Supporting Information

Inhibition of Glucose Dependent Insulin Secretion by a Potent, Selective sst3 Antagonist

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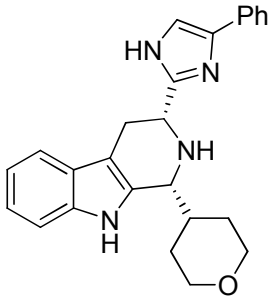
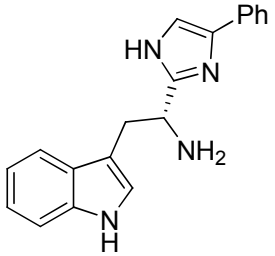


5a

Preparation of (1*R*)-2-(1*H*-indol-3-yl)-1-(4-phenyl-1*H*-imidazol-2-yl)ethanamine



Details for the preparation of (1*R*)-2-(1*H*-indol-3-yl)-1-(4-phenyl-1*H*-imidazol-2-yl)ethanamine are described below and in reference 15: Poitout, L.; Roubert, P.; Contour-Galcera, M.-O.; Moinet, C.; Lannoy, J.; Pommier, J.; Plas, P.; Bigg, D.; Thurieau, C. Identification of Potent Non-Peptide Somatostatin Antagonists with sst3 Selectivity. *J. Med. Chem.* **2001**, *44*, 2990-3000.

Step A: *tert*-butyl[(1*R*)-2-(1*H*-indol-3-yl)-1-(5-phenyl-1*H*-imidazol-2-yl)ethyl]carbamate

To a solution of *N*-(*tert*-butoxycarbonyl)-D-tryptophan (5.00 g 16.4 mmol) in DMF (33 mL), were added 2-bromo-1-phenylethanone (3.30 g 16.4 mmol) and cesium carbonate (5.8 g 18 mmol), and the resulting mixture was stirred at room temperature overnight. The reaction mixture was diluted with EtOAc (60 mL) and washed twice with brine (30 mL). The organic phase was dried over anhydrous sodium sulfate. The solvents were removed and the residue was dissolved in toluene (30 mL). Ammonium acetate (3.80 g 49.5 mmol) was added to reaction mixture. The reaction mixture was then stirred at reflux for 3 h using a Dean-Stark trap to remove water. The reaction mixture was cooled to room temperature, diluted with EtOAc (200 mL) and washed twice with water (50 mL), then once with $NaHCO_3$ solution (50 mL) and twice with brine (25 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by silica gel column (Ethyl acetate - Hexanes 4:6 to 9:1 gradient) to afford *tert*-butyl[(1*R*)-2-(1*H*-indol-3-yl)-1-(5-phenyl-1*H*-imidazol-2-yl)ethyl]carbamate (4.1 g 10.1 mmol, 62%).

^{1}H NMR (DMSO-d_6) δ 7.75 (d, J = 7.5 Hz, 1H), 6.93-7.55 (m, 10H), 4.85 (m, 1H), 3.35 (m, 1H), 3.16 (m, 1H), 1.32 (s, 9H);

LC/MS m/e 403 ($M+H^+$, calcd. for C24H27N4O2, 403).

Step B: (1*R*)-2-(1*H*-indol-3-yl)-1-(5-phenyl-1*H*-imidazol-2-yl)ethanamine

The *tert*-butyl[(1)-2-(1*H*-indol-3-yl)-1-(5-phenyl-1*H*-imidazol-2-yl)ethyl]carbamate (4 g 10 mmol) was dissolved in 4M HCl in dioxane (50 mL) at room temperature and stirred overnight. All the solvents were removed under reduced to afford (1*R*)-2-(1*H*-indol-3-yl)-1-(5-phenyl-1*H*-imidazol-2-yl)ethanamine.

^{1}H NMR (CD3OD) δ 7.87 (s, 1H), 7.73 (m, 2H), 7.47-7.52 (m, 4H), 7.35 (d, J = 8 Hz, 1H), 7.19 (s, 1H), 7.10 (t, J = 7.5 Hz, 1H), 7.00 (t, J = 7.5 Hz, 1H), 5.11 (m, 1H), 3.84-3.89 (m, 1H), 3.73-3.79 (m, 1H);

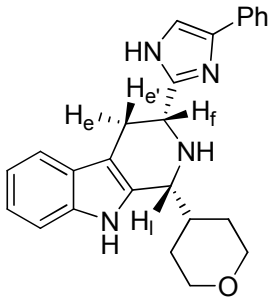
LC/MS m/e 303 ($M+H^+$, calcd. for C19H19N4, 303).

(1*R*,3*R*)-3-(4-phenyl-1*H*-imidazol-2-yl)-1-(tetrahydro-2*H*-pyran-4-yl)-2,3,4,9-tetrahydro-1*H*-carboline (**5a**)

A solution of (1*R*)-2-(1*H*-indol-3-yl)-1-(4-phenyl-1*H*-imidazol-2-yl)ethanamine (3.02 g, 10.0 mmol) in DCM-TFA (1:1, 20mL) at room temperature was treated with tetrahydro-2*H*-pyran-4-carbaldehyde (1.20 g 10.5 mmol) in DCM 20 mL at room temperature and the resulting mixture was stirred for 5h. The reaction mixture was diluted with DCM 100 mL, washed twice with water (100 mL), twice with saturated sodium bicarbonate solution (100ml) and the organic phase was dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography EtOAC-Hex (8:2) to afford (1*R*,3*R*)-3-(4-phenyl-1*H*-imidazol-2-yl)-1-(tetrahydro-2*H*-pyran-4-yl)-2,3,4,9-tetrahydro-1*H*-carboline (**5a**, 2.8 g, 7.0 mmol, 70%) as the major diastereomer product.

^{13}C NMR (CDCl3) δ 26.8, 28.0, 29.3, 39.6, 52.8, 57.3, 68.2, 110.1, 110.7, 118.2, 119.6, 121.9, 124.8, 126.9, 127.2, 128.7, 133.9, 136.1, 150.5.
^{1}H NMR (CDCl3) δ 8.09 (1H, s), 7.22 (2H, d J= 6Hz), 7.46 (1H, d J=7Hz), 7.38 (2H, t J=8Hz), 7.33 (1H, d J=6Hz), 7.30 (1H, s), 7.26 (2H, m), 7.16 (1H, t J=5Hz),7.10 (1H, d J=5Hz), 2.48 (1H, dd J=4Hz J=9Hz), 4.23 (1H, s), 4.04 (1H, dd J=4Hz J=9Hz), 3.93 (1H, dd J=4Hz J=9Hz), 3.42 (1H, t J=11Hz), 3.45-3.31 (2H, m), 2.95 (1H, t J=11Hz), 2.10-2.05 (1H, m), 2.10-2.05 (1H, m), 1.87-1.78 (1H, m), 1.63--1.58 (2H, m), 1.26 (1H, d J=6Hz)
HRMS m/e 399.2188 ($M+H^+$, calcd. For C25H27N4O, 399.2185).

The stereochemistry of **5a** was determined by NMR (CD_3OD) to be *cis*. Selective excitation of the methine signal at 4.31 ppm (labeled as H-l) led to a strong NOE in the 1D NOE NMR spectrum to the methine signal at 4.23 ppm (labeled as H-f). Additionally, five-bond homoallylic coupling was observed in the 1D 1H NMR spectrum between the protons H-e and H-l, with coupling constants of 5Je',l (cis) = 1.7 Hz and Je,l (trans) = 2.4 Hz. The larger homoallylic coupling between the trans protons H-e/H-l together with the large coupling between protons H-e/H-f supports the cis stereochemistry determined by NOESY.



5a

2) COMPOUND PURITY/HPLC DATA

Compound purities were determined by two diverse HPLC conditions. Final compounds all had purities >95%. Two conditions are as follows:

HPLC conditions #1:

Column: Waters Sunfire 20 mm X 2.1 mm X 3.5 μM
Solvents
A = 0.1% formic acid in water

B = 0.1% formic acid in acetonitrile

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HP1100 LC Pump Initial Conditions

Solvents
A% 0.1% formic acid in water        95.0
B% 0.1% formic acid in acetonitrile 5.0
C%                                  0.0
D%                                  0.0
Valve A set to channel
Valve B set to channel
Flow (ml/min)                             3.000
Stop Time (mins)                    1.8
Min Pressure (bar)                        0
Max Pressure (bar)                        400
Oven Temperature Left(°C)                 30.0
Oven Temperature Right(°C)                50.0

HP1100 LC Pump Gradient Timetable

The gradient Timetable contains 6 entries which are :

Time     A%  B%     C%    D%  Flow  Pressure
0.00   95.0   5.0   0.0   0.0 3.000   400
1.00    5.0  95.0   0.0   0.0 3.000   400
1.25    2.0  98.0   0.0   0.0 3.000   400
1.59    2.0  98.0   0.0   0.0 3.000   400
1.60   95.0   5.0   0.0   0.0 3.000   400
1.75   95.0   5.0   0.0   0.0 3.000   400


HP1100 PDA Spectrum
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Storage type : All
Start Range (nm)                    220
End Range (nm)                            400
Range Interval (nm)                       4.0
Threshold (mAU)                           0.1
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HPLC conditions #2:

Column: Waters Xterra 50 mm X 3 mm X 3.5 μM

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HP1100 LC Pump Initial Conditions

Solvents
A = 0.05% TFA in water
B = 0.05% TFA in acetonitrile

Solvents
A%                                  90.0
B%                                  10.0
C%                                  0.0
D%                                  0.0
Valve A set to channel                    1
Valve B set to channel                    1
Flow (ml/min)                             1.000
Stop Time (mins)                    5.5
Min Pressure (bar)                        0
Max Pressure (bar)                        400
Oven Temperature Left(°C)                 40.0
Oven Temperature Right(°C)                30.0

HP1100 LC Pump Gradient Timetable

The gradient Timetable contains 5 entries which are :

   Time          A%        B%    C%    D%  Flow (ml/min)     Pressure
    0.00   90.0    10.0   0.0   0.0 1.000   400
    3.75    2.0    98.0   0.0   0.0 1.000   400
    4.75    2.0    98.0   0.0   0.0 1.000   400
    4.76   90.0    10.0   0.0   0.0 1.000   400
    5.50   90.0    10.0   0.0   0.0 1.000   400
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3) IN VITRO ASSAYS

Cell line Generation:

Human, mouse, rat, dog and Rhesus SSTR3 stable cell-lines were generated in regular CHO or the Flp-In CHO cells (Invitrogen, Cat# R758-07), and were used as single cell clone or stable pools for each species in cAMP assays. The full-length cDNA of SSTR3 from all species [NCBI Accession#: NM_001051 (human), NM_009218 (mouse), NM_133522 (rat), NM_001025620 (dog) and XM_001086007 (Rhesus)] were cloned into the pSC015 (for human) or pEF5/FRT/V5/Dest vector (for rat, mouse, dog and monkey SSTR3) using conventional molecular biology cloning techniques. For generation of Flp-In stable lines, the expression plasmids were transfected using Lipofectamine 2000 (Invitrogen Life Technologies) following manufacturer's

instructions followed by antibiotic selection. The stable CHO cell lines for human SSTR1, SSTR2, SSTR4 and SSTR5 were made by transfecting the CHO cells with the pSC015 vectors carrying corresponding full-length cDNA followed by antibiotic selection.

SSTR Functional Assay Methods:

PerkinElmer Lance cAMP assay was used to determine the agonist (in the presence of 3.5 µM forskolin) and antagonist activities (in the presence of 3.5 µM forskolin plus SS-14 (0.2 nM, without serum or 0.4 nM, with 10% serum) of compounds on recombinant SSTR3 expressed in CHO cells. In brief, stable cell lines expressing all SSTR3 were grown in media as described in the Method section for the binding assays. On the day of the cAMP assays, cells were detached with Cellstriper (Cellgro, Cat#25-056-CI) and resuspended in the stimulation assay buffer, containing HBSS with calcium and magnesium, (Invitrogen, Cat# 14025-076), 0.05% DTPA Purified BSA (7.5%, PerkinElmer # CR84-100), 0.5µM IBMX (Sigma-Aldrich# I7018) and 5 mM HEPES buffer (Invitrogen # 15630080). Each well (384 Optiplate, PerkinElmer) was incubated with 5,000 cells and increasing concentrations of compound for 15 min at 37°C and 5% CO_2, followed by addition of challenge buffer (stimulation assay buffer plus 3.5 µM forskolin and SS-14 at 0.2 or 0.4 nM according to assay type). Plates were incubated for 30 min at 37°C and 5% CO_2, followed by addition of detection buffer and 1 hour incubation at room temperature. Results were acquired using Envision plate reader (PerkinElmer Life and Analytical Sciences, Inc. Waltham, MA) as time-resolved fluorescence (TRF) at 665 nm and 615 nm. Data were analyzed as 4-parameter fits using Assay Data Analyzer (Merck and Co.).

SSTR Binding Assay Methods:

Chinese Hamster Ovary (CHO) cells expressing recombinant human, mouse, rat, dog or Rhesus monkey SSTR3 receptors were grown in growth media containing alpha-MEM (Gibco#12571), 10 mM HEPES (Gibco#15630-080), 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin, G418 (0.5mg/mL, Gibco#10131) for human SSTR3; alpha-MEM (Hyclone SH30265.02), 10% fetal bovine serum, 10 mM HEPES, 1% penicillin-streptomycin, hygromycin (500 µg/mL) for mouse SSTR3; or Ham's F12 (Gibco #11765), 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin-streptomycin, hygromycin (500 µg/mL) for rat, dog and Rhesus SSTR3. Cells were seeded and grown to near-confluence in 10-layer cell factories for three days. The medium was removed and the monolayers were washed twice with 400 mL PBS, then the cells were detached in 600 mL Enzyme-free dissociation buffer (Specialty Media). The cell suspension was centrifuged at 2,200 rpm for 10 min and the cell pellet was washed with 40mL/factory of assay buffer (50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM EGTA, 10 µg/mL leupeptin, 200 µg/mL bacitracin, 0.5 µg/mL aprotinin, 10 µg/mL pepstatin) then homogenized in 40 mL assay buffer/factory with 8-10 strokes using glass-teflon Glas-Col grinder at setting 40. After gently rocking for 15 min at room temperature, they were re-homogenized as per previous step then centrifuged at 2,200 rpm for 10 min at 4°C. The resulting supernatant was collected and centrifuged in a Sorvall SS-34 rotor for 20 min at 18,000 rpm at 4°C. The final pellet was resuspended in 10 mL assay buffer/factory with five passages sequentially through a syringe with 18-, 23- and 25-gauge needles. Protein

content was determined using the Bradford Protein Assay (BioRad) with BSA as a standard. Aliquots were stored at -80 °C.

For competition binding studies, membranes expressing human, mouse, rat, dog or Rhesus SSTR3 receptors were incubated for 60-90 min at room temperature in 250 µL assay buffer containing with 5 µL of serial dilutions of compounds and 40 µL of [^{125}I]SS-14 (100 pM final). Non-specific binding was generally determined in wells with the addition of 2 µM unlabeled SSTR3 antagonist 5a. The assay was terminated by vacuum filtration over GF/C filters presoaked in 0.2% PEI and washing with ice cold wash buffer containing 50 mM Tris, pH 7.8. After the filters dried, 50 µL Microscint20 was added prior to sealing the plates for counting the radioactivity using a TopCount (Packard). Data were analyzed using Assay Data Analyzer software (Merck and Co.).

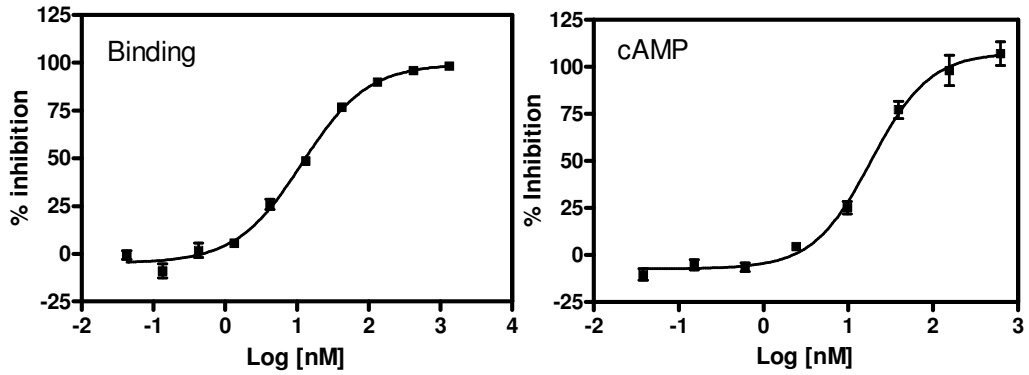

Figure 1: Representative dose titrations for Compound 5a in the SSTR3 competition binding and SSTR3 cAMP functional antagonism assays. Compound **5a** was titrated in the 125I-SS-14 competition binding assay for SSTR3 affinity determination (left) and the cAMP functional assay for antagonism of SS-14-induced inhibition of forskolin-stimulated cAMP production in SSTR3-expressing cells (right) as described in the section above. The curves shown are an aggregate of 8 representative studies each.

4) GLUCOSE STIMULATED INSULIN SECRETION ASSAY WITH ISOLATED PANCREATIC ISLETS

Isolation of pancreatic islets and the static GSIS assay. Mouse islets were isolated by collagenase digestion and discontinuous Ficoll gradient separation (Lacy PE, Kostianovsky M: Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967;16:35–39) and cultured overnight in RPMI 1640 medium with 11 mM glucose to facilitate recovery from the isolation process. Insulin secretion was determined by 1-h static incubation in the Krebs-Ringer bicarbonate (KRB) buffer in 96-well format as previously described (Herrington J, Zhou YP, Bugianesi RM, Dulski PM, Feng Y, Warren VA, Smith MM, Kohler MG, Garsky VM, Sanchez M, Wagner M, Raphaelli K, Banerjee P, Ahaghotu C, Wunderler D, Priest BT, Mehl JT, Garcia ML, McManus OB, Kaczorowski GJ, Slaughter RS: Blockers of the delayed-rectifier potassium current in pancreatic beta-cells enhance glucose-dependent insulin secretion. Diabetes 2006;55:1034-1042). Briefly, islets were preincubated in KRB medium with 2 mM glucose for 30 min and then transferred to a 96-well plate (one islet/well) and

incubated in 200 µl of KRB medium with 2 or 16 mM glucose in the presence or absence of test compounds for 60 min. At the end of the incubation, the medium was assayed for insulin levels with the ultrasensitive rat insulin ELISA kit (ALPCO Diagnostic, Salem, NH).

5) GLUCOSE TOLERANCE TEST

Glucose Tolerance Test (GTT) in wild-type and SSTR3 knockout (KO) mice. Male C57BL/6N mice (8-11 weeks of age) were obtained from Taconic Farms (Germantown, NY). The SSTR3 knockout mice were licensed from Deltgen Inc. and were backcrossed onto a C57BL/6 background for 8 generations. Mice were acclimated in our laboratory for at least one week prior to experiments, and were given free access to rodent chow (Teklad 7012) and water. All procedures were approved by the local Institutional Animal Care and Use Committee. GTT was performed with mice (n=5-7/group) that had been fasted 5 to 6 hours, as previously described (Tan CP, Feng Y, Zhou YP, Eiermann GJ, Petrov A, Zhou C, Lin S, Salituro G, Meinke P, Mosley R, Akiyama TE, Einstein M, Kumar S, Berger JP, Mills SG, Thornberry NA, Yang L, Howard AD. Selective small-molecule agonists of G protein-coupled receptor 40 promote glucose-dependent insulin secretion and reduce blood glucose in mice. Diabetes. 2008;57:2211-2219). Animals were then treated orally with vehicle (0.25% methylcellulose, 10 mL/kg) or **5a** 60 min prior to the GTT (dextrose 5 g/kg, oral gavage for OGTT; or 3g/kg, intraperitoneal injection for IPGTT). The blood glucose excursion profile from t = 0 to t = 90 min was used to calculate the area under the curve (AUC_{Glu}) for each treatment. Percent inhibition values for each treatment were generated from the area under the curve of glucose levels (AUC_{Glu}) data after the subtraction of the AUC_{Glu} of the control group that received vehicle (not drug) at -60 min and water (not dextrose) at 0 min.

Statistical analysis. Data are expressed as mean ±SE. Statistical analysis was conducted using either single-factor ANOVA or Student's t test, as appropriate. Statistical significance was defined as two-tailed $P < 0.05$.