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The effects of dual GLP-1/GCG receptor agonists with different receptor selectivity in mouse models of obesity and NASH

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Running title: Preclinical effects of GLP-1/GCG receptor agonists

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Number of text pages:
Number of tables: 2
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Abbreviations

ALP Alkaline phosphatase
ALT Alanine transaminase
AST Aspartate transaminase
chol Cholesterol
FBG Fasting blood glucose
GCG Glucagon
GLP-1 Glucagon-like peptide 1
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<tr>
<td>iAUC</td>
<td>Incremental area under the curve</td>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
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<tr>
<td>NASH</td>
<td>Non-alcoholic steato hepatitis</td>
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<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<tr>
<td>tAUC</td>
<td>Total area under the curve</td>
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<td>TG</td>
<td>Triglyceride</td>
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**Section assignment:** Drug Discovery and Translational Medicine
Abstract

There is an unmet need for NASH therapeutics, considering the increase in global obesity. Dual GLP-1/GCG receptor agonists have shown beneficial effects in circumventing the pathophysiology linked to NASH. However, dual GLP-1/GCG receptor agonists as a treatment for metabolic diseases need delicate optimisation to maximise metabolism effects. The impacts of increased relative GLP-1/GCG receptor activity in NASH settings must be addressed to unleash the full potential. In this study, we investigated the potential of OXM-104 and OXM-101, two dual GLP-1/GCG receptor agonists with different receptor selectivity in the setting of NASH, to establish the relative receptor activities leading to the best metabolic outcome efficacies to reduce the gap between surgery and pharmacological interventions. We developed dual GLP-1/GCG receptor agonists with selective agonism. Despite the improved metabolic effects of OXM-101, we explored a hyperglycemic risk attached to increased relative GCG receptor agonism. Thirty-eight days of treatment with a dual GLP-1/GCG receptor agonist OXM-104 with increased GLP-1 receptor agonism in obese NASH mice was found to ameliorate the development of NASH by lowering body weight, improving liver and lipid profiles, reducing the levels of the fibrosis marker PRO-C4, and improve glucose control. Similarly, dual GLP-1/GCG receptor agonist OXM-101 with increased relative GCG receptor agonism ameliorated NASH by eliciting dramatic bodyweight reductions to OXM-104, reflected in the improvement of liver and lipid enzymes and reduced PRO-C4 levels. Optimising dual GLP-1/GCG agonists with increased relative GCG receptor agonism can provide the setting for future agonists to treat obesity, type 2 diabetes, and NASH without having a hyperglycemic risk.
Significant statement
There is an unmet need for NASH therapeutics considering the increase in global obesity. Dual GLP-1/GCG receptor agonists have shown beneficial effects in circumventing the pathophysiology linked to NASH. Therefore, we have examined OXM-104 and OXM-101, two dual GLP-1/GCG receptor agonists in the setting of NASH, to establish the relative receptor activities leading to the best metabolic outcome efficacies to reduce the gap between surgery and pharmacological interventions.

Introduction
The prevalence of overweight and obesity has been increasing for the past 50 years resulting in increased incidences of metabolic syndrome, a cluster of metabolic abnormalities covering insulin resistance, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), hypertension, and central obesity (Younossi et al., 2018; World Health Organization. Diabetes, 2020; World Health Organization. Obesity and overweight, 2020). In recent years, mono receptor-acting incretins such as glucagon-like peptide 1 (GLP-1) receptor agonists have been extensively investigated as anti-obesogenic and anti-diabetic therapeutics. The GLP-1 receptor agonists induce postprandial insulin secretion, decrease glucagon secretion while increasing satiety, slow gastric motility, and enhance β-cell health (Holst, 2007). Recently, GLP-1 receptor agonists have emerged as a potential treatment for NAFLD’s progressive form known as non-alcoholic steatohepatitis (NASH) due to beneficial effects on metabolism leading to NASH resolution (Armstrong et al., 2016; Sanyal et al., 2020).

The evolution has previously inspired the development of drugs targeting diseases such as arthritis, osteoporosis, obesity and type 2 diabetes (Sexton et al., 1999; Henriksen et al., 2010; Gydesen et al., 2016; Katri et al., 2019). The idea of using evolution as an inspirational source has previously
shown that paddlefish glucagon (GCG) is structurally similar to the GLP-1 receptor agonist exendin-4 and improves glucose control (Graham et al., 2018). Moreover, the European dogfish GCG exhibited promising insulinotropic effects in vitro and in mice (O’Harte et al., 2016). GCG is a peptide hormone known to increase glucose through gluconeogenesis and glycogenolysis. Furthermore, GCG drives body weight reductions through its effects on satiety and energy expenditure (Müller et al., 2019).

Oxyntomodulin, a peptide hormone released from the gut with dual GLP-1 and GCG receptor activity, has been an inspiration source for therapeutics targeting obesity, type 2 diabetes NASH (Henderson et al., 2016; Boland et al., 2020). Dual GLP-1/GCG receptor agonists have been shown to induce more significant body weight-lowering effects than selective GLP-1 receptor agonists, thus highlighting the potential of dual GLP-1/GCG receptor agonism (Elvert et al., 2018a). Moreover, combinational treatments enhancing metabolism have attracted more attention in recent years due to their beneficial effects on lowering body weight.

In this context, the direct effects of dual GLP-1/GCG agonists on GCG receptor activation in the liver have shown great promise as a potential treatment for NASH (Boland et al., 2020). The classical view of GCG as the opposing insulin hormone has limited its use as a therapeutic agent. Previously, relative receptor activity was crucial as it may deteriorate glucose control (Day et al., 2012). Hence a thorough understanding of relative GCG receptor activity to relative GLP-1 receptor activity is required to gain knowledge about peptide designs to treat obesity, type 2 diabetes, and especially NASH.

Therefore, we developed two long-acting GLP-1/GCG receptor agonists, OXM-104 and OXM-101. OXM-104 was designed with an increased relative GLP-1 receptor activity, whereas OXM-101 was designed with an increased relative GCG receptor activity. These ligands were examined in obese
mice and obese NASH mice to determine the effects on body weight reductions, glucose control, liver and lipid profiles, and NASH resolution.

**Materials and Methods**

**Peptide therapy**

Synthetic oxyntomodulin mimetics (Synpeptide, Shanghai, China) were dissolved in Phosphate-buffered Saline at a stock concentration of 1 mM. For in vivo test, stocks were further diluted to the desired concentration in 0.9 % NaCl.

**In vitro peptide screening**

The dual GLP-1/GCG receptor agonists' potencies at the GCG receptor and GLP-1 receptor were determined using the PathHunter® β-Arrestin GPCR assay. Cell lines heterologously expressing the GLP-1 receptor: (CHO-K1 GLP1R DiscoveRx: cat. no.: 93-0300C2) and GCG receptor (CHO-K1 GCGR, DiscoveRx: cat. no.: 93-0241C2) were used. All experiments were conducted using 2500 cells per well in 10 μL cell-type in Gibco's Ham's F-12 Nutrient Mix (cat. 21765-037) from Invitrogen with the addition of fetal bovine serum (10 % cultivation and 0.1 % during experimentation), 400 μg/mL Hygromycin B, 800 μg/mL Geneticin, and Penicillin/Streptomycin 1 unit. To quantify the GPCR-mediated β-arrestin recruitment, the PathHunter® Detection Kit (93-0001, DiscoverX) was used, and the assay was performed according to the manufacturer's instructions. In vitro screening of peptides was performed with ligand concentrations starting from 20 μM.

**Animal experiments**

Animal studies were conducted according to the institutional license issued to Nordic Bioscience (2020-15-0201-00614) by the Animal Experiment Inspectorate under the Ministry of Environment and Food of Denmark. Twelve weeks old Female C57BL/6JOlaHsd mice (Envigo, Netherlands)
and 5-7 weeks old male MSNASH/PcoJ (The Jackson Laboratory, USA) were quad-wise housed under controlled temperature (21-22°C) on a standard 12-hour light-dark cycle (lights on 1900) with ad libitum access to water and food in a standard TYPE III H cage. C57BL/6JOlaHsd were fed a standard High fat diet (D12495, Research Diets Inc), and MSNASH/PcoJ mice were fed a GAN-diet consistent with 40 kcal% Fat (Palm Oil), 20 kcal% Fructose, and 2% cholesterol (D09100310, Research Diets Inc). All animals were allowed ad libitum access to food and water for 8-16 weeks before study initiation and for treatment groups throughout the rest of the study period. Pair-feeding groups had restricted access to food throughout the treatment period, where the amount of food provided matched that consumed by the matched treatment group.

Acute potency test

According to body weight, 40 female C57BL/6JOlaHsd mice (~ 60 weeks old) were allocated into treatment groups (n = 8/group). Mice received a single subcutaneous administration of either vehicle (0.9 % NaCl), OXM-104 and OXM-101 at 12.5 and 25 nmol/kg. Body weight and food intake were monitored every 24 hours for 96 hours.

Chronic study in High-fat diet (obese) mice

According to body weight, 96 female C57BL/6JOlaHsd mice (~ 26 weeks old) were allocated into treatment groups (n = 8-16 mice/group). For 35 days, mice received subcutaneous administration of either vehicle (0.9 % NaCl) or OXM-101 at 12.5-25 nmol/kg or OXM-104 25 nmol/kg every third day (QW.III). Pair-feeding groups to OXM-104 and OXM-101 were introduced as a surrogate marker for energy expenditure. The pair-fed groups received administrations of 0.9 % NaCl (QW.III). Body weight and food intake were monitored every 24 hours, and five weeks into the study, an oral glucose tolerance test (OGTT) was performed. Animals were euthanised, followed by
exsanguination, and dissection at study termination. Inguinal and perirenal adipose tissue and liver were surgically removed and weighed.

**A chronic intervention study in obese/NASH mice**

Twenty-eight male MSNASH/PcoJ (FATZO ~16-18 weeks old) were allocated into treatment groups according to body weight (n= 8-10 mice/group). For 38 days mice received subcutaneous administrations of either vehicle (0.9 % NaCl), OXM-101 30 nmol/kg, and OXM-104 30 nmol/kg (QW.III). All mice initiated a four-step dose escalation regimen. At study start, all groups received 7.5 nmol/kg; on day 7, all groups escalated to 15nmol/kg; on day 13, all groups escalated to 22.5nmol/kg; and on day 19, all groups escalated to full dose. Body weight and food intake were monitored every 24 hours, and six weeks into the study, an OGTT was performed. Animals were euthanised, followed by exsanguination, and dissection at study termination. Inguinal and perirenal adipose tissue and liver were surgically removed and weighed. Five hours FBG was measured one week before the study started, again at week five, and at termination (week 8).

**Oral glucose tolerance test (OGTT):**

Overnight fasted mice received glucose by oral gavage (5g/kg in C57BL/6JolaHsd, and 2g/kg in MSNASH/PcoJ, Blood glucose was measured by the Accu-Check® Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland) at 0, 15, 30, 60, 120, and 180 mins using a drop of tail blood.

**Biochemical analysis**

Blood samples were collected in EDTA tubes and centrifuged at 5,000 rpm for 10 min at 4°C. Plasma insulin levels (Mercodia mouse Insulin ELISA, Mercodia AB, Uppsala, Sweden) were
analysed according to manufacturer instructions. ALT, AST, ALP, -TG, LDL, HDL, and chol levels were measured in-house (ADVIA® 1800, Siemens Healthineer, Germany). Basement membrane type IV collagen, PRO-C4 (Nordic Bioscience A/S, Herlev) was assessed by a validated competitive ELISA (Leeming et al., 2012). A streptavidin-coated 96 well plate with the appropriate biotinylated peptide was incubated for 30 min in dark at 20°C while shaking (300 rpm) and subsequently washed in washing buffer. Twenty microliters of controls and samples was added to the wells together with 100µL horseradish peroxidase conjugated monoclonal antibody and incubated for 1 hour at 20°C in dark while shaking and washed in washing buffer. Hundred microliters of substrate solution were added to each well for 15 minutes left in the dark while shaking following 100µL of stopping solution. Plates were measured at 450nm with 650nm as reference.

Histological analysis

Liver tissue fixed in 10 % neutral-buffered formalin for 48 hours was paraffin-embedded using a Sakura Tissue Tek VIP 5 Jr. Tissue Processor and cut on an HM 360 microtome in 5 μm-thick slides. The staining procedure started with one hour of melting paraffin followed by deparaffinisation and rehydration. Stained slides were analysed with an Olympus BX60 microscope attached to an Olympus DP71 camera at 2x-20x magnification.

Sirius red staining: Liver slides were incubated in Weigert's Haematoxylin for 8 minutes. Slides were next placed in running tap water for 5 minutes and stained with a 0.1 % Sirius Red (36554, Sigma Aldrich) solution dissolved in aqueous saturated picric acid for 1 hour, followed by dehydration and mounting with a toluene-based glue.

Hematoxylin and eosin staining: Following rehydration, liver slides were incubated in Mayer's hematoxylin for 5 minutes. Slides were placed in running tap water for 5 minutes, counterstained
with eosin for 4 minutes, and washed in running tap water for 5 minutes. Slides were next dehydrated and mounted with a toluene-based glue.

A modified NAFLD activity score (NAS) composed of a Steatosis (0-3), microvesicular steatosis (0-1), inflammation (0-1), and fibrosis stage (0-4) were used to evaluate the effects on NASH. We completed scoring of liver health under blinded conditions (one slide per animal at three depths 150 µm apart). Slides were examined using an Olympus BX60 microscope attached with an Olympus DP71 camera at 4x-20x magnification. Specific magnification for each picture is stated in the respective figures.

Statistical analysis
Data were statistically analysed by one-way ANOVA, and Bonferroni's multiple comparison post-hoc test or by Kruskal-Wallis test comparison, and Dunn's post-hoc test. All analyses and graphical presentations were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA). Results are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. A $P$-value of < 0.05 was considered statistically significant shown with one symbols, a $P$-value of <0.01 is shown with two symbols, and a $P$-value of $p<0.001$ is shown with three symbols. *: significant from the vehicle. #: significant from pair-fed groups or comparison between agonists with increased relative activity.
Results

In vitro characterisation and acute in vivo testing of OXM-104 and OXM-101. The sequences and modifications of OXM-104 and OXM-101 are shown in (Table 1). Potency (EC50) for OXM-104 was 47.1 ± 16.4 nM at the GLP-1 receptor and 2.1 ± 1.1 µM at the GCG receptor, whereas OXM-101 potency at the GLP-1 and GCG receptor was 22.3 ± 8.4 nM and 0.2 ± 0.2 µM, respectively. For OXM-104 and OXM-101, approximately equivalent potencies were observed at the GLP-1 receptor, whereas OXM-101 showed a 9.5-fold borderline significant (p=0.07) potency increase at the GCG receptor compared to OXM-104. These findings indicated that OXM-101 had a strong GCG receptor selectivity, whereas OXM-104 favoured the GLP-1 receptor. Moreover, the acute in vivo effects of OXM-104 and OXM-101 on body weight reductions and food intake were examined in obese mice to establish the applicable dose range for chronic tests applied in this study. During the acute test, the lowest dose of OXM-104 and OXM-101 reduced body weight by 5.0 ± 1.0 % and 4.2± 0.4 % compared to vehicle (Figure 1SA). Furthermore, the highest dose of OXM-104 and OXM-101 led to a 7.4 ± 0.9 % and 8.3 ± 0.7 % reduction in body weight compared to vehicle (Figure 1SA). Body weight reductions were reflected in an immediate suppression of food intake, which lasted for 24 hours, as seen in the mice receiving low doses of OXM-104 and OXM-101. In mice treated with the highest dose of OXM-104 and OXM-101, food suppression lasted for 48 hours, after which it normalised (Figure 1SB).

Head-to-head comparison of OXM-104 and OXM-101 on caloric intake, body weight and energy expenditure. OXM-104 and OXM-101 were synthesised and tested in obese mice to investigate the effects of different receptor selectivities. OXM-104 and OXM-101 significantly lowered body weight compared to vehicle (Figure 1A and C). A 17.1 ± 4.2 % and 32.0 ± 2.8 % body weight loss was observed in mice on OXM-104 and OXM-101 treatment. The absolute body
weight in OXM-104 and OXM-101 mice was 33.7 ± 2.2 g, and 24.8 ± 0.7 g, respectively, at the end of the study (Figure 1B). OXM-101 drove dramatic reductions in body weight, which for ethical considerations, required a dose halving of OXM-101 from day 13 (Figure 1A). Pair feeding groups were included to investigate the effects of selective agonism beyond energy intake (Figure 1A). Matching of food intake resulted in evident body weight reductions compared to vehicle mice (Figure 1A and C). Pair-fed OXM-104 (PF-OXM-104) and Pair-fed OXM-101 (PF-OXM-101) mice significantly (p<0.05) reduced body weight by 21.4 ± 3.2 % and 24.8 ± 6.7 % compared to vehicle mice (Figure 1C). Effects on body weight reductions beyond energy intake were examined by comparing OXM-104 and OXM-101 to their corresponding pair-fed treatment groups. By comparing OXM-104 to PF-OXM-104, and OXM-101 to PF-OXM-101, a tendency for increased reduction in body weight was observed in both OXM-104 and OXM-101 (Figure 1C). A further increased body weight reduction of 7.9 ± 4.7 % was observed in OXM-104 mice compared to PF-OXM-104, whereas OXM-101 treatment showed a borderline significant (p=0.06) 23.1 ± 3.2 % reduction in body weight compared to PF-OXM-101 (Figure 1C). Reductions in body weight were reflected in the suppression of caloric intake compared to vehicle (Figure 1D). Caloric intake was reduced by 22.7 % and 37.4 % in OXM-104 and OXM-101 mice compared to vehicle. In line with the apparent reductions in body weight, treatment with OXM-104 and OXM-101 significantly reduced the absolute weights of inguinal adipose tissue in OXM-104 and OXM-101 mice compared to vehicle (Figure 1E). Absolute weights of perirenal adipose tissue in OXM-104 mice showed a tendency for reduction, whereas a significant decrease was found in OXM-101 mice compared to vehicle (Figure 1G). Inguinal and perirenal adipose tissue weights relative to body weight for OXM-104 mice were almost equal to relative inguinal and perirenal vehicle weights. In contrast, relative inguinal and perirenal adipose tissue was significantly reduced compared to vehicle. In
addition, relative perirenal adipose tissue showed a borderline significant (p=0.05) reduction compared to PF-OXM-101 (Figure 1F and H).

Repeated administration of OXM-101 followed by an OGTT impaired glucose tolerance in obese mice. The day before the study ended, an OGTT was conducted to investigate the effects of glucose tolerance and glucose control (Figure 2B and C). Fasting blood glucose (FBG) levels at OGTT baseline indicated a significant treatment effect of OXM-104 and a slight tendency for a treatment effect of OXM-101 compared to vehicle (Figure 2A). OXM-104 and OXM-101 mice had a significantly lower baseline FBG than their respective pair-fed groups (Figure 2A). The total glucose area under the curve (tAUC), an index of glucose excursion during an OGTT, was significantly decreased by OXM-104 (Figure 2D). The tAUC decreased by 24.5 ± 1.9 % and 21.7 ± 5.3 % compared to vehicle, and PF-OXM-104, respectively (Figure 2D). In contrast, OXM-101 mice increased the tAUC by 7.6 ± 5.3 % and 8.2 ± 7.6 % compared to vehicle, and PF-OXM-101, respectively (Figure 2D). Incremental AUCs (iAUC) showed a tendency for improved glucose tolerance in OXM-104 mice, whereas OXM-101 mice showed a tendency for glucose intolerance compared to vehicle (Figure 2E).

Effects of selective GLP-1/GCG receptor agonists on body weight reductions and plasma lipid profiles in obese NASH mice. Treatment with OXM-104 and OXM-101 in obese NASH mice significantly lowered body weight compared to vehicle (Figure 3A, B and C). OXM-104 mice reduced body weight by 10.5 ± 4.2 % from baseline, whereas OXM-101 mice reduced body weight by 46.3 % ± 0.9 %. Furthermore, tAUC for body weight changes was reduced by OXM-104 and OXM-101 compared to vehicle (Figure 3C). tAUCs in the OXM-104, and OXM-101 groups reduced by 7.7 ± 0.6 %, and 21.6 ± 0.6 % respectively. Interestingly, body weight reductions were
not driven by caloric intake reductions among the treatment groups (Figure 3C). Absolute weights of inguinal, epididymal, and perirenal adipose tissue were measured at study end (Figure 3E-G). In line with the apparent reductions in body weight, OXM-104 and OXM-101 significantly reduced inguinal, epididymal, and perirenal adipose tissue compared to vehicle (Figure 3E-G). Furthermore, OXM-101 significantly reduced inguinal, epididymal, and perirenal adipose tissue compared to OXM-104. Adipose tissue weights of inguinal, epididymal, and perirenal depots relative to body weight are presented in supplementary (Figure S2). Interestingly, changes in plasma cholesterol (chol) levels from baseline were significantly reduced in OXM-104 and OXM-101 compared to vehicle. Plasma chol levels in OXM-104 and OXM-101 mice were reduced by -0.3 ± 0.3 mmol/L and -3.5 ± 0.2 mmol/L, respectively (Table 2). In conjunction, changes in plasma low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels showed opposing patterns (Table 2). OXM-104 and OXM-101 mice significantly reduced plasma LDL levels compared to vehicle (Table 2). Interestingly, OXM-104 mice showed a 0.1 ± 0.1 mmol/L modest increase in plasma LDL levels from the baseline, while OXM-101 decreased plasma LDL levels by -0.2 ± 0.1 mmol/L, again, from baseline (Table 2). Changes in plasma HDL levels from baseline increased by 0.6 ± 0.1 mmol/L in OXM-104 mice, whereas plasma HDL levels decreased in OXM-101 mice by -1.1 ± 0.1 mmol/L (Table 2). Changes in plasma triglyceride (TG) levels indicated a borderline significant (p=0.060) reduction for OXM-104 mice and a significant reduction in OXM-101 mice compared to vehicle (Table 2). Plasma TG levels decreased from baseline by -0.7 ± 0.3 mmol/L and -0.9 ± 0.2 mmol/L in OXM-104 and OXM-101 mice, respectively (Table 2).

Effects of selective GLP-1/GCG receptor agonists on liver metabolism. The effects of OXM-104 and OXM-101 on liver metabolism were examined through the assessment of liver enzymes and liver histology (Figure 4). Absolute liver weights of OXM-104 and OXM-101 mice were 3.2 ±
0.1 g and 1.8 ± 0.1 g, which corresponded to a 1.4-fold and 2.5-fold reduction in size compared to vehicle (Figure 4A). Liver weights relative to body weight were approximately similar in size in OXM-104 and OXM-101 mice; both were significantly reduced from the vehicle, but no difference were observed between the two (Figure 4B). Changes in liver enzymes, alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) from baseline were also investigated throughout the study (Figure 4C, D, and E). ALT levels were significantly lowered when compared to vehicle. ALT levels in OXM-104 mice reduced ALT levels by 16.0 ± 11.4 %, whereas OXM-101 mice decreased by 36.2 ± 4.8 % (Figure 4C). AST levels increased in all treatment groups, but progression was significantly lower in OXM-104 mice than in the vehicle and OXM-101 group (Figure 4D). AST levels increased respectively by 53.5 ± 16.9 % and 149.0 ± 17.4 %, in OXM-104, and OXM-101 mice. ALP levels increased in all treatment groups from baseline, but only OXM-101 significantly differed from vehicle. ALP levels in OXM-104, and OXM-101 increased by 77.4 ± 27.7 %, and 245.8 ± 186.7 % respectively (Figure 4E).

The basement type IV collagen formation marker PRO-C4 has previously been shown to reflect liver fibrosis in NASH rodents and was therefore measured at study end14,15. PRO-C4 levels were significantly reduced in OXM-104 and OXM-101 mice compared to vehicle (Figure 4F). PRO-C4 levels decreased by 26.6 ± 5.3 % in OXM-104 mice and 38.6 ± 7.7 % in OXM-101 mice compared to vehicle. Histological macro and micro steatosis scores were significantly reduced in OXM-104 (only a non-significant tendency for micro steatosis lowering) and OXM-101 mice compared to vehicle (Figure 4G and I). No significant differences were observed in inflammation and fibrosis scores, but fibrosis scores showed a tendency for reduction in OXM-104 and OXM-101 mice compared to vehicle (Figure 4G and H).
Effects of selective GLP-1/GCG receptor agonists on glucose control in obese NASH mice.

Glucose control was examined in the obese NASH mice throughout the study. Repeated administration of OXM-104 and OXM-101 significantly lowered FBG levels compared to vehicle (Figure 5A). OXM-104 and OXM-101 mice reduced FBG levels by 33.5 ± 3.9 % and 34.4 ± 3.7 % from baseline (Figure 5B). Examination of body weight independent effects of dual GLP-1/GCG receptor agonists on glucose control indicated a superiority of OXM-104 compared to OXM-101 (Figure 5C). Plasma insulin levels measured at the study end were mildly reduced in OXM-104 mice, whereas a significant reduction was observed in OXM-101 mice compared to vehicle mice (Figure 5D). Plasma insulin levels in vehicle, OXM-104 and OXM-101, was 11.0 ± 0.8 ng/mL, 9.5 ± 1.2 ng/mL, and 1.5 ± 0.1 ng/mL respectively (Figure 5D).

Effects of selective GLP-1/GCG receptor agonists on glucose tolerance in obese NASH mice.

An OGTT was performed at the study end to investigate effects on glucose tolerance. Chronic treatment effects were reflected in the baseline FBG levels (Figure 6A). Repeated administration of OXM-101 and OXM-104 affected glucose as indicated by differences in baseline FBG levels. At baseline, the mean FBG level was 7.5 ± 1.1 mmol/L in OXM-104 administrated mice, 4.2 ± 0.9 mmol/L in OXM-101 administrated mice and 9.7 ± 1.6 mmol/L in vehicle mice. tAUC levels in OXM-104 and OXM-101 mice were significantly lowered compared to vehicle (Figure 6B). The substantial reductions on body weight could drive the improved glucose tolerance observed in OXM-101 mice (Figure 3A). Interestingly, iAUCs comparison between OXM-104 and OXM-101 showed no statistical difference but was significantly reduced compared to vehicle (Figure 6C). This strongly suggests that the effects are derived from the weight improvements.
Discussion

Global health has been heavily burdened by the western lifestyle for a long time, promoting metabolic dysfunctions such as obesity, type 2 diabetes, and NASH. Currently, no FDA-approved drugs for the treatment of NASH exist. Dual GLP-1/GCG receptor agonists have shown promise as anti-obesogenic, anti-diabetic, and anti-NASH treatments [15,16,17]. However, receptor selectivity in the context of NASH needs to be explored to maximise metabolic outcomes. The combination of direct liver action and insights on peptide selectivity can optimise outcome efficacies, thus reducing the gap between body weight-lowering effects of pharmacological interventions and surgery.

Dual GLP-1/GCG receptor agonists OXM-104 and OXM-101 were developed as a peptide program using evolutionary GCG and oxyntomodulin sequences. The peptide lengths of OXM-104 were chosen to mimic native oxyntomodulin and GCG for OXM-101. OXM-104 and OXM-101 were designed to provide approximal effects on glucose control, as shown in the in vitro screening. In contrast, we designed the GCG receptor component of OXM-104 and OXM-101 differently to provide shifted effects on energy expenditure (Nair, 1987; Salem et al., 2016). OXM-104 was designed with an increased relative GLP-1 receptor activity (~44:1), whereas OXM-101 showed much greater GCG receptor potency (~10:1). Therapeutic candidates have been previously developed with both a balanced GLP-1/GCG receptor agonism (e.g., ALT-801 (Harris, 2020), and HM12525A (Jung et al., 2014)) and with an increased GLP-1 receptor activity (e.g., cotadutide SAR425899, and BI 456906 (Hamprecht et al., 2018; PD Ambery et al., 2018; Tillner et al., 2019)).

We observed that OXM-104 and OXM-101 induced reductions in body weight in obese and obese NASH mice throughout both studies. Previously, body weight-lowering effects of dual GLP1/GCG
receptor agonists have been reported (P Ambery et al., 2018; Tillner et al., 2019). Interestingly, OXM-101 significantly induced body weight reductions from OXM-104, possibly due to the increased relative GCG receptor activity. In line with the anti-obesity effects observed using OXM-101, GCG receptor agonists have previously been shown to elicit marked reductions in body weight (Kim et al., 2019). Effects on body weight lowering beyond energy intake by OXM-101 were more significant than for OXM-104, possibly due to increased resting energy expenditure. Interestingly, infusions studies in humans have shown that GLP-1 does not affect resting energy intake, whereas infusions of dual GLP-1/GCG and GCG-alone increase resting energy expenditure (Scott et al., 2018).

We also observed reductions in inguinal and perirenal adipose tissue in both models, corresponding to a previous finding using balanced dual GLP-1/GCG receptor agonists (Day et al., 2009). Glucose control and glucose tolerance during an OGTT were improved in OXM-104 mice in both obese and obese NASH mice. Interestingly, SAR425899, and cotadutide, two dual GLP-1/GCG receptor agonists with increased relative GLP-1 receptor agonism (5:1), were found to improve glucose control in clinical trials (PD Ambery et al., 2018; Tillner et al., 2019). The hyperglycemic risk associated with increased relative GCG receptor activity can have serious negative consequences if not correctly balanced. In obese mice, we found an impaired glucose tolerance using OXM-101. In contrast, OXM-101 in obese NASH mice showed improved glucose control and tolerance. This finding appeared to be driven by the considerable reduction in body weight, which improved insulin sensitivity, as indicated by the apparent insulin levels.

Similar to our findings, others used dual GLP-1/GCG receptor agonists with extreme receptor selectivity, reporting apparent body weight lowering effects and suppression of caloric intake (Day...
et al., 2012). They reported that GCG selectivity had less impact on caloric intake and that adding 10 % relative GLP-1 receptor agonism tripled the body weight-lowering effects and improved the hyperglycemic risk (Day et al., 2012). In obese NASH mice, body weight reductions during the dose-escalation period were similar between OXM-104 and OXM-101. Interestingly, treatment with OXM-101 elicited dramatic body weight reductions compared to OXM-104 at full dose. Our findings suggest the existence of a GCG receptor activity threshold, which upon subthreshold doses, produces similar body weight lowering effects regardless of selectivity. In contrast, suprathreshold doses may result in additional activation of direct GCG receptor-mediated anorexic and lipolytic pathways. In line with this finding, GCG effects at low doses (~14 nmol/kg) have been shown to induce hyperphagia, whereas at high doses (~115 nmol/kg), has been shown to reduce food intake in rats (Geary and Smith, 1983; Hell and Timo-Iaria, 1985).

Dual GLP-1/GCG receptor agonists show promise as a potential NASH treatment due to the direct liver action. In obese NASH mice, OXM-104 and OXM-101 reduced PRO-C4 levels to a similar extent compared to vehicle. Collagen formation marker PRO-C4 recognises the 7S domain of collagen type IV (P4NP7S), which was previously shown to be associated with liver fibrosis (Leeming et al., 2012). Furthermore, the effects of OXM-104 and OXM-101 on liver function showed reduced ALT levels compared to vehicle. Similarly, six weeks of cotadutide treatment in ob/ob mice reduced levels of P4NP7S and ALT (Boland et al., 2020). AST levels in OXM-104 mice decreased compared to the vehicle, whereas OXM-101 mice maintained similar AST levels as the vehicle. In contrast, cotadutide treatment in ob/ob mice increased AST levels compared to vehicle (Boland et al., 2020). Additionally, a tendency for reduction in ALP levels was observed in OXM-104 mice compared to vehicle. OXM-101 mice reduced ALP levels compared to control. In line with this finding, ALP levels were reduced in liraglutide-treated mice with acute liver injury.
Overall improved NAS scores were observed using both OXM-104 and OXM-101. The improved liver fat reductions have been suggested to be due to the direct impact of GCG agonism to inhibit lipogenesis while enhancing mitochondrial turnover and oxidative capacity (Boland et al., 2020). This finding underlines the importance of GCG agonism and its potential as future anti-NASH therapy. Therefore, we suggest that future dual GLP-1/GCG receptor agonists can be developed in a more personalised manner. Lean subjects with NASH can potentially be introduced to therapy with increased relative GLP-1 receptor agonism. In contrast, morbidly obese NASH subjects could receive treatment with increased relative GCG receptor activity to induce a more significant reduction in body weight. This reasoning requires the development of selective GCG receptor-agonists with improved glucose control to test the hypothesis.

Based on our findings, we confirm the potential of dual GLP-1/GCG receptor agonists as a treatment for NASH as both peptides improved liver health and steatosis. The most crucial consideration is the hyperglycemic risk associated with increased GCG receptor activity. The safest developmental choice is to initiate a peptide program focused on peptides with increased relative GLP-1 receptor agonism. Moreover, increased body weight lowering is favourable for metabolism. With the marked increase in obesity prevalence, we suggest that future peptide designs should be arranged with a slightly increased GCG receptor activity without disrupting glucose control to reduce the gap between surgery vs pharmacological treatment.
Conclusion

The current study indicates that an agonist with increased relative GLP-1 receptor activity is favoured over an agonist with increased relative GCG receptor activity due to lower hyperglycemic risk. Furthermore, dual GLP-1/GCG receptor agonist as potential therapies for NASH is kept intact as both peptides provide healthy liver and lipid profiles.

Limitations

This study included pair-feeding groups as a surrogate marker for energy expenditure. In future studies, metabolic cages should be used to directly measure energy expenditure. Furthermore, diet compositions can be further enhanced to provide a more translational milieu. Future studies should have a natural reference, such as oxyntomodulin, as it may indicate what an optimal receptor selectivity is to obtain maximised metabolic effects.
Authorship contributions

Participated in research design: Kayed, Henriksen

Conducted experiments: Kayed, Melander, Khan

Performed data analysis: Kayed, Melander, Andreassen, Henriksen

Wrote or contributes to the writing of the manuscript: Kayed, Melander, Andreassen, Karsdal, Henriksen
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Footnotes

Funding information

This work was supported by the Danish Research Foundation (Den danske forskningsfond); and the Danish Innovation Fund (Innovationsfonden).

Conflict of interest

Karsdal and Henriksen own stock in Nordic Bioscience A/S. All authors are employed by Nordic Bioscience A/S.

Data availability statement

The data supporting this manuscript’s findings are available from the corresponding author upon reasonable request.

Ethics approval statement:

All animal procedures were performed under guidelines from the Animal Welfare Division of the Danish Ministry of Justice under the institutional licenses issued to Nordic Bioscience (2020-15-0201-00614).
Figure legends and legends

Figure 1. Chronic effects of OXM-104, and OXM-101 on body weight, food intake, and adipose tissue weights in obese mice after 35 days with vehicle (n=16), OXM-104 (n=8), OXM-101 (n=4), PF-OXM-104 (n=8), and PF-OXM-101 (n=4). A) Body weight change (% of initial body weight) throughout the study. B) Endpoint body weights (g). C) Column graphs illustrating study-end body weight changes (% of vehicle). D) Accumulated caloric intake expressed in kcal/4 mice. E) Absolute weight of Inguinal adipose tissue. F) Relative weight of inguinal adipose tissue. G) Absolute weight of perirenal adipose tissue. H) Relative weight of perirenal adipose tissue. Dotted lines shown in A) designate dosing days. Statistical analysis between groups was evaluated by one-way ANOVA, and Bonferroni's multiple comparison post-hoc test except for E), G), and H) analyzed by a Kruskal Wallis, and Dunn's multiple comparison post-hoc test. * p<0.05 **p<0.01, and *** p<0.001 compared to vehicle mice. # p<0.05 when compared to OXM-101. Data are mean ± SEM.

Figure 2. Chronic administration of OXM-104 and OXM-101 significantly improves glucose tolerance in obese mice. A) FBG levels at OGTT baseline. B) Plasma glucose levels during end OGTT in mice dosed 24 hours before testing with vehicle (n=16), OXM-104 (n=8), and PF-OXM-104 (n=8). C) Plasma glucose levels during OGTT in mice dosed with vehicle (n=16), OXM-101 (n=4), and PF-OXM-101 (n=4). D) Total AUC for blood glucose (mmol/L*min) in all treatment groups. E) Incremental AUC for blood glucose (mmol/L*min) in all treatment groups. Statistical analysis between groups was evaluated by one-way ANOVA and Bonferroni's multiple comparison post-hoc test except for A) which was analyzed by a Kruskal Wallis and Dunn's multiple comparison post-hoc test. * p<0.05, ** p<0.01 compared to vehicle and # p<0.05, ## p<0.01 compared to the pair-fed groups. Data are mean ± SEM.
Figure 3. Effects of OXM-104 and OXM-101 on body weight, food intake, and adipose tissue depots in obese NASH mice. A) Body weight and B) Body weight change (% of initial body weight) in obese NASH mice after 38 days of treatment with vehicle (n=8), OXM-104 30nmol/kg (n=10), and OXM-101 30nmol/kg (n=10). C) tAUC for body weight change (% of initial body weight) in all treatment groups. D) Accumulated caloric intake expressed in kcal/4 mice. E) Absolute inguinal adipose tissue weight. F) Absolute epididymal adipose tissue weight. G) Absolute perirenal adipose tissue weight. Dotted lines designate escalation timepoint. Statistical analysis between groups was evaluated by one-way ANOVA and Bonferroni’s multiple comparison post-hoc test except for E) and G), which were evaluated by a Kruskal Wallis test and Dunn's multiple comparison test. ** p<0.01 *** p<0.001 vs Vehicle mice. # p<0.05, and ### p<0.001 vs. OXM-104. Data are mean ± SEM.

Figure 4. Effects of OXM-104 and OXM-101 on liver metabolism in obese NASH. Following 38 days of administration with vehicle (n=8), OXM-104 30nmol/kg (n=10), and OXM-101 30nmol/kg (n=10). A) Absolute liver weight (g). B) Relative liver weight. C) Changes in liver enzymes from baseline are shown in C) ALT (U/L), D) AST (U/L), and E) ALP (U/L). F) PRO-C4 levels (ng/mL) at study end. G) Steatosis, inflammation and fibrosis scores. H) Representative Sirus red-stained liver sections, scale bar 100µM. I) Representative Hematoxylin and Eosin liver sections, scale bar 100µM. Statistical analysis between groups was evaluated by one-way ANOVA and Bonferroni’s multiple comparison post-hoc test except for A) and B), analyzed by Kruskal Wallis, and Dunn's multiple comparison post-hoc test. ** p<0.01, ** p<0.01 compared to vehicle, ## p<0.01 compared to OXM-104. Data are mean ± SEM.
**Figure 5. Effects of OXM-104 and OXM-101 on glucose regulation in obese NASH mice.** A) Change in 5 hours FBG levels from baseline shown as mmol/L, and B) Change in 5 hours FBG levels from baseline shown as % of initial FBG. C) Effects on glucose control beyond body weight shown as ΔFBG_{1-31}/ΔBW_{1-31}. D) Plasma insulin levels (ng/mL) at study end. The dotted line shown in A and B designates the study start. Treatments are indicated as follows vehicle (n=8), OXM-104 30nmol/kg (n=10), and OXM-101 30nmol/kg (n=10). Statistical analysis between groups was evaluated by a Kruskal Wallis test, and Dunn's multiple comparison test in C), and one-way ANOVA, and Bonferroni's multiple comparison post-hoc test for D). *** p<0.001 vs vehicle mice. ## <0.01 vs OXM-104, ### p<0.001 vs OXM-104. Data are mean ± SEM.

**Figure 6. Effects of OXM-104 and OXM-101 on glucose tolerance in obese NASH mice.** A) Plasma glucose levels during end OGTT in mice dosed 24 hours before testing with vehicle (n=8), OXM-104 30nmol/kg (n=10), and OXM-101 30nmol/kg (n=10). B) Total AUC for blood glucose (mmol/L*min) in all treatment groups. Incremental AUC for blood glucose (mmol/L*min) in all treatment groups. Statistical analysis between groups was evaluated by one-way ANOVA and Bonferroni's multiple comparison post-hoc test. *** p<0.001 vs vehicle , and # p<0.05 vs OXM-104. Data are mean ± SEM.
Table 1. Sequence overview of OXM-104 and OXM-101 and \textit{in vitro} potency at the GLP-1 and GCG receptor. β-arrestin recruitment assays were used to establish EC50 (nanomolar) values from n ≥ 3 independent experiments, which formed the basis for GLP-1/GCG receptor ratio calculations. A two-tailed unpaired T-test analysed potencies. Data are mean ± SEM.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Peptide sequences of selective GLP-1/GCG receptor agonists & GLP-1 receptor & GCG-receptor & GCG receptor/GLP-1 receptor ratio \\
\hline
\textbf{OXM-104} & HXQGTFSDYKLDK(Ac)RRAFVQWLMNTKRNQGQ-QNH2 & 47.1 & 2057.9 & 43.7 \\
\textbf{OXM-101} & HXQGTFSDYKLDK(Ac)RRARDFVQWLMNT-NH2 & 22.3 & 216.1 & \textit{p=0.07} \\
\hline
\end{tabular}
\end{table}

\textit{X}: Aminoisobutyric acid

\textit{K(Ac)}: y-Glu-y-Glu-C20 diacid conjugated to lysine
Table 2. Lipid profiles of the vehicle, OXM-104 (30nmol/kg) and OXM-101 (30nmol/kg), presented as % change from baseline. Levels of chol, LDL, HDL, and TG are presented as mean ± SEM. With the following annotations: ** p<0.01 *** p<0.001 vs vehicle (change). Statistical analysis between groups was evaluated by one-way ANOVA and Bonferroni’s multiple comparison post-hoc test.

<table>
<thead>
<tr>
<th>Lipid profile</th>
<th>Vehicle</th>
<th>OXM-104</th>
<th>OXM-101</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mmol/L) Baseline</td>
<td>Study end</td>
<td>Change %</td>
<td>Baseline</td>
</tr>
<tr>
<td>Chol</td>
<td>5.1 ±0.2</td>
<td>7.75 ±0.39</td>
<td>21.9 ±11.7</td>
</tr>
<tr>
<td>LDL</td>
<td>0.3 ±0.02</td>
<td>0.76 ±0.06</td>
<td>84.2 ±45.2</td>
</tr>
<tr>
<td>HDL</td>
<td>1.6 ±0.1</td>
<td>3.16 ±0.12</td>
<td>70.2 ±33.9</td>
</tr>
<tr>
<td>TG</td>
<td>1.8 ±0.1</td>
<td>1.42 ±0.03</td>
<td>-13.2 ±7.1</td>
</tr>
</tbody>
</table>
Figure 3

A) Body weight (g) over days with different dose concentrations (nmol/kg) for Vehicle, OXM-104, and OXM-101.

B) BW change from baseline over days with different dose concentrations (nmol/kg) for Vehicle, OXM-104, and OXM-101.

C) Graph showing AUC values for Vehicle, OXM-104, and OXM-101.

D) Total food intake (Kcal) over days with different dose concentrations (nmol/kg) for Vehicle, OXM-104, and OXM-101.

E) Inguinal AT (g) for Vehicle, OXM-104, and OXM-101.

F) Epididymal AT (g) for Vehicle, OXM-104, and OXM-101.

G) Perirenal AT (g) for Vehicle, OXM-104, and OXM-101.
**Figure 4**

A) Liver weight (g)

B) Relative liver weight (g/g bw)

C) Change in ALT UL

D) Change in AST UL

E) Change in ALP UL

F) PRO-C4 ng/mL

G) NASH Score ± SEM

H) Sirius Red

I) Hematoxylin & Eosin
Figure 5

**A)**

5h FBG (mmol/L) vs. Weeks

- **Vehicle**
- OXM-104, 30 nmol/kg
- OXM-101, 30 nmol/kg

**B)**

Change in 5h FBG % from baseline vs. Weeks

**C)**

ΔFBG13±/ABW1± vs. Vehicle, OXM-104, OXM-101

**D)**

Fasting plasma insulin (μg/mL)

- Vehicle
- OXM-104
- OXM-101

***p < 0.001
Figure 6

A) Blood Glucose (mmol·L⁻¹) over time for Vehicle, OXM-101, and OXM-104 treatments.

B) tAUC₀⁻¹₈₀ min comparison for Vehicle, OXM-104, and OXM-101 treatments.

C) iAUC₀⁻¹₈₀ min comparison for Vehicle, OXM-104, and OXM-101 treatments.