Supplemental data

Reagents and Instrumentation

The EG1, EG2, and EG4 GIP ligands were custom-synthesized by Peptide Specialty Laboratories GmbH. All reagents were of the best grade available and were purchased from common suppliers. $^{68}Ge/^{68}Ga$-generator IGG100 was available from Eckert and Ziegler. All culture reagents were from Gibco BRL, Life Technologies. Gelofusine (GF) is commercially available as succinylated gelatine solution (40 g/L) (Brown). The inhibitor of the serine protease DDP IV vildapliptin is commercially available (Galvus, 50 mg) (Novartis).

The quality control of EG1, EG2, and EG4, as well as the radiolabeled compounds, was performed by analytical RP-HPLC on an analytical 120-5 C18 Nucleosil column (250 × 4.5 mm) applying a linear gradient of 15%–90% solvent B in 25 min at a flow rate of 1 mL/min. (solvent A, 0.1% TFA/H_2O; solvent B, 0.1%TFA/acetonitrile). Ultraviolet detection was performed using a Knauer detector at 280 nm. For radioactivity measurement, a Na(TI) well-type scintillation Gina star was used. The radiotracer solutions were prepared by dilution with 0.9% NaCl.

MALDI mass spectra were acquired on an Ultraflex TOFTOF I instrument (Bruker Daltonik GmbH) equipped with a nitrogen laser (20 Hz). Peptides were analyzed in the positive-ion reflector mode with the following settings: ion acceleration voltage, 25.0 kV; reflector voltage, 26.3 kV; first extraction plate, 21.75 kV. Mass spectra were obtained by accumulation of up to 100 individual laser shots. Calibration of spectra was performed externally by a quadratic fit using the peptide calibration standard II (Bruker Daltonik GmbH). FlexControl version 2.4 was used for instrument control and FlexAnalysis version 2.4 for spectrum processing. (Bruker Daltonik GmbH).

Quantitative γ counting was performed with a COBRA 5003 γ-system well counter from Packard Instruments. All experiments were performed 2 times in triplicate.

For PET studies, a dedicated small-animal PET scanner (Focus 120 microPET scanner; Concorde Microsystems Inc.) was used.

Mass spectrometry of synthetic peptides

Synthetic peptides were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Lyophilized peptides were dissolved in 0.1% trifluoroacetic acid (TFA) containing various amounts of acetonitrile (ACN). Peptide fractions from preparative HPLC runs were diluted with 0.1% TFA before analysis. For MALDI-MS, peptide samples were prepared on a stainless steel target with α-cyano-4-hydroxycinnamic (HCCA) acid as matrix. Briefly, 0.7 μL of peptide samples

were co-crystallized with 0.7 μL of a saturated solution of HCCA in ACN/0.1% TFA (50:50, v/v) directly on individual spots of the target plate. Samples were dried at ambient temperature before mass spectrometry analysis.

Radiolabeling with ^{111}In and ^{68}Ga

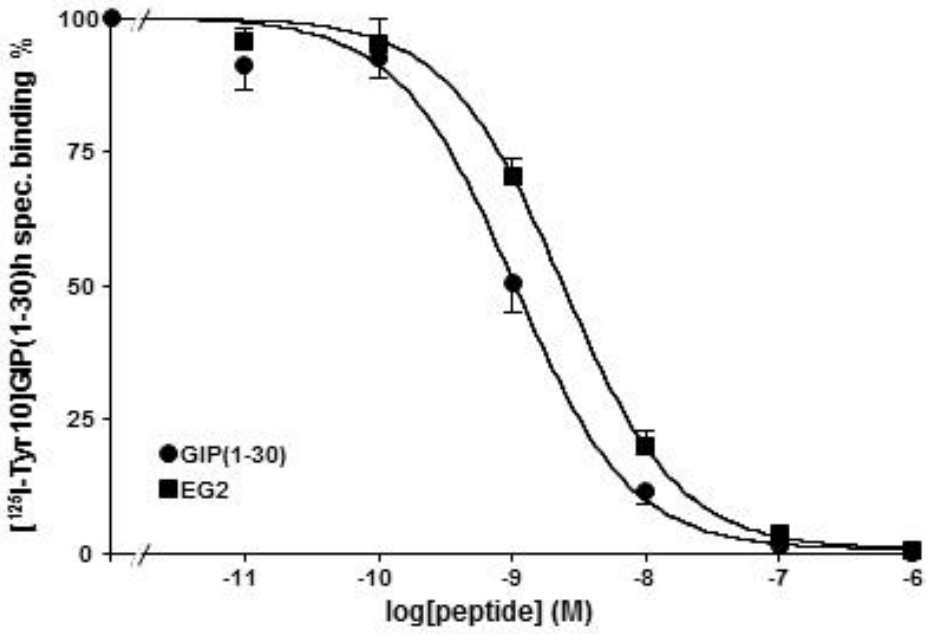
^{111}In-EG2 and ^{111}In-EG4: The radioligands were prepared by dissolving 20 μg of peptides in 250 μL of ammonium acetate buffer (0.5 mol/L, pH 5.4), followed by incubation with $^{111}InCl_3$ (35–40 MBq) for 30 min at 95°C. For EG2, the radiolabeling took place in the presence of 20 μL of selenomethionine (5 mg/250 μL) to prevent the oxidation of methionine in the amino acid sequence of the peptides.

^{68}Ga-EG2 and ^{68}Ga-EG4: The new radiotracers ^{68}Ga-EG2 and ^{68}Ga-EG4 were prepared within 10 min at 95°C, followed by purification, using the Modular-Lab PharmTracer module by Eckert and Ziegler. Briefly, the ^{68}Ge/^{68}Ga generator was eluted with 7 mL of HCl 0.1N and the eluate (~450 MBq) was loaded onto a cation exchange column (Strata-XC; Phenomenex). ^{68}Ga was eluted with 800 μL of a mixture of acetone/HCl (97.6%/0.02N) directly in a vial containing 2 mL of sodium acetate buffer (0.2 M, pH 4.0) and the minimum necessary amount (40 μg) of the conjugates, followed by SepPak C-18 purification to remove uncomplexed radiometal. For EG2, the radiolabeling took place in the presence of 200 μL of selenomethionine (5 mg/250 μL) to prevent the oxidation of methionine in the amino acid sequence of the peptides.

All radiopeptides were analyzed with (analytic) RP-HPLC. The radiotracer solutions were prepared by dilution with 0.9% NaCl.

Binding Affinity Studies

The IC_{50} values were estimated by in vitro GIPr autoradiography on cryostat sections of human insulinoma tissue using the radioligand [^{125}I-Tyr^{10}]GIP(1–30) in the presence or absence of each peptide in concentrations ranging from 10^{-12} to 10^{-6} M.



Supplemental Figure 1: Displacement curves of [^{125}I-Tyr^{10}]GIP(1–30) by GIP(1–30) and EG2.

Saturation Binding Studies

For receptor saturation analysis, INR1G9-hGIPr cells were seeded at a density of 0.8–1 million cells per well in 6-well plates and incubated overnight with medium (DMEM containing 1% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and 500 μg/mL G418). The next day, the medium was removed and the cells washed and incubated for 1 h at 37°C with fresh medium. Afterward, the plates were placed on ice for 30 min followed by incubation with increasing concentrations of either $^{111/nat}$In-EG2 or $^{111/nat}$In-EG4 (1–100 nM) in phosphate-buffered saline binding buffer, pH 7.4. After the addition of the radioligands, the cells were incubated for 120 min at 4°C. Nonspecific binding was determined in the presence of GIP(1–30) at a final concentration of 1 μM. Then, the cells were washed twice with ice-cold PBS, followed by solubilization with 1N NaOH, and the cell-associated radioactivity was measured using a γ counter. Specific binding was plotted against the total molar concentration of the added radiotracer. The K_d value and the concentration of the radiotracer required to saturate the receptors (B_{max}) were determined by nonlinear regression using GraphPad Prism 5 (Graph Pad Software). For all the cell studies, the values are normalized for 1×10^6 cells per well, and all data are from two independent experiments with triplicates in each experiment.

Calculation of receptors per cell from B_{max}:

B_{max} is the radioligand concentration required to saturate all the binding sites. The average of B_{max} for both radioligands is 1 nM (Fig. 1), meaning that the concentration of the receptors is 1 nM. Taking into account that the incubation volume of the cells was 1 mL and we have approximately 1 million cells and that 1 mol consists of $6{,}023 \times 10^{23}$ molecules, a density of 6×10^5 receptors per cell was calculated (6×10^{23} divided by $10^6 \times 10^9 \times 10^3 = 6 \times 10^5$).

Internalization Studies

For internalization experiments, INR1G9-hGIPr cells were seeded into 6-well plates and treated as described above. Approximately 0.25 pmol of the respective radiopeptide was added to the medium, and the cells were incubated (in triplicates) for 0.5, 1, 2, 4, and 6 h at 37°C, 5% CO_2. To determine nonspecific membrane binding and internalization, an excess of GIP(1–30) (final concentration, 1 μM) was added to selected wells. At each time point, the internalization was stopped by removing the medium and washing the cells twice with ice-cold PBS. To remove the receptor-bound radioligand, an acid wash was performed twice with a 0.1 M glycine buffer, pH 2.8, for 5 min on ice. Finally, cells were solubilized with 1N NaOH. The radioactivity of the culture medium, the receptor-bound fraction and the internalized fraction, was measured in a γ counter.

Biodistribution Studies

Ten picomoles/0.03–0.04 MBq of ^{111}In-EG4 and ^{68}Ga-EG4 in 100 μL of NaCl 0.9% were injected into the tail vein of INR1G9-hGIPr cell xenografted mice. Animals were sacrificed by isoflurane anesthesia at 1, 4, and 24 h after injection in the case of ^{111}In-EG4 and at 1 and 2 h after injection in the case of ^{68}Ga-EG4, and biodistribution studies were conducted as described in detail here.

The organs of interest were dissected, and the radioactivity in weighed tissue samples was counted in a γ counter. Biodistribution data are given as percentage injected activity per gram of tissue (%IA/g) and are means ± SD (n = 3-5).

To demonstrate the specificity of binding, INR1G9-hGIPr mice were injected first with 20 nmol of GIP(1–30) and 3–5 min later with 10 pmol of the respective radiopeptide. Animals were sacrificed at 1 h after injection by isoflurane anesthesia.

Lipophilicity

The lipophilicity ($LogD_{(pH=7.4)}$) was determined by the "shake-flask" method: to a solution of 500 μL of 1-octanol and 500 μL of PBS (pH 7.4), 10 μL of 100 nM of the labeled conjugates were added. The solution were vortexed for 1 h to reach equilibrium and then centrifuged (3,000 rpm) for 10 min. From each phase, 100 μL of the aliquot was pipetted out and measured in a γ counter. Each measurement was repeated 3 times. Care was taken to avoid cross-contamination between the phases. The partition coefficient was calculated as the average log ratio of the radioactivity in the organic fraction and the PBS fraction.

Supplemental Table 1: LogD Values of EG2 and EG4 Conjugates Labeled with ^{111}In and ^{68}Ga.

	^{111}In	^{68}Ga
EG2	–2.64 ± 0.04	–2.23 ± 0.15
EG4	–2.31 ± 0.07	–1.91 ± 0.07

Supplemental Table 2: Biodistribution Data of ^{68}Ga-EG4 after the Administration of Vildagliptin in Combination with Gelofusin

	^{68}Ga-EG4
Organ	**1 h/ Vildagliptin+GF**
Blood	0.58±0.40
Heart	0.38±0.18
Liver	1.32±0.07
Spleen	2.08±0.69
Lung	2.31±0.37
Kidney	182.23±29
Stomach	0.71±0.32
Intestine	0.67±0.43
Adrenal glands	0.71±0.03
Pancreas	0.43±0.15
Muscle	0.06±0.01
Bone	0.57±0.41
INR1G9 tumor	13.49±2.79

Data are presented as %IA/g of tissue and as mean ± SD (n = 3).