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# Human GIP(3-30)$NH_2$ inhibits G protein-dependent as well as G protein-independent signaling and is selective for the GIP receptor with high-affinity binding to primate but not rodent GIP receptors

Maria Buur Nordskov Gabe[a,b], Alexander Hovard Sparre-Ulrich[b], Mie Fabricius Pedersen[c], Lærke Smidt Gasbjerg[a,d,e], Asuka Inoue[f], Hans Bräuner-Osborne[c], Bolette Hartmann[a,e], Mette Marie Rosenkilde[a,e,*]

[a] Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[b] Antag Therapeutics ApS, Copenhagen, Denmark
[c] Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[d] Center for Diabetes Research, Gentofte Hospital, University of Copenhagen, Denmark
[e] NNF Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[f] Laboratory of Molecular and Cellular Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Japan

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ABSTRACT

GIP(3-30)$NH_2$ is a high affinity antagonist of the GIP receptor (GIPR) in humans inhibiting insulin secretion via G protein-dependent pathways. However, its ability to inhibit G protein-independent signaling is unknown. Here we determine its action on arrestin-recruitment and receptor internalization in recombinant cells. As GIP is adipogenic, we evaluate the inhibitory actions of GIP(3-30)$NH_2$ in human adipocytes. Finally, we determine the receptor selectivity of GIP(3-30)$NH_2$ among other human and animal GPCRs.

cAMP accumulation and β-arrestin 1 and 2 recruitment were studied in transiently transfected HEK293 cells and real-time internalization in transiently transfected HEK293A and in HEK293A β-arrestin 1 and 2 knockout cells. Furthermore, human subcutaneous adipocytes were assessed for cAMP accumulation following ligand stimulation. Competition binding was examined in transiently transfected COS-7 cells using human $^{125}$I-GIP (3–30)$NH_2$. The selectivity of human GIP(3-30)$NH_2$ was examined by testing for agonistic and antagonistic properties on 62 human GPCRs.

Human GIP(3-30)$NH_2$ inhibited GIP(1-42)-induced cAMP and β-arrestin 1 and 2 recruitment on the human GIPR and Schild plot analysis showed competitive antagonism with a $pA_2$ and Hill slope of 16.8 nM and 1.11 ± 0.02 in cAMP, 10.6 nM and 1.15 ± 0.05 in β-arrestin 1 recruitment, and 10.2 nM and 1.06 ± 0.05 in β-arrestin 2 recruitment. Efficient internalization of the GIPR was dependent on the presence of either β-arrestin 1 or 2. Moreover, GIP(3-30)$NH_2$ inhibited GIP(1-42)-induced internalization in a concentration-dependent manner and notably also inhibited GIP-mediated signaling in human subcutaneous adipocytes. Finally, the antagonist was established as GIPR selective among 62 human GPCRs being species-specific with high affinity binding to the human and non-human primate (*Macaca fascicularis*) GIPRs, and low affinity binding to the rat and mouse GIPRs ($K_d$ values of 2.0, 2.5, 31.6 and 100 nM, respectively).

In conclusion, human GIP(3–30)$NH_2$ is a selective and species-specific GIPR antagonist with broad inhibition of signaling and internalization in transfected cells as well as in human adipocytes.

## 1. Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is a hormone secreted postprandially from enteroendocrine K cells in response to ingestion of either fat [1], protein [2], or carbohydrates [3]. GIP is primarily known for its glucose-dependent insulinotropic actions mediated via the GIP receptor (GIPR) expressed on pancreatic β-cells [4–6], a property it shares with the other incretin hormone glucagon-like peptide-1 (GLP-1), secreted from enteroendocrine L cells [7]. GIP regulates glucagon secretion from the pancreatic α-cells in a glucose-dependent manner by stimulating glucagon secretion at hypoglycemic conditions [8,9], but not during hyperglycemia [10]. Thus, there is a

* Corresponding author at: Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.
*E-mail address:* rosenkilde@sund.ku.dk (M.M. Rosenkilde).

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clear glucose-dependent duality regarding its stimulation of both insulin and glucagon secretion from the pancreatic islets. The lipogenic effect of GIP has drawn much attention after the generation of the GIPR knockout ($GIPR^{-/-}$) mouse which was resistant to diet-induced obesity [11]. Consistent with this, a study of mice lacking intestinal GIP secreting cells demonstrated resistance to diet-induced obesity [12]. However, studies of the effects on adipocyte metabolism have shown conflicting results as GIP has been shown both to have anabolic and catabolic effects in adipose tissue [13–23].

The GIPR is widely expressed in various tissues such as the pancreas, adipose tissue, bone, lungs and brain [24] and belongs to class B GPCRs which is structurally characterized by a large N-terminal extracellular domain which is important for ligand binding [25]. GPCRs are especially known for their interaction with G proteins resulting in either activation or inhibition of second messengers [26]. They also signal through G protein-independent signaling pathways by interaction with e.g. β-arrestins [27] which has given rise to the study of biased signaling. Coupling to β-arrestins is primarily associated with the termination of G protein-dependent signaling and subsequent receptor desensitization and internalization, but may also initiate signaling pathways [28]. In patients with type 2 diabetes (T2D), the incretin effect of GIP is attenuated [29,30]. As the secretion of GIP in these patients seems to be unaltered [31] this may point towards an alteration of GIPR signaling pathways such as e.g. an increased receptor desensitization. However, this still remains to be clarified.

We have previously described that the naturally occurring GIPR antagonist, GIP(3-30)$NH_2$, is a highly potent competitive antagonist of the human GIPR with regard to GIP-mediated G protein-dependent signaling [32]. Importantly, GIP(3-30)$NH_2$, is a more potent antagonist compared to the other naturally occurring GIPR antagonist GIP(3-42) [32,33]. Like exendin(9-39), being a specific GLP-1 receptor (GLP-1R) antagonist with an indispensable role in the study of human GLP-1 physiology, GIP(3-30)$NH_2$ represents a comparable tool for the studies of human GIP physiology and pathophysiology. As such, we have shown that GIP(3-30)$NH_2$ efficiently inhibits GIP-potentiated glucose-stimulated insulin secretion [34] and triacylglycerol (TAG) uptake in adipose tissue in humans [35].

Until now, GIP(3-30)$NH_2$'s ability to inhibit different GIPR-mediated signaling pathways has not been studied. Understanding the GIPR inhibition mechanism of GIP(3-30)$NH_2$ is of considerable importance when using it as a tool to study GIP physiology in humans but also in relation to potential drug development strategies. In the present study we examine if GIP(3-30)$NH_2$ inhibits different G protein-independent pathways. We also investigate if it inhibits the GIP-mediated effects in adipocytes to probe for cell signaling differences. Finally, using a palette of metabolically relevant GPCRs, we screen for whether GIP(3-30)$NH_2$ is a specific antagonist for the human GIPR. Taken together, these experiments contribute to the understanding of the different physiological actions of GIP such as its lipogenic effects and attenuated effects in patients with T2D.

## 2. Materials and methods

### 2.1. Materials

Human GIP(1-42) was purchased from Bachem, Bubendorf, Switzerland (H5645). Human GIP(3-30)$NH_2$ was 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, rat, and mouse GIPR were purchased from Origene, Rockville, Maryland, USA and cloned into the pCMV-Script vector (SC110906, RN212314, and MC216211, respectively). B-arrestin 1 and 2 knockout HEK293A cells (Δβ-Arr1/2) were generated as described in [36]. Vectors containing the human β-arrestin 1 (transcript variant 1, Ref.seq. NM_004041.4) and β-arrestin 2 (transcript variant 1, Ref.seq. NM_004313.3) were cloned from in-house vectors into the pcDNA3.1(+) vector between restriction sites HindIII and XbaI. The restriction sites were inserted with the following primers: fwd β-arr1 CTTCTTAAGCTTGCCACCATGGGCGA-CAAAGGGAC, rev β-arr1 CGGGCCCTCTAGACTATCTGTTG, fwd β-arr2 CTTCTTAA-GCTTG CCTCCATGGGGGAGAAACCCGGG, rev β-arr2 CGGGCCCTCTAGACTA GCA-GAG. Furthermore, the full kozak-sequence (GCCACCATG) was inserted upstream of the ORF. The human N-terminally SNAP-tagged GIPR [37] and *Macaca fascicularis* GIPR (NCBI reference sequence: XP_005589662.1) were synthesized by GenScript, Piscataway, STATE, USA. As tracer we used $^{125}$I-labeled human GIP(3-30)$NH_2$. Human subcutaneous preadipocytes were purchased from Lonza, Walkersville, Maryland, USA (PT-5020).

### 2.2. Transfection and tissue cultures

HEK293, HEK293A and HEK293A Δβ-Arr1/2 cells were cultured at 5% or 10% $CO_2$ and 37 °C in DMEM-GlutaMAX™-I supplemented with 10% foetal bovine serum (FBS), 180 units/ml penicillin, and 45 g/ml streptomycin. COS-7 cells were cultured at 10% $CO_2$ and 37 °C in Dulbecco's modified Eagles medium 1885 supplemented with 10% FBS, 2 mM glutamine, 180 units/ml penicillin, and 45 g/ml streptomycin. Transient transfection of HEK293 cells for BRET cAMP, β-arrestin 1, and β-arrestin 2 and COS-7 cells for competition binding was performed using the calcium phosphate precipitation method [38]. For the BRET cAMP assay the cells were co-transfected with the Epac-based BRET sensor for cAMP, CAMYEL (cAMP sensor using YFP-Epac-RLuc) [39]. For the BRET β-arrestin 1 and 2 assay the cells were co-transfected with either the donor Rluc8-Arrestin-2-Sp1 (β-arrestin 1) or Rluc8-Arrestin-3-Sp1 (β-arrestin 2), the acceptor mem-linker-citrine-SH3 and GPCR kinase 2 (GRK2) to facilitate β-arrestin 1 or 2 recruitment [40]. Transient transfection of HEK293A and HEK293A Δβ-Arr1/2 cells for real-time internalization was performed with Lipofectamine according to the manufacturer's instructions.

Human subcutaneous preadipocytes were cultured at 5% $CO_2$ and 37 °C in Preadipocyte Growth Medium-2 (PGM-2) supplemented with 10% FBS, 2 mM L-glutamine and 30 μg/ml gentamicin, and 15 ng/ml amphotericin-B. For initiating differentiation of the preadipocytes the PGM-2 medium was added insulin, dexamethasone, indomethacin and IBMX.

### 2.3. BRET cAMP and β-arrestin 1 and 2 assay

Two days after transfection, transiently transfected HEK293 cells co-expressing the human GIPR and CAMYAL for cAMP or the human GIPR and Rluc8-Arrestin-2-Sp1 (donor, β-arrestin 1) or Rluc8-Arrestin-3-Sp1 (donor, β-arrestin 2), mem-linker-citrine-SH3 (acceptor) and GRK2 (helper protein) for β-arrestin 1 or 2 recruitment were washed with PBS and resuspended in PBS with 5 mM glucose. 85 μl of the cell suspension solution were added to each well of a black-white 96-well isoplate followed by the addition of PBS with 50 μM coelenterazine-h. Following a 10 min incubation, fixed concentrations of human GIP(3-30)$NH_2$ (10 nM, 100 nM and 1 μM) were added and preincubated for 10 min before subsequent addition of increasing concentrations of human GIP (1-42) (0.1 pM to 10 nM in the cAMP assay and 100 pM to 10 μM in the β-arrestin 1 and 2 assay). Following 20 min incubation after agonist addition, luminescence (Rluc8 at 485 nm and YFP at 535 nm) was measured by the Berthold Technologies Mithras Multilabel Reader.

### 2.4. Real-time internalization assay

Real-time internalization assays were essentially performed as previously published [41,42]. Briefly, HEK293A wild-type or Δβ-Arr1/2 knockout cells [43] transiently expressing the human SNAP-tagged GIPR and in relevant experiments also β-arrestin 1 or 2 were seeded in white 384-well plates the day after transfection at a density of $2 * 10^4$ cells/well. The following day, the media was removed and the