Title: GIP receptor antagonist treatment causes weight loss in ovariectomized high fat diet-fed mice

Running title: a GIP receptor antagonist causes weight loss

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Word count (excluding figure legends and references): 3403 words

Acknowledgements: We would like to thank Tabatha Emilia de A Constantini, Lene Brus Albæk, Christine Rasmussen, Maibritt Sigvardt Baggesen, and Søren Petersen for their excellent technical assistance. This work was supported by a grant from AP Møller Fonden (17-L-0366) to GAB, a grant from the Novo Nordisk Foundation (NNF15OC0016574) and an ERC Advanced Grant (695069) to JJH.

Conflicts of interest statement: MBNG, AHSU, JJH and MMR are co-founders of Antag Therapeutics ApS. The remaining authors declare that they do not have a conflict of interest.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

ABSTRACT

Background and purpose The incretin hormone, glucose-dependent insulinotropic polypeptide (GIP), secreted by the enteroendocrine K-cells in the proximal intestine, may regulate lipid metabolism and adiposity but its exact role in these processes is unclear.

Experimental approach We characterized *in vitro* and *in vivo* antagonistic properties of a novel GIP analogue, mGIPAnt-1. We further assessed the *in vivo* pharmacokinetic profile of this antagonist, as well as its ability to affect high-fat diet (HFD)-induced body weight gain in ovariectomized mice during an 8-week treatment period.

Key results mGIPAnt-1 showed competitive antagonistic properties to the GIP receptor (GIPR) *in vitro* as it inhibited GIP-induced cAMP accumulation in COS-7 cells. Furthermore, mGIPAnt-1 was capable of inhibiting GIP-induced glucoregulatory and insulinotropic effects *in vivo* and has a favourable pharmacokinetic profile with a half-life of 7.2 hours in C57Bl6 female mice. Finally, sub-chronic treatment with mGIPAnt-1 in ovariectomized HFD mice resulted in a reduction of body weight and fat mass.

Conclusion and Implications mGIPAnt-1 successfully inhibited acute GIP-induced effects *in vitro* and *in vivo* and sub-chronically induces resistance to HFD-induced weight gain in ovariectomized mice. Our results support the development of GIP antagonists for the therapy of obesity.

KEYWORDS

Glucose-dependent insulinotropic polypeptide (GIP); GIP receptor; Antagonist; Pharmacokinetics; Obesity

MAIN TEXT

INTRODUCTION

Following ingestion of a meal, the enteroendocrine K-cells in the proximal gut secrete the 42-amino acid hormone, glucose-dependent insulinotropic polypeptide (GIP) (Buffa et al., 1975; Jörnvall et al., 1981). GIP is an incretin hormone, stimulating insulin production through activation of its receptor, GIPR, on pancreatic β-cells (McIntyre, Holdsworth, & Turner, 1964). Additionally, the receptor is expressed in various other tissues, including adipose tissue, bone, the central nervous system (CNS) and the heart, suggesting that GIP has other functions in the body (Adriaenssens et al., 2019; Bollag et al., 2000; Usdin, Mezey, Button, Brownstein, & Bonner, 1993; Yip, Boylan, Kieffer, & Wolfe, 1998). Research has especially been directed towards elucidating the effect of GIP on adipose tissue and its role in the pathogenesis of obesity.

Besides GIPR expression on adipocytes (Yip et al., 1998), other indications for a link between GIP and obesity include findings of increased circulating GIP levels in both obese individuals (Calanna et al., 2013) and subjects given a high-fat diet (HFD) (Brøns et al., 2009). *In vitro* studies have shown that GIP increases fatty acid uptake in adipocytes (Kim, Nian, & McIntosh, 2007; Yip et al., 1998), and in humans infusion of GIP during a high-insulin, high-glucose clamp increased triglyceride deposition in subcutaneous adipose tissue (Asmar et al., 2010). Furthermore, GIPR knockout (GIPR KO) mice are resistant to diet-induced obesity (Miyawaki et al., 2002), and transgenic rescue of the GIPR in adipose tissue in the same mouse model counteracted this resistance (Ugleholdt et al., 2011).

Following these findings, considerable effort has been exerted towards the development of antagonists of the GIPR for the treatment of obesity. Both GIP and GIPR neutralizing antibodies and treatment with SKL-14959, a GIPR antagonistic small-molecule compound, induced resistance to weight gain in HFD-fed mice (Boylan, Glazebrook, Tatalovic, & Wolfe, 2015; E. A. Killion et al., 2018; Nakamura et al., 2018). Peptide analogues based on a naturally occurring C-

and N-terminally truncated GIP variant, GIP(3-30)NH_2, were found to antagonize the GIPR and reduced obesity and improved metabolic control in mice (Hansen et al., 2016; Pathak, Gault, Flatt, & Irwin, 2015). The most potent antagonist of the truncated GIP variants, GIP(3-30)NH_2 was shown to inhibit GIPR signalling in human adipocytes (Gabe et al., 2018) and to inhibit triglyceride deposition in subcutaneous adipose tissue in humans during *in vivo* infusions (Asmar et al., 2017). Interestingly, treatment with GIPR *agonists* has also been shown to reduce body weight or weight gain in preclinical studies; both when given alone and in combination with an agonist of the glucagon-like peptide-1 (GLP-1) receptor (Mroz et al., 2019; Nørregaard et al., 2018). It was, however, shown recently that chronic treatment with a GIPR agonist resulted in desensitization of the GIPR, resulting in functional antagonism of the receptor (Elizabeth A. Killion et al., 2020).

In this study, we have characterized the *in vitro* antagonistic properties of a novel GIP analogue that was optimized for chronic use in murine studies, hereafter indicated as mGIPAnt-1. We also assessed the pharmacokinetic profile of this antagonist, as well as its ability to acutely inhibit GIP-induced glucoregulatory actions *in vivo.* Finally, we studied how sub-chronic treatment with mGIPAnt-1 affected HFD-induced body weight gain in ovariectomized mice. The ovariectomized model resembles the post-menopausal state and the estrogen deficiency-induced in this model results in increased body weight, abdominal fat mass and insulin resistance compared to sham-operated mice (McElroy & Wade, 1987). GIPR KO mice are resistant to weight gain (F. Isken et al., 2008) induced by ovariectomy. With this study we also used the ovariectomized mouse model to assess whether our antagonist was able to induce similar beneficial effects.

MATERIALS AND METHODS

Peptide

mGIPAnt-1, which is a peptide based on the structure of truncated GIP, was custom synthesized by Peptides&Elephants (Hennigsdorf, Germany). Prior to delivery, the purity of the

peptide was determined to be 95.7%, and the correct molecular weight was ascertained by mass spectrometry. The peptide was dissolved in 100 mM $NaHCO_3$ containing 0.1% casein (solution from bovine milk – 5% in water, Sigma Aldrich, St Louis, USA) prior to use. cDNAs of human and mouse GIPR were purchased from OriGene, Rockville, Maryland, USA (SC110906 and MC216211, respectively).

***In vitro* studies**

COS-7 cells were cultured at 10% CO_2 and 37°C in Dulbecco's Modified Eagle Medium 1885 supplemented with 10% fetal bovine serum, 2 mM glutamine, 180 units/mL penicillin, and 45 g/mL streptomycin. Transient transfection of the cells with either human or mouse GIPR was performed using the calcium phosphate precipitation method as previously described (van der Velden et al., 2021). Following the day after transfection, the cells were seeded in white 96-well plates with a density of 35.000 cells/well. The next day the assay was initiated by washing the cells with HEPES buffered saline (HPS) followed by an incubation step with HPS and 1 mM 3-isobutyl-1-methylxanthine for 30 minutes at 37°C. For agonist studies, the ligands were then added and incubated for an additional 30 minutes at 37°C. To test for antagonistic properties, the cells were first preincubated for 10 minutes with the antagonist with subsequent addition of the agonist and incubated for an additional 20 minutes. The HitHunter® cAMP assay (Eurofins DiscoverX, Fremont, USA) was carried out according to the manufacturer's protocol. Luminescence was measured by PerkinElmer™ Envision 2014 Multilabel Reader.

Animal studies

Animal experiments were performed with permission from the Danish Animal Experiments Inspectorate (license 2013-15-2934-00833) and the local ethical committee following the guidelines of Danish legislation governing animal experimentation (1987) and the National Institutes of Health (publication number 85-23). Female C57Bl/6Jrj mice (8 weeks old) were obtained from Janvier Labs (Saint Berthevin Cedex, France) and allowed to acclimatize prior to

procedures in groups of 4 for 1 week in individually ventilated cages with a 12-hour light cycle with *ad libitum* access to standard chow and water.

Pharmacokinetic analysis

To assess mGIPAnt-1 half-life and bioavailability, mice (n=6) received a single subcutaneous (SC) administration of 6 nmol mGIPAnt-1. Retro-orbital blood samples (50 µL) were taken using EDTA coated glass capillaries (Vitrex, Vasekær, Denmark) at 1) t=0, 0.5, 1, 1.5, 2 and 2.5 hours, 2) t=2.5, 3, 4, 6, 8 and 22 hours or 3) t=22, 24, 26, 28, 30 and 32 hours and immediately added to Eppendorf tubes (Eppendorf, Hamburg, Germany) and centrifuged at 3500 *g*, 20 min, 4˚C. Plasma was transferred into Eppendorf tubes on dry ice and stored at -20˚C for analysis.

In vivo biological activity of mGIPAnt-1

To assess the biological activity of mGIPAnt-1, we performed an intraperitoneal glucose tolerance test (IPGTT). Mice were given either mGIPAnt-1 (25 µmol/kg body weight; BW, SC) or vehicle at t=-90 minutes. Group sizes were as follows: Vehicle + Vehicle, n=10, Vehicle + GIP, n=12 and mGIPAnt-1 + GIP, n=8. At t=-10 all mice were given an intraperitoneal (IP) injection with dipeptidyl peptidase-4 (DPP-4) inhibitor valine pyrrolidide (val-pyr, a gift from Novo Nordisk, Bagsværd, Denmark), followed by a SC injection of either 25 nmol/kg BW GIP (Synthetic mouse GIP(1-42), Caslo peptides, Lyngby, Denmark) or vehicle. At t=0 minutes, mice received an IP injection with 0.5 g/kg BW glucose. Blood glucose was measured from the tail vein using a hand-held glucometer (Accu-Chek Mobile, Roche, Basel, Switzerland) at t=0, 10, 30, 45, 60, 120 and 180 minutes. Retro-orbital blood samples (50 µL) were taken using EDTA coated glass capillaries (Vitrex, Vasekær, Denmark) at t=0, 15, 30 and 60, 120 and 180 minutes and centrifuged at 3500 *g*, 20 min, 4˚C. Plasma was transferred into Eppendorf tubes on dry ice and stored at -20˚C for later insulin measurements.

Ovariectomy

Prior to bilateral ovariectomy surgery and once daily for 48 hours following, mice received analgesics through SC injection of carprofen (Rimadyl, 5 mg/kg BW, Pfizer, New York, NY, USA). Anaesthesia was induced in an induction chamber with 5% isoflurane (Baxter, Søborg, Denmark) and maintained with 2% isoflurane through a nose cone during the procedure. Mice were placed in a prone position on a sterile surgery pad and the area of incision was shaved and sterilized with 70% ethanol. Two dorsal-lateral incisions were made and the ovary and attached fat pad were individually pulled out of the abdominal cavity. The fallopian tubes were ligated and the ovaries were dissected, and the fat pad was carefully placed back in the abdominal cavity. The skin incision was closed using wound clips (Reflex, Alzet, Cupertino, CA, USA). In half of the animals, a microtransponder (Datamars, Lamone, Switzerland) was inserted and animals were placed in a HM-2 cage (MBrose, Faaborg, Denmark) and left to recover and acclimatize for a week. The remaining animals were placed back in their home cages to recover for a week. Uterine atrophy was confirmed in all mice upon termination of the study, indicating successful ovariectomy.

HFD studies

A week after ovariectomy, at 10 weeks of age, animals were placed on a 60 kcal% HFD (HFD, D12492, Research Diets, New Brunswick, NJ, USA) or a control diet (CD, 10 kcal% fat, D12450J, Research Diets) for 8 weeks. Group sizes were as follows: Vehicle/CD, n=12; mGIPAnt-1/CD, n=12; Vehicle/HFD, n=11; mGIPAnt-1/HFD, n=12. Animals received a daily SC injection at 4:30 pm with mGIPAnt-1 (300 nmol/kg BW) or vehicle. Body weight and body composition were assessed weekly (LF90II body composition analyzer, Bruker, Billerica, MA, USA). On the final experimental day, animals were anaesthetized with an intraperitoneal injection of 90 mg/kg ketamine (Ketaminol Vet., MSD Animal Health, Madison, NJ, USA) and 10 mg/kg xylazine (Rompun Vet., Bayer Animal Health, Leverkusen, Germany). The microchip was quickly removed

from HM-2 cage animals and a final body composition measurement was performed on all animals. A terminal retro-orbital blood sample was taken, mice were euthanized and tissues were quickly dissected and weighed.

Glucose tolerance test

After 7 weeks HFD and mGIPAnt-1 treatment, an oral glucose tolerance test (OGTT) was performed. Animals were fasted from 08:00 am to 11:00 am and received 2 g/kg BW glucose through oral gavage. Blood glucose was measured in the tail vein samples using a hand-held glucometer (Accu-Chek Mobile, Roche) at t=0, 15, 30, 45, 60, 120 and 180 minutes. Retro-orbital blood samples (75 µL) were taken using EDTA coated glass capillaries (Vitrex, Vasekær, Denmark) at t=0, 15, 30 and 60 minutes and centrifuged at 3500 *g*, 20 min, 4˚C. Plasma was transferred into Eppendorf tubes on dry ice and stored at -20˚C for later analysis of insulin measurements.

Biochemical analysis

Radioimmunoassay

mGIPAnt-1 levels were measured by an in-house radioimmunoassay (RIA) using a polyclonal antibody recognizing a mid-regional part of GIP (code no. 95234) (Gasbjerg et al., 2017). mGIPAnt-1 was used as standard and the assay buffer was a 80mM phosphate buffer pH 7.5, containing (in final concentrations) 0.1 % HSA, 10mM EDTA (Titriplex, Merck Millipore, Burlington, MA, USA), 500 KIU/ml aprotinin (Trasylol, Nordic Group, Hoofddorp, The Netherlands) and 0.01mM valine pyrrolidide. Samples were carefully diluted to ensure that samples would fall within the measureable range of the assay. The tracer was ^{125}I-labelled human GIP(1-42) (catalogue no. NEX402; Perkin Elmer, Skovlunde, Denmark).

ELISA

Insulin levels in IPGTT and OGTT plasma samples were measured using a mouse insulin ELISA (catalogue no. 10-1247-10; Mercodia AB, Uppsala, Sweden). Adiponectin levels in terminal plasma samples were measured using a mouse adiponectin ELISA kit (catalogue no. 80569, Crystal Chem, Elk Grove Village, IL, USA). Adiponectin samples were carefully diluted in kit-specific assay buffer. The manufacturer's instructions were followed closely.

Triglyceride and glycerol

Triglyceride and free glycerol levels in terminal plasma samples were determined using a triglyceride and free glycerol kit (TR0100-1KT, Sigma-Aldrich, St. Louis, MO, USA)

Calculations and statistical analysis

Half-life (t½) of mGIPAnt-1 administered SC was, determined from the concentration vs time curves after semi-logarithmical transformation and calculated with the following formulas: $k= (\ln(C_{t1})-\ln(C_{t2}))/\Delta t$ and $t½= \ln(2)/k$ with C_{t1} at t=1.5 hours and C_{t2} at t=32 hours. Results are presented as mean ± SE. Data were analyzed with 1-way and 2-way ANOVA with Tukey's or Sidak multiple comparisons test and unpaired t-tests where appropriate. Statistical significance was accepted at $P<0.05$. For the *in vitro* data IC_{50} values were determined by nonlinear regression using GraphPad Prism 9 (San Diego, California, USA). Sigmoid curves were fitted with a Hill slope of -1.0 for the inhibition curves.

RESULTS

In vitro properties of mGIPAnt-1

The antagonistic properties of mGIPAnt-1 was determined *in vitro* in cAMP accumulation before initiation of the *in vivo* studies in comparison to the naturally occurring GIPR antagonist GIP(3-30)NH_2. The experiments were done on both the human and mouse GIPR to examine the translatability between species. To keep the experiments species specific, mGIPAnt-1 was

compared to mouse GIP(3-30)NH_2 (mGIP(3-30)NH_2) on the mouse GIPR and human GIP(3-30)NH_2 (hGIP(3-30)NH_2) on the human GIPR. We also determined whether mGIPAnt-1 had any intrinsic activity. Here we observed no activation of the mouse or human GIPR and neither did mGIP(3-30)NH_2 activate the mouse GIPR (Fig.1A and B) as previously shown for hGIP(3-30)NH_2 on the human GIPR (Gabe et al., 2018; Gasbjerg et al., 2017; Hansen et al., 2016). mGIPAnt-1 inhibited the mouse GIPR with a 3-fold better potency than mGIP(3-30)NH_2 with an IC_{50} of 269 nM compared to 813 nM, respectively (Fig.1A). Focusing on the human GIPR, mGIPAnt-1 antagonized with an IC_{50} of 11 nM (Fig.1B) which is similar to that of hGIP(3-30)NH_2 previously shown to antagonize with an IC_{50} of 12 nM (Hansen et al., 2016).

Pharmacokinetics of mGIPAnt-1 *in mice*

Fig. 2A shows plasma concentrations of mGIPAnt-1 following SC administration (6 nmol/mouse). Plasma concentrations were measured by in-house RIA. To obtain the full elimination curve mice were studied in groups with sampling at different time intervals: 0-2.5 hours, 2.5-22 h and 22-32 h. Peak concentration was reached 1.5 h following administration and half-life was calculated to be approximately 7.2 hours.

In vivo mGIPAnt-1 *bioavailability*

We performed an IPGTT in mice treated with 1) vehicle and vehicle, 2) vehicle and GIP and 3) mGIPAnt-1 and GIP to determine if mGIPAnt-1 could inhibit the glucose-lowering actions of GIP following IP glucose administration *in vivo*. Fig. 2B shows the glucose curve following glucose administration at t=0 minutes. Treatment with GIP alone lowered glucose levels significantly from t=45 minutes and onwards compared to mice treated with both mGIPAnt-1 and GIP. The glucose curves of mice treated with vehicle alone did not differ from that of mice treated with both mGIPAnt-1 and GIP. The incremental area under the curve (iAUC) (Fig. 2C) was

significantly different for mice treated with vehicle and GIP compared to the double vehicle group and for mice treated both with mGIPAnt-1 and GIP.

Body weight and body composition of ovariectomized mice following mGIPAnt-1 treatment

Two weeks following ovariectomy, mice were placed on either CD or HFD and daily treatment with mGIPAnt-1 or vehicle (control) commenced. Fig. 3A shows that from 4 weeks onwards, body weight in the control, HFD group was significantly higher compared to the mGIPAnt-1/HFD group, whereas absolute body weight was not affected by mGIPAnt-1 treatment in animals fed CD. We observed an overall treatment effect of mGIPAnt-1, reducing total body weight gain over the course of the experiment (Fig. 3A). As expected, we also observed an overall diet effect (Fig. 3B). Final lean body mass was not affected by mGIPAnt-1 treatment, whereas at 6 and 8 weeks, Control/HFD animals significantly increased body fat percentage compared to mGIPAnt-1/HFD animals (Fig. 3C). Weight of both eWAT and iWAT were reduced in mGIPAnt-1/HFD mice compared to Vehicle/HFD mice, whereas mGIPAnt-1 treatment did not affect eWAT and iWAT weight in CD-fed mice (Fig. 3D-E). BAT and liver weight was not affected by mGIPAnt-1 treatment (Fig. 3F-G).

Glycemic control following mGIPAnt-1 *treatment*

Whereas HFD feeding increased glucose and insulin levels during an OGTT, mGIPAnt-1 treatment for 7 weeks did not affect glucose or insulin curves, nor the respective iAUCs (Fig 4A-B and D-E). However, AT/HFD mice did have significantly decreased fasting glucose levels compared to Vehicle/HFD mice (Fig 4C). Fasting insulin levels were not affected by mGIPAnt-1 treatment, although HFD did significantly increase fasting insulin levels (Fig 4F).

Terminal plasma levels of adiponectin, triglycerides and glycerol

mGIPAnt-1 treatment did not significantly affect plasma adiponectin or triglyceride levels or glycerol levels (Fig. 5). mGIPAnt-1/HFD mice showed a trend towards increased glycerol levels when compared to Vehicle/HFD mice, but this did not reach significance

DISCUSSION AND CONCLUSIONS

Antagonism of the GIPR has received much attention as a potential target for obesity therapy. In this study, we characterized the *in vitro* and *in vivo* pharmacological properties of a novel GIPR antagonist, mGIPAnt-1. Secondly, we show that sub-chronic treatment with mGIPAnt-1 results in a reduction of body weight gain in ovariectomized mice.

mGIPAnt-1 is a truncation of the GIP sequence which was acylated to enhance *in vivo* half-life. A similar strategy has been applied to prolong the activity of other GIPR antagonists. (Pro^3)GIP, in which Glu^3 is substituted with Pro^3, was demonstrated to be resistant to enzymatic degradation (Gault, O'Harte, Harriott, & Flatt, 2002). This compound was acylated (e.g. Pro^3 GIPLys^{16}PAL) or mPEGylated (Pro^3GIP[mPEG]) to enhance bioactivity of the antagonist, although this was only achieved after mPEGylation (Gault et al., 2007; McClean, Irwin, Hunter, Gault, & Flatt, 2008). (Pro^3)GIP and its variants showed promising effects in murine models; treatment induced a decrease in body weight gain in HFD-fed mice, improved glucose tolerance and enhanced insulin sensitivity (McClean et al., 2008). It was, however, later discovered that Pro^3GIP is not a full antagonist, but rather a partial agonist of the rodent GIPR and a full *agonist* of the human GIPR (Sparre-Ulrich et al., 2016). This finding also highlighted the importance of interspecies differences in the GIP system (Sparre-Ulrich et al., 2016). Human GIP(3-30)NH_2 is a specific and competitive antagonist of the human GIPR and has been used as a tool to study GIP physiology in humans (Gasbjerg et al., 2021; Gasbjerg et al., 2017) and a species-specific variant was used in rodent studies (Perry et al., 2019). However, due to its short half-life, GIP(3-30)NH_2 has so far only been used in acute studies in humans (Lynggaard, Gasbjerg, Christensen, & Knop, 2020).

In this study, we first confirmed *in vitro* that mGIPAnt-1 was capable of inhibiting cAMP accumulation induced by bio-active, species-specific GIP(1-42) at both the human and mouse GIPR, and that it did so to a similar degree as mGIP(3-30)NH_2 on the mouse GIPR. Furthermore, our pharmacokinetic *in vivo* studies showed that following SC administration, peak values were reached after 1.5 hours and half-life of the compound was approximately 7.2 hours. *In vivo* GIPR antagonism by mGIPAnt-1 at a dose 1000 times that of mGIP(1-42) was confirmed with an IPGTT. As mice consume the majority of their food during the dark-phase (Kurokawa, Akino, & Kanda, 2000), we decided from our data that a once-daily injection, 1.5 hours before the dark phase in the animal would be sufficient to induce antagonism of secreted GIP following food ingestion during studies with sub-chronic treatment with mGIPAnt-1.

We utilized an ovariectomized mouse model to study the effects of sub-chronic daily treatment of mGIPAnt-1 on body weight and composition and glucose regulation. Rodent ovariectomy models mimic menopause, and the drop of plasma estrogen levels following removal of the ovaries results in increased fat mass and body weight gain (McElroy & Wade, 1987). Interestingly, GIPR KO was reported to prevent ovariectomized-induced weight gain in mice (Frank Isken et al., 2008; Shimazu-Kuwahara et al., 2019). Daily treatment with mGIPAnt-1 significantly reduced body weight in HFD-fed ovariectomized mice after 4 weeks of treatment and when assessing overall body weight gain over the 9-week treatment period, we observed an overall reducing effect of mGIPAnt-1 treatment on body weight gain. This increase was due to a change in fat mass, rather than due to a change in lean body mass. This was further confirmed by decreased tissue weights of both eWAT and iWAT. Due to technical difficulties, we were, unfortunately, unable to obtain food-intake data. Previous studies using GIPR antagonists have shown contrasting effects of treatment with the different antagonists on food intake. Treatment of diet-induced obese mice with a GIPR antibody reduced food consumption (E. A. Killion et al., 2018), whereas the small-

molecule compound SKL-14959 suppressed weight gain without affecting food consumption (Nakamura et al., 2018).

Whereas mGIPAnt-1 acutely inhibited the glucose-lowering and insulin-releasing actions of exogenously administered GIP, no effects of sub-chronic mGIPAnt-1 treatment on glucose and insulin curves during an OGTT were observed. We did, however, observe reduced fasting levels in HFD-fed female mice treated with the antagonist compared to vehicle-treated animals on the same diet. These results are similar to those observed in mice treated with a GIPR antibody, which also exhibited lower fasting glucose levels but no change in glucose curves during an OGTT (E. A. Killion et al., 2018). However, those mice also exhibited lower fasting insulin levels, whereas fasting insulin levels were unchanged in the current study.

Now that we have established that mGIPAnt-1 functions as an antagonist of the murine GIPR, has a half-life that allows for once-daily dosing, and induces a weight reduction in mice, it is of great interest to further explore the mechanism behind these anti-obesogenic effects. In a recent study, we showed that the resistance to diet-induced weight gain more commonly shown in GIPR KO mice can be at least in part be explained by enhanced energy expenditure and activity levels, as well as increased lipolysis in iWAT and eWAT (Boer, Keenan, Miotto, Holst, & Watt, 2021). Interestingly, we found that GIPR KO had increased postprandial lipid storage in iWAT. It would be highly interesting to investigate whether the resistance to weight gain that we observed following GIPR antagonist treatment in this study is due to similar mechanisms.

Killion et al. (E. A. Killion et al., 2018) showed that in both obese mice and non-human primates, treatment with a combination of an antagonistic GIPR antibody and the GLP-1 receptor (GLP-1R) agonist dulaglutide resulted in greater weight loss compared to either treatment alone. Paradoxically, however, in both clinical and pre-clinical trials, combining a GLP-1R agonist with a GIPR agonist likewise resulted in a more positive treatment outcome compared to treatment with the GLP-1R agonist alone (Frias et al., 2018; Nørregaard et al., 2018). With the development of

mGIPAnt-1, we have created a novel tool compound to further study the effects of GIPR antagonism in the context of obesity.

In conclusion, we demonstrate that the tool compound developed to antagonize the GIPR in mice, mGIPAnt-1, is capable of acutely inhibiting GIP's glucoregulatory and insulinotropic effects *in vivo* and induces resistance to HFD-induced weight gain in ovariectomized mice.

FIGURE LEGENDS

Figure 1. *In vitro* signalling profile of mGIPAnt-1 on the mouse and human GIPR. COS-7 cells were transiently transfected with (A) mouse GIPR or (B) human GIPR and assayed for cAMP accumulation. To test for agonism, increasing concentration of the ligand was added. To test for antagonism, increasing concentrations of the antagonist were added together with a fixed concentration of either mouse GIP(1-42) or human GIP(1-42) corresponding to 50-80% of E_{max} on the mouse GIPR or human GIPR, respectively. Data are shown as mean ± SE from n=5 independent experiments.

Figure 2. mGIPAnt-1 pharmacokinetics and *in vivo* bioavailability. (A) mGIPAnt-1 pharmacokinetics following SC administration of 6 nmol mGIPAnt-1, (B) glucose levels during intraperitoneal glucose tolerance test (IPGTT) following mGIPAnt-1 (25 µmol/kg BW)/vehicle and GIP (25 nmol/kg BW)/vehicle administration, (C) incremental AUC. Statistical analysis: (B) 2-way ANOVA with Tukey Multiple Comparisons test, *P<0.05, **P<0.01, ****P<0.0001, Vehicle + GIP *vs. mGIPAnt-1* + GIP; (C) 1-way ANOVA with Tukey Multiple Comparisons test **P<0.01, ***P<0.001. Female C57Bl6/J mice, Vehicle + Vehicle, n=10, Vehicle + GIP, n=12, mGIPAnt-1 + GIP, n=8. Data are presented as mean ± SE.

Figure 3. Body weight and composition following high fat diet (HFD) and mGIPAnt-1 treatment. (A) Body weight, (B) body weight gain (%), (C) Body fat (%), and terminal tissue weight (D) epididymal white adipose tissue (eWAT), (E) inguinal white adipose tissue (iWAT), (F) brown adipose tissue (BAT) and (G) Liver. Statistical analysis: (A, C) 2-way ANOVA with Tukey Multiple Comparisons test *P<0.05, **P<0.01, Vehicle/HFD *vs.* mGIPAnt-1/HFD; (B, D-G) 2-way ANOVA with Sidak Multiple Comparison Test; ***P<0.001. Female ovariectomized C57Bl6/J mice,

Vehicle/CD, n=12; mGIPAnt-1/CD, n=12; Vehicle/HFD, n=11; mGIPAnt-1/HFD, n=12 (n=6 for C). Data are presented as mean ± SE.

Figure 4. Oral glucose tolerance test (OGTT). (A) glucose and (D) insulin levels, and (B) glucose and (E) incremental area under the curve following 2 g/kg BW oral glucose, (C) fasting glucose and (F) insulin levels. Statistical analysis: (A, D) 2-way ANOVA with Tukey Multiple Comparisons test; (B, C, E, F) 2-way ANOVA with Sidak Multiple Comparisons test, *P<0.05. Female ovariectomized C57Bl6/J mice, Vehicle/CD, n=12; mGIPAnt-1/CD, n=12; Vehicle/HFD, n=11; mGIPAnt-1/HFD, n=12. Data are presented as mean ± SE.

Figure 5. Biochemical analysis. Terminal plasma levels of (A) adiponectin, (B) triglyceride and (C) glycerol. Statistical analysis: 2-way ANOVA with Sidak Multiple Comparisons test. Female ovariectomized C57Bl6/J mice, Vehicle/CD, n=12; mGIPAnt-1/CD, n=12; Vehicle/HFD, n=11; mGIPAnt-1/HFD, n=12. Data are presented as mean ± SE.

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