

RESEARCH PAPER

Species-specific action of (Pro3)GIP – a full agonist at human GIP receptors, but a partial agonist and competitive antagonist at rat and mouse GIP receptors

A H Sparre-Ulrich^{1,2†}, L S Hansen^{1,2,4†}, B Svendsen², M Christensen⁴, F K Knop⁴, B Hartmann^{2,3}, J J Holst^{2,3} and M M Rosenkilde¹

¹Department of Neuroscience and Pharmacology, Faculty of Health and Medical Sciences, The Panum Institute, University of Copenhagen, Copenhagen, Denmark, ²NNF Center for Basic Metabolic Research, Copenhagen, Denmark, ³Department of Biomedical Sciences Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, and ⁴Center for Diabetes Research, Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark

Correspondence

Mette M. Rosenkilde Department of Neuroscience, Pharmacology, Faculty of Health, Medical Sciences, The Panum Institute, University of Copenhagen, Copenhagen, Denmark.

E-mail: rosenkilde@sund.ku.dk

†Both authors shared first authorship.

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BACKGROUND AND PURPOSE

Specific, high potency receptor antagonists are valuable tools when evaluating animal and human physiology. Within the glucose-dependent, insulinotropic polypeptide (GIP) system, considerable attention has been given to the presumed GIP receptor antagonist, (Pro3)GIP, and its effect in murine studies. We conducted a pharmacological analysis of this ligand including interspecies differences between the rodent and human GIP system.

EXPERIMENTAL APPROACH

Transiently transfected COS-7 cells were assessed for cAMP accumulation upon ligand stimulation and assayed in competition binding using ¹²⁵I-human GIP. Using isolated perfused pancreata both from wild type and GIP receptor-deficient rodents, insulin-releasing, glucagon-releasing and somatostatin-releasing properties in response to species-specific GIP and (Pro3)GIP analogues were evaluated.

KEY RESULTS

Human (Pro3)GIP is a full agonist at human GIP receptors with similar efficacy (E_{max}) for cAMP production as human GIP, while both rat and mouse (Pro3)GIP were partial agonists on their corresponding receptors. Rodent GIPs are more potent and efficacious at their receptors than human GIP. In perfused pancreata in the presence of 7 mM glucose, both rodent (Pro3)GIP analogues induced modest insulin, glucagon and somatostatin secretion, corresponding to the partial agonist activities observed in cAMP production.

CONCLUSIONS AND IMPLICATIONS

When evaluating new compounds, it is important to consider interspecies differences both at the receptor and ligand level. Thus, in rodent models, human GIP is a comparatively weak partial agonist. Human (Pro3)GIP was not an antagonist at human GIP receptors, so there is still a need for a potent antagonist in order to elucidate the physiology of human GIP.

Abbreviations

GIP, glucose-dependent insulinotropic polypeptide (gastric inhibitory peptide); GLP-1, glucagon-like peptide-1; HBS, HEPES-buffered saline

Tables of Links

TARGETS
GPCRs
GIP receptor
GLP-1 receptor

LIGANDS	
GIP	(Pro3)GIP
Glucagon	
Insulin	
Somatostatin	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Glucose-dependent insulintropic polypeptide (also gastric inhibitory peptide; GIP) is a hormone secreted from the K cells of the gut following a meal (Baggio and Drucker, 2007). Like the related hormone glucagon-like peptide-1 (GLP-1), GIP is a potent insulin secretagogue (Holst, 2004). In contrast to the glucagonostatic effect of GLP-1 (Gutniak *et al.*, 1992; de Heer *et al.*, 2008), GIP has glucagon-releasing properties (Kreymann *et al.*, 1987; Christensen *et al.*, 2011). The incentive to understand the biology of GIP was intensified by the discovery of an association between rodent GIP receptors and adiposity (Miyawaki *et al.*, 1999; Miyawaki *et al.*, 2002; Nasteska *et al.*, 2014). In humans, although less clear, there is likewise evidence for a role of GIP in fat metabolism with the demonstration of GIP receptor expression in adipose tissue (Ahlqvist *et al.*, 2013), an association between high body mass index and increased GIP levels (Ahlqvist *et al.*, 2013; Calanna *et al.*, 2013), and increased adipose tissue blood flow and triacylglycerol deposition following GIP administration in a state of high insulin and high glucose (Asmar *et al.*, 2010). Furthermore, obese children decrease their basal and postprandial GIP levels, when put on a low-calorie diet (Deschamps *et al.*, 1980), and healthy young men increase their fasting GIP levels, when put on a high-fat diet (Brøns *et al.*, 2009).

The availability of the GLP-1 receptor antagonist exendin-(9–39) (Raufman *et al.*, 1991; Jørgensen *et al.*, 2013) has been helpful for our understanding of the physiology of GLP-1, which underlies the pharmaceutical development of GLP-1 receptor agonists as anti-diabetic and anti-obesity agents. Inspired by this, researchers have tried to develop a potent GIP receptor antagonist. Many different strategies have been undertaken in order to inhibit GIP function, including *prepro*-GIP gene truncations (Nasteska *et al.*, 2014), administration of low MW receptor antagonists (Nakamura *et al.*, 2012), immunization against GIP (Ebert *et al.*, 1979; Fulurija *et al.*, 2008; Irwin *et al.*, 2009), various truncations and amino acid substitutions of the GIP molecule thought to provide antagonistic properties (Tseng *et al.*, 1996; Gelling *et al.*, 1997; Hinke *et al.*, 2001; Gault *et al.*, 2002; Deacon *et al.*, 2006; Irwin *et al.*, 2006; Kerr *et al.*, 2011), and recently, a potent antagonistic antibody against the GIP receptor was reported (Ravn *et al.*, 2013). However, most attention has been given to the analogue (Pro3)GIP following demonstrations that chronic treatment with this

peptide improved diabetic parameters in *ob/ob* mice (Irwin *et al.*, 2007), improved diabetic parameters and induced weight loss in obese mice previously on a high-fat diet (McClellan, 2007), and reduced weight gain and improved diabetic parameters following the induction of a high-fat diet (Gault *et al.*, 2007).

Animal models are widely used as basis for predicting biological properties of given receptor-ligand systems to human (patho)physiology. Often, rodent models are the first choice due to reduced costs and space requirements compared with larger animal models (pigs, dogs and primates). Initial drug screening are similarly often carried out in rodent models. This creates the classical dilemma of whether the chosen rodent model is representative for human physiology or whether the inter-species differences (stemming from structural, functional, spatial or temporal differences) are too great. Among the seven-transmembrane receptor systems, such differences have been described for instance for the lipid-activated G protein-coupled receptor 119 (GPR119), where a series of compounds, with similar binding properties to human and mouse GPR119, displayed large differences in potency and efficacy (Scott *et al.*, 2013). A similar example can be found within the 5-HT receptor system, where a single amino acid difference between the human and mouse receptor can account for large differences in binding affinities and potencies (Canal *et al.*, 2013).

Other receptor-ligand systems, however, display very little interspecies variations, as seen in the GLP-1 system with a large degree of sequence homology both in terms of the ligand (GLP-1) and its receptor in humans, mice and rats and where both liraglutide (a long-acting GLP-1 receptor agonist) and native GLP-1 have similar potencies across species-specific receptors (Knudsen *et al.*, 2012) (Figure 1). Even though the GIP system is closely related to GLP-1 and the GLP-1 receptor, the GIP system is noticeably less conserved across species (Figure 1).

In an attempt to develop GIP receptor antagonists to be used in humans, for instance, as a putative therapeutic against obesity (Asmar *et al.*, 2010; Ahlqvist *et al.*, 2013), we set out to investigate functional differences between rodents and human within the GIP system in terms of ligand binding and signalling properties. As agonists, we chose the endogenous agonist GIP(1–42) from human, rat and mouse, and characterized this in parallel with the species-corresponding previously described antagonist (Pro3)GIP (Gault *et al.*, 2002) on all three GIP receptors (from human, rat and

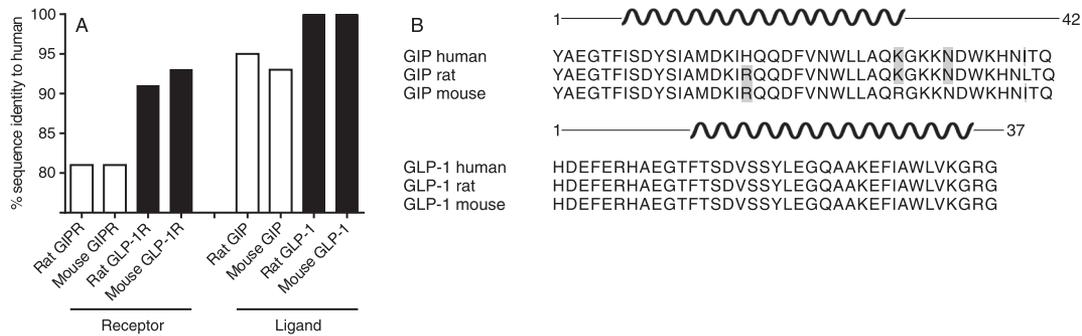


Figure 1

The GIP system is less conserved than the GLP-1 hormone system. A) Sequence identity between rodent and human GIP receptors (GIPR) and GLP-1 receptors (GLP-1R), as well as GIP and GLP-1 given in percent. The receptors were compared without signalling peptide sequences. The alignment was done in Geneious 6.0.5. B) Sequence alignment between human, rat, and mouse GIP (top) and GLP-1 (bottom). Grey indicates a mismatch and the black spiral indicates predicted alpha helix structure.

mouse). The goal was to determine whether the beneficial antagonistic effects of (Pro3)GIP in rodents could possibly be transferred to humans.

Methods

Animals

All animal care and experimental procedures complied with institutional guidelines and were approved by the Danish Animal Experiments Inspectorate (2013-15-2934-00833). Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 18 animals were used in the experiments described here.

Male C57Black/6 (B6) mice (25–30 g) or male Wistar rats (220–250 g) were purchased from Taconic (Lille Skensved, Denmark). The animals were housed in plastic-bottomed, wire-lidded cages in air-conditioned (21°C) and humidity-controlled (55%) rooms with a 12:12 h light–dark cycle and free access to standard rat chow and water. Animals were acclimatized for at least 1 week before use.

Transfections and tissue culture

COS-7 cells (ATCC, Virginia, USA) were cultured at 10% CO₂ and 37 °C in DMEM 1885 supplemented with 10% FBS, 2 mM glutamine, 180 units mL⁻¹ penicillin and 45 g mL⁻¹ streptomycin. Transient transfection of the COS-7 cells for cAMP accumulation and competition binding was performed using the calcium phosphate precipitation method with the addition of chloroquine (Kissow *et al.*, 2012).

cAMP assay

Transiently transfected COS-7 cells were seeded out in white 96-well plates at a density of 3 × 10⁴ cells per well. The day after, the cells were washed twice with HEPES-buffered saline (HBS) buffer and incubated with HBS and 1 mM IBMX for 30 min at 37 °C. To test agonists, ligands were added and incubated for 30 min at

37 °C. In order to test for antagonistic properties, the antagonist was pre-incubated for 10 min, and then the agonist was added and incubated for an additional 20 min. The HitHunterTM cAMP XS assay (DiscoverX, Herlev, Denmark) was carried out according to the manufacturer's instructions.

Competition binding assay

COS-7 cells were seeded in clear 96-well plates the day after transient transfection. The number of cells seeded per well, which is determined by the apparent expression efficiency of the receptor, was aimed to result in 5–10% specific binding of the added radioactive ligand (¹²⁵I-human GIP). The following day, cells were assayed by competition binding for 4 h at 4 °C using 15–40 pM ¹²⁵I-human GIP as well as unlabelled ligand in 50 mM HEPES buffer (pH 7.2) supplemented with 0.5% BSA (binding buffer). After incubation, the cells were washed twice in ice-cold binding buffer and lysed using 200 mM NaOH with 1% SDS for 30 min. Nonspecific binding was determined as the binding in the presence of 100 nM unlabelled human GIP. The samples were analysed by the Wallac Wizard 1470 Gamma Counter.

Rat or mouse isolated perfused pancreas

Male C57B6 mice (25–30 g) or male Wistar rats (220–250 g) were anaesthetized (mice: ketamine/xylazine 100/10 mg·kg⁻¹ i.p.; rats: 0.0158 mg fentanyl citrate + 0.5 mg fluanisone + 0.25 mg midazolam/100 g; Pharmacy Service, Denmark), and the pancreas was dissected and perfused *in situ* as described previously (Deacon *et al.*, 2006). Briefly, the pancreas was perfused in a single-pass system through both the coeliac and the superior mesenteric artery via a catheter inserted into the aorta. All other aortic branches were ligated. The venous effluent was collected for 1 min intervals via a catheter in the portal vein, and stored at –20 °C until analysis. The pancreas was perfused with a modified Krebs Ringer bicarbonate buffer [composition: 118 mM NaCl, 3.0 mM KCl, 2.6 mM CaCl₂ 2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ 2H₂O, 25 mM NaHCO₃, 5 mM fumarate, 5 mM pyruvate, 5 mM glutamate, 7 mM glucose,

0.1% BSA, 5% dextran (Pharmacosmos, Holbaek, Denmark)]. Flow rate was kept constant at $1.5 \text{ mL}\cdot\text{min}^{-1}$ (mouse) or $4 \text{ mL}\cdot\text{min}^{-1}$ (rat), perfusion buffer was heated and oxygenated (95% O_2 , 5% CO_2), and pressure was continuously measured throughout the experiment. Rodent (rat/mouse) forms of (Pro3)GIP and of GIP were infused as test substances through a sidearm infusion pump at a flow rate of $0.075 \text{ mL}\cdot\text{min}^{-1}$ (mouse) or $0.2 \text{ mL}\cdot\text{min}^{-1}$ (rat). Arginine (10 mM) was infused at the end of each experiment as a positive control.

Hormone analysis

Hormone concentrations in the perfusion effluent were measured using in-house radioimmunoassays. Glucagon was measured using a midregion-directed glucagon antiserum (code no. 4304) or a COOH-terminally directed antiserum (code no. 4305), which measures fully processed glucagon as well as N-terminally extended molecular forms, in addition, a synthetic glucagon for standards, and ^{125}I -labelled glucagon (a gift from Novo Nordisk A/S) as tracer (Orskov *et al.*, 1991). Insulin was measured using an antibody cross-reacting strongly with rat insulin I and II (code no. 2006–3). As the standard, we used human insulin, and the tracer was ^{125}I -labelled human insulin (gift from Novo Nordisk A/S) (Brand *et al.*, 1995). Somatostatin concentrations were determined using a rabbit antiserum (code no. 1758) raised against synthetic cyclic somatostatin, recognizing both somatostatin 14 and somatostatin 28 (Baldissera *et al.*, 1983), somatostatin 14 as standard and ^{125}I -labelled Tyr¹¹-somatostatin (NEX389, Perkin-Elmer, Skovlunde, Denmark) as tracer.

Sequence alignments

The amino acid sequences of the GIP and GLP-1 systems were acquired from GenBank of NCBI. The alignment was performed in Geneious 6.0.5 using MAFFT v6.814b. The BLOSUM62 matrix was applied with gap open penalty and offset value of 1.53 and 0.123 respectively.

Data analysis

Pharmacological analyses including Schild plot analyses and statistical analyses were carried out with the GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA) and Microsoft ExcelTM. Sigmoid curves were fitted logistically with a Hill slope of 1.0. The calculations of K_i values were based on the Cheng-Prusoff formula (DeBlasi *et al.*, 1989). Dose ratios (DR) for the Schild analyses (Lazareno and Birdsall, 1993) were based on the potency shift of GIP(1–42) in the presence of a given antagonist concentration, relative to the absence of antagonist.

Materials

Rat and mouse (Pro3)GIP (cat. no. 027–29 and 027–49, respectively) and rat and mouse GIP (cat. no. 027–12 and 027–27, respectively) were purchased from Phoenix Pharmaceuticals (Karlsruhe, Germany). Human (Pro3)GIP was synthesized by CASLO ApS (Lyngby, Denmark) and human GIP was purchased from Bachem (H5645: Bubendorf, Switzerland). All peptides had a purity of more than 95% by HPLC analyses and an MS-controlled molecular weight. cDNAs of the human, rat and mouse GIPR were

purchased from Origene (Rockville, MD, USA) (SC110906, RN212314 and MC216211, respectively) and cloned into the pCMV-Script vector. ^{125}I -labelled human GIP was purchased from PerkinElmer Life Sciences (Skovlunde, Denmark; NEX402).

Results

Larger structural diversity in the GIP system compared with the GLP-1 system

It is not clear whether the GLP-1 and GIP systems in rodents are equally transferable to human physiology. As a crude overview of the potential differences between the systems, the amino acid sequences of both the ligands and the receptors were analysed. At the receptor level, it is apparent that the GIP receptor is less conserved between human and rodents (81%) than the GLP-1 receptor (91% and 93% for rat and mouse, respectively) (Figure 1A). Also, in regard to ligand sequences, the GLP-1 system is more conserved than GIP (Figure 1B). Thus, the sequence of GLP-1 is identical between the analysed species, while human GIP has one non-conservative amino acid substitution (Arg to His) at position 18 and one additional conservative substitution compared with rat (position 40: Ile to Leu) and two compared with mouse GIP (position 30: Lys to Arg and 34: Asn to Ser).

(Pro3)GIP is an agonist with efficacies dependent on the species providing GIP receptors

Due to the reported antagonistic properties and the effects observed in obese and/or diabetic animal models (Gault *et al.*, 2007; Irwin *et al.*, 2007; McClean, 2007), the Pro3-analogues were investigated on the human, rat and mouse GIP receptors, in parallel with the respective endogenous GIP agonists. As GIP receptor-induced cAMP accumulation is well established as an important signalling pathway for GIP function, the effects of species-specific GIP and (Pro3)GIP analogues were evaluated by measuring cAMP accumulation. Compared with human GIP, human (Pro3)GIP was an efficacious agonist on the human GIP receptor approaching the E_{max} of human GIP (Figure 2A), while both rat (Pro3)GIP (Figure 2B) and mouse (Pro3)GIP (Figure 2C) were partial agonists with E_{max} values of ~50 and ~30%, respectively, on their respective receptors. In addition to the differences in efficacy, there was a dramatic shift in potency between GIP and the corresponding (Pro3)GIP within the three species. On the human GIP receptor, we observed 181-fold difference between human GIP and human (Pro3)GIP, while much larger differences (1300-fold and 2636-fold in rat and mouse, respectively) were observed in the rodent systems. These differences were due to both *higher* species-specific GIP potencies (from 26 pM for human GIP on human GIP receptors to 10 and 11 pM for rat and mouse GIP on their GIP receptors) and *lower* (Pro3)GIP species-specific potencies (from 4.7 nM on human GIP receptors to 13 and 29 nM on rat and mouse GIP receptors respectively).

(Pro3)GIP is a partial agonist with competitive antagonistic properties in rodent GIP receptors

To determine the potential of the two partial agonists (rat and mouse (Pro3)GIP) as antagonists of GIP-induced activation,

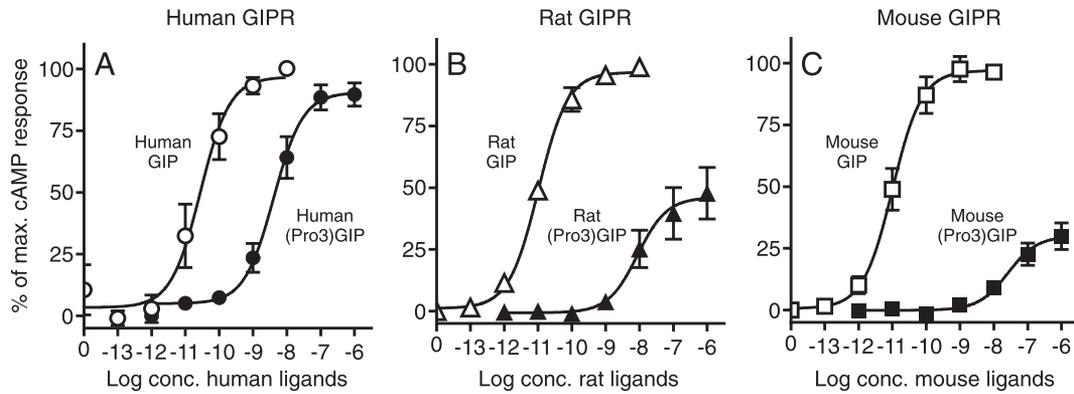


Figure 2

Human (Pro3)GIP is a full agonist, rat and mouse (Pro3)GIP are partial agonists. COS-7 cells were transiently transfected with either human GIP receptors (GIPR; A), rat GIPR (B), or mouse GIPR (C) and assayed for cAMP accumulation following increasing concentrations of WT GIP and (Pro3)GIP from the corresponding species. The data was normalised to E_{max} of the endogenous GIP on every receptor and shown as mean \pm SEM, $N \geq 3$. Nonlinear regression was used to calculate the EC_{50} value and E_{max} .

cAMP production was measured as a function of increasing concentration of rat and mouse GIP in the absence or presence of various concentrations of rat and mouse (Pro3)GIP on the corresponding GIP receptors (Figure 3A and B respectively). Reflected by the agonistic properties, (Pro3)GIP increased the cAMP production in the absence or at low GIP concentrations on both receptors. However, a concomitant rightward shift in potency of GIP was observed, which is an indication of a competitive antagonistic nature. At 10, 100, and 1000 nM of rat (Pro3)GIP, the potency of rat GIP was shifted 2-fold, 4-fold and 16-fold compared with the absence of (Pro3)GIP (Figure 3A). Using the calculated EC_{50} values from these curves, a Schild plot analysis was made (Figure 3C). The Hill coefficient was 0.55 ± 0.20 , and the X-axis intercept, which represents the dissociation constants (K_i) for rat (Pro3)GIP, was 13 nM. A similar pattern was observed in the mouse system. Here, the highest concentration of mouse (Pro3)GIP (1 μ M) resulted in an 11-fold right-shift in the potency of mouse GIP. The Hill coefficient in the Schild Plot in the mouse system was 0.79 ± 0.16 and K_i of rat (Pro3)GIP was calculated to be 61 nM (Figure 3D). As the Hill coefficient was <1 in both cases, this indicates confounding factors, presumably determined by the concomitant (partial) agonist and antagonist effects of (Pro3)GIP.

Lower activities of all three (Pro3)GIP in the rodent GIP systems compared with the human system

To determine whether the species-specific differences of (Pro3)GIPs were due to ligand or receptor differences, we investigated the effects of the three (Pro3)GIP ligands across all three GIP receptors. The approaching full agonism as observed for human (Pro3)GIP at the human GIP receptor was not seen with the two rodent (Pro3)GIP ligands, which both demonstrated an E_{max} of $\sim 70\%$ as compared with human GIP (Figure 4A). In contrast, the (Pro3)GIP analogues were equally potent at the human GIP receptor (Table 1). At the rat GIP receptor, there was a lower efficacy of all three (Pro3)GIP analogues, with human

(Pro3)GIP again showing the highest efficacy (67 vs. $\sim 40\%$), but with similar potencies for the three (Pro3)GIP analogues (Figure 4B, Table 1). The same tendency was seen at the mouse GIP receptor with an even lower efficacy of human (Pro3)GIP (41%) and rodent (Pro3)GIP ($\sim 30\%$) compared with mouse GIP (Figure 4C).

Higher activities of GIP in the rodent GIP system as compared with the human system

Inspired by the findings that the glutamic acid to proline substitution at the third position in GIP resulted in a lower activity in the rodent systems, we decided to examine whether such species-dependent differences were present for native GIP. Interestingly, the opposite picture arose here, as the human GIP displayed a lower potency and efficacy on all three receptors compared with the rodent GIP ligands (Figure 5A–C respectively). The rodent GIPs were equally potent and efficacious on all tested receptors, without any considerable change in potency between the receptors ($EC_{50} \sim 10$ pM) (Table 1). In contrast, human GIP had a lower efficacy on all three receptors with the largest difference in the rodent receptors with an E_{max} of 75% on the rat GIP receptor and 60% on mouse GIP receptors compared with the corresponding rodent GIP. Likewise, human GIP had a lower potency than the rodent GIPs on all three receptors with the largest difference being 23-fold on the mouse GIP receptor followed by 10-fold and 5-fold on the rat and human GIP receptors respectively.

No inter-species differences in the binding affinities of GIP analogues or (Pro3)GIP analogues

In order to determine whether the differences at both the receptor and the ligand level in terms of cAMP accumulation were due to differences in binding affinity, competition binding experiments were conducted using 125 I-labelled human GIP as the radioligand. Human GIP was found to bind to all three

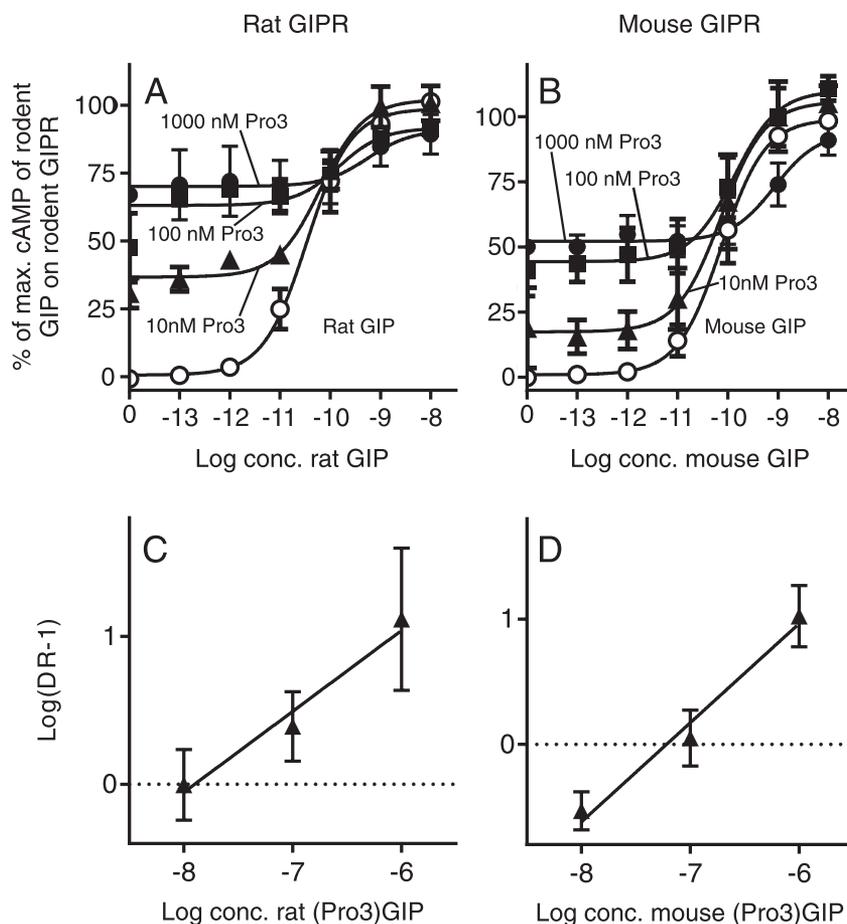


Figure 3

The rodent partial agonist peptides, (Pro3)GIP, are functional antagonists at their corresponding GIP receptors (GIPR). (A) rat GIP and (B) mouse GIP cAMP accumulation concentration–response curves with or without increasing concentrations (10, 100 and 1000nM) of rat (Pro3)GIP or mouse (Pro3)GIP. The results were normalized to E_{max} of the species-specific GIP in the absence of (Pro3)GIP and shown as mean + SEM, $N = 3$. Nonlinear regression was used to calculate EC_{50} values. Schild plot analysis of the dose–response curves of (C) rat GIP and (D) mouse GIP revealed K_i -values of 13 and 61 nM respectively.

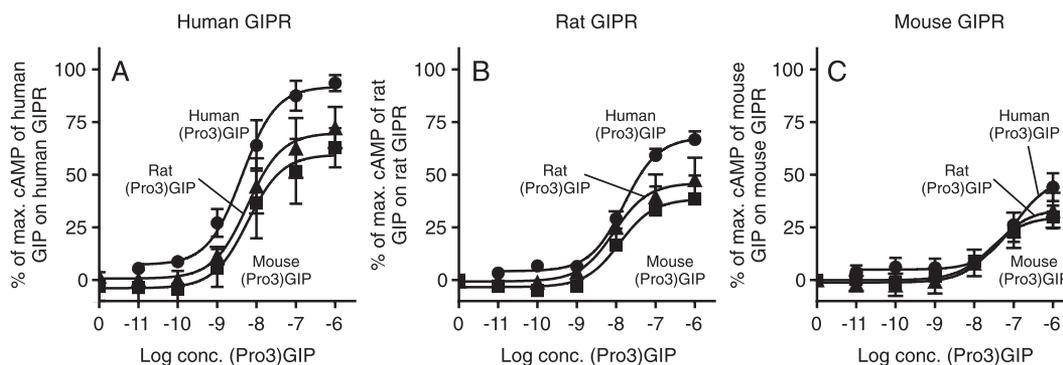


Figure 4

Human (Pro3)GIP is a partial agonist on rodent receptors. COS-7 cells were transiently transfected with either human GIPR (A), rat GIPR (B), or mouse GIPR (C) and assayed for cAMP accumulation following increasing concentrations of human, rat, and mouse (Pro3)GIP. The data was normalised to E_{max} of the species specific GIP on every receptor and shown as mean±SEM, $N \geq 3$. Nonlinear regression was used to calculate the EC_{50} value and E_{max} .

Table 1

cAMP accumulation induced by human, rat, and mouse GIP and (Pro3)GIP

	Human GIPR				Rat GIPR				Mouse GIPR			
	logEC ₅₀ ±SEM	EC ₅₀ (nM)	E _{max} ±SEM	(N)	logEC ₅₀ ±SEM	EC ₅₀ (nM)	E _{max} ±SEM	(N)	logEC ₅₀ ±SEM	EC ₅₀ (nM)	E _{max} ±SEM	(N)
Human GIP	-11 ± 0.26	0.026	100 ± 0	7	-10 ± 0.34	0.071	100 ± 0	7	-9.6 ± 0.26	0.25	100 ± 0	7
Rat GIP	-11 ± 0.11	0.0076	113 ± 9	4	-11 ± 0.06	0.010	117 ± 12	4	-11 ± 0.16	0.011	144 ± 12	4
Mouse GIP	-11 ± 0.11	0.0052	99 ± 3	4	-11 ± 0.13	0.0072	120 ± 10	4	-11 ± 0.16	0.011	156 ± 13	4
Human (Pro3) GIP	-8.3 ± 0.25	4.7	90 ± 4	4	-7.8 ± 0.01	15	80 ± 4	3	-7.0 ± 0.054	92	71 ± 8	3
Rat (Pro3) GIP	-7.9 ± 0.32	12	73 ± 11	5	-7.9 ± 0.21	13	64 ± 15	5	-7.4 ± 0.24	38	57 ± 14	5
Mouse (Pro3) GIP	-8.0 ± 0.31	11	67 ± 8	5	-7.8 ± 0.16	15	61 ± 12	5	-7.5 ± 0.30	29	59 ± 8	4

cAMP accumulation is shown following ligand stimulation of COS-7 cells transiently transfected with either human, rat, or mouse GIPR cDNA. Data are presented as mean logEC₅₀±SEM and E_{max}±SEM. N, number of experiments. Data in bold indicate that the receptor and ligands are from the same species.

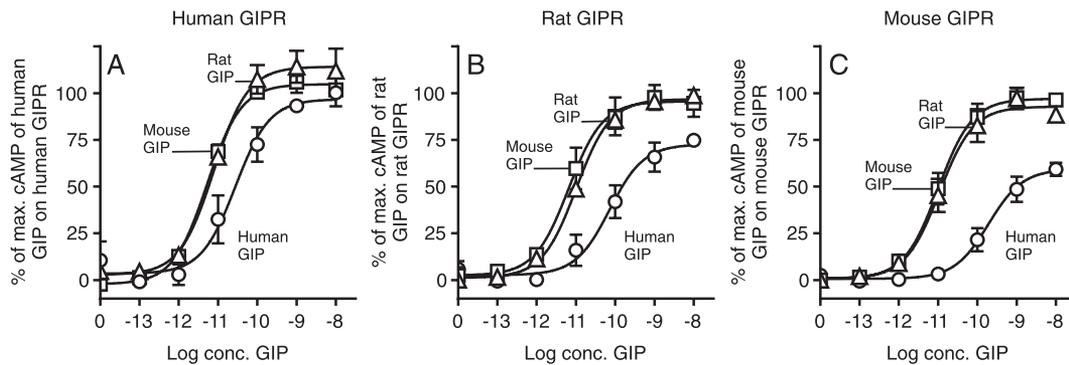


Figure 5

Human GIP is less efficacious and has a lower potency than rodent GIP. COS-7 cells were transiently transfected with either human GIP receptors (GIPR; A), rat GIPR (B), or mouse GIPR (C) and assayed for cAMP accumulation following increasing concentrations of human, rat and mouse GIP. The data was normalized to E_{max} of the species specific GIP on every receptor and shown as mean±SEM, N≥3. Nonlinear regression was used to calculate the EC₅₀ value and E_{max}.

receptors with equally high affinities (K_d of 0.90 to 1.1 nM) (Table 2). Likewise, both rodent GIP analogues were similar to human GIP on the tested receptors with a K_i in the nM range from 0.27 nM of rat GIP on the mouse GIP receptor to 2.0 nM for mouse GIP on the human GIP receptor. Thus, the functional discrepancies between human and rodent GIP are not due to actual differences in affinity. The (Pro3)GIP analogues displayed in average 10-fold lower affinities than the native GIP analogues on all three receptors with the lowest affinity observed for the human ligand on mouse GIP receptor (K_i of 39 nM) whereas the rest displayed affinities (K_i) between 4.8 and 14 nM. Thus, similar to the native GIP ligands, the functional differences between the human (Pro3)GIP and the rodent counterparts are not caused by differences in binding affinities.

(Pro3)GIP stimulates insulin, glucagon and somatostatin release

As most of the studies describing (Pro3)GIP were carried out in rodent models (Gault *et al.*, 2007; Irwin *et al.*, 2007;

McClellan, 2007), we determined how the receptor binding and activation would translate into hormone secretion, using perfused pancreata from rodents. In brief, the tested analogues were perfused through the pancreata of the rodents, and the venous effluent was collected at 1 min interval and analysed for insulin, glucagon and somatostatin content. In both perfusion models, we used 100-fold higher concentrations of (Pro3)GIP (100 nM), compared with that of GIP (1 nM), because of the 1000-fold difference in potencies (Table 1) and the 10-fold difference in affinity (Table 2). In the mouse pancreas, (Pro3)GIP as well as GIP-induced secretion of insulin, glucagon and somatostatin (Figure 6A–C) as expected from the agonistic nature of (Pro3)GIP (Figure 2C). However, compared with GIP, there were differences in the magnitude of secretion. Although given at 100-fold higher concentration, (Pro3)GIP did not reach the same levels of insulin secretion as GIP (Figure 6A), while there was no difference in somatostatin and glucagon release (Figure 6B and C).

Table 2Similar displacement of ^{125}I -labeled hGIP by GIP analogues or (Pro3)GIP analogues.

	Human GIPR			Rat GIPR			Mouse GIPR		
	$\log\text{IC}_{50} \pm \text{SEM}$	K_i/K_d (nM)	(N)	$\log\text{IC}_{50} \pm \text{SEM}$	K_i/K_d (nM)	(N)	$\log\text{IC}_{50} \pm \text{SEM}$	K_i/K_d (nM)	(N)
Human GIP	-9.0 ± 0.093	0.90	7	-8.9 ± 0.095	1.1	3	-9.1 ± 0.17	0.72	3
Rat GIP	-8.9 ± 0.12	1.2	3	-9.2 ± 0.094	0.67	3	-9.6 ± 0.099	0.27	3
Mouse GIP	-8.7 ± 0.30	2.0	3	-8.9 ± 0.37	1.2	3	-9.1 ± 0.42	0.72	3
Human (Pro3)GIP	-8.0 ± 0.36	10	4	-7.8 ± 0.42	14	3	-7.4 ± 0.59	39	3
Rat (Pro3)GIP	-7.9 ± 0.20	12	3	-7.9 ± 0.13	12	3	-8.1 ± 0.077	8.8	3
Mouse (Pro3)GIP	-8.1 ± 0.06	7.7	3	-8.1 ± 0.093	8.8	3	-8.3 ± 0.26	4.8	3

The binding of ^{125}I -labeled hGIP to the transiently transfected COS-7 cells with either human, rat, or mouse GIPR cDNA, was tested in the presence of increasing amounts of human, rat, or mouse GIP and (Pro3)GIP. Data are presented as mean $\log\text{IC}_{50} \pm \text{SEM}$. N, number of experiments. Data in bold indicate that the receptor and ligands are from the same species.

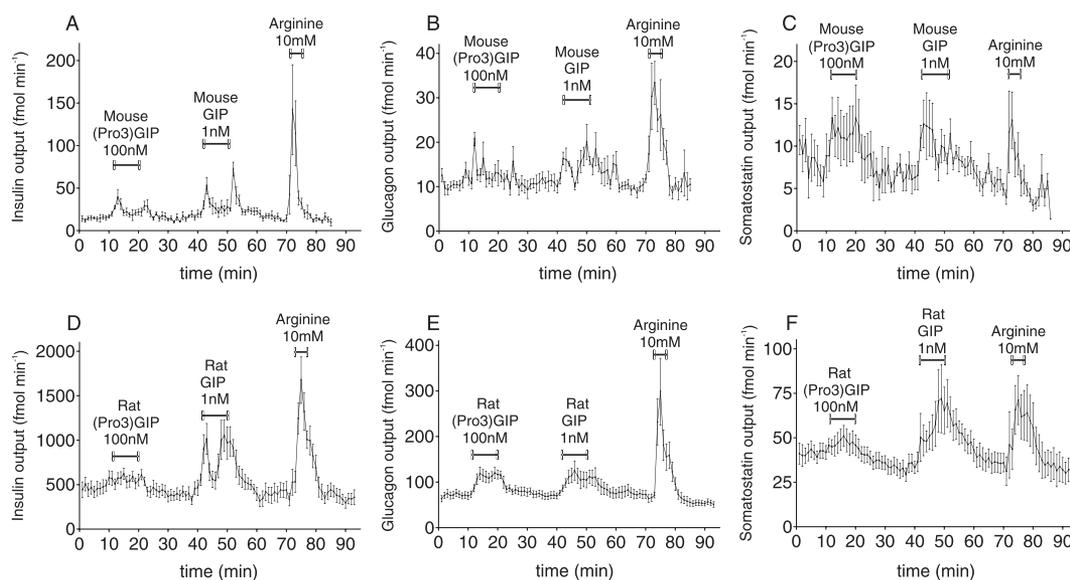
Similar results were obtained in the rat perfused pancreas model with a significantly lower insulin and somatostatin secretion by (Pro3)GIP compared with GIP (Figure 6D and F, respectively) but with an equal glucagon release by both ligands (Figure 6E).

In order to confirm that the observed hormone secretion from perfused pancreata was mediated through the GIP receptors, the same experimental set-up was repeated using GIP receptor KO mice. As seen in Figure 7, GIP receptor-deficient mice did not respond to mouse GIP or mouse (Pro3)GIP with either insulin, glucagon or somatostatin secretion (Figure 7A–C respectively). As with the pancreata from wild-type mice and rats, the positive control, arginine,

induced secretion of the respective hormones, validating the experimental set-up.

Discussion

Our study demonstrated that human (Pro3)GIP was not an antagonist at human GIP receptors, but rather approaches full agonism. At rodent GIP receptors, (Pro3)GIP was a partial agonist with competitive antagonistic properties when evaluated in terms of cAMP accumulation. In terms of its effect on pancreatic hormone secretion, (Pro3)GIP followed the partial agonistic pattern seen in cAMP with regard to insulin, glucagon and somatostatin secretion. However,

**Figure 6**

(Pro3)GIP is an agonist on rodent pancreata. (A/D) insulin, (B/E) glucagon, and (C/F) somatostatin secretion from perfused mouse (A, B, C) and rat (D, E, F) pancreata following stimulation with either species specific 100 nM (Pro3)GIP, 1 nM GIP, or 10 mM arginine (N=6–9). The glucose concentration was 7 mM and data are mean \pm SEM.

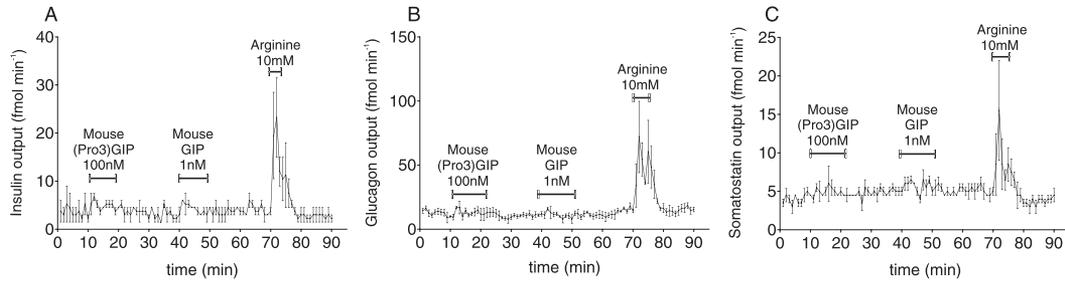


Figure 7

The effects seen in perfused pancreata are mediated through GIP receptors (GIPR). A) insulin, B) glucagon, and C) somatostatin secretion following stimulation of perfused pancreata from GIPR KO mice by either 100 nM mouse (Pro3)GIP, 1 nM mouse GIP, or 10 mM arginine (N=3). The glucose concentration was 7 mM and data are mean \pm SEM.

(Pro3)GIP induced a glucagon release equal to that induced by native GIP in rats.

(Pro3)GIP is not an effective GIP receptor antagonist for future studies of the GIP system

The first GIP receptor antagonists that showed promise were the truncated and amidated forms, GIP(6–30)-NH₂ and GIP(7–30)-NH₂ with affinities (IC₅₀ values) between 3 and 200 nM obtained in heterologous binding experiments (Tseng *et al.*, 1996; Gelling *et al.*, 1997; Hinke *et al.*, 2001). However, elaborate *in vivo* characterization has not been carried out. The naturally occurring GIP metabolite GIP(3–42)

binds with similar affinity (IC₅₀ of 22 nM); however, no antagonistic effect could be demonstrated *in vivo* in pigs (Deacon *et al.*, 2006). Low MW compounds have also been presented, e.g. the antagonist SKL-14959 with an affinity (K_i value) of 55 nM and the ability to increase blood glucose levels and inhibit insulin secretion during an oral glucose tolerance test in mice (Nakamura *et al.*, 2012). Further development of this compound has not been published. Recently, a promising GIP receptor antibody, Gipg013, with a K_i of 6.8 nM was reported (Ravn *et al.*, 2013). Based on the numerous rodent studies that displayed promising improvements in the diabetic and/or obese state, (Pro3)GIP showed potential to fill the role as a promising antagonist in the context of

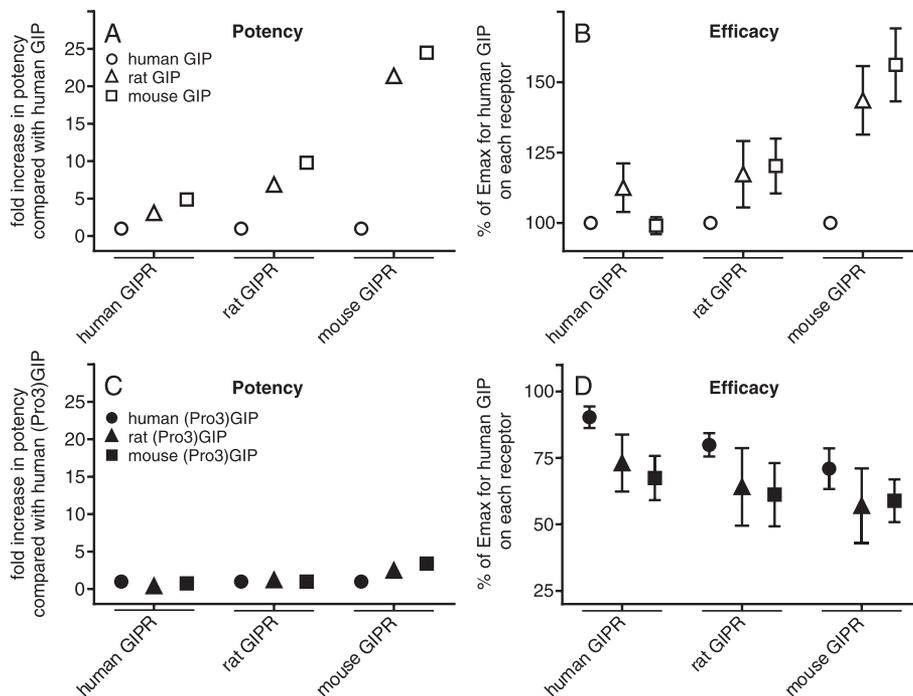


Figure 8

The changes in potency and efficacy of the tested GIP receptors (GIPR) and GIP analogues. The calculated EC₅₀ (A/C) and E_{max} (B/D) values determined from the cAMP accumulation assays and normalised to human GIP on each species receptor (A, B, and D) or human (Pro3)GIP on each species receptor.

GIP physiology (Gault *et al.*, 2007; Irwin *et al.*, 2007; McClean, 2007; De Toro-Martin *et al.*, 2014). In *ob/ob* mice, chronic treatment with (Pro3)GIP improved glucose tolerance and insulin sensitivity (Irwin *et al.*, 2007), while treatment in mice previously on a high-fat diet resulted in weight loss, improved insulin sensitivity and glucose tolerance (McClean, 2007).

Consistent with previous rodent studies (Gault *et al.*, 2002; Gault *et al.*, 2003; Gault Victor *et al.*, 2007), we find that rodent (Pro3)GIP ligands act as competitive antagonists on the rodent receptors (Figure 3). But when it comes to the human GIP system, human (Pro3)GIP acted as a efficacious agonist (Figure 2A), which is in line with very recent studies that reported substantial agonist activity of human (Pro3)GIP with efficacies up to 83% of human GIP in cAMP release from transiently transfected HEK293 cells (Ravn *et al.*, 2013) or CHL cells (Pathak *et al.*, 2015), and in the reporter gene expression for cAMP-response element (Al-Sabah *et al.*, 2014). However, this contrasts to a previous study demonstrating (Pro3)GIP to have 9% of GIP's efficacy on transiently transfected Chinese hamster lung cells (CHL) expressing the human GIP receptor (Gault Victor *et al.*, 2007). These efficacy discrepancies may rely on differences in cell types (CHL, HEK283 and COS-7 cells); however, consistent for all studies is the finding that (Pro3)GIP was not a neutral antagonist, but has agonist properties in cAMP-dependent pathways.

Structural GIP divergence between species has markedly affects the pharmacology

Our study emphasizes important interspecies variations within the GIP system. The rodent GIP receptor ligands were more potent and efficacious than human GIP on all the tested receptors (Figure 5), with up to 22-fold and 25-fold increase in potency of rat GIP and mouse GIP on the mouse GIP receptors (Figure 8A and B). These changes were not matched by a similar increase in binding affinities (Table 2). Only few amino acids are altered among the three GIP receptor ligands (Figure 1A). The most dramatic change is located at position 18 where human GIP has a histidine instead of an arginine, which is found in rat and mouse GIP. Among the few non-identities observed (in positions 18, 30, 34 and 40), this is the only case where the rodent sequences are identical and, because of the non-conservative nature (Arg/His), a significant structural divergence is possible. A previous study supports the functional importance of this position, as an alanine substitution in human GIP increased insulin secretion from BRIN-BD11 cells compared with native GIP (Venneti *et al.*, 2011). Thus, position 18 seems to have a role in the activation mechanism of the GIP receptor, as also supported by a recent study predicting a direct interaction between His18 of human GIP and Ala32 of the N-terminus of the GIP receptor (Tikhele *et al.*, 2010). Thus, these findings add some detail to the general view that hydrophobic interactions between the C-terminal part of GIP and the N-terminus receptor parts account for binding, while the N-terminus of GIP interacts with other extracellular receptor domains inducing activation (Malde *et al.*, 2007; Parthier *et al.*, 2007). This so-called two-step receptor activation not only describes GIP receptor activation but presents a global mechanism for

ligand-binding and receptor activation among class B1 receptors (e.g. glucagon, GLP-1, secretin, vasoactive intestinal polypeptide and parathyroid hormone receptor) (Gourlet *et al.*, 1996; Hjorth and Schwartz, 1996; Gardella and Jüppner, 2001; Castro *et al.*, 2005; Vilardaga *et al.*, 2011). It also extends beyond class B1, as also some class A seven-transmembrane receptors (e.g. chemokine, C3a and C5a receptors) are activated by their endogenous peptide ligands in a two (or multi)-step model involving receptor recognition and activation by different regions in the ligands (Allen *et al.*, 2007; Klos *et al.*, 2013; Thiele and Rosenkilde, 2014).

Taken together, the rodent GIP systems seem more active compared with the human system, in terms of GIP (1–42)-mediated activation and more sensitive to GIP ligand modifications, as judged by the lower activity of (Pro3)GIP. This is relevant and should be considered in studies using rodent models. As our study indicates, when using human GIP in rodent models, researchers are in fact injecting a less potent partial agonist (compared with rodent GIP) that may lead to underestimation of the true GIP activity in such experimental systems.

The therapeutic potential of targeting the GIP receptor

In the wake of the GIP receptor KO studies demonstrating resistance to diet-induced obesity (Miyawaki *et al.*, 2002), many have tried to antagonize the GIP receptors as a potential target for treating obesity. Accumulating evidence links GIP biology to adiposity and due to the lack of an effective GIP receptor antagonist, the therapeutic potential of antagonizing this receptor in obese patients remains to be assessed. In this context, our study brings attention to a significant interspecies difference within the GIP system, and thus, pharmacological evaluation of novel compounds in rodent models should be interpreted cautiously with respect to their relevance for humans.

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Conflicts of interest

A. H. S. U., L. S. H., B. S., M. C., B. H. and M. M. R. declare that they have no conflict of interest. J. J. H. has served as a consultant or advisor to Novartis Pharmaceuticals, Novo Nordisk, Merck Sharp & Dohme and Roche and has received fees for lectures from Novo Nordisk, Merck Sharp & Dohme and GlaxoSmithKline. F. K. K. has received fees for consultancy, lectures or being part of an advisory board from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, Gilead, Merck Sharp & Dohme, Novo Nordisk, Sanofi and Zealand Pharma, and has received research support from Sanofi and Novo Nordisk.

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