Supplementary materials

Synthesis and Agonistic Activity at the GPR35 of 5,6-Dihydroxyindole-2-carboxylic Acid Analogs

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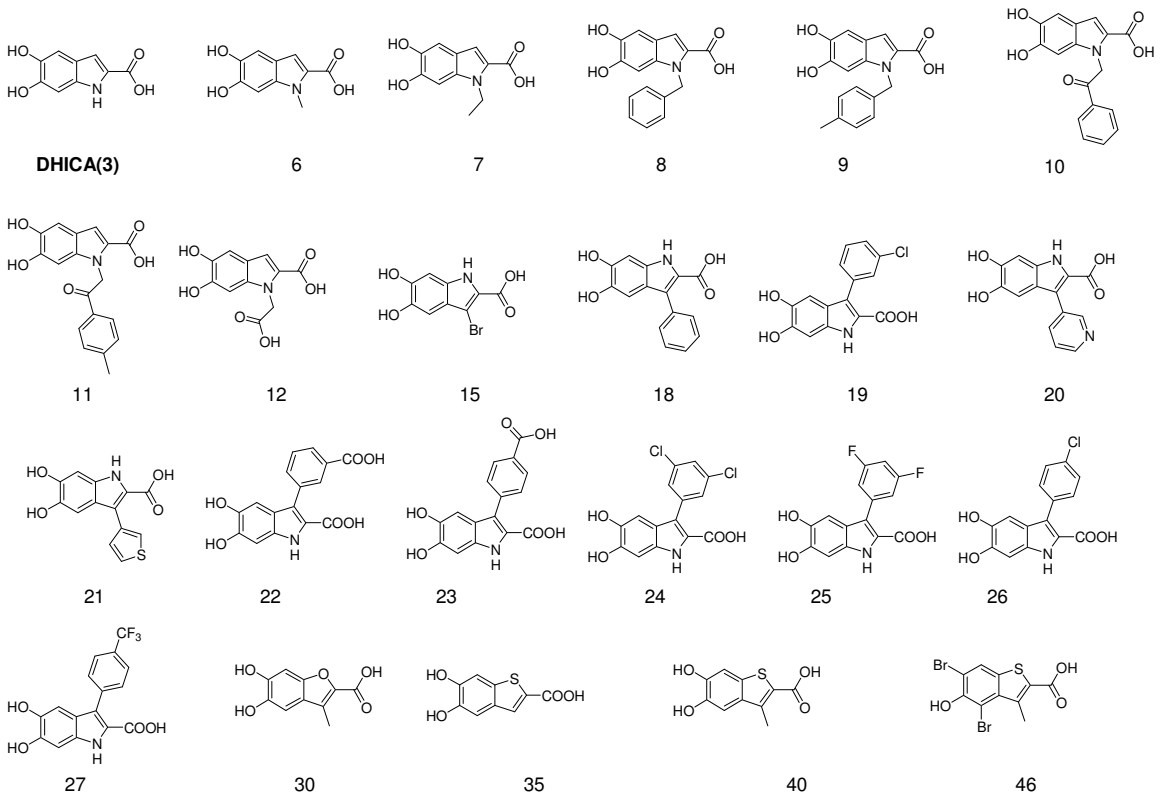

Supplementary Figure 1 Structures, full names and abbreviations of DHICA analogs.

3 (5,6-Dihydroxyindole-2-carboxylic acid, DHICA)

6 (N-methyl-5,6-dihydroxyindole-2-carboxylic acid, N-methyl-DHICA)

7 (N-Ethyl-5,6-dihydroxyindole-2-carboxylic acid, N-ethyl-DHICA)

8 (N-benzyl-5,6-dihydroxyindole-2-carboxylic acid, N-benzyl-DHICA)

9 (N-(4-methylbenzyl)-5,6-dihydroxyindole-2-carboxylic acid, N-(4-methylbenzyl)-DHICA)

10 (N-(2-oxo-2-phenylethyl)-5,6-dihydroxyindole-2-carboxylic acid, N-(2-oxo-2phenylethyl)-DHICA)

11 (N-(2-oxo-2-p-tolylethyl)- 5,6-dihydroxyindole-2-carboxylic acid, N-(2-oxo-2-p-tolylethyl)-DHICA)

12 (N-carboxymethyl-5,6-dihydroxyindole-2-carboxylic acid, N-carboxymethyl-DHICA)

15 (3-Bromo-5,6-Dihydroxy-1H-indole-2-carboxylic acid, 3-bromo-DHICA)

18 (5,6-Dihydroxy-3-phenyl-1H-indole-2-carboxylic acid, 3-phenyl-DHICA)

19 (3-(3-chlorophenyl)-5,6-dihydroxy-1H-indole-2-carboxylic acid, 3-(3-chlorophenyl)-DHICA)

20 (5,6-Dihydroxy-3-(pyridin-3-yl)-1H-indole-2-carboxylic acid, 3-(pyridin-3-yl)-DHICA)

21 (5,6-Dihydroxy-3-(thiophen-3-yl)-1H-indole-2-carboxylic acid, 3-(thiophen-3-yl)-DHICA)

22 (3-(3-carboxyphenyl)- 5,6-dihydroxy-1H-indole-2-carboxylic acid, , 3-(3-carboxyphenyl)-DHICA)

23 (3-(4-carboxyphenyl)- 5,6-dihydroxy-1H-indole-2-carboxylic acid, , 3-(4-carboxyphenyl)-DHICA)

24 (3-(3,5-dichlorophenyl)-5,6-dihydroxy-1H-indole-2-carboxylic acid, , 3-(3,5-dichlorophenyl)-DHICA)

25 (3-(3,5-difluorophenyl)-5,6-dihydroxy-1H-indole-2-carboxylic acid, , 3-(3,5-difluorophenyl)-DHICA)

26 (3-(4-chlorophenyl)-5,6-dihydroxy-1H-indole-2-carboxylic acid, 3-(4-chlorophenyl)-DHICA)

27 (3-(4-trifluoromethyl)phenyl)-5,6-dihydroxy-1H-indole-2-carboxylic acid, 3-(4-trifluoromethyl)-phenyl)-DHICA)

32 (5,6-Dihydroxy-3-methylbenzofuran-2-carboxylic acid, 3-methyl-DHFCA)

35 (5,6-dihydroxybenzo[b]thiophene-2-carboxylic acid, DHTCA)

40 (5,6-dihydroxy-3-methylbenzo[b]thiophene-2-carboxylic acid, 3-methyl-DHTCA)

46 (4,6-dibromo-5-hydroxy-3-methylbenzo[b]thiophene-2-carboxylic acid)

Supplementary Table 1. Compounds, NMR and mass spectroscopy characterization.

Compound	^{1}H NMR (300 MHz, δ ppm)	Mass Spec (LCMS)
3(DHICA)	DMSO-d_6, 12.41 (s,1H), 11.11 (s, 1H), 8.07 (s, 1H), 8.56 (s, 1H), 6.87 (m, 3H)	ES- 192 (M-H)
6	DMSO-d_6, 12.37 (s, 1H), 9.17(s, 1H), 8.85 (s, 1H), 6.98 (s, 1H), 6.92(s, 1H), 6.80 (s, 1H), 3.89 (s, 3H)	ES+ 208 $(M+H)^+$
7	CD_3OD, 8.85 (s, 1H), 7.07 (s, 1H), 6.94 (s, 1H), 6.80 (s, 1H), 4.50 (q, *J*=7.2Hz, 1H), 3.89 (s, 1H), 1.31 (t, *J*=7.2Hz, 1H)	ES+ 222 $(M+H)^+$
8	CD_3OD, 7.26-7.17 (m, 4H), 7.03-6.98 (m, 3H), 6.71 (s, 1H), 3.31 (s, 3H)	ES+ 284 $(M+H)^+$
9	CD_3OD, 7.17 (s, 1H), 7.08 (d, *J*=8.1Hz, 2H), 6.99 (s, 1H), 6.95 (d, *J*=8.1Hz, 2H), 6.74 (s, 2H), 2.29 (s, 3H)	ES+ 298 $(M+H)^+$
10	CD_3OD, 8.09-8.07 (m, 2H), 7.69-7.64 (m, 1H), 7.57-7.52 (m, 2H), 7.16 (s, 1H), 7.10 (s, 1H) 6.67 (s, 1H), 5.96 (s, 1H)	ES+ 312 $[M+H]^+$
11	CD_3OD, 7.97 (d, *J*=7.8Hz, 2H), 7.35 (d, *J*=7.8Hz, 2H), 7.16 (s, 1H), 6.99 (s, 1H), 6.67 (s, 1H), 5.92 (s, 1H), 2.42(s, 3H)	ES+ 326 $[M+H]^+$
12	DMSO-d_6, 12.68 (s, 2H), 9.11 (s , 1H), 8.87 (s, 1H), 7.01(s, 1H), 6.91 (s, 1H), 6.78 (s, 1.H), 5.14 (s, 2.H), 3.36 (s, 3.H)	ES+ 251 $[M+H]^+$
15	DMSO-d_6, 12.88 (m, 1H), 11.49 (s, 1H), 9.35 (s, 1H), 8.98 (s, 1H), 6.81 (s, 1H), 6.75 (s, 1H)	ES- 270, 272 (M-H)
18	DMSO-d_6, 12.35 (m, 1H), 11.20 (s, 1H), 9.12 (s, 1H), 8.66 (s, 1H), 7.46-7.22 (m, 5H), 6.94 (s, 1H), 6.76 (s, 1H)	ES- 268, (M-H)
19	CD_3OD, 7.41 (m, 4H), 6.86 (s, 1H), 6.84 (s, 1H)	ES- 302 (M-H)
20	DMSO-d_6, 11.74 (m, 1H), 9.09 (s, 1H), 8.83 (m, 1H), 8.69 (m, 1H), 8.09 (m, 1H), 6.92 (s, 1H), 6.85 (s, 1H)	ES- 269, (M-H)
21	DMSO-d_6, 12.58 (m, 1H), 11.17 (s, 1H), 9.20 (m, 1H), 7.57 (m, 1H), 7.34 (m, 1H), 6.90 (s, 1H), 6.83 (s, 1H)	ES- 274 (M-H)
22	CD_3OD, 8.18 (s, 1H), 7.97 (m, 1H), 7.74 (m, 1H), 7.52 (m, 1H), 6.87 (s, 1H), 6.85 (s, 1H)	ES- 312 (M-H)
23	CD_3OD, 8.07 (d, 2H), 7.63 (d, 2H), 6.97 (m, 2H)	ES- 312 (M-H)
24	CD_3OD, 7.42 (m, 3H), 6.87 (s, 1H), 6.84 (s, 1H)	ES- 336 (M-H)
25	DMSO-d_6, 12.61 (m, 1H), 11.41 (s, 1H), 9.20 (s, 1H), 8.80 (s, 1H), 7.17 (m, 3H), 6.86 (s, 1H), 6.81 (s, 1H)	ES- 304 (M-H)
26	CD_3OD, 7.48 (d, 2H), 7.40 (d, 2H), 6.86 (s, 1H), 6.83 (s, 1H)	ES- 302 (M-H)
27	CD_3OD, 7.59 (m, 4H), 6.85 (m, 2H)	ES- 336 (M-H)
32	DMSO-d_6, 12.90 (s, 1H), 9.59 (s, 1H), 9.09 (s, 1H), 6.92 (m, 2H), 2.41 (s, 3H)	ES- 207 (M-H).
35	DMSO-d_6, 12.98 (s, 1H), 9.69 (m, 1H), 9.37 (s, 1H), 7.83 (s, 1H), 7.25 (m, 2H)	ES- 209 (M-H)
40	DMSO-d_6, 12.89 (m, 1H), 9.71 (m, 1H), 9.38 (m, 1H), 7.22 (s, 1H), 7.17 (s, 1H) , 2.59 (s, 3H)	ES- 223 (M-H)
46	DMSO-d_6, 13.62 (m, 1H), 9.92 (s, 1H), 8.34 (s, 1H), 3.04 (s, 3H)	ES- 363, 365(M-H)

Materials and Methods

Compounds and Reagents

All DHICA analogs were synthesized through CRO with BioDuro Chemical Co. (Beijing, P.R.China), and were received with a purity >95%. Zaprinast was obtained from Enzo Life Sciences (Plymouth Meeting, PA). Kynurenic acid was obtained from Sigma Chemical Co. (St. Louis, MO). SPB05142 was obtained from Ryan Scientific, Inc. (Mt. Pleasant, SC). YE210 and Epic® 384-well biosensor microplates were obtained from Corning Inc. (Corning, NY, USA).

Cell Culture

Human colorectal adenocarcinoma HT-29 was obtained from American Type Cell Culture (Manassas, VA, USA). The cells were cultured in McCoy's 5a Medium Modified supplemented with 10% fetal bovine serum, 4.5g/liter glucose, 2 mM glutamine, and antibiotics at 37^{o}C under air/5% CO_2. Tango™ GPR35-bla U2OS cells were purchased from Invitrogen. The cells were cultured according to the protocols recommended by the supplier. Briefly, the cells were passed using McCoy's 5A medium (Invitrogen 16600-082) supplemented with 10% dialyzed fetal bovine serum, 0.1 μM NEAA, 25 μM Hepes (pH 7.3), 1mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml zeocin, 50 μg/ml hygromycin, and 100 μg/ml geneticin in a humidified 37°C/5% CO_2 incubator.

Dynamic mass redistribution (DMR) assays

Epic® system (Corning Inc., Corning, NY), a wavelength interrogation reader system tailored for resonant waveguide grating biosensors in microtiter plates, was used for dynamic mass redistribution (DMR) assays. This system consists of a temperature-control unit (26^{o}C), an optical detection unit, and an on-board liquid handling unit with robotics. The detection unit is centered on integrated fiber optics, and enables kinetic measures of cellular responses with a time interval of ~15sec. For whole cell DMR assays, a common cell preparation procedure was used – that is, cells were directly seeded in Epic® plates with a seeding density of 32,000 cells per well and cultured overnight to form confluent monolayer in the corresponding cell culture medium; and the cells were then washed twice using plate washer, and maintained with Hank's balanced salt solution (1x HBSS) and further incubated inside the system for 1hr before measurements.

For DMR agonism profiling, a 2-min baseline was first established after the cell preparation step. Immediately after the compound addition using the onboard liquid handler, the cellular responses were recorded in real time for about 1hr. For DMR antagonist profiling, the cells were pretreated with SPB05142 at different doses for 5min, followed by stimulation with a compound at a fixed dose. All

EC_{50} or IC_{50} described in the main text were calculated based on the amplitudes of DMR signals at 10 min post agonist stimulation. All DMR signals were background corrected.

Tango™ β-arrestin translocation gene reporter assays

Tango™ GPR35-*bla* U2OS cells were obtained from Invitrogen. This cell line stably expresses two fusion proteins: human GPR35 linked to a TEV protease site and a Gal4-VP16 transcription factor, and β-arrestin/TEV protease fusion protein. The cell line also stably expresses the β-lactamase reporter gene under the control of a UAS response element. The cells were cultured according to the protocols recommended by the supplier. The activation of GPR35 by agonists leads to the recruitment of β-arrestin/TEV protease fusion proteins to the activated GPR35. As a result, the protease cleaves the Gal4-VP16 transcription factor from the receptor, which then translocates to the nucleus and activates the expression of β-lactamase.

For Tango assays, 10000 cells per well were seeded in 384-well, black-wall, clear bottom assay plates with low fluorescence background (Corning), and cultured in Dulbecco's modified eagle medium (Invitrogen,10569-010) supplemented with 10% dialyzed fetal bovine serum, 0.1 μM non-essential amino acids, 25 μM Hepes (pH 7.3), 100 U/ml penicillin, and 100 μg/ml streptomycin. After overnight culture, the cells were stimulated with ligands for 5 hrs in a humidified 37°C/5% CO_2, and then loaded with the cell permeable LiveBLAzer™ FRET B/G substrate. After the two hour incubation the coumarin to fluorescein ratio was measured using Tecan Safire II microplate reader (Männedorf, Switzerland). In the absence of β-lactamase expression (i.e., no GPR35 activation), cells generate green fluorescence. In the presence of β-lactamase expression upon receptor activation, the substrate is cleaved and the cells generate blue fluorescence. The coumarin to fluorescein ratio was calculated. All results obtained were normalized to the zaprinast maximal responses using an intra-plate referencing protocol; that is, a dose response of zaprinast was obtained within the same plate, and a compound response was then normalized to the maximal response of zaprinast which was set to be 100%.

Synthesis and characterization of compound 26

Compound **26** (3-(4-chlorophenyl)-5,6-dihydroxy-1H-indole-2-carboxylic acid, 3-(4-chlorophenyl)-DHICA) was synthesized as follows.

First, ethyl 3-(4-chlorophenyl)-5,6-dimethoxy-1H-indole-2-carboxylate was synthesized by added Na_2CO_3 (200 mg, 1.88 mmol) and $Pd(dppf)_2Cl_2$ (37 mg, 0.05 mmol) under N_2 to a solution of ethyl 3-bromo-5,6-dimethoxy-1H-indole-2-carboxylate (300 mg, 0.92 mmol), 4-chlorophenylboronic acid (190 mg, 1.2 mmol) in dioxane (10 mL) and water (2 mL). The mixture was stirred for 16 hour at 100 ^oC and concentrated. The resulting mixture was puri-6fied by flash column chromatography on silica gel

(petroleum ether/EtOAc =4:1) to give ethyl 3-(4-chlorophenyl)-5,6-dimethoxy-1H-indole-2-carboxylate (230 mg, 70% yield) as yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 9.90 (s, 1H), 7.46 (m, 4H), 6.89 (s, 1H), 6.88 (s, 1H), 4.28 (q, 2H), 3.96 (s, 3H), 3.86 (s, 3H), 1.26 (t, 3H).

Second, 3-(4-chlorophenyl)-5,6-dimethoxy-1H-indole-2-carboxylic acid was synthesized by adding LiOH (68 mg, 2.96 mmol) to the solution of ethyl 3-(4-chlorophenyl)-5,6-dimethoxy-1H-indole-2-carboxylate (230 mg, 0.64 mmol) in EtOH/H_2O(5 mL/5 mL). The mixture was stirred for 16 hours at 50 ^{o}C. After removal of the solvent, the residue was adjusted pH to 2~3, filtered, the filter cake was dried to give 3-(4-chlorophenyl)-5,6-dimethoxy-1H-indole-2-carboxylic acid (180 mg, 85% yield) as off-white solid.

Third, compound **26** was synthesized by adding BBr_3 (0.1 mL, 0.18mmol) dropwise at -78 ^{o}C to the solution of 3-(4-chlorophenyl)-5,6-dimethoxy-1H-indole-2-carboxylic acid (180mg, 0.54 mmol) in CH_2Cl_2 (10 mL). The mixture was stirred for 18 hours at room temperature. The mixture was quenched with water (5 mL), filtered, the filter cake was purified by pre-HPLC to give compound **26** (10 mg, 6% yield) as off-white solid: ^{1}H NMR (300 MHz, CD3OD) δ ppm 7.48 (d, 2H), 7.40 (d, 2H), 6.86 (s, 1H), 6.83 (s, 1H). LCMS (m/z) ES- 302 (M-H).

Synthesis and characterization of compound 46

To a solution of 2-bromo-5-methoxybenzaldehyde (4.0 g, 18.6 mmol) in THF (80 ml) was added methylmagnesium bromide (20 mL, 20 mmol) dropwise at -78 ^{o}C under N_2. After stirring for 30 minutes, the reaction was quenched with water (200 mL) and extracted with EtOAc (80 mL*3). After removal of the solvent, the residue was purified by flash column chromatography on *silica gel* (petroleum ether/EtOAc =10:1) to give 1-(2-bromo-5-methoxyphenyl)ethanol (3.8 g, 88% yield) as yellow oil.

Next, to a solution of 1-(2-bromo-5-methoxyphenyl)ethanol (3.8 g, 16.5 mmol) in CH_2Cl_2 (100 mL) was added PCC (8.4 g, 33.0 mmol). The mixture was stirred for 16 hours at room temperature. The reaction was filtered and the filtrate was concentrated and purified by flash column chromatography on *silica gel* (petroleum ether/EtOAc =5:1) to give 1-(2-bromo-5-methoxyphenyl)ethanone (3.6 g, 95% yield) as off-white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.45 (d, 1H), 6.96 (d, 1H), 6.82 (dd, 1H), 4.38 (q, 2H), 3.80(1, 3H), 2.62 (s, 3H).

Next, to a solution of 1-(2-bromo-5-methoxyphenyl)ethanone (1.4 g, 6.1 mmol) and K_2CO_3 (1.7 g, 12.2 mmol) in DMF (30 mL) was added ethyl 2-mercaptoacetate (0.96 g, 7.93 mmol) dropwise at 70 ^{o}C, a catalytic amount of 18-crown-6 was added. After stirring continued for 16 hours at 80 ^{o}C, the reaction was quenched with water (200 mL) and extracted with EtOAc (50 mL*3). The combined organic layers were dried over Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography on *silica gel* (petroleum ether/EtOAc =10:1) to give ethyl 5-methoxy-3-

methylbenzo[b]thiophene-2-carboxylate (900 mg, 63% yield) as off-white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.67 (d, 1H), 7.21 (d, 1H), 7.11 (dd, 1H), 4.38 (q, 2H), 3.89(1, 3H), 2.73 (s, 3H), 1.41 (t, 3H).

Next, to the solution of ethyl 5-methoxy-3-methylbenzo[b]thiophene-2-carboxylate (300mg, 1.2 mmol) in DCM (10 mL) was added BBr_3 (0.2 mL, 2.4 mmol) dropwise at -78 ^{o}C. The mixture was stirred for 6 hours at room temperature. The mixture was quenched with water (10 mL), extracted with EtOAc (10 mL*3). The combined organic layers were dried over Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography on *silica gel* (petroleum ether/EtOAc =2:1) to give ethyl 5-hydroxy-3-methylbenzo[b]thiophene-2-carboxylate (60 mg, 21% yield) as off-white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 13.26 (m, 1H), 9.67 (s, 1H), 7.79 (d, 1H), 7.22 (s, 1H), 7.07 (d, 1H), 2.64 (s, 3H).

Next, to the solution of ethyl 5-hydroxy-3-methylbenzo[b]thiophene-2-carboxylate (60mg, 0.25 mmol) in HOAc (5 mL) was added Br_2 (120 mg, 0.75 mmol) at 0 ^{o}C. The mixture was stirred for 16 hours at room temperature. The mixture was quenched with water (10 mL), extracted with EtOAc (10 mL*3). The combined organic layers were dried over Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography on *silica gel* (petroleum ether/EtOAc =2:1) to compound ethyl 4,6-dibromo-5-hydroxy-3-methylbenzo[b]thiophene-2-carboxylate (30 mg, 30% yield) as off-white solid.

Lastly, to the solution of ethyl 4,6-dibromo-5-hydroxy-3-methylbenzo[b]thiophene-2-carboxylate (30 mg, 0.076 mmol) in EtOH/H_2O (5 mL/5 mL) was added LiOH (10 mg, 0.38 mmol). The mixture was stirred for 16 hours at room temperature. After removal of the solvent, the residue was adjusted to pH 2~3, extracted with EtOAc (10 mL*3). The combined organic layers were dried over Na_2SO_4. After removal of the solvent, the residue was purified by pre-HPLC to compound 46 (4,6-dibromo-5-hydroxy-3-methylbenzo[b]thiophene-2-carboxylic acid) (8 mg, 29% yield) as off-white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 13.62 (m, 1H), 9.92 (s, 1H), 8.34 (s, 1H), 3.04 (s, 3H). LCMS (m/z) ES- 363, 365(M-H).