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*Published in:*
Biochemical Pharmacology

*DOI:*
10.1016/j.bcp.2017.02.012

*Publication date:*
2017

*Document version*
Publisher's PDF, also known as Version of record

Biochemical Pharmacology 131 (2017) 78–88

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

# GIP(3–30)$NH_2$ is a potent competitive antagonist of the GIP receptor and effectively inhibits GIP-mediated insulin, glucagon, and somatostatin release

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ARTICLE INFO

*Article history:*
Received 28 December 2016
Accepted 14 February 2017
Available online 22 February 2017

*Keywords:*
Glucose-dependent insulinotropic polypeptide receptor
Antagonist
cAMP
Competition binding
Pancreatic hormone secretion

ABSTRACT

Alternative processing of the precursor protein pro-GIP results in endogenously produced GIP(1–30)$NH_2$, that by DPP-4 cleavage *in vivo* results in the metabolite GIP(3–30)$NH_2$. We showed previously that GIP(3–30)$NH_2$ is a high affinity antagonist of the human GIPR *in vitro*. Here we determine whether it is suitable for studies of GIP physiology in rats since effects of GIP agonists and antagonists are strictly species-dependent. Transiently transfected COS-7 cells were assessed for cAMP accumulation upon ligand stimulation or assayed in competition binding using human $^{125}$I-GIP(1–42) as radioligand. In isolated perfused rat pancreata, insulin, glucagon, and somatostatin-releasing properties were evaluated. Competition binding demonstrated that on the rat GIP receptor (GIPR), rat GIP(3–30)$NH_2$ bound with high affinity ($K_i$ of 17 nM), in contrast to human GIP(3–30)$NH_2$ ($K_i$ of 250 nM). In cAMP studies, rat GIP(3–30)$NH_2$ inhibited GIP(1–42)-induced rat GIPR activation and schild-plot analysis showed competitive antagonism with a $pA_2$ of 13 nM and a slope of 0.9 ± 0.09. Alone, rat GIP(3–30)$NH_2$ displayed weak, low-potent partial agonistic properties ($EC_{50}$ > 1 μM) with an efficacy of 9.4% at 0.32 μM compared to GIP(1–42). In perfused rat pancreata, rat GIP(3–30)$NH_2$ efficiently antagonized rat GIP(1–42)-induced insulin, somatostatin, and glucagon secretion. In summary, rat GIP(3–30)$NH_2$ is a high affinity competitive GIPR antagonist and effectively antagonizes GIP-mediated G protein-signaling as well as pancreatic hormone release, while human GIP(3–30)$NH_2$, despite a difference of only one amino acid between the two (arginine in position 18 in rat GIP(3–30)$NH_2$; histidine in human), is unsuitable in the rat system. This underlines the importance of species differences in the GIP system, and the limitations of testing human peptides in rodent systems.



## 1. Introduction

GIP(1–42) is known as a postprandial gut hormone secreted from enteroendocrine K cells of the small intestine [1] together with other gut hormones [2,3]. Following a meal, GIP(1–42) enters the circulation and potentiates glucose-mediated insulin secretion from the pancreas [4]. Additional pancreatic effects may include stimulation of glucagon secretion from the α-cells [5,6] and somatostatin release from δ-cells [7,8]. The GIP receptor (GIPR) is widely expressed in various tissues besides the pancreas including adipose, bone, and lung tissue [9,10]. Particularly, the relationship between adipose tissue biology and the GIP system has received much interest. GIPR knock out mice are resistant to diet-induced obesity and crossing this mouse with the leptin mutant (*ob*/*ob*) mouse, which is an established mouse model for hyperphagic obesity, reduced weight gain by 23% [11], whereas transgenic GIPR expression in adipose tissue in global GIPR knock out mice restores diet-induced body weight gain [12]. Moreover, a recent study showed that heterogeneous abrogation of the GIP gene displays

*Abbreviations:* BSA, bovine serum albumin; DPP-4, dipeptidyl peptidase-4; GIP, glucose-dependent insulinotropic polypeptide; GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide-1; HBS, HEPES-buffered saline.

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http://dx.doi.org/10.1016/j.bcp.2017.02.012
0006-2952/

an intermediate phenotype in regard to high fat diet-induced insulin resistance and weight gain when compared to wild type and homogenous abrogation [13]. If GIP's physiology in rodents is mirrored in humans, these results support the use of GIPR antagonists as potential therapeutics for the treatment of obesity.

Various strategies have been pursued in the search for GIPR antagonists. Antibodies raised against both GIP(1–42) [14,15] or the GIPR [16,17], a small molecule antagonist [18], amino acid substitutions of GIP(1–42) [19], and various GIP(1–42) truncations and modifications such as e.g. Pro3(GIP) [20–24] have all been reported to be effective, but none have been found suitable for human studies. In 2006, we showed that the dipeptidyl peptidase-4 (DPP-4)-mediated metabolite, porcine GIP(3–42), antagonized porcine GIP (1–42)-mediated cAMP accumulation, but had no antagonistic effects in anesthetized pigs at physiological concentrations [22]. Recently, an alternative processing of the precursor protein pro-GIP was shown to occur in the α-cells of the pancreas and in a subset of the K-cells of the small intestine, which potentially leads to the secretion of GIP(1–30)$NH_2$ [25,26]. We combined the previously reported N-terminal truncation GIP(3–42) with this C-terminally truncated GIP(1–30)$NH_2$ to design the GIP(3–30)$NH_2$ (which is a naturally occurring metabolite of the DPP-4 cleaved GIP(1–30)$NH_2$), and demonstrated that GIP(3–30)$NH_2$ is an effective competitive antagonist on the human GIPR [27]. In fact, it was superior to other truncations of the N-terminus (GIP(2-, 4-, 5-, 6-, 7-, 8-, and 9–30)$NH_2$) and to GIP(3–42) in terms of basic binding affinity and antagonistic properties of the human GIPR *in vitro*. In the present study, we determine whether GIP(3–30)$NH_2$ is sufficiently active in the rat model system to be used for studies elucidating the role of GIP in physiology and pathophysiology.

## 2. Materials and methods

### 2.1. Materials

Rat GIP(1–42) (cat. No. 027-12) was purchased from Phoenix Pharmaceuticals, Karlsruhe, Germany. Human GIP(1–42) (H5645) was purchased from Bachem, Bubendorf, Switzerland. Human and rat GIP(3–30)$NH_2$ and GIP(1–30)$NH_2$ were synthesized by CASLO ApS, Lyngby, Denmark. All peptides had a purity of more than 95% by HPLC analysis and had the correct mass spectrometry controlled molecular weight. cDNAs of the human and rat GIPR were purchased from Origene, Rockville, Maryland, USA (SC110906, RN212314, and MC216211, respectively) and cloned into the pCMV-Script vector. Human $^{125}$I-labeled GIP(1–42) and $^{125}$I-labeled Tyr$^{11}$-somatostatin were purchased from PerkinElmer Life Sciences, Skovlunde, Denmark (NEX402 and NEX389, respectively). $^{125}$I-labeled glucagon and human $^{125}$I-insulin were kind gifts from Novo Nordisk A/S.

### 2.2. Animals

All animal care and experimental procedures were complied with institutional guidelines and 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 [28].

Male Wistar rats (220–250 g) were purchased from Janvier, Le Genest-Saint-Isle, France. 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 acclimatised for at least one week before use.

### 2.3. Transfections and tissue culture

COS-7 cells were cultured at 10% $CO_2$ and 37 °C in Dulbecco's modified Eagle's medium 1885 supplemented with 10% foetal bovine serum, 2 mM glutamine, 180 units/ml penicillin, and 45 g/ml 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 [29].

### 2.4. cAMP assay

Transiently transfected COS-7 cells were seeded in white 96-well plates at a density of 3 * $10^4$cells/well. One day after, the cells were washed twice with Hepes-buffered saline (HBS) buffer and incubated with HBS and 1 mM 3-isobutyl-1-methylxanthine (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 cells were preincubated for 10 min with the antagonist with subsequent addition of the agonist and incubated for a further 20 min. The HitHunter™ cAMP XS assay (DiscoveRx, Herlev, Denmark) was carried out according to the manufacturer's instructions.

### 2.5. Competition binding assay

COS-7 cells were seeded in clear 96-well plates the day after transient transfection. The number of cells added per well was governed by the apparent expression efficiency of the receptor, aiming for 5–10% specific binding of the radioactive ligand. The following day, cells were assayed by competition binding for 4 h at 4 °C using 15–40 pM of $^{125}$I-labeled GIP(1–42) as well as unlabeled ligand in a total volume of 100 μl per well in 50 mM Hepes buffer (pH 7.2) supplemented with 0.5% bovine serum albumin (BSA) (binding-buffer). After incubation, the cells were washed twice in 100 μl per well ice-cold binding buffer and lysed using 175 μl per well of 200 mM NaOH with 1% SDS for 30 min. Nonspecific binding was determined as the binding in the presence of 100 nM unlabeled ligand. The samples were analysed by the Wallac Wizard 1470 Gamma Counter.

### 2.6. Isolated perfused rat pancreas

Male Wistar rats (220–250 g) were anaesthetized (0.0158 mg fentanyl citrat + 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 [22]. 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 containing in addition of 5% dextran (Pharmacosmos, Holbaek, Denmark), 0.1% BSA, fumarate, glutamate, and pyruvate (5 mM of each), and 7 mM glucose. Flow rate was kept constant at 4 ml/min, perfusion buffer was heated and oxygenated (95% $O_2$, 5% $CO_2$), and pressure was continuously measured throughout the experiment. Rat GIP (3–30)$NH_2$ and rat GIP(1–42) were infused as test substances through a sidearm infusion pump at a flow rate of 0.2 ml/min. Arginine (10 mM) was infused at the end of each experiment as a positive control.

### 2.7. Hormone analysis

Hormone concentrations in the perfusion effluent were measured using in-house radioimmunoassays. Glucagon was measured using a side viewing antiserum (code no 4304) recognizing a mid sequence of glucagon, using synthetic glucagon for standards and $^{125}$I-labeled glucagon as tracer [30]. Insulin was measured using an antibody cross-reacting strongly with rat insulin I and II (code no. 2006-3). As standard we used human insulin and the tracer was $^{125}$I-labeled human insulin [31]. Somatostatin concentrations were determined using a rabbit antiserum (code no. 1758) raised against synthetic cyclic somatostatin, recognizing both somatostatin 14 and -28 [32], somatostatin 14 as standard and $^{125}$I-labeled Tyr$^{11}$-somatostatin as tracer.

### 2.8. Data- and statistical analysis

$IC_{50}$, $EC_{50}$, and $K_i$ values were determined by nonlinear regression using GraphPad Prism 7 (San Diego, California, United States of America). Sigmoid curves were fitted logistically with a Hillslope of 1.0 for the activation curves and -1.0 for the inhibition of cAMP and binding. $K_i$ values were calculated using the Cheng-Prusoff formula under the assumption of one class of binding sites. Dose ratios (DR) for the Schild analyses were based on the potency shift of rat GIP(1–42) in the presence of a given rat GIP(3–30)$NH_2$ concentration, relative to the absence of GIP(3–30)$NH_2$. Schild plots were performed with log(DR-1) (ordinate) and log(antagonist concentration) (abscissa) to estimate the slopes and $K_i$ values. For the rat pancreas perfusion data, baseline subtracted hormone output responses were evaluated using one-way ANOVA for repeated measurements. All calculations were performed using the software GraphPad Prism 7 with p-values <0.05 being considered significantly different.

### 2.9. Sequence alignments

The amino acid sequences of the rat/human GIP were acquired from GenBank of NCBI. The alignment was done 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. The sequence logo was generated using the web-based program WebLogo (http://weblogo.berkeley.edu) and the various mammalian GIP sequences were acquired from ensembl.org and uniprot.org.

## 3. Results

### 3.1. Human GIP(3–30)$NH_2$ displays a surprisingly low affinity on the rat GIPR compared to rat GIP(3–30)$NH_2$

In order to determine whether the double truncation of GIP(1–42), which leads to GIP(3–30)$NH_2$, is an effective antagonist *in vivo* using the rat as a model system, we initially evaluated the affinity of the ligand *in vitro*. Competition binding was conducted on transiently transfected COS-7 cells expressing the rat GIPR with $^{125}$I-labeled GIP(1–42) as the radioligand. GIP(1–30)$NH_2$ was included to enable assessment of the significance of the C-terminus in terms of GIPR binding. In light of our recent study identifying major interspecies differences between rodents and humans within the GIP system [24], both rat and human GIP(1–42), GIP(1–30)$NH_2$, and GIP(3–30)$NH_2$ were included. Human and rat GIP(1–42) were found to bind to the rat GIPR with equally high affinities ($K_i$ of 1.1 nM for human GIP(1–42) and $K_i$ of 0.88 nM for rat GIP(1–42)) (Fig. 1A). In contrast, rat GIP(1–30)$NH_2$ displayed a statistically significant improved affinity compared to human GIP(1–30)$NH_2$ with $K_i$ of 0.4 and 1.5, respectively (Fig. 1B). This species difference became even more pronounced for GIP(3–30)$NH_2$ which showed a 15-fold shift in affinity. For rat GIP(3–30)$NH_2$, the $K_i$ was 17 nM, which is a 19-fold reduction compared to rat GIP(1–42). In contrast, human GIP(3–30)$NH_2$ had a $K_i$ of 250 nM and thus a 227-fold lower affinity for the rat GIPR compared to human GIP(1–42). When looking at the sequence differences between species (Fig. 1D), only one amino acid (position 18 with arginine in rat and histidine in human GIP) differs between rat and human GIP(1–30)$NH_2$/GIP(3–30)$NH_2$. In fact, among the 42 sequences of GIP identified so far, a histidine is found at this position in all human and non-human primates (10 sequences), whereas GIP in the remaining 32 species has an arginine (Fig. 2A, B). Importantly, this alteration of position 18 had a large effect on the binding properties of GIP(3–30)$NH_2$, a minor effect on GIP(1–30)$NH_2$ binding, whereas it did not affect the binding affinity of GIP(1–42).

### 3.2. GIP(3–30)$NH_2$ is an antagonist of the rat GIPR

To investigate whether the high affinity of GIP(3–30)$NH_2$ reflects high antagonistic potency, as observed in the human GIP system [27], we chose the GIPR-induced cAMP accumulation, a well-established signaling pathway for GIPR activation [33,34], in transiently transfected COS-7 cells. GIP(1–42) and GIP(1–30)$NH_2$ were included to examine whether both forms activate the rat GIPR in a similar manner. For GIP(3–30)$NH_2$, the evaluation was conducted both in the absence and presence of rat or human GIP (1–42) in amounts corresponding to ~60% $E_{max}$. As previously shown [24], rat GIP(1–42) was more potent and efficacious on the rat GIPR compared to human GIP(1–42) with $EC_{50}$ values of 11 and 58 pM and $E_{max}$ values of 100 and 76%, respectively (Fig. 3A). In contrast, rat and human GIP(1–30)$NH_2$ were more similar to $EC_{50}$ values of 18 and 38 pM and $E_{max}$ values of 92 and 87%, respectively (Fig. 3B). Due to the higher potency and efficacy of rat GIP(1–42) compared to human GIP(1–42), 10 pM and 316 pM were chosen to achieve ~60% of $E_{max}$ for the evaluation of GIP(3–30)$NH_2$ antagonism of rat and human GIP(1–42), respectively. In the absence of GIP(1–42), rat GIP(3–30)$NH_2$ displayed a low-potent partial agonistic profile with an efficacy of 9.4% at 0.32 μM (Fig. 3C), while no agonistic properties were observed for human GIP(3–30)$NH_2$ even at the highest concentration (0.32 μM). In the presence of rat GIP(1–42), rat GIP(3–30)$NH_2$ antagonized rat GIPR-induced cAMP accumulation dose-dependently with an estimated $EC_{50}$-value of 118 nM. A similar pattern was observed for human GIP(3–30)$NH_2$ which dose-dependently inhibited human GIP(1–42), however, with an estimated $EC_{50}$-value of 380 nM (Fig. 3D). Thus, rat GIP(3–30)$NH_2$ is more potent as an antagonist on the rat GIPR than human GIP(3–30)$NH_2$, a pattern that mimicked the low affinity obtained for human GIP(3–30)$NH_2$ as compared to the rat counterpart (Fig. 1C), and rat GIP(3–30)$NH_2$ was therefore chosen for further investigation.

### 3.3. Rat GIP(3–30)$NH_2$ is a high affinity competitive antagonist of the rat GIPR

To determine the nature of the antagonistic properties of rat GIP (3–30)$NH_2$ on the rat GIPR, cAMP accumulation was measured as a function of increasing concentrations of rat GIP(1–42) in the absence or presence of fixed concentrations of rat GIP(3–30)$NH_2$ (Fig. 4A). Rightward shifts in potency of rat GIP(1–42) were observed with increased rat GIP(3–30)$NH_2$ concentration which is in line with the antagonistic properties (Fig. 3C). At concentrations from 17.8 to 316 nM of rat GIP(3–30)$NH_2$, the potency ($EC_{50}$) of rat GIP(1–42) decreased 2.6 to 28-fold compared to in the absence of rat GIP(3–30)$NH_2$. Based on these $EC_{50}$ values for

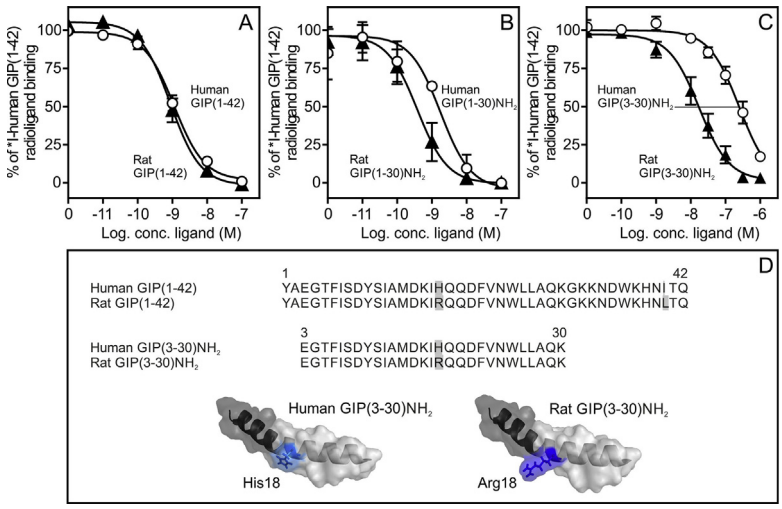


**Fig. 1.** Rat GIP(3–30)$NH_2$ binds to the rat GIPR with high affinity. The binding of $^{125}$I-labeled human GIP(1–42) to the transiently transfected COS-7 cells with rat GIPR cDNA, was tested in the presence of increasing amounts of (A) rat GIP(1–42) (▲), human GIP(1–42) (○), (B) rat GIP(1–30)$NH_2$ (▲), human GIP(1–30)$NH_2$ (○), (C) rat GIP(3–30)$NH_2$ (▲) or human GIP(3–30)$NH_2$ (○). The data were normalized to maximal specific binding and shown as mean ± SEM, n ≥ 3 independent experiments carried out in duplicates. Nonlinear regression was used to calculate the $IC_{50}$ value. (D) Structure overview with nonconserved residues highlighted of full length GIP(1–42) and GIP(3–30)$NH_2$ for both human and rat GIP(3–30)$NH_2$. The N-terminus not involved in binding to extracellular receptor domains is emphasized with a darker grey and position 18 is highlighted in the peptide model.

rat GIP(1–42), a Schild plot analysis was conducted (Fig. 4B). This analysis determines whether an antagonist acts competitively; if so, the equilibrium inhibitory constant ($K_i$) can be determined from the X-axis intercept ($pA_2$). A straight line relating the potency shifts with a Hill slope of 1.0 indicates competitive antagonism and the X-intercept or $pA_2$-value of the Schild plot corresponds to the $K_i$ of the antagonist. The Hill coefficient was 0.9 ± 0.09 which proves the competitive nature of rat GIP(3–30)$NH_2$ and the $pA_2$-value was 13 nM. This corresponds well with the $K_i$ of 17 nM for rat GIP(3–30)$NH_2$ observed from the competition binding (Fig. 1C), and thereby confirms the high affinity binding of GIP(3–30)$NH_2$ to the GIP receptor.

### *3.4. Rat GIP(3–30)$NH_2$ inhibits GIP(1–42)-induced hormone secretion from the perfused pancreas*

To determine whether the antagonistic properties from the cAMP measurements reflected a physiological antagonism of GIP-mediated pancreatic output, we used isolated perfused rat pancreata. In this model, rat GIP(1–42) and rat GIP(3–30)$NH_2$ were added to the arterial perfusate. The venous effluent was collected at 1 min intervals and the pancreatic output in terms of insulin, glucagon, and somatostatin was determined. To ensure detection of possible antagonistic properties of rat GIP(3–30)$NH_2$, a rat GIP(1–42) concentration, which still elicited a prominent release of each of the three pancreatic hormones of interest, was determined in the perfusion model. Three different concentrations of rat GIP(1–42) were tested (10 pM, 100 pM and 1 nM). From these experiments, 1 nM rat GIP(1–42) was chosen due to a significant release of all three hormones at this concentration (data not shown). 1 μM rat GIP (3–30)$NH_2$ was applied to ensure adequate antagonism. A preincubation with rat GIP(3–30)$NH_2$ and subsequent co-perfusion with both rat GIP(3–30)$NH_2$ and rat GIP(1–42) resulted in a clear, effective reduction in both the insulin, glucagon, and somatostatin output from the rat pancreata. Rat GIP(3–30)$NH_2$ demonstrated clear antagonism on the pancreatic β-, δ-, and α-cells, respectively (Fig. 5A–C). The same hormone release pattern was observed when the stimulation order was switched giving the mono-perfusion of rat GIP(1–42) before the co-perfusion with rat GIP(3–30)$NH_2$ and rat GIP(1–42) (data not shown). Furthermore, the partial agonism of rat GIP(3–30)$NH_2$ observed *in vitro* (Fig. 3C) was not reproduced for any of the hormonal responses during the single perfusion with rat GIP(3–30)$NH_2$ (Fig. 5A–C). Thus, a significant GIPR antagonism by GIP(3–30)$NH_2$ on pancreatic insulin, glucagon, and somatostatin secretion was confirmed (Fig. 5A–C, total output shown as columns).

## 4. Discussion

Our study demonstrates that rat GIP(3–30)$NH_2$ is a high affinity competitive antagonist on the rat GIPR *in vitro* and in the surviving perfused rat pancreas, whereas human GIP(3–30)$NH_2$ displays much lower affinity and a consequent lower antagonistic potency on the rat GIPR. This indicates that human GIP(3–30)$NH_2$ is irrelevant in the rat GIP system, whereas rat GIP(3–30)$NH_2$ can be used as a tool to study the GIP physiology when using the rat as a model system. To substantiate this, we show that rat GIP(3–30)$NH_2$ inhibits GIP(1–42)-mediated hormone release from the intact pancreas as evident from the strong inhibition of GIP(1–42)-mediated insulin, glucagon, and somatostatin release from β-, α-, and δ-cells of the pancreas (Fig. 5). This establishes GIP(3–30)$NH_2$ as an effective antagonist in a physiological system.

### *4.1. The C-terminal truncation improves antagonistic properties of GIP (3–30)$NH_2$ versus GIP(3–42), but not agonism of GIP(1–30)$NH_2$ versus GIP(1–42)*

The function of the 12 amino acids of the C-terminus of GIP(1–42) remains elusive. We previously showed that human GIP(3–30)$NH_2$ inhibited the human GIPR more potently compared to human GIP(3–42) [27]. Moreover, a recent study reported that palmitoylated human GIP(3–30)$NH_2$Cex (where Cex is a C-terminal extension of exendin) was able to antagonize GIP(1–42)-mediated insulin release *in vitro* from a rat β-cell line (BRIN-BD11 cells) [35]. However, when comparing the corresponding agonists (GIP (1–42) and GIP(1–30)$NH_2$) the differences are indistinguishable in terms of cAMP accumulation [27,36–38]. From a physiological perspective, both molecular forms have been shown to stimulate insulin secretion [26,39] and β-cell survival equipotently [40]; however, GIP(1–30)$NH_2$ has a reduced effect on lipoprotein lipase

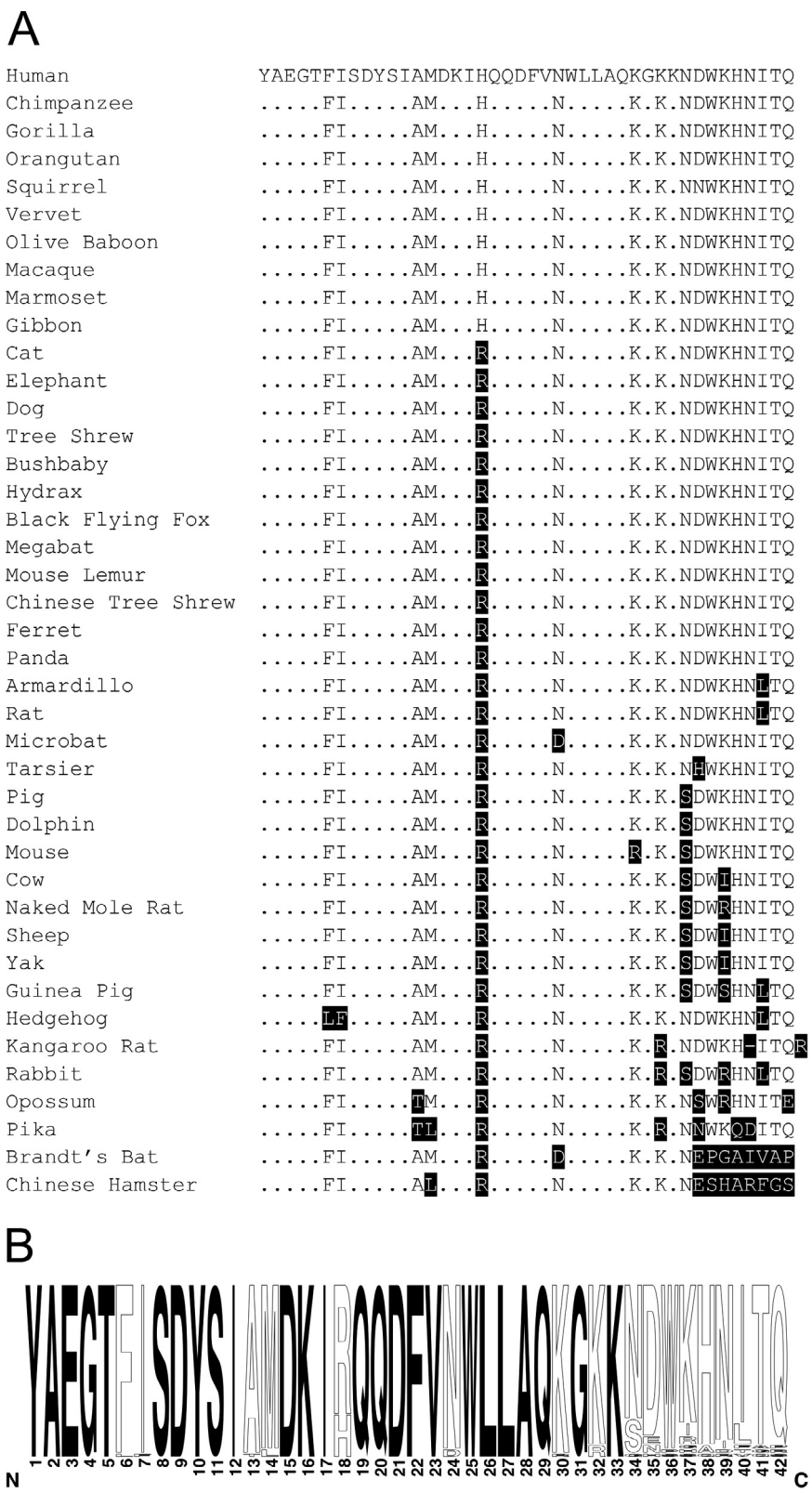


**Fig. 2.** Conservation of GIP(1–42) among mammals. (A) 41 of the available mammal sequences of the GIP(1–42) protein from ensembl.org and uniprot.org aligned with mismatches from the human sequence highlighted in black. Especially the C-terminus and position 18 of GIP(1–42) is less conserved. (B) The degree of conservation of the GIP (1–42) protein sequence between available mammal sequences displayed as sequence logo. The amino acids in black are fully conserved.

activity [40] and a reduced inhibitory effect on gastric acid secretion in rats [41]. In addition, a controversy exists in terms of gastric somatostatin release as GIP(1–30)$NH_2$ has been shown to be equipotent in mice [25] and less potent in rats [42], compared to GIP(1–42). In structure-activity studies, all the pivotal amino acids involved in the GIPR interaction are found within the first 30 amino acids [38,43], with the C-terminal part initiating binding with the extracellular receptor domains (ECD) while the N-

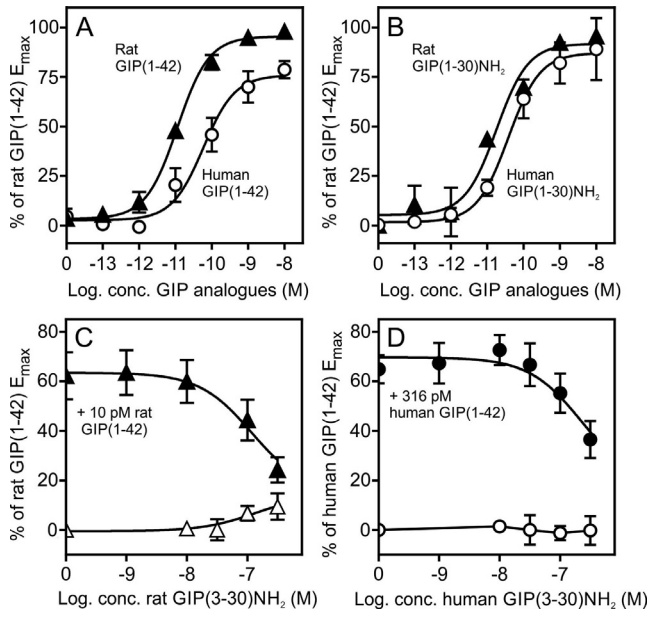


**Fig. 3.** Human and rat GIP(3–30)$NH_2$ are antagonists of the rat GIPR. COS-7 cells were transiently transfected with rat GIPR cDNA and assayed for cAMP accumulation following stimulation of (A) human GIP(1–42) (○) or rat GIP(1–42) (▲), (B) human GIP(1–30)$NH_2$ (○) or rat GIP(1–30)$NH_2$ (▲), (C) increasing concentrations of rat GIP(3–30)$NH_2$ in the absence (△) and presence of 10 pM rat GIP(1–42) (▲), and (D) increasing concentrations of human GIP(3–30)$NH_2$ in the absence (○) and presence of 316 pM human GIP(1–42) (●). The data were normalized to $E_{max}$ of rat GIP(1–42) (A, B, C) or $E_{max}$ of human GIP(1–42) (D) and shown as mean ± SEM, n ≥ 3 independent experiments carried out in duplicates. Nonlinear regression was used to calculate the $IC_{50}$ value.

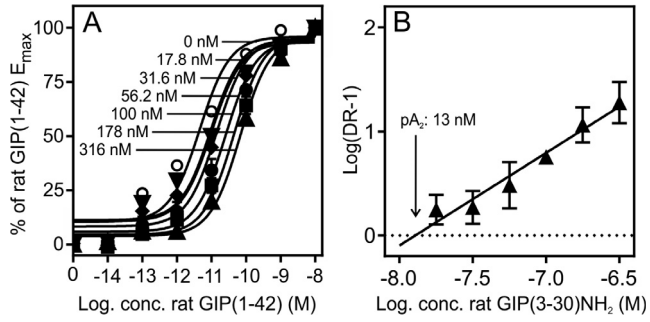


**Fig. 4.** Rat GIP(3–30)$NH_2$ is a high affinity competitive antagonist of the rat GIPR. (A) Rat GIP(1–42) cAMP accumulation dose–response curves in the absence of and with increasing concentrations of rat GIP(3–30)$NH_2$: 0 nM (○), 17.8 nM (*), 31.6 nM (▼), 56.2 nM (◆), 100 nM (●), 178 nM (■), and 316 nM (▲) are displayed. The data were normalized to $E_{max}$ of each curve and shown as mean ± SEM, n = 3 independent experiments carried out in duplicates. Nonlinear regression was used to calculate $EC_{50}$ values. (B) The corresponding Schild plot of the dose-response curves revealed a $pA_2/K_i$-value of 13 nM.

terminal part is thought to interact with the transmembrane receptor segments [43,44]. Particularly important GIP(1–42) residues involved in the binding to the ECD include Phe22, Val23, Leu26, and Leu27 which participate in hydrophobic interactions with ECD and the hydrophilic Asp15, Gln19, and Gln20 which interact with the ECD through hydrogen bonds [43]. In support of this, the truncations GIP(15–30)$NH_2$ and GIP(15–30) still retain binding, albeit with a ~400-fold reduction compared to GIP(1–42) [38]. Following binding of GIP(1–42)'s C-terminal to the ECD, Tyr1 appears to interact with multiple amino acids of the transmembrane receptor segments of the GIPR [45,46] and is pivotal for receptor activation [47], which is in line with the inactivation of GIP by DPP-4 and with the present study showing GIP(3–30)$NH_2$ as an effective antagonist [22,48]. Furthermore, alanine screening of the N-terminus of human GIP(1–42) has identified Glu3, Gly4, Thr5, and Ile7 as highly important for GIP(1–42)-induced insulin secretion in a rat cell line [47]. This so-called *two step receptor activation* not only describes GIPR activation, but is thought as a general activation mechanism for secretin-like receptors [44,49–52], and even for receptors outside the secretin-like receptors, such as the chemokine receptors, which belong to the class of rhodopsin-like receptors [44,53]. Moreover, when determining the degree of conservation of GIP between mammalian species, most of the variation is found among residues 30– 42 (Fig. 2B). In the present study, we found that human and rat GIP(1–42) bound with equal affinity to the rat GIPR, and only minor differences between human and rat GIP(1–30)$NH_2$ were observed. As previously shown, rat GIP (1–42) had greater agonistic potency and efficacy with respect to cAMP accumulation compared to human GIP(1–42) on the rat GIPR [24]. Surprisingly, a similar species difference was not observed between human and rat GIP(1–30)$NH_2$ indicating that the activity via $G_{\alpha s}$ is independent of whether an arginine (rat) or a histidine (human) is found in position 18. When looking at the rat ligands only, GIP(1–42) and GIP(1–30)$NH_2$ activated the rat GIPR in a completely identical manner confirming that the C-terminus does not have an impact on the agonistic properties. This is in agreement with previous work showing that human GIP(1–42) and GIP(1–30)$NH_2$ activate the human GIPR in an identical manner [27].

### *4.2. Position 18 impacts the antagonistic potential of GIP(3–30)$NH_2$ on the rat GIPR*

In contrast to the single C-terminally truncated GIP(1–30)$NH_2$, the double truncated GIP(3–30)$NH_2$ showed species-dependent variation as the rat GIP(3–30)$NH_2$ displayed both higher affinity and antagonistic potency of GIP-induced cAMP accumulation for the rat GIPR compared to human GIP(3–30)$NH_2$. Since position 18 is the only difference between human and rat GIP(1–30)$NH_2$/GIP(3–30)$NH_2$, the N-terminal truncation very likely exposes position 18 differently to the extracellular GIPR binding domain and thereby explains the shift in affinity. When taking a closer look at the GIP sequence among 41 species, most variations are observed in the C-terminal region (Fig. 2) and in comparison to the other incretin hormone, Glucagon-Like Peptide-1 (GLP-1), GIP is much less conserved between species [54]. However, position 18 remains conserved showing only a conservative substitution between histidine (in primates) and arginine (in rodents) - providing stronger antagonism with an arginine at this position. Confirming this, we observed a better antagonistic potency with porcine GIP(3–42), (arginine at position 18) compared to human GIP(3–42) (histidine at position 18) on the human GIPR [27]. Thus despite another variation in position 34 (serine vs. asparagine in pig vs. human, respectively), it is likely that this will also apply for the C-terminal truncated forms, such as GIP(3–30)$NH_2$.

### *4.3. Caution should be exercised when testing human GIP analogues in rodents*

Researchers striving for the elucidation of human GIP physiology have used rodent *in vivo* models extensively. Much attention has been given to the presumed GIPR antagonist, (Pro3)GIP, in rodent models [19,55–57], however, we recently discovered that human (Pro3)GIP is a partial agonist on the rodent GIPRs but a full agonist on the human GIPR [24]. Thus, interspecies differences at both the receptor and ligand level are important to consider when evaluating the potential of a new compound, a fact that has been neglected in previous studies (Tables 1 and 2). In addition, there are many differences related to the organ of interest between

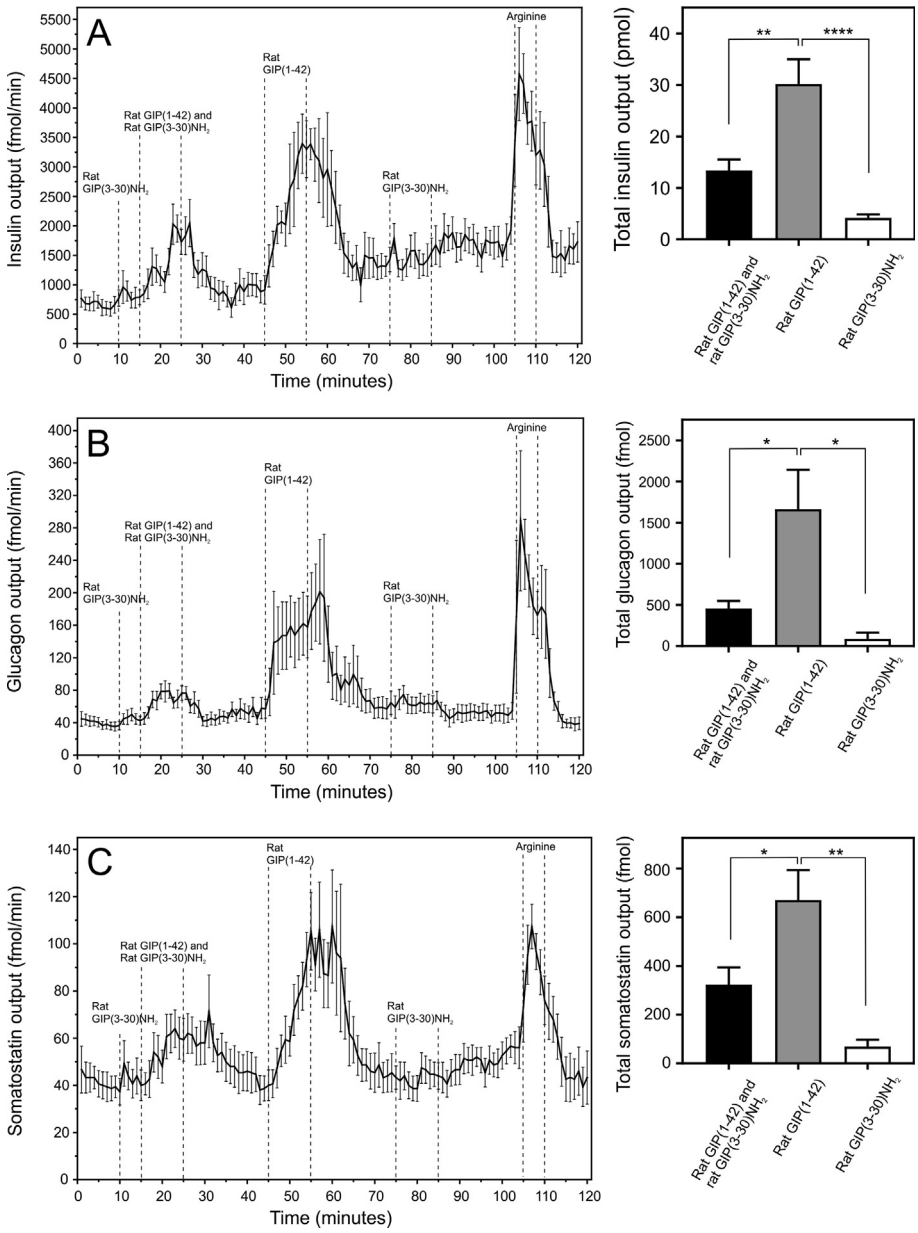


**Fig. 5.** Antagonistic effects of rat GIP(3–30)$NH_2$ on insulin, glucagon, and somatostatin secretion are observed in perfused rat pancreata. Insulin (A), glucagon (B), and somatostatin (C) secretion following stimulation of perfused rat pancreata by either 1 nM rat GIP(1–42) and 1 μM rat GIP(3–30)$NH_2$ with a 5 min preincubation of 1 μM rat GIP(3–30)$NH_2$, 1 nM rat GIP(1–42), 1 μM rat GIP(3–30)$NH_2$ or 10 mM arginine, n = 6 independent experiments. The glucose concentration was 7 mM and data are mean ± SEM. Baseline subtracted total outputs of insulin (A), glucagon (B), and somatostatin (C) during the 10 min infusion of rat GIP(1–42)/rat GIP(3–30)$NH_2$ are compared using a one-way ANOVA with multiple comparisons; mean ± S.E.M., $^{****}P < 0.0001$, $^{**}P < 0.01$, and $^{*}P < 0.05$.

rodents and humans, as for instance within the endocrine pancreas. This includes the topographical organization the rodent islets with the β-cells concentrated in the core surrounded by a mantle of α-cells and δ-cells. In contrast, the different cells of the human islet are highly dispersed [58–61], which may promote different potential paracrine effects and different intra-islet communication. On a structural level, our study emphasizes differences between GIPR, GIP(1–42), and GIP(3–30)$NH_2$ of rodent or human sequences (Figs. 1 and 3) and establishes that application of the DPP-4 metabolite of human GIP(1–30)$NH_2$, human GIP(3–30)$NH_2$, in rodent models would be misleading. Thus, a characterization of this antagonist and variants hereof should be carried out in human studies. Importantly, such studies could lead to the establishment of an effective tool for the elucidation of human GIP physiology and putatively result in a novel therapeutic possibility for the treatment of obesity. The rodent counterparts, on the other hand, must be used to characterize the GIP system in rodents.

## Conflicts of interest

A.H.S.U., M.N.G., L.S.G., C.B.C., B.S., 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.

**Table 1**
Overview of truncated GIP variants tested on the rat GIPR. The table displays an overview of truncated GIP variants of various different species tested on the rat GIPR both *in vitro* and *in vivo*. Percentages compared to full agonist GIP(1–42) or GIP(1–30) results. Results showing no binding, agonism or antagonism are in *italic*. GIP = species sequence of ligand, B = bovine, P = porcine, Hu = human, US = unknown species, UC = unknown concentration.

| Truncation | GIP | Rat GIPR | | | |
|---|---|---|---|---|---|
| | | Species | Function | Binding | References |
| N- and C-terminal (mainly antagoanists) | (3–30)$NH_2$ | R | COS cells: cAMP, antagonism ($EC_{50}$ 120 nM), low partial agonist, Emax 9.4% activity (0.32 μM) vs. GIP(1-42)<br>Pancreas perfusion: inhibition of GIP-mediated insulin, glucagon, and somatostatin release | COS cells: $IC_{50}$ 17nM | Figs. 1C, 3C, and 5 |
| | (6–30)$NH_2$ | P | CHO cells: cAMP: 58% inhibition (0.1 μM) | CHO cells: Equal $IC_{50}$ to GIP(1-42) | [20] |
| | (7–30)$NH_2$ | P | CHO cells: 25% cAMP inhibition (0.1 μM)<br>L293 cells: cAMP inhibition $IC_{50}$ 0.1 μM<br>In vivo: 100 nmol/kg abolishes 1.5 nmol/kg GIP-insulin secretion and reduces meal-induced insulin with 55%. | CHO cells: $IC_{50}$ 24 nM<br>L293 cells: $IC_{50}$ 200 nM | [38,62] |
| | | P | CHO cells: 10 μM decreases GIP(1-42) cAMP by 34%<br>3 μg/250 g: 30% inhibition of glucose-induced insulin, 15% plasma glucose, 54% meal-induced insulin. 20 μg/kg reduced glucose-uptake 30%. | CHO cells: $IC_{50}$ 177 nM | [63,20] |
| | (10–30) | P | CHO cells: 50%-inhibition in cAMP (10 μM) | CHO cells: 187 fold decreased $IC_{50}$ | [20] |
| | (15–30)$NH_2$ | P | CHO cells: cAMP, antagonism (10 μM): 30 % of GIP(7–30)<br>*Pancreas perfusion: No insulin release (1 ng/ml)* | CHO cells: $IC_{50}$ 1400 nM | [38,64] |
| | (16–30)$NH_2$ | P | CHO cells: cAMP, antagonism(10 μM): 20 % of GIP(7–30)<br>*L293 cells: No cAMP or GIP-inhib.(10 nM)* | CHO cells: $IC_{50}$ 2530 nM | [38,62] |
| | (17–30)$NH_2$ | P | CHO cells: cAMP, antagonism (10 μM): 30 % of GIP(7–30)<br>Pancreas perfusion: 38% insulin release (1 ng/ml) | CHO cells: $IC_{50}$ 1540 nM | [38,64] |
| | (19–30)$NH_2$ | P | CHO cells: 40% cAMP production (20 μM)<br>Pancreas perfusion: 60% insulin release (1 ng/ml)<br>In vivo: Significant glucose decrease (100 pmol/min/100g) | CHO cells: 52% binding (10 μM) | [38,64] |
| | | P | *CHO cells: No cAMP (1 μM)* | *CHO cells: No binding (1 μM)* | [65] |
| | (21–30)$NH_2$ | P | *L293 cells: No activity, no inhibition of pGIP(1–42)* | | [62] |
| N-terminal (mainly antagonists) | (3–42) | US | BRIN-BD11: 30% cAMP ($EC_{50} \approx$ nM) and 1 μM inhibits 45%. 70% inhibition of insulin release by 1 μM | | [23] |
| | | P | Pan. perfusion: Inhibition of insulin, $IC_{50}$ 138nM | | [66] |
| | (4–42) | US | BRIN-BD11: 50% cAMP, 30% inhibition of insulin (1 μM) | | [23] |
| | (5–42) | US | BRIN-BD11: 35% cAMP (1 μM), insulin inhibition 70% | | |
| | (6–42) | US | BRIN-BD11: 80% cAMP (1 μM), insulin inhibition 40% | | |
| | (7–42) | US | BRIN-BD11: 65% cAMP (1 μM), insulin inhibition 55% | | |
| | (8–42) | US | RINm5F: 30-fold decrease of GIP(1–42) potency (5 μM) | | [67] |
| | | US | BRIN-BD11: 16% cAMP (1 μM), insulin inhibition 65% | | [23] |
| | (9–42) | US | BRIN-BD11: 100% cAMP (1 μM), 100% insulin secretion | | |
| | (15–42) | P | CHO cells: cAMP, antagonism (10 μM): 40 % of GIP(7–30)<br>Pancreas perfusion: 40% insulin response (10 ng/ml) | CHO cells: $IC_{50}$ 1270 nM | [23,38,68] |
| | (17–42) | B | Pancreas perfusion: 32% insulin response (5 ng/ml) | | [69] |
| | | US | *RINm5F cells: No cAMP production (1 μM)* | *RINm5F cells: $IC_{50}$ 0.4 μM* | [23] |
| | (31–44) | P | *L293 cells: No cAMP or pGIP inhibition (10 nM)* | | [62] |
| C-terminal (agonists) | (1–6)$NH_2$ | US | *CHO cells: No cAMP production* | *CHO cells: No binding (10 μM)* | [38] |
| | (1–7)$NH_2$ | US | *CHO cells: No cAMP production* | *CHO cells: No binding (10 μM)* | |
| | (1–13)$NH_2$ | P | CHO cells: 13% cAMP (20 μM) | CHO cells: 5% binding (10 μM) | |
| | (1–14)$NH_2$ | P | CHO cells: 73% cAMP (20 μM)<br>26% insulin release (5 nM), sig. glucose decrease | CHO cells: 28% binding (10 μM) | |
| | | P | *Pancreas perfusion: No insulin release (10 ng/ml)* | | [68] |
| | (1–14) | H | CHO cells: Full agonist (cAMP) | CHO cells: 51% binding (10 μM) | [38] |
| | (1–15)$NH_2$ | P | CHO cells: 4% cAMP (20 μM) | CHO cells: 4% binding (10 μM) | |
| | (1–15) | P | *CHO cells: No cAMP production* | *CHO cells: No binding (10 μM)* | |
| | (1–16) | US | CHL: 35% cAMP, BRIN-BD11: 10–50% insulin (1 μM) | | [70] |
| | | US | BRIN-BD11: Insulin 1.4 fold of basal (1 μM) | | [71] |
| | (1–30)$NH_2$ | R | COS cells: Full agonist (cAMP), $EC_{50}$ equal to GIP | COS cells: $IC_{50}$ 0.4nM | Figs. 1B and 3B |
| | | P | CHO and L293 cells: Full agonist (cAMP), $EC_{50}$ like GIP(1–42)<br>RINm5F: cAMP full agonism, 10-fold decreased $EC_{50}$<br>Equal glucose decrease (1 pmol/min/100 g)<br>Pancreas perfusion: Full insulin response (5 nM)<br>Pancreas perfusion: Full insulin response, 65% reduced somatostatin response (1 ng/ml) | CHO and L293 cells: $IC_{50}$ 2.0 nM<br>RINm5F : 10-fold decreased $IC_{50}$ | [38,62,65,72] |
| | | US | Pancreas: Full insulin response (1 nmol/kg/h)<br>Stomach: 10-fold lower gastric acid-inhibition (10 nmol/kg/h) | | [41] |
| | (1–30)OH | Hu | Pancreas perfusion: Full insulin response (1 nM) | | [65] |
| | (1–38) | P | Pancreas perfusion: Full insulin response (UC) | | [73] |
| | (1–39) | B | Pancreas perfusion: Full insulin response (1 nM) | | [74] |

**Table 2**
Overview of truncated GIP variants tested on various GIPR species. The table displays an overview of truncated GIP variants of various different species tested on different species of GIPR both *in vitro* and *in vivo*. Percentages compared to full agonist GIP(1–42) or GIP(1–30) results. Results showing no binding, agonism or antagonism are in *italic*. GIP = species sequence of ligand, Hu = human, P = porcine, B = bovine, M = mouse, Ha = hamster, US = unknown species.

| Truncation | GIP | GIPR from other species (human, mouse, porcine, bovine, hamster) | | | | |
|---|---|---|---|---|---|---|
| | | Species | GIPR | Function | Binding | References |
| N- and C-terminal (mainly antagonists) | (2–30)$NH_2$ | Hu | Hu | COS cells: 20% efficacy in cAMP, $EC_{50}$ of 3.7 nM, Antagonism (1 μM): 50 % inhibition of GIP(1–42) with $EC_{50}$ of 22 nM | COS cells: $IC_{50}$ of 14 nM | [27] |
| | (3–30)$NH_2$ | Hu | Hu | COS cells: Antagonism (0.1 μM): 100 % inhibition of GIP(1–42) with $EC_{50}$ of 12 nM | COS cells: $IC_{50}$ of 2.3 nM | |
| | (4–30)$NH_2$ | Hu | Hu | COS cells: Antagonism (1 μM): 90 % inhibition of GIP(1–42) with $EC_{50}$ of 108 nM | COS cells: $IC_{50}$ of 22 nM | |
| | (5–30)$NH_2$ | Hu | Hu | COS cells: Antagonism (0.1 μM): 85% inhibition of GIP(1–42) with $EC_{50}$ of 12 nM | COS cells: $IC_{50}$ of 5.9 nM | |
| | (6–30)$NH_2$ | Hu | Hu | COS cells: Antagonism (0.1 μM): 75% inhibition of GIP(1–42) with $EC_{50}$ of 342 nM | COS cells: $IC_{50}$ of 347 nM | |
| | (7–30)$NH_2$ | Hu | Hu | COS cells: Antagonism (0.1 μM): 75% inhibition of GIP(1–42) with $EC_{50}$ of 137 nM | COS cells: $IC_{50}$ of 26 nM | |
| | (8–30)$NH_2$ | Hu | Hu | COS cells: *No intrinsic cAMP*. Antagonism (0.1 μM): 50% inhibition of GIP(1–42) with $EC_{50}$ of 133 nM | COS cells: $IC_{50}$ of 79 nM | |
| | (9–30)$NH_2$ | Hu | Hu | COS cells: *No intrinsic cAMP*. Antagonism (0.1 μM): 25% inhibition of GIP(1–42) with $EC_{50}$ of 450 nM | COS cells: $IC_{50}$ of 307 nM | |
| | (17–30)$NH_2$ | US | US | CHL cells: 1 μM inhibits cAMP (0.1 μM GIP) 15% BRIN-BD11: 1 μM inhibits insulin (0.1 μM GIP) 11% | | [70] |
| | (18–28) | Hu | P | | *CHO cells: No binding (1 μM)* | [65] |
| | (19–30)$NH_2$ | B | Ha | | *Pancreas membranes: No binding* | [69] |
| | (21–26) | Hu | P | | *CHO cells: No binding (1 μM)* | [65] |
| N-terminal (mainly antagonists) | (3–42) | Hu | Hu | COS cells: cAMP, antagonism (1μM): 60 % inhibition of GIP(1–42) with $EC_{50}$ of 671 nM | | [27] |
| | | P | Hu | COS cells: cAMP, antagonism (1μM): 75 % inhibition of GIP(1–42) with $EC_{50}$ of 32 nM | | |
| | | P | P | Anaesthetized pig: No inhibitory effect on insulinotropic effects of GIP | | [22] |
| | (4–42) | B | Ha | | Pancreas membranes: $IC_{50}$ of 5 nM | [69] |
| | | US | US | CHL cells: 1 μM inhibits cAMP (0.1 μM GIP) 40% BRIN-BD11: 1 μM inhibits insulin (0.1 μM GIP) 23% | | [70] |
| | | US | M | Ob/ob: Glucose increase (25 nmol/kg) | | [23] |
| | (5–42) | US | M | Ob/ob: Glucose increase (25 nmol/kg) | | |
| | (7–42) | US | M | Ob/ob: Glucose increase (25 nmol/kg) | | |
| | (8–42) | US | M | Ob/ob: Glucose increase, sig. insulin decrease (25 nmol/kg) | | |
| | (13–42) | Hu | Hu | | Titration calorimetry: 6-fold lower affinity | [43] |
| | (15–42) | Hu | Hu | | Titration calorimetry: 10-fold lower affinity | |
| | (17–42) | B | Ha | | Pancreas membranes: $IC_{50}$ 0.5 μM | [69] |
| | | Hu | Hu | | Titration calorimetry: 15-fold lower affinity | [43] |
| | (19–42) | Hu | Hu | | Titration calorimetry: 17-fold lower affinity | |
| | (21–42) | Hu | Hu | | Titration calorimetry: 100-fold lower affinity | |
| | (23–42) | Hu | Hu | | *Titration calorimetry: No binding* | |
| C-terminal (agonists) | (1–3) | B | Ha | | *Pancreas membranes: No binding (10 μM)* | [69] |
| | (1–16) | B | Ha | | *Pancreas membranes: No binding (10 μM)* | |
| | | US | M | *Ob/ob: No effect on glucose/insulin (25 nmol/kg)* | | [71] |
| | (1–30)$NH_2$ | Hu | Hu | COS cells: Full agonist (cAMP), $EC_{50}$ equal to GIP(1–42) | COS cells: $IC_{50}$ equal to GIP(1–42) | [27] |
| | (1–30)OH | Hu | P | | CHO cells: 11-fold lower affinity than GIP(1-42) | [65] |

## Acknowledgements

This work was supported by the Novo Nordisk Foundation Center for Basic Metabolic Research and the University of Copenhagen.

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