3 receptor, human	>30	>30	>30
Melanocortin 3 receptor, human	>30	>30	>30
Motilin receptor, human	>30	>30	>30
Muscarinic M1 receptor, human	>30	>30	>30
Muscarinic M3 receptor, human	>30	>30	>30
Neurotensin 1 receptor, human	**>30**	**>30**	**>30**
Opiate delta receptor, human	>30	>30	>30
Opiate kappa receptor, human	8.1	>30	>30
Opiate mu receptor, human	>30		
Thromboxane A2 receptor, human	>30	>30	
Neuropeptide Y1 receptor, human	>30	>30	>30
Neuropeptide Y2 receptor, human	>30	>30	
Vasopressin V1a receptor, human	>30	>30	>30
Vasopressin V2 receptor, human	>30	>30	>30
Benzodiazepine receptor, rat	>30	>30	>30
GABA A receptor, rat	>30	>30	>30
Nicotinic receptor, human	>30	>30	>30
NMDA channel, rat	>30	>30	>30
Serotonin 5HT3 receptor, human	>30	>30	>30
Ca channel L-type, rat	>30		
Estrogen alpha receptor, human	>30	>30	>30
Pregnane X receptor, human	>30	>30	>30
Glucocorticoid receptor, human	>30	>30	>30
Adenosin transporter, human	>30	>30	17
Dopamine transporter, human	>30	>30	15
Norepinephrine transporter, human	>30	>30	>30
COX-1 receptor, bovine	>30		>30
COX-2 receptor, human	>30	>30	>30
Monooamine Oxidase A, human	26	5.6	15
Phosphodiesterase 3, human	>30	>30	25
Phosphodiesterase 4D, human	4.6	5.4	4

5HT1A calcium assay, agonist	>30	>30	>30
5HT1A calcium assay, antagonist	>30		>30
5HT2A calcium assay, agonist	>30	>30	>30
5HT2A calcium assay, antagonist	13.9	14	10.1
5HT2B calcium assay, agonist	>30	>30	>30
5HT2B calcium assay, antagonist	>30	>30	>30
Alpha 1A calcium assay, agonist	>30	>30	>30
Alpha 1A calcium assay, antagonist	>30	>30	6.8
Alpha 2A calcium assay, agonist	>30	>30	>30
Alpha 2A calcium assay, antagonist	4.7	>30	>30
Beta 2 assay, agonist		>30	>30
Cannabinoid 1 assay, agonist	>30	>30	>30
Cannabinoid 1 assay, antagonist	>30	>30	>30
Dopamine D1 agonist	>30	>10	>30
GABA A agonist		>30	>30
GABA A antagonist		>30	>30
Histamine 1 agonist			>30
Histamine 1 antagonist			>30
Histamine 2 agonist	>30	>30	>30
Histamine 2 antagonist			
Muscarinic 2 agonist	>30	>30	>10
Muscarinic 2 antagonist	>30	>30	>30
Androgen receptor agonist	>30	>30	>30
Androgen receptor antagonist	>30	>30	>30
PPARg receptor agonist		>30	>30
PPARg receptor antagonist		>30	>30
Progesterone receptor agonist	>30	>30	>30
Progesterone receptor antagonist	>30	>30	>30
PXR receptor agonist	>30	>30	>30
PXR receptor antagonist	>30	>30	>30
Caspase 3, human	>100	>100	>100
Cathepsin D, human	>100	>100	>100
MMP8, human	>100	>100	>100
Thrombin, human	>100	>100	>100
ABL1 kinase	>10	>10	>10
ALK kinase	>10	>10	>10
AURKA kinase	>10	>10	>10
AXL kinase	>10	>10	>10
BTK kinase	>10	>10	>10
CDK2A kinase	>10	>10	>10
CDK4D1 kinase	>10	>10	>10
EGFR kinase	>10	>10	>10
EPHA4 kinase	>10	>10	>10
EPHB4 kinase	>10	>10	>10
FGFR3 kinase	>10	>10	>10

GSK3b kinase	>10	>10	>10
IGF1R kinase	>10	>10	>10
INSR kinase	>10	>10	>10
JAK1 kinase	>10	>10	>10
JAK2 kinase	>10	>10	>10
JAK3 kinase	>10	>10	>10
KDR kinase	>10	>10	>10
KIT kinase	>10	>10	>10
LCK kinase	>10	>10	>10
MAP3K8 kinase	>10	>10	>10
MAPK1 kinase	>10	>10	>10
MAPK14 kinase	>10	>10	>10
MET kinase	>10	>10	>10
PDGFRa kinase	>10	>10	>10
PDPK1 kinase	>10	>10	>10
PKN1 kinase	>10	>10	>10
PKN2 kinase	>10	>10	>10
PRKACA kinase	>10	>10	>10
PRKCA kinase	>10	>10	>10
PRKCQ kinase	>10	>10	>10
RET kinase	>10	>10	>10
ROCK2 kinase	>10	>10	>10
SYK kinase	>10	>10	>10
TYK2 kinase	>10	>10	>10
ZAP70 kinase	>10	>10	>10

Pharmacokinetic studies

Rat i.v. arm: Male Sprague-Dawley rats (*n= 2 for each compound*) were administered intravenously at 2 mg/kg as a solution of 10% NMP, 30% PEG200 and 5% solutol in water (compound **2**) and at 1 mg/kg as as solution of 2 eq. 0.1 N HCl, 10% PG, 25(20%) solutol, PBS (compound **20**). **p.o. arm**: Male Sprague-Dawley rats (*n= 3 for each compound*) were administered orally by gavage at 5 mg/kg as a solution of 10% NMP, 30% PEG200 and 5% solutol in water (compound **2**) and at 3 mg/kg as a solution of 2 eq. 0.1 N HCl, 10% PG, 25(20%) solutol, PBS (compound **20**).

At the allotted times over 7 h after dosing blood was collected via the tail vein. The plasma was separated by centrifugation. The concentration of the compound was measured in plasma by the LC/MS/MS method after protein precipitation with acetonitrile. Relevant estimated pharmacokinetic parameters for plasma such as clearance and volume of distribution half-life were derived by noncompartmental analysis.

Compound **2**: Cl = 42 ml min^{-1} kg^{-1}, Vss = 11.7 l kg^{-1}, $t_{1/2}$ = 5.8 h, F = 25%.

Compound **20**: Cl = 18 ml min^{-1} kg^{-1}, Vss = 1.1 l kg^{-1}, $t_{1/2}$ = 1.0 h, F = 45%.

Mouse: Male C57/Bl6 mice (*n = 10 for each dose*) were administered at 10 mg/kg, 30 mg/kg and 100 mg/kg by gavage as a suspension of compound **3** in 0.5% methylcellulose/0.1 tween80 in water. At the allotted times between 1 h and 1.5h after dosing blood was collected via tail vein (between 5 and 10 samples per dose and time point). The plasma was separated by centrifugation. The concentration of the compound was measured in plasma by the LC/MS/MS method after protein precipitation with acetonitrile and averaged for each time point and dose.
10 mg/kg: 1.21 μM (60 min), 1.36 μM (65 min), 1.11 μM (90 min)
30 mg/kg: 6.13 μM (60 min), 5.55 μM (65 min), not detrmined
100 mg/kg: 25.34 μM (60 min), 23.25 μM (65 min), 15.13 μM (90 min)

Pharmacodynamic studies
Male C57BL/6 mice (Taconic Laboratories, 11-15 week old, *n = 10 for each dose group*) were dosed by gavage with either vehicle or 30 mg/kg of a suspension of test compound in 0.5% methylcellulose/0.1% tween80 in water concurrently with DPP4 inhibitor PKF275-055 (3 mg /kg, Akarte A.S. et al, *Biochem. Pharmacol.* **2012**, *83*, 241-252). The animals were challenged orally after 1 hour with a glucose bolus (3 g/kg) and active GLP-1 levels were measured 30 min later by MSD Active GLP-1 Assay kit (K150JWC). Curve fitting was performed using MSD DISCOVERY WORKBENCH Data Analysis Tools 3.0 Software. Standards were fitted using the 4-parameter logistical curve with a 1/y2 weighting function. Sample pg/ml concentrations were interpolated from the fit curve. Mean ± SEM for n=10 mice is reported in graph. Data were statistically evaluated with One-way ANOVA followed by Dunnett's post-test. $P<0.001$ versus PKF275-055 alone group. The concentrations of the compounds were measured in the plasma samples as described above.
Vehicle: active GLP-1 = 4.77 pg/ml (± 0.74)
Compound **21**: active GLP-1 = 8.94 pg/ml (± 2.13); 2.05 μM (90 min)
Compound **3**: active GLP-1 = 30.24 pg/ml (± 3.97); 1.57 μM (90 min)