nature chemical biology

Supplementary information

https://doi.org/10.1038/s41589-026-02195-0

Development of a clinically viable MRGPRX4 inverse agonist for cholestatic itch treatment

In the format provided by the authors and unedited

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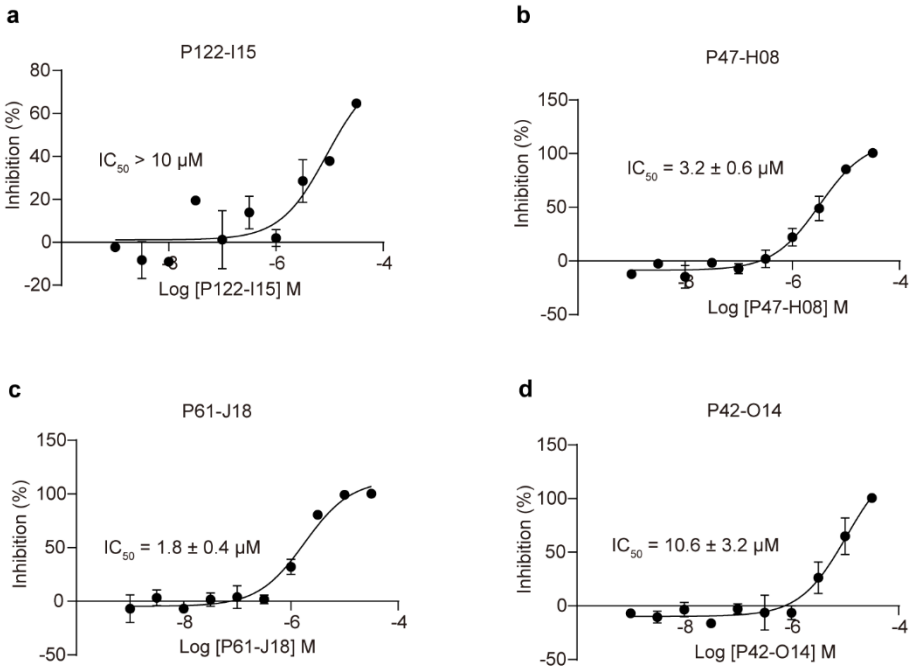
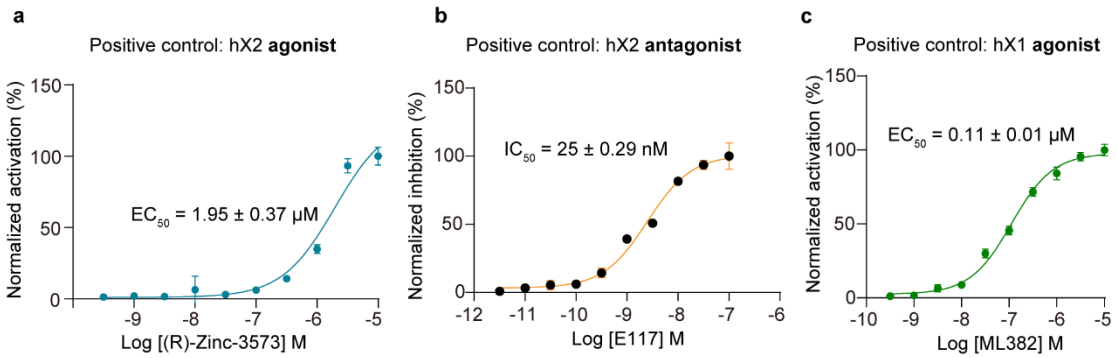
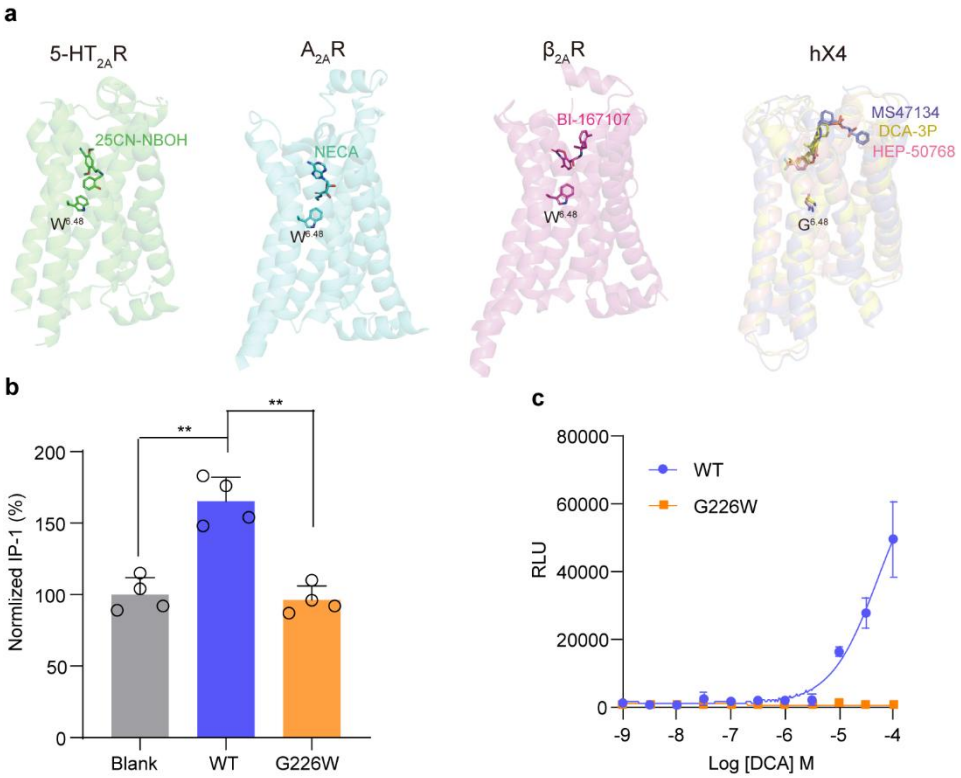
Supplementary Figures



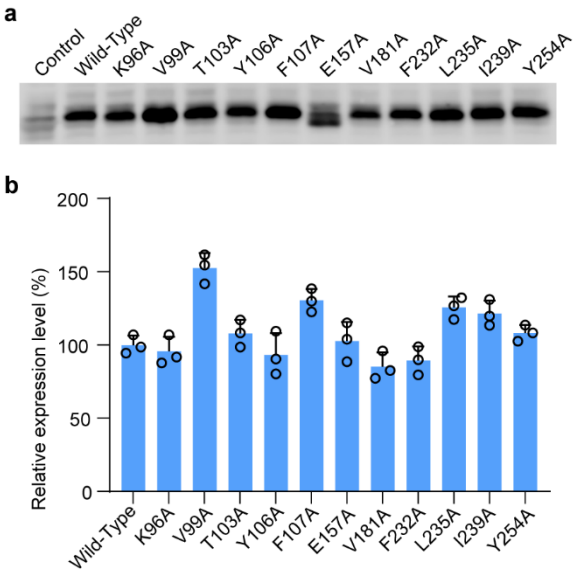
Supplementary Figure 1. Dose-dependent inhibition of hX4 by hit compound P122-I15 (a), P47-H08 (b), P61-J18 (c) and P42-O14 (d). All plots are representative of three biologically independent experiments, with each experimental data point collected with three technical replicates. Data are represented as mean ± SEM.



Supplementary Figure 2. Positive control for hX1 and hX2 activation and inhibition. a, (R)-Zinc-3573 was used as a positive control for hX2 activation. **b,** E117 served as a positive control for hX2 inhibition. **c,** ML382 was used as a positive control for hX1 activation. The activation and inhibition of these controls were evaluated via FLIPR assay. All plots are representative of three biologically independent experiments, with each experimental data point collected with three technical replicates. Data are shown as mean ± SEM.



Supplementary Figure 3. Comparison of residue G6.48 in hX4 with other classical GPCs and function of G6.48 in hX4's activation. a, Agonists of 5-HT_{2A}R, A_{2A}R, and β_{2A}R bind close to the toggle switch $W^{6.48}$, while in hX4, the equivalent position is $G^{6.48}$, which does not directly contact the ligand and has not previously been implicated as a toggle switch. **b-c,** Variant G226W inhibited the basal activity of hX4 **(b)** and the activity of DCA **(c)**. All plots are representative of three biologically independent experiments, with each experimental data point collected with three technical replicates. Data are shown as mean ± SEM.



Supplementary Figure 4. Expression levels of hX4 mutants in HEK293T cells. a, Representative western blot showing the expression of hX4 mutations in HEK293T cells. The plasmids containing the genes of hX4 mutants were transiently transfected into HEK293T cells, and the cells were collected after 48 hours for analysis. **b,** Quantification of relative expression levels of hX4 variants using ImageJ. All the mutations displayed comparable expression levels, indicating that the mutations did not significantly affect protein expression. All plots are representative of three biologically independent experiments, with each experimental data point collected with three technical replicates. Data are shown as mean ± SEM.

Supplementary Tables

Supplementary Table 1. Small molecule screening data.

Category	Parameter	Description
Assay	Type of assay	Cell-based
	Target	Human MRGPRX4 (Uniprot entry: Q96LA9)
	Primary measurement	Detection of compound inhibiting DCA-induced calcium mobilization.
	Key reagents	Fluo-8 No Wash Calcium Assay Kit (AAT Bioquest; Cat No. 36316)
	Assay protocol	Method section
Library	Library size	100,000
	Library composition	About 5000 natural products, 3500 FDA-approval drugs and about 91500 drug-like compounds.
	Source	This study
Screen	Format	384-well plates
	Concentration(s) tested	10 μM, 0.5 % DMSO
	Plate controls	0.5 % DMSO
	Reagent/ compound dispensing system	ECHO
	Detection instrument and software	FLIPR® Tetra (Molecular device)
	Assay validation/QC	Z' score
	Normalization	Inhibition was calculated as the calcium mobilization response of compound-treated samples relative to that of DMSO-treated controls
Post-HTS analysis	Hit criteria	Inhibition higher than 90 %
	Hit rate	0.007 %
	Additional assay(s)	Dose-dependent inhibition of the hits.
	Confirmation of hit purity and structure	Compounds were resynthesized and purity were verified analytically.

Supplementary Table 2. Cryo-EM data collection, refinement and validation statistics

	HEP-50768-bound hX4-Gq-scFv16 complex (Global) (PDB: 9V81)	HEP-50768-bound hX4-Gq-scFv16 complex (Local) (PDB: 9V82)
Data collection and processing		
Magnification	105,000	105,000
Voltage (kV)	300	300
Electron exposure (e-/Å2)	50	50
Defocus range (μm)	-1.0 to -2.2	-1.0 to -2.2
Pixel size (Å)	0.82	0.82
Symmetry imposed	C1	C1
Total micrographs	1758	1758
Initial particle projections	1,712,286	1,712,286
Final particle projections	244,199	244,199
Map resolution (Å)	2.63	2.56
FSC threshold	0.143	0.143
Refinement		
Initial model used (PDB code)	8YRG	8YRG
Model resolution range (Å)	-91.7	-88.5
Model composition		
Non-hydrogen atoms	8139	1905
Protein residues	1085	244
Ligand	1	1
B factors (Å2)		
Protein	37.06	44.46
Ligand	42.90	44.64
R.M.S. deviations		
Bond lengths (Å)	0.006	0.007
Bond angles (°)	0.681	0.726
Validation		
MolProbity score	1.85	1.84
Clashscore	11.52	10.95
Poor rotamer (%)	0.00	0.00
Ramachandran plot		
Favored (%)	95.97	96.83
Allowed (%)	4.03	4.17
Disallowed (%)	0.00	0.00

Supplementary Table 3. IC_{50} of hX4 variants determined via FLIPR assay.

Variants	IC_{50}
WT	0.014 ± 0.001 μM
Y254A	> 10 μM
I235A	0.98 ± 0.18 μM
E157A	> 10 μM
F107A	> 10 μM
V181A	0.11 ± 0.01 μM
Y106A	1.26 ± 0.26 μM
F232A	> 10 μM
T103A	0.14 ± 0.01 μM
V99A	1.12 ± 0.13 μM
K96A	1.04 ± 0.11 μM

Supplementary Table 4. The PK parameters of HEP-50768 in rat plasma/tissue after a single oral gavage of 5 mpk HEP-50768.

Matrix	Sex					
	Male			Female		
	T_{max} (h)	C_{max} (ng/g)	AUC_{last} (h·ng/g)	T_{max} (h)	C_{max} (ng/g)	AUC_{last} (h·ng/g)
Bone Marrow	6	733.83	2201.5	0.5	877.5	5037.83
Brain	6	133.07	399.2	6	168.97	848.36
Fat	6	633.43	5691.74	0.5	1131.33	7185.85
Heart	6	3001.2	33524.5	6	3335.87	39567.13
Kidney	6	26799.13	340105.72	6	26122.27	418617.53
Large Intestine	6	4100.57	58709.76	6	4400	59145.4
Liver	6	32046.2	438679.55	6	34563.13	467258.22
Lung	6	3577.2	39342.6	0.5	4525.97	48199.79
Muscle	6	1337.43	17272.64	0.5	1885.73	17610.98
Ovary	NA	NA	NA	6	2381.83	22722.54
Skin	6	1865.03	18452.84	6	1801.43	18410.84
Small Intestine	0.5	10026.63	80338.96	0.5	32329.5	150555.86
Spinal Cord	NA	NA	NA	NA	NA	NA
Spleen	6	1803.5	21801.33	6	1989.57	24148.41
Stomach	0.5	32996.13	118972.31	0.5	108006.33	388189.74
Testis	6	385.13	3746.42	NA	NA	NA
Plasma*	6	5177.69	61846.47	6	6244.42	73685.85

Supplementary Table 5. Summary of toxicokinetic parameters of HEP-50768 across dosing groups in rhesus monkeys.

	Gender	Dose	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/mL)	$AUC_{0\text{-}24\ h}$ (h·μg/mL)	AUC_{last} (h·μg/mL)
Day 1	Male	5 mpk	5.30	1.60	23.13	171.84	171.84
		25 mpk	6.90	5.20	95.29	1171.28	1171.28
		120 mpk	-	6.00	345.67	4879.51	4897.51
	Female	5 mpk	5.82	1.80	16.83	124.02	124.02
		25 mpk	7.76	3.20	90.86	1031.42	1031.42
		60 mpk	10.83	5.20	205.94	2813.51	2813.51
Day 8	Male	60 mpk	24.86	3.00	298.97	4658.16	4658.16
Day 28	Male	5 mpk	7.66	1.90	15.89	152.43	165.35
		25 mpk	10.35	4.00	86.47	1108.42	1366.39
		60 mpk	19.96	5.50	184.00	2524.11	3608.85
	Female	5 mpk	11.27	2.40	20.76	174.19	198.85
		25 mpk	11.58	2.40	96.93	960.01	1150.10
		60 mpk	10.07	8.40	142.47	251.58	345.43

Supplementary Protocols

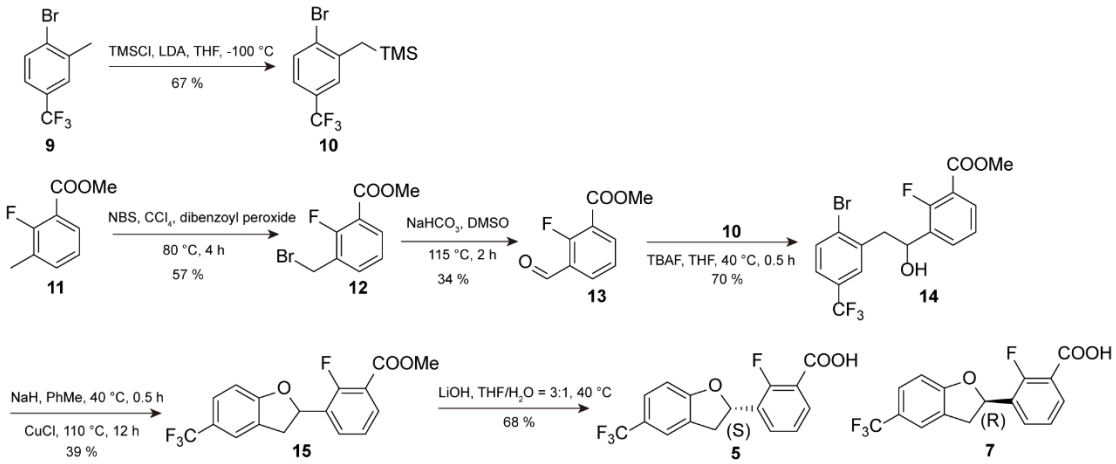
Chemical synthesis of HEP-50768

General information

^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer at ambient temperature with D2O, Chloroform-d, and Methanol-d_4 as the solvent unless otherwise stated. ^{13}C NMR spectra were recorded on a Bruker 100 MHz spectrometer (with complete proton decoupling) at ambient temperature. Data for $_{1}H$ NMR are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, m = multiplet), and coupling constants. Analytical thin layer chromatography was performed using 0.25 mm silica gel 60-F plates. Flash chromatography was performed using 200-300 mesh silica gel. Yields refer to chromatographically and spectroscopically pure materials unless otherwise stated. Tetrahydrofuran was distilled from sodium/benzophenone ketyl prior to use; the other solvents were distilled from calcium hydride unless otherwise noted. Reagents were purchased at the highest commercial quality and used without further purification unless otherwise stated. All reactions were carried out in oven-dried glassware under an argon atmosphere with dry solvents unless otherwise noted.

Synthesis of HEP-21189

Route for chemical synthesis of HEP-21189:



Step 1:

To a solution of LDA (2.09 mL, 0.4 mmol) in THF/n-hexane (1:1) at -100 °C was added a solution of 1-bromo-2-methyl-4-(trifluoromethyl)benzene (**9**) (1.0 g, 0.4 mmol) and chlorotrimethylsilane (0.55 g, 0.5 mmol) in THF (10 mL) over 20 min. After the addition was complete, the mixture was stirred for an additional 20 min at -100 °C, warmed to room temperature over 1 h, and stirred at room temperature for 24 h. The reaction was quenched with 10 mL of water. The organic fraction was separated, and the aqueous fraction was extracted three times with diethyl ether. The combined organic layer was washed with 2 M HCl, 1 M $NaHCO_3$, and water. The organic fraction was dried with $MgSO_4$ and concentrated. The residue was subjected to fractional distillation. The product **10** (874 mg, 67 % yield) was collected at 90 °C at 9 mbar as a colorless oil.

Step 2:

To a mixture of methyl 2-fluoro-3-methylbenzoate (**11**) (100 mg, 0.59 mmol) and N-bromosuccinimide (117 mg, 0.65 mmol) in CCl_4 (2 mL) at room temperature, benzoyl peroxide (2.9 mg, 0.01 mmol) was added, and the mixture was heated at reflux for 2.5 h. After the starting material was consumed, the mixture was concentrated under reduced pressure. The residue was purified by column chromatography to afford **12** (84 mg, 57% yield) as a yellow oil.

^{1}H NMR (400 MHz, Chloroform-d) δ 7.97-7.82 (m, 1H), 7.63-7.51 (m, 1H), 7.19 (t, J = 7.7 Hz, 1H), 4.52 (s, 2H), 3.93 (s, 3H).

Step 3:

To a solution of methyl 3-(bromomethyl)-2-fluorobenzoate (**12**) (84 mg, 0.34 mmol) in DMSO (2 mL) was added $NaHCO_3$ (245mg, 2.9 mmol). The mixture was heated at 115 °C under argon for 2 h. The reaction mixture was diluted with ethyl acetate and washed with brine. The organic layer was dried with Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography to afford **13** (21 mg, 34% yield) as a yellow oil.

^{1}H NMR (400 MHz, Chloroform-d) δ 10.42 (s, 1H), 8.27-8.16 (m, 1H), 8.11-8.00 (m, 1H), 7.34 (t, J = 8.0 Hz, 1H), 3.97 (s, 3H).

Step 4:

Methyl 2-fluoro-3-formylbenzoate (**13**) (21 mg, 0.12 mmol) and (2-bromo-5-(trifluoromethyl)benzyl) trimethylsilane (**10**) (109 mg, 0.35 mmol) were dissolved in THF (2 mL). TBAF solution (1 M in THF, 0.018 mL) was added in one portion. The mixture was stirred at 35–40 °C for 30 min. After the addition of 0.12 mL of TBAF to the system, the solvent was evaporated under vacuum. The crude was acidified with 1 M HCl and extracted with EtOAc three times. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by preparative TLC to afford **14** (35.7 mg, 70% yield) as a yellow solid.

^{1}H NMR (400 MHz, Chloroform-d) δ 7.91-7.84 (m, 1H), 7.76-7.65 (m, 2H), 7.47 (s, 1H), 7.36 (d, J = 8.2 Hz, 1H), 7.22 (d, J = 7.7 Hz, 1H), 5.40 (d, J = 4.2 Hz, 1H), 3.92 (s, 3H), 3.35-3.26 (m, 1H), 3.19-3.08 (m, 1H).

Step 5:

Methyl 3-(2-(2-bromo-5-(trifluoromethyl)phenyl)-1-hydroxyethyl)-2-fluorobenzoate (**14**) (35.7 mg, 0.085 mmol) was dissolved in 2 mL of toluene. Under the nitrogen atmosphere, NaH (6.8 mg, 0.17 mmol) was added to the system with stirring. After stirring at 35-40 °C for 30 min, CuCl (0.4 mg, 0.004 mmol) was added. The system was refluxed for 6-8 h and then cooled to r.t. The reaction mixture was diluted with 5 mL EtOAc and washed with H_2O (2 mL) and brine (5 mL). The organic layer was dried with $MgSO_4$ and concentrated. The residue was purified by silica gel chromatography to give **15** (11.3 mg, 39% yield) as a yellow oil.

^{1}H NMR (400 MHz, Chloroform-d) δ 7.94-7.85 (m, 1H), 7.68-7.59 (m, 1H), 7.49-7.40 (m, 2H), 7.24-7.18 (m, 1H), 6.95 (d, J = 8.3 Hz, 1H), 6.12 (s, 1H), 3.93 (s, 3H), 3.85-3.73 (m, 1H), 3.33-3.04 (m, 1H).

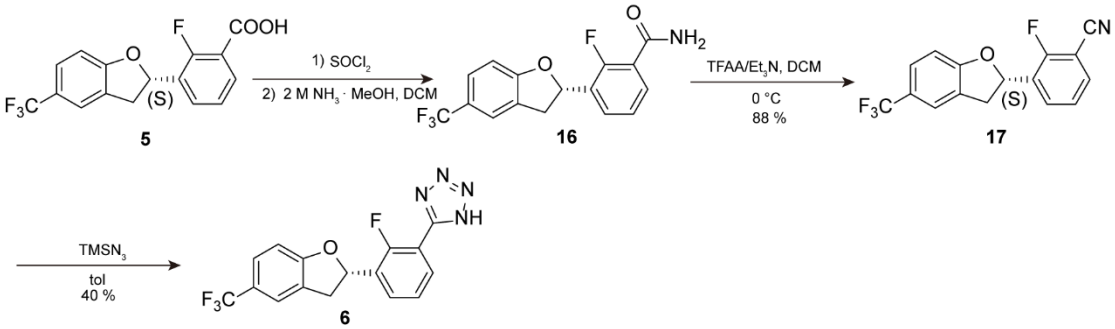
Step 6:

To a solution of **15** (11.3 mg, 0.033 mmol) in THF (0.9 mL) and H2O (0.3 mL), LiOH (4.0 mg, 0.166 mmol) was added, and the mixture was stirred at 40°C overnight. The reaction mixture was acidified with 1 M HCl and extracted with EtOAc three times. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum to give racemic HEP-21189 (7.4 mg, 68% yield) as a red solid. Compounds **5** and **7** were obtained by HPLC chiral separation of racemic HEP-21189. Chiral Pak IE, n-Hexane / EtOH 0.1%TFA=90 / 10(V / V), Rt (**5**) = 4.0 min; Rt (**7**) = 3.7 min.

^{1}H NMR (400 MHz, Methanol-d$_4$) δ 7.88 (s, 1H), 7.65 (s, 1H), 7.55-7.43 (m, 2H), 7.25 (t, J = 7.7 Hz, 1H), 6.98 (d, J = 8.3 Hz, 1H), 6.22-6.05 (m, 1H), 3.91-3.72 (m, 1H), 3.26-3.12 (m, 1H).

Synthesis of HEP-50768

Route for chemical synthesis of HEP-50768



Step 1:

To a solution of **5** (500 mg, 1.533 mmol) in $SOCl_2$ (5 mL), the mixture was stirred at 60 ℃ overnight. The reaction mixture was then concentrated under reduced pressure to yield the residue. And then to (S)-2-fluoro-3-(5-(trifluoromethyl)-2,3-dihydrobenzofuran-2-yl) benzoyl chloride in DCM (5 mL) was added 2M NH_3·MeOH (1.5mL, 3.067 mmol) at 0 ℃. The mixture was stirred at room temperature (RT) for 10 min. Then, the reaction mixture was washed with H_2O and extracted with EtOAc three times. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give the residue. The residue was not purified to provide the crude Compound **16** (526 mg, 105% yield) as a colorless oil.

Step 2:

To a solution of **16** (526 mg, 1.618 mmol) in DCM (5 mL), was added TEA (706 mg, 6.472 mmol) and TFAA (548 mg, 1.942 mmol) at 0 ℃. The mixture was stirred at RT for 2 hours. Then the reaction mixture was washed with H_2O and extracted with EtOAc for three times, the combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give the residue. The residue was purified by column chromatography to yield Compound **17** (440 mg, 88% yield) as a yellow oil. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.77 (dddd, *J* = 7.8, 7.0, 1.8, 0.7 Hz, 1H), 7.65 (ddd, *J* = 7.7, 6.0, 1.7 Hz, 1H), 7.55 – 7.47 (m, 2H), 7.37 – 7.28 (m, 1H), 7.01 (d, *J* = 8.3 Hz, 1H), 6.13 (dd, *J* = 9.8, 7.5 Hz, 1H), 3.86 (dd, *J* = 16.0, 9.8 Hz, 1H), 3.27 – 3.17 (m, 1H).

Step 3:

To a solution of **17** (440 mg, 1.429 mmol) in Tol (5 mL), was added $TMSN_3$ (332 mg, 2.858 mmol) and Dibutyltin oxide (36 mg, 0.1429 mmol) under N_2. The mixture was stirred at 110 ℃ for 12 hours. Then the reaction mixture was stirred in MeOH (5mL) for 30 min and then was washed with H_2O and extracted with EtOAc three times, the combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give the residue. The residue was purified by column chromatography to give the HEP-50768 (200 mg, 40 % yield) as white solid.

^{1}H NMR (400 MHz, D_2O) δ 8.19 – 8.11 (m, 1H), 7.75 (td, *J* = 7.5, 1.8 Hz, 1H), 7.60 – 7.42 (m, 3H), 7.04 (d, *J* = 8.4 Hz, 1H), 6.24 (dd, *J* = 9.9, 7.6 Hz, 1H), 3.91 (dd, *J* = 16.2, 9.9 Hz, 1H), 3.38 (dd, *J* = 8.5 Hz, 1H).

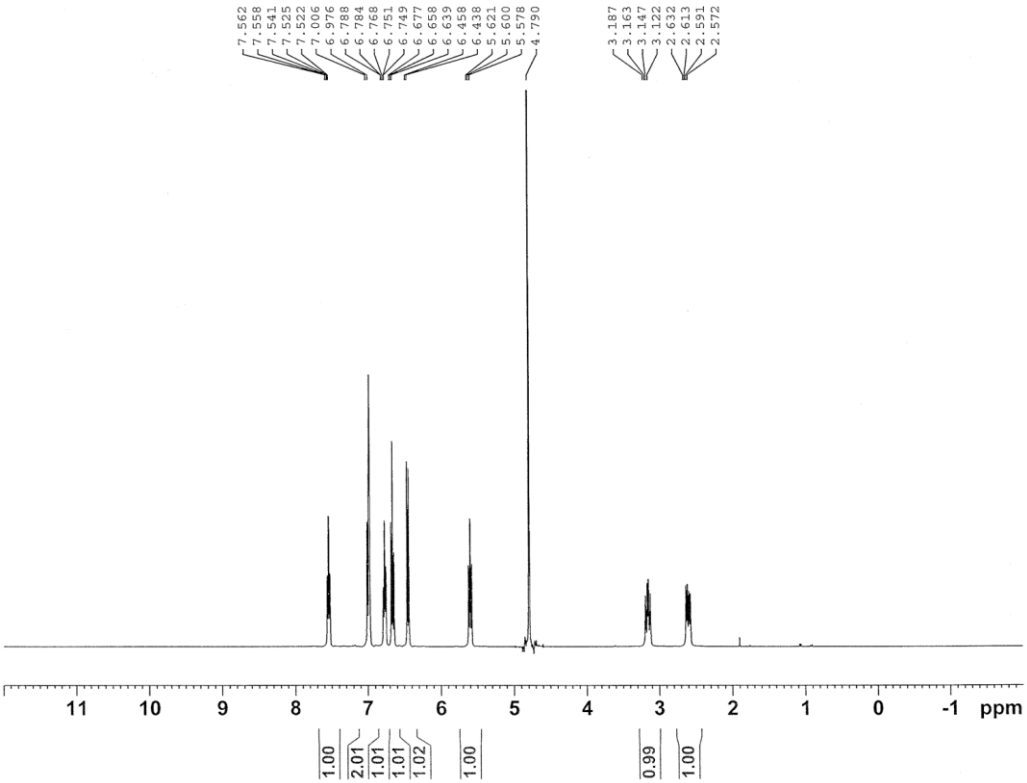
^{13}C NMR (100 MHz, D_2O) δ 160.79, 157.46, 157.41, 154.94, 129.09, 128.68, 128.55, 128.34, 126.89, 126.76, 126.73, 125.71, 125.67, 125.65, 124.18, 124.14, 123.14, 122.95, 122.82, 122.51, 122.19, 121.93, 121.90, 121.86, 121.83, 120.26, 116.81, 116.68, 108.71, 78.79, 78.75, 35.43

^{19}F NMR (400 MHz, D_2O) δ 61.254, 119.114

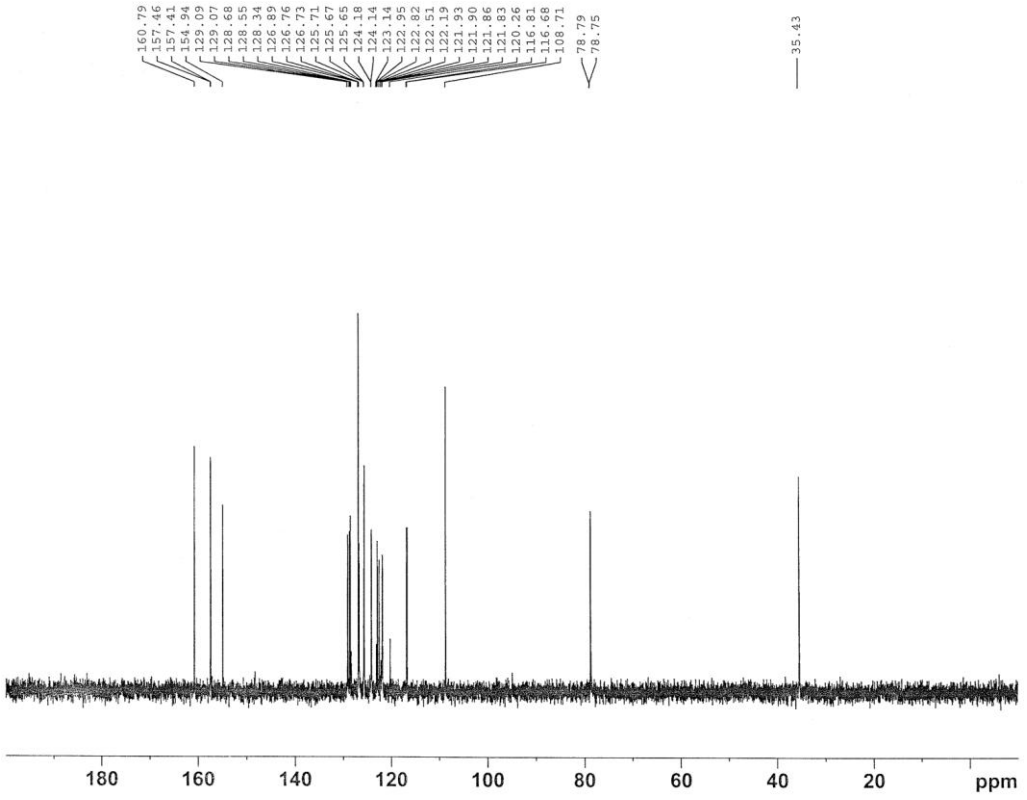
HRMS (ESI) m/z: $[M + H]^-$ calculated for $C_{16}H_9F_4N_4O$: 349.08, found: 349.0722.

NMR spectrum

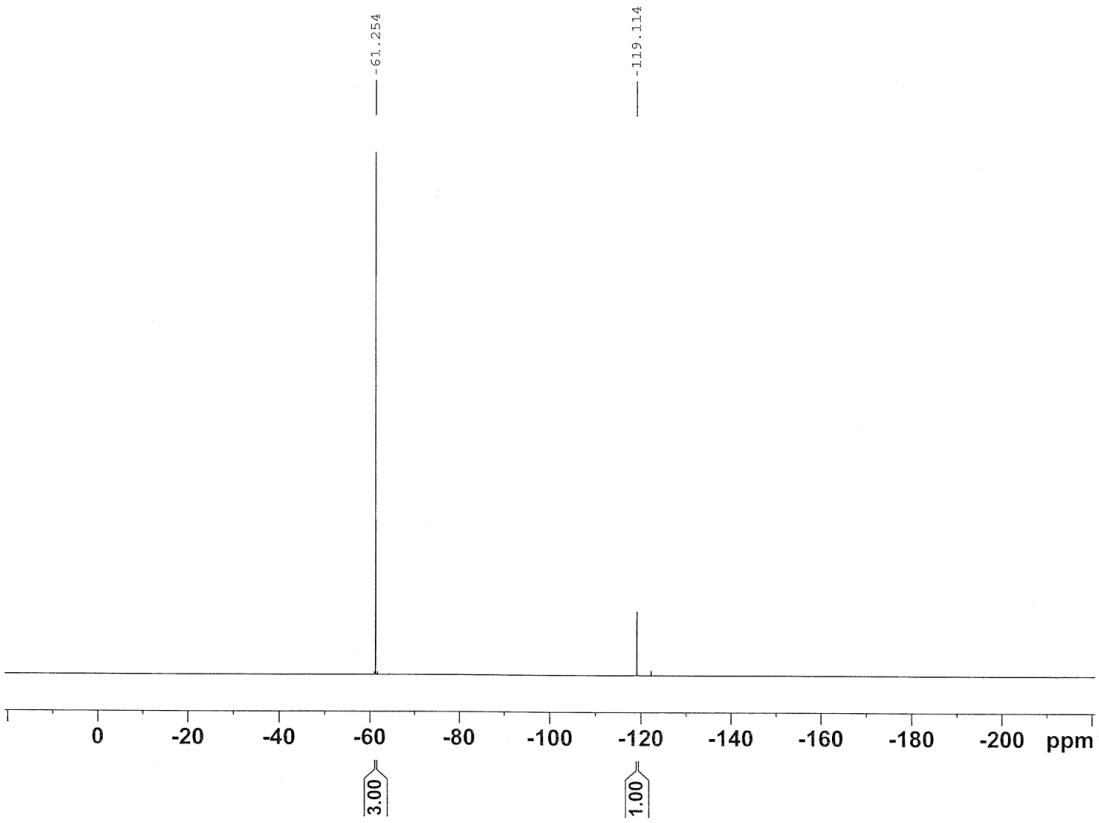
^{1}H NMR spectrum of HEP-50768:



^{13}C NMR spectrum of HEP-50768:



^{19}F NMR spectrum of HEP-50768:



Protocols for assessing a broad panel of 48 potential off-targets.

1.1. GPCR Target Protocol

1.1.1. Assay to test activation (inhibition) of phospholipase C-type Gq pathway receptors

1. 1× Stimulation Buffer was prepared according to the IP-One - Gq kit instructions.
2. 10× working solution of the positive article and the test article were prepared.
3. Cells were digested with trypsin, centrifuged followed by resuspended in 1× Stimulation Buffer. After counting, the cells were cultured in 384-well plate.
4. Added the diluted compound in step 2 to the corresponding experimental well, centrifuged and incubated at 37 ℃ for 1 hour. (In the case of inhibitor mode: added compound after 10 min of incubation, added a step of agonist, and then incubated at 37 ℃ for 1 h to induce IP1 production).
5. IP1-d2 working solution diluted by Lysis & Detection Buffer was added to all wells.
6. Anti-IP1-Cryptate working solution diluted by Lysis & Detection Buffer was added to all wells, and the plate was centrifuged, followed by incubation at room temperature.
7. After incubation, the 665 nm and 620 nm readings were detected by a microplate reader.

Receptors tested by this assay were as follows: V1a, CCK1, H1, ETA, alpha 1A & alpha 2A.

1.1.2. Assay to test activation (inhibition) of phospholipase C-type Gq pathway receptors (Adhesive treatment)

1. Cell plating: Digested and collected cells, resuspend and count in culture medium, and seed into 384-well cell plates. The cell plate was then placed in a 37 ℃, 5 % CO_2 incubator overnight.

2. 1× Stimulation Buffer was prepared according to the IP-One - Gq kit instructions.
3. 10× working solution of the positive article and the test article were prepared.
4. Took out the cell plate, invert the centrifuge to discard the medium, and then added 1× Stimulation Buffer to each experimental well.
5. Added the diluted compound in step 2 to the corresponding experimental well, centrifuged and incubated at 37 ℃ for 1 h. (In the case of inhibitor mode: added compound after 10 minutes of incubation, added a step of agonist, and then incubated at 37 ℃ for 1 h to induce IP1 production).
6. IP1-d2 working solution diluted by Lysis & Detection Buffer was added to all wells.
7. Anti-IP1-Cryptate was diluted to working concentration with Lysis & Detection Buffer, then added to all experimental wells, centrifuged and incubated at room temperature.
8. After incubation, the 665 nm and 620 nm readings were detected by a microplate reader.

Receptors tested by this assay were as follows: M1, M2, M3, 5-HT2A & 5-HT2B.

1.1.3. Assay to test activation (inhibition) of adenylate cyclase-type Gs pathway receptors

1. 1×Stimulation Buffer was prepared according to the LANCE Ultra cAMP kit instructions.
2. 10× working solution of the positive compound and the test compound were prepared.
3. Cells were digested with trypsin, centrifuged followed by resuspended in 1× Stimulation Buffer. After counting, the cells were cultured in 384-well plate.
4. Added the diluted compounds in step 2 to the corresponding experimental wells, centrifuged and incubated at 37 ℃ for 30 min.
   (In the case of inhibitor mode: added compound and incubated for 10 minutes, added a step of agonist, then incubated at 37 ℃ for 30 min to induce cAMP production)
5. Diluted Eu-cAMP to 4× working concentration with Detection buffer, and then added it to the corresponding experimental wells.

6. Diluted ULight-anti-cAMP to 4× working concentration with Detection buffer, then added it to the corresponding experimental wells, and incubated at room temperature for 1 hour after centrifugation.
7. After incubation, reading board with emission wavelength of 665 nm and 620 nm on microplate reader.

Receptors tested by this assay were as follows: β1, β2, D1, H2 & A2A.

1.1.4. Assay to test activation (inhibition) of adenylate cyclase-type Gi pathway receptors

1. 1×Stimulation Buffer was prepared according to the LANCE Ultra cAMP kit instructions.
2. 10× working solution of the positive compound and the test compound were prepared.
3. Cells were digested using trypsin, centrifuged followed by resuspended in 1× Stimulation Buffer. After counting, the cells were cultured in 384-well plate.
4. Then compound in step 2 was added to the wells, and incubation at 37 ℃ for 10 minutes.
5. Forskolin solution was added and incubated at 37 ℃ for 30 min to induce cAMP production.
   (In the case of inhibitor mode: Forskolin & agonist buffer were added, and incubated for 30 minutes to induce cAMP production)
6. Diluted Eu-cAMP to 4× working concentration with Detection buffer, and then added it to the corresponding experimental wells.
7. Diluted ULight-anti-cAMP to 4× working concentration with Detection buffer, then added it to the corresponding experimental wells, and incubated at room temperature after centrifugation.
8. After incubation, reading board with emission wavelength of 665 nm and 620 nm on microplate reader.

Receptors tested by this assay were as follows: CB1, CB2, 5-HT1A, 5-HT1B, D2S, op-delta, op-kappa & op-mu.

1.2. Ion channel Targets Protocol

1.2.1. Manual Patch Clamp Detection Method

Table 3.2.1-1 Manual Patch Clamp Detection Method

Channel	Extracellular solution	Pipette solution	Protocol
Cav1.2	140 mM TEA-Cl, 2 mM $MgCl_2$•$6H_2O$, 10 mM $CaCl_2$•$2H_2O$, 10 mM HEPES, 5 mM D-Glucose, pH=7.4 (TEA OH)	120 mM CsCl, 1 mM $MgCl_2$•$6H_2O$, 10 mM HEPES, 4 mM Mg-ATP, 10 mM EGTA, 0.3 mM Na2-GTP pH=7.2 (CsOH)	When a whole cell seal was formed the cell membrane voltage was clamped at -80 mV. The clamped voltage was depolarized from -80 mV to +10 mV for 0.3 s (refer to the pilot IV test for the specific depolarization voltage) and the data were collected at repeated 20 s intervals.
I_{Ks}	140 mM NaCl, 2.5 mM KCl, 1mM $MgCl_2$•$6H_2O$, 2 mM $CaCl_2$•$2H_2O$, 5 mM D-Glucose, 10 mM HEPES pH=7.4 adjust by NaOH	120 mM K-Aspartic, 5 mM $MgCl_2$•$6H_2O$, 5 mM EGTA, 10 mM HEPES, 4 mM Na_2-ATP, 0.3 mM Na_2-GTP, 14 mM Phosphocreatine disodium salt, pH=7.2(KOH)	When a whole cell seal was formed the cell membrane voltage was clamped at -80 mV. The clamped voltage was depolarized from -80 mV to +60 mV for 5 s and then stepped to -40 mV for 4 s. The current in the I_{Ks} channel could be detected. The data acquisition was repeated every 20 s.
NR1/NR2B	140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$•$2H_2O$, 5 mM D-Glucose, 10 mM HEPES, pH=7.4(NaOH)	110 mM CsMeS, 10 mM NaCl, 2 mM $MgCl_2$•$6H_2O$, 10 mM HEPES, 10 mM EGTA, 2 mM Na_2-ATP, 0.2 mM Na_2-GTP, pH=7.2 (CsOH)	When a whole cell seal was formed the cell membrane voltage was clamped at -70 mV and recorded in Gap-free mode.
$GABA_A$(α1 β2γ2)	140 mM NaCl, 5 mM CsCl, 2 mM $CaCl_2$•$2H_2O$, 1 mM $MgCl_2$•$6H_2O$, 5 mM HEPES,10 mM D-Glucose, pH=7.4 (NaOH)	130 mM CsCl, 0.1 mM $CaCl_2$•$2H_2O$, 2 mM $MgCl_2$•$6H_2O$, 1.1 mM EGTA, 5 mM Na_2-ATP, 10 mM HEPES, pH=7.2 (CsOH)	When a whole cell seal was formed the cell membrane voltage was clamped at -70 mV and recorded in Gap-free mode.

The data of Cav1.2, $I_{Ks,}$ NR1/NR2B and $GABA_A$(α1β2γ2) were collected by EPC-10 amplifier and stored in PatchMaster (HEKA) software. The glass pipette was manipulated using a micro-manipulator under the microscope. After touching the cell, a slight suction was applied to achieve high seal resistance (GΩ). Fast capacitance (in pF) compensation was made after achieving a high seal, and the membrane was broken. Cell capacitance compensation was made from whole-cell capacitance compensation

after the whole-cell mode was achieved. No leak subtraction was made. The cell covered on slides was placed in a chamber under the microscope. The test and control solutions flowed into a recording chamber mounted on the stage of an inverted microscope via a gravity-fed solution delivery system. During the experiment, solutions were withdrawn from the recording chamber by vacuum aspiration. Multiple cells were assayed in independent replicates. All manual electrophysiology experiments were performed at room temperature.

1.2.2. Auomatic Patch Clamp Detection Method

Table 3.2.2-1 Auomatic Patch Clamp Detection Method

Ion channel	Extracellular solution	Pipette solution	Protocol
Nav1.5	137 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2•6H_2O$, 2 mM $CaCl_2•2H_2O$, 5 mM D-Glucose, 10 mM HEPES, pH=7.4(NaOH)	10 mM CsCl, 5 mM NaCl, 10 mM HEPES, 135 mM CsF, 5 mM EGTA, pH=7.2 (CsOH)	When a whole cell seal was formed the cell membrane voltage was clamped at -120 mV. the clamped voltage was stepped from -120 mV to -30 mV for 50 ms (refer to the pilot IV test for the specific voltage) and the data was repeated every 10 s.
hERG	140 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2•6H_2O$, 2 mM $CaCl_2•2H_2O$, 10 mM, D-Glucose, 10mM HEPES, 1.25 mM $NaH_2PO_4•2H_2O$, pH=7.4(NaOH)	20 mM KCl, 115 mM K-Aspartic, 1mM $MgCl_2•6H_2O$, 5 mM EGTA, 10 mM HEPES, 2 mM Na_2-ATP, pH=7.2 (KOH)	When a whole-cell seal was formed the cell membrane voltage was clamped at -80 mV. The clamped voltage was depolarized from -80 mV to -50 mV for 0.5 s (as a leakage current detection), then stepped to 30 mV for 2.5 s, and then rapidly recovered to -50 mV for 4 s to excite the tail current of the hERG channel. The data acquisition was repeated every 10 s.

hERG and Nav1.5 data were recorded by Automated Patch Clamp (APC) system, QPatch 48 X (Sophion), which was used for electrophysiological assay.
Placed the prepared cells on the centrifuge of the Qpatch work plane, washed the cells with multiple centrifugation/suspension times, and replaced the cell culture medium with extracellular solution. Took out an MTP-96 plate and placed it on the MTP source position. Took out the QPlate chip, and then put the QPlate in the Qplate source position. The barcode reader scans the barcode of MTP-96 board and QPlate chip and grabbed it to the measurement slot. Took the reference, intracellular and extracellular solution from the saline reservoir and added them to the intracellular and extracellular fluid pool, the cell and the test article pool of the QPlate chip. For the measuring, all the measuring points of QPlate were under the initial quality control. The quality control process

included sucking the cell suspension from the cell container of the centrifuge, positioning the cells on the chip hole by the pressure controller, establishing a high-impedance seal, and forming a whole-cell recording mode. Once a stable control current baseline was obtained, the test article could be drawn from the MTP-96 plate according to the multiple concentration and applied to the cells. The current detected in each cell in the external solution without compound served as its own control, and the assay was repeated independently for two cells. All Qpatch electrophysiology experiments were performed at 24 ℃.

1.2.3. FLIPR assay for 5-HT3A and nAChRα4β2 targets

1. Digested and collected cells. After counting, inoculated cells in black bottom transparent 384-well plates and cultured overnight.
2. Prepared 1× buffer according to the kit instructions, and used 1× buffer to prepared 2× dyes for use;
3. Invert the centrifuge to remove the medium in the 384-well plate, and immediately added 18 μL 1× buffer.
4. Added the 18 μL dye prepared in step 2 to the corresponding experimental wells and incubated at 37℃ in the dark.
5. Prepared 10× positive control and test article intermediate solutions, transferred it to the corresponding 384 source plate.
6. Prepared 5× agonist intermediate solutions, transferred it to the corresponding 384 source plate.
7. After the incubation, 4 μL of the test substance prepared in step 5 was added to the test wells using the FLIPR instrument, and the data was recorded for 5 minutes; then 10 μL of the agonist prepared in step 6 was incubated for 15 minutes and the data was recorded for 5 minutes. The calcium flow detection excitation light is 470-515 nm and emission light is 515-575 nm.
   Note: nAChRα4β2, 5-HT3A were detected with calcium flow kit.

1.3. Enzyme Assay

1.3.1. Test compounds inhibition on COX1 and COX2 enzyme activity

1. Diluted the test compound in ultrapure water or pure water.
2. Used the Echo to transfer 200× compound to the reaction plate and centrifuged at 1000 rpm for 1 minute.
3. Transferred 2.5 μL of 4× enzyme to the plate, centrifuged at 1000 rpm for 1 min, added 2.5 μL of 4× Hemin mix to the reaction plate, centrifuged at 1000 rpm for 1 min, and incubated at 25℃ for 5 minutes.
4. Transferred 2.5 μL of 4× ADHP to a 384 reactions plate and centrifuged at 1000 rpm for 1 min.
5. Transferred 2.5 μL of 4× AA (arachidonic acid) mixed solution with KOH to a 384 reactions plate and centrifuged at 1000 rpm for 1 min.
6. Read the signal with a microplate reader (excitation wavelength 540 nm, emission wavelength 590 nm).

1.3.2. Test compounds inhibition on PDE3Aand PDE4D2 enzyme activity

1. The test compound was diluted with ultrapure water or pure water, and 0.1 μL of the compound was transferred to 384 Wells and centrifuged at 1000rpm for 1 min.
2. Added 2.5 μL of 4× PDE enzyme into 384-well plate, centrifuge at 1000 rpm for 1 min.
3. Added 2.5 μL 4× cAMP to 384-well plate, centrifuge at 1000 rpm for 1 min, and incubate at 25 ℃ for 75 min.
4. Added 5 μL Anti-cAMP-d2 (20×) and cAMP-Cryptate (20×) mixture to 384-well plate, centrifuged at 1000 rpm for 1 minute, and incubated at 25 ℃ for 60 min.
5. Read HTRF signal with BMG (ratio 665/620 nm).

1.3.3. Test compounds inhibition on LCK enzyme activity

1. Diluted the test compound in ultrapure water or pure water.
2. Transferred 200× of the test compound to the target plate using an Echo, and centrifuged at 1000 rpm for 1 min.
3. Added 2.5 μL of 2× kinase/metal ion solution to the plate and incubated at 25℃ for 10 min.
4. Added 2.5 μL/well of 2× substrate /ATP solution to the assay plate and incubated at 25℃ for 60 min.
5. Configured XL665 and antibody detection reagent with detection buffer and set aside.
6. Added 5 μL of Kinase Detection Reagent to the plate and incubated at 25℃ for 60 min.
7. The fluorescence signals of 620 nm (Cryptate) and 665 nm (XL665) were read by BMG microtiter-plate reader.

1.3.4. Test compounds inhibition on AchE enzyme activity

1. Diluted the test compound in ultrapure water or pure water.
2. Transferred 200× compound to the reaction plate using the Echo and centrifuged at 1000 rpm for 1 min.
3. Added 10 μL/well 2× AchE mixture to assay plate, centrifuged at 1000 rpm for 1 min, and incubated at 25℃ for 10 min.
4. Transferred 10 μL of 2× (ATC, DNTB) mixture to the reaction plate, centrifuged at 1000 rpm for 1 min, and incubated at 25℃ for 90 min.
5. Read 405 nm OD signal with BMG microplate reader.

1.3.5. Test compounds inhibition on MAO A enzyme activity

1. Diluted the test compound in ultrapure water or pure water.
2. Transferred 200× compound to the reaction plate using the Echo and centrifuged at 1000 rpm for 1 min.

3. Added 2.5 μL of 2× MAO-Substrate solution to the plate and centrifuged at 1000 rpm for 1 min.
4. Transferred 2.5 μL of 2× MAO enzyme to the plate and centrifuged at 1000 rpm for 1 min. Incubate at 25 ℃ for 20 min.
5. Transferred 5 μL of MAO-Glo and centrifuged at 1000 rpm for 1 min. Incubated at 25 ℃ for 20 min.
6. Reading on a microplate reader (Luminescence).

1.3.6. Determination of compound inhibition of INSR enzyme activity

1. Diluted the subjects with ultrapure water or pure water.
2. Used Echo to transfer 200× subjects to the target plate and centrifuged at 1000 rpm for 1 minute.
3. Added 2 μL of 2× kinase/metal ion solution to the plate and incubated for 10 minutes at 25℃.
4. Added 2 μL of 2× substrate/ATP solution to the plate and incubated at 25℃ for 60 minutes.
5. Added 4 μL of ADP-Glo assay reagent to the plate and incubated at 25℃ for 40 minutes.
6. Added 8 μL Detection reagent to the plate and incubated at 25℃ for 40 minutes.
7. Read the chemiluminescence signal (Luminescence) with an enzyme marker.

1.3.7. Test compounds inhibition on ROCK1 enzyme activity

1. Diluted the subjects with ultrapure water or pure water.
2. Used Echo to transfer 200× subjects to the target plate and centrifuged at 1000 rpm for 1 minute.
3. Added 2 μL of 2× kinase/metal ion solution to the plate and incubated for 10 minutes at 25℃.
4. Added 2 μL of 2× substrate/ATP solution to the plate and incubated at 25℃ for 60 minutes.
5. Added 4 μL of ADP-Glo assay reagent to the plate and incubated at 25℃ for 40 minutes.
6. Added 8 μL Detection reagent to the plate and incubated at 25℃ for 40 minutes.
7. Read the chemiluminescence signal (Luminescence) with an enzyme marker.

1.3.8. Test compounds inhibition on VEGFR2 enzyme activity

1. Diluted the subjects with ultrapure water or pure water.
2. Used Echo to transfer 200× subjects to the target plate and centrifuged at 1000 rpm for 1 minute.
3. Added 2 μL of 2× kinase/metal ion solution to the plate and incubated for 10 minutes at 25℃.
4. Added 2 μL of 2× substrate/ATP solution to the plate and incubated at 25℃ for 60 minutes.
5. Added 4 μL of ADP-Glo assay reagent to the plate and incubated at 25℃ for 40 minutes.
6. Added 8 μL Detection reagent to the plate and incubated at 25℃ for 40 minutes.
7. Read the chemiluminescence signal (Luminescence) with an enzyme marker.

1.4. Luciferase Reporter Assays for Nuclear Receptors

1. The first day: HEK-293 cells or luc2P-GAL4-HEK293 cells were seeded at 6 cm dish.
2. The second day: Transfection. HEK-293 cells were transfected with pBIND-AR /pGL4.35/AR ORF plasmids, and luc2P-GAL4-HEK293 cells were transfected with pBIND-GR plasmid.
3. The third day: Cells were digested, resuspended and counted in phenol red-free DMEM medium containing 5% carbon-adsorbed serum, and seeded into white cell culture plates. Compounds were then added and incubated overnight.
   (Inhibitor mode: first added compound and incubated for 30 minutes, then added agonist solution and continued to incubate overnight).
4. The fourth day: Luminescence activity was assayed 20-24h post-induction with Bright-Glo Luciferase Assay System. 96-well plates containing cells were removed from the incubator. Bright-Glo Reagent was added to each well, vibrated to allow complete cell lysis, and then measured luminescence value using multi-function enzyme-labeled instrument.

Receptors tested by this assay were as follows: AR & GR.

1.5. Reuptake Assay of Transporters

1. Cells were seeded in 384-well plate, and incubated overnight.
2. Prepared 1× Assay Buffer according to the instructions of Neurotransmitter Transporter Uptake Assay Kit.
3. Prepared 2× working solution of positive compound and test substance.
4. Remove the medium from the 384-well plate by centrifugation. Added the diluted compounds in step 3 to the corresponding experimental wells, centrifuged and incubated at 37℃ for 30 minutes.
5. Prepared detection reagent with HBSS solution, added an equal volume of detection reagent to each well, and incubated at 37℃ after centrifugation.
6. After incubation, used a multi-plate reader to detect the reading at 528 nm, and the excitation light is 450 nm.

Receptors tested by this assay were as follows: DAT, NET, 5-HTT.

2. Data Analysis

1) M1, M2, M3, 5-HT2A, 5-HT2B, V1a, CCK1, H1, ETA, alpha 1A, alpha 2A, β1, β2, D1, H2, A2A, CB1, CB2, 5-HT1A, 5-HT1B, D2S, op-delta, op-kappa, op-mu, AR, GR, $GABA_A$(α1β2γ2), 5-HT3A & nAChRα4β2:

$$\% \text{ Activation} = \left|\frac{Ratio_{cmpd} - \overline{Ratio}_{vehicle}}{\overline{Ratio}_{pozitive} - \overline{Ratio}_{vehicle}}\right| * 100$$

$$\% \text{ Inhibition} = \left|\frac{Ratio_{cmpd} - \overline{Ratio}_{vehicle}}{\overline{Ratio}_{pozitive} - \overline{Ratio}_{vehicle}}\right| * 100$$

2) DAT, NET, 5-HTT, COX1, COX2, MAO A, PDE3A, PDE4D2, ACHE, INSR, ROCK1, VEGFR2 & LCK:

$$\% \, Inhibition = \left|\frac{Ratio_{cmpd} - \overline{Ratio}_{vehicle}}{\overline{Ratio}_{pozitive} - \overline{Ratio}_{vehicle}}\right| * 100$$

3) Calculate IC_{50} or EC_{50} by fitting to non-linear regression (dose response – variable slope) with GraphPad

Y=Bottom + (Top-Bottom)/(1+10^((LogIC_{50}-X) ×HillSlope))

Y=Bottom + (Top-Bottom)/(1+10^((LogEC_{50}-X) ×HillSlope))

4) Data analysis of hERG channel current

Within each recording, the current responses to the test compound were normalized

to control $\left(\frac{Peak\ tail\ current_{compound}}{Peak\ tail\ current_{vehicle}}\right)$ and the ratios of inhibition were calculated $\left(1-\left(\frac{Peak\ tail\ current_{compound}}{Peak\ tail\ current_{vehicle}}\right)\right)$.

5) Data analysis of Nav1.5, I_{Ks} and Cav1.2 channels current

Within each recording, the current responses to the test compound were normalized to control $\left(\frac{Peak\ current\ compound}{Peak\ current\ vehicle}\right)$ and the ratios of inhibition were calculated$\left(1-\frac{Peak\ current\ compound}{Peak\ current\ vehicle}\right)$.

6) Data analysis of NR1/NR2B receptors agonist effect

First within each recording, the current responses to the test compound were normalized to the agonist $\left(\frac{Peak\ current\ compound}{Peak\ current\ agonist}\right)$. The agonist of NR1/NR2B was Glycine+L-Gluatemate, and its agonistation effect formual was $\left(\frac{Peak\ current\ (compound+Glycine)}{Peak\ current\ agonist}\right)$. Means and standard errors were calculated for each test concentration ratio.

7) Data analysis of NR1/NR2B receptors antagonist effect

First within each recording, the current responses to the test compound were normalized to the agonist $\left(\frac{Peak\ current\ agonist+compound}{Peak\ current\ agonist}\right)$, and the ratios of inhibition were calculated$\left(1-\frac{Peak\ current\ agonist+compound}{Peak\ current\ agonist}\right)$, then calculated the average.