Supplemental Material

In vitro cellular assays for GIPR, GLP1R and GCGR efficacy

Agonism of compounds at the human glucose-dependent insulinotropic polypeptide (GIPR), glucagon-like peptide-1 (GLP1R), or glucagon (GCGR) receptors was determined by functional assays measuring cAMP response of recombinant PSC-HEK-293 cell lines stably expressing human, rat and non-human primate (Cynomolgus monkey) GIPR, GLP1R or GCGR, respectively.

384-well format

The cells were grown in a T-175 culture flask placed at 37°C to near confluence in medium (DMEM/ 10% FBS) and collected in 2 ml vials in cell culture medium containing 10% DMSO in concentration of 10-50 million cells /ml. Each vial contained 1.8 ml cells. The vials were slowly frozen to −80 °C in isopropanol, and then transferred in liquid nitrogen for storage. Prior to their use, frozen cells were thawed quickly at 37 °C and washed (5 min at 900 rpm) with 20 ml cell buffer (1x HBSS; 20 mM HEPES, with 0.1% BSA). Cells were resuspended in assay buffer (cell buffer plus 2 mM IBMX) and adjusted to a cell density of 1 million cells/ml. For measurement of cAMP generation, 5 µl cells (final 5000 cells/well) and 5 µl of test compound were added to a 384-well plate, followed by incubation for 30 min at room temperature. The cAMP generated was determined using a kit from Cisbio Corp. based on HTRF (Homogenous Time Resolved Fluorescence). The cAMP assay was performed according to manufacturer's instructions (Cisbio).

After addition of HTRF reagents diluted in lysis buffer (kit components), the plates were incubated for 1 h, followed by measurement of the fluorescence ratio at 665 / 620 nm. In vitro potency of agonists was quantified by determining the concentrations that caused 50% activation of the maximal response (EC50).

96-well format

cAMP content of cells was determined using a kit from Cisbio Corp. (cat. no. 62AM4PEC) based on HTRF (Homogenous Time Resolved Fluorescence). For preparation, cells were split into T-175 culture flasks and grown overnight to near confluency in medium (DMEM / 10% FBS). Medium was then removed and cells washed with PBS lacking calcium and magnesium, followed by proteinase treatment

with accutase (Sigma-Aldrich cat. no. A6964). Detached cells were washed and resuspended in assay buffer (1 x HBSS; 20 mM HEPES, 0.1% BSA, 2 mM IBMX) and cellular density determined. They were then diluted to 400000 cells/ml and 25 µl-aliquots dispensed into the wells of 96-well plates. For measurement, 25 µl of test compound in assay buffer was added to the wells, followed by incubation for 30 minutes at room temperature. After addition of HTRF reagents diluted in lysis buffer (kit components), the plates were incubated for 1 hr, followed by measurement of the fluorescence ratio at 665 / 620 nm. In vitro potency of agonists was quantified by determining the concentrations that caused 50% activation of maximal response (EC50).

In vitro assays for binding to the human GIP receptor

Preparation of membranes from HEK-293 cells over-expressing GIPR

HEK-293 cells recombinantly over-expressing GIPR were grown to 50% confluency, washed with warm 1xPBS (Gibco) and detached in HEPES/EDTA-buffer (100 mM HEPES pH 7.5, 5 mM EDTA). Cells were harvested by centrifugation at 4°C and 3000xg and the pellets were stored at -80°C until further processing.

After thawing on ice, pellets were resuspended in HEPES/EDTA-buffer and homogenized on ice for 1 min using Ultra-Turray T25. After subsequent sonification the cell debris was removed by centrifugation at 1000xg and 4°C. Supernatants were then ultra-centrifuged at 100000xg and 4°C under vacuum for 30 min. Pellets were resuspended in HEPES/EDTA/NaCl-buffer (20 mM HEPES, 1 mM EDTA, 150 mM NaCl; add 1 Complete Mini Protease inhibitor cocktail to 10 ml buffer) and protein content was determined via BCA-Protein assay.

Measurement of binding activity of test compounds to human GIPR

For the measurement of the binding activity to GIPR, [^{125}I]GIP (PerkinElmer), in a final concentration of 100 pM and a test compound in 10 concentrations were mixed with PVT-WGA SPA beads (0.125 mg/well; Perkin-Elmer) coated with HEK-293 cell membranes (1 µg/well of protein) expressing the GIPR in assay buffer [50 mM HEPES (pH 7.4, WAKO), 5 mM EGTA (WAKO), 5 mM MgCI2 (WAKO), and 0.005% Tween 20 (BioRad)] and incubated at room temperature for 2 h. Specific binding was calculated as the difference between the amount of [^{125}I]labeled hot

ligand bound in the absence (total binding) and presence (nonspecific binding) of 1 μM unlabeled cold reference ligand, respectively.

NHP handling and housing

The NHPs were housed in a colony of 10 individuals at the Astrid Fagraeus Laboratory (Karolinska Institutet, Solna, Sweden) with a total cage volume of 50 m^3, with access to an outdoor partition. The housing is enriched with platforms, ropes etc. according to the needs of the particular species. The NHPs are regularly trained to cooperate with the housing staff for sampling and similar procedures.

Anaesthesia was induced by an intramuscular injection of ketamine hydrochloride (10 mg/kg). Intravenous catheters were placed in each hind leg, for intravenous anaesthesia, PET tracer administration, study drug administration, blood sampling and measurements of radioactivity. Anaesthesia was maintained by intravenous Propofol as needed, until the NHP was placed on the bed of the PET/CT scanner intubated and connected to a respirator and maintained on 1.4-4% sevoflurane inhalation anesthesia and artificial ventilation. The animal was fixed and body temperature maintained by a Bair Hugger model 505 (Arizant Healthcare, MN). Blood glucose, body temperature, heart rate, ECG, respiratory rate, oxygen saturation and blood pressure were monitored throughout the study. Fluid balance was maintained by a continuous infusion of saline (with added glucose to prevent hypoglycemia due to the long anesthesia).

NHP dose escalation study design

PET in vivo evaluation of [^{68}Ga]S02-GIP-T4 was performed by a dose escalation design in NHPs. The in vivo dose escalation study design was used in order to evaluate:

1) At which mass dose the PET ligand behaves as a PET “tracer” (defined as incurring <5% receptor occupancy).
2) If there is a proportional decrease in PET tracer binding in the target organ as the co-administered peptide mass is increased
3) The remaining background off-target /non-specific binding remaining after all target receptors have been saturated

Dose escalation in this case means that up to 3 PET examinations are performed in each NHP each experimental day. Progressively increasing radioactivity corresponding to increasing peptide mass is administered for each examination. This is analogous to progressively decreasing the specific radioactivity (MBq/nmol).
In some cases, the passage of time itself is sufficient to reduce the specific radioactivity to a suitable level for the next PET examination. In some cases, unlabelled S02-GIP-T4 must be added to the [^{68}Ga]Ga-S02-GIP-T4 injection solution in order to reach the desired specific radioactivity.
The increase in radioactivity means that eventual residual radioactivity in the NHP from the previous examination will be negligible. The increasing peptide mass means that the already administered peptide mass during previous examinations during the day will be negligible or very low in comparison.

Stability in NHP plasma

Blood plasma from cynomolgus NHPs (0.5 mL) was incubated with [^{68}Ga]Ga-S02-GIP-T4 (3-5 MBq) for 0, 10, 45, and 90 min at 37 °C. Then, 0.5 mL of acetonitrile was added to precipitate proteins, and the vials (Eppendorf 5415R centrifuge, Eppendorf AG, Hamburg, Germany) were centrifuged at 13200 rpm for 1 min at 4 °C. The supernatant was transferred into 0.2 µm nylon membrane filter (Corning Incorporated, Corning, NY, USA) that was centrifuged at 13200 rpm and 4 °C for 1 min. The radioactivity of the supernatant, pellet, and filter was measured in well-type in-house built NaI(Tl) scintillation counter and corrected for dead-time and decay. The data was used to calculate the recovery of the sample (>95%). The supernatant (0.5 mL) was diluted with 1.5 mL of deionized water and spiked with 10 µL of standard reference (S02-GIP-T4 solution of 1 mg/mL concentration) and analyzed (1.8 mL) on UV-radio-HPLC (Gilson, Middleton, USA) using an automated solid phase extraction controller (ASPEC Gilson) connected to a dilutor (Gilson), and a radio detector (Radiomatic 610TR, Packard, USA) coupled in series with a UV detector. The separation was performed on an Xbridge Prep BEH130 C18 (peptide separation technology) 250mm x 10mm, 5µm with a 10x10 mm C18 security guard from the same supplier. The HPLC system was operated at a flow rate of 6 ml/min. The mobile phase consisted of 0.1% TFA in MilliQ: 0.1% TFA in Acetonitrile. Gradient elution mode was used for the separation (Gradient: 0-5 min: 20-45%, 5-7

min: 45%, 7-10 min: 45-80%, 10-15 min: 80%). The outlet from the detector was connected to a switching valve on the arm of the ASPEC to enable automatic fraction collection. Five fractions were collected, and the radioactivity in the fractions was measured by a well-type scintillation counter.