Investigating the glucagon receptor and GLP-1 receptor activity of oxyntomodulin-like analogues in male Wistar rats

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Abstract

Background
Oxyntomodulin is a natural agonist at both the glucagon receptor (GCGr) and the GLP-1 receptor (GLP-1r), and peripheral administration reduces food intake and increases energy expenditure in rodents and humans. Substituting the native glutamine (Gln/Q) at amino acid position 3 of oxyntomodulin for glutamate (Glu/E) has previously been shown to diminish GCGr activity without affecting GLP-1r activity. The effects of Glu-3 oxyntomodulin analogues have not been investigated in rats.

Methods
The effect of two Glu-3 substituted oxyntomodulin-like analogues (OXM14E3 and OXM15E3) on food intake and bodyweight was investigated in male Wistar rats during 6 days of daily SC administration. The effects of Glu-3 substitution on analogue binding and activity at the rat glucagon receptor and rat GLP-1 receptor were investigated in vitro using CHO or CHL cells.

Results
This study reports the novel finding that 25nmol/kg Glu-3 oxyntomodulin-like analogues (OXM14E3 & OXM15E3) significantly increased rat bodyweight by up to 4% compared to the equivalent non Glu-3 analogues (OXM14 & OXM15), without affecting food intake. The effect of OXM15E3 on bodyweight was dose dependent. Glu-3 analogues, including Glu-3 oxyntomodulin, decreased glucagon mediated cAMP accumulation in CHO cells expressing the rat glucagon receptor, suggesting they may be acting as antagonists.

Conclusion
The results indicate Glu-3 OXM analogues might not be suitable tools to investigate the mechanism of oxyntomodulin analogue action in a rat model as they significantly increase bodyweight independently of food intake. Glu-3 OXM analogues are partial agonists at the rat GCGr and may also act as antagonists, possibly resulting in the observed increase in bodyweight.

Keywords
Oxyntomodulin, analogue, bodyweight, glucagon

Introduction
The gut-derived hormone oxyntomodulin is a naturally occurring dual agonist of both the glucagon receptor (GCGr) and GLP-1 receptor (GLP-1r) [1]. Structurally oxyntomodulin is the 29 amino acids...
of glucagon with a C-terminal octapeptide tail [2]. Administration of oxyntomodulin to rodents and humans reduces food intake and increases energy expenditure, generating significant weight loss and highlighting oxyntomodulin as a potential pharmacological treatment for obesity [3, 4]. Due to the short in vivo half-life of native oxyntomodulin [5] it is necessary to produce long lasting analogues for clinical use.

The dual agonist properties of oxyntomodulin make investigating its mechanism(s) of action difficult. Using GCGr or GLP-1r antagonists can interfere with the actions of endogenous glucagon and GLP-1, hormones which play critical roles in glucose homeostasis, therefore giving an inaccurate picture of oxyntomodulin actions. Alternatively, substituting glutamine at position 3 of oxyntomodulin (Gln/Q) with glutamate (Glu/E) has previously been reported to diminish GCGr activity without affecting GLP-1r activity [6, 7] enabling investigation of mechanisms of action relating to exogenous oxyntomodulin administration.

These Glu-3 oxyntomodulin analogues have been utilised in mice to investigate the contribution of GCGr and GLP-1r activity to the function of both native oxyntomodulin [7, 8] and a derivatised analogue [6]. Previous results indicate that oxyntomodulin activity at the GLP-1r is responsible for the reduction in food intake and activity at the GCGr increases energy expenditure [6, 7].

Previous work in our lab designed oxyntomodulin-like dual analogues of the GCGr and GLP-1r based on the 29 amino acids of glucagon. A number of conservative amino acid substitutions were made to increase GLP-1r activity, improve peptide stability and extended circulatory half-life, with a view to developing a potential treatment for obesity. Several equivalent Glu-3 analogues were also designed to investigate the contribution of GCGr and GLP-1r activity to the effects of the oxyntomodulin-like dual analogues. Our pilot experiments suggested that Glu-3 oxyntomodulin-like analogues increased bodyweight in rats without affecting food intake, therefore further experiments were undertaken to clarify these findings. The two Glu-3 analogues OXM14E3 and OXM15E3 were chosen for investigation as they increased bodyweight in a pilot experiment and had similar conservative amino acid substitutions and pharmacokinetic profiles.
The results of the current study suggest that Glu-3 substituted oxyntomodulin analogues may be partial agonists which can act as competitive antagonists in vitro and increase bodyweight independently of food intake in vivo. Therefore Glu-3 oxyntomodulin analogues are not a suitable tool to investigate the effects of oxyntomodulin-like dual analogues in a rat model.

**Methods**

**Compounds**

All peptides (human glucagon, GLP-1 and oxyntomodulin; analogues OXM14 and OXM15 and their respective Glu-3 analogues OXM14E3 and OXM15E3; Glu-3 oxyntomodulin OXME3) were custom synthesised by Insight Biotechnology Limited (Middlesex, UK) and were >90% pure. The structure of analogues OXM14E3 and OXM15E3 were based on the 29 amino acids of glucagon and, other than the Glu-3 substitution, contained 6 single amino acid substitutions between residues 12-29. Both analogues had additional amino acids at residues 30 and 31 and OXME3 was C-terminally amidated (Figure 1). These analogues were chosen for investigation as they had similar pharmacokinetic profiles but different affinity and potency at the rat glucagon and GLP-1 receptors.

**Animals**

Male Wistar rats (Charles River, Margate, UK) were maintained in individual cages under controlled temperature (21-23C) and light conditions (12:12 light-dark cycle, lights on at 0700 hours) with ad-libitum access to food (RM1 diet; SDS, Witham, UK) and water unless otherwise stated. All animal procedures were approved under the British Home Office Animals (Scientific Procedures) Act 1986 (Project Licence 70/7236).

**In vivo food intake and bodyweight studies**

Prior to study commencement, rats were randomised to treatment groups with equal mean bodyweights. An injection of peptide (maximum volume 50μl, reconstituted in ZnCl₂ to enhance peptide depot formation [9, 10]) or vehicle (saline) was administered subcutaneously at 1600 hours daily and food and body weight recorded.

**Cell culture**
All cell culture reagents were obtained from Invitrogen (Life Technologies Ltd, Paisley, UK) unless otherwise stated. CHO-K1 cells over expressing the rGCGr were routinely maintained in DMEM with 25mM glucose and 1mM sodium pyruvate supplemented with 10% FBS, 25mM HEPES, 1% antibiotic (100U/ml Penicillin and 100µg/ml Streptomycin) and 1 X nonessential amino acids. CHL cells over expressing the rGLP-1r were maintained in the same DMEM but without additional HEPES or nonessential amino acids.

**In vitro cAMP accumulation**

Levels of cAMP accumulation was measured in chinese hamster ovary (CHO-K1) cells over expressing the rat GCGr (rGCGr; cDNA from Origene, Cambridge Bioscience, Cambridge, UK) or chinese hamster lung (CHL) cells over expressing the rat GLP-1r (rGLP-1r; a kind gift from Professor Bernard Thorens, University of Lausanne, Switzerland).

Cells were seeded in a 48 well plate (Nunc, VWR International Inc, Chicago, USA) in their respective supplemented DMEM, at a density of 37 500 cells/well (250µl/well), then incubated for 24 hours. Cells were serum starved in DMEM + 1% antibiotic for 1 hour prior to 30 minutes incubation at room temperature with peptide concentrations diluted in serum free DMEM with 1mM IBMX (Sigma-Aldrich, Dorset, UK). Each peptide concentration was applied in duplicate or triplicate per experiment.

For rat GCGr antagonism studies, cells were incubated for 30 minutes with peptide concentrations diluted in serum free DMEM with 1mM IBMX and 0.1nM glucagon. Five wells of each concentration and 12 wells of 0.1nM glucagon alone were applied per experiment.

Post incubation, medium was removed and cells lysed with 120µl/well of 0.1M HCL + 0.5% Triton-X. cAMP levels were measured using an indirect ELISA according to the manufacturer’s instructions (ADI-900-066, Enzo Life Sciences, UK), optical density was read at 405nm on a Biotek ELx808 (Wolf Laboratories, York, UK). Data was plotted and EC₅₀ values calculated.

**In vitro receptor binding- supplementary**
Refer to O’Shea [11] for detailed methods. Briefly, membranes were prepared from CHO-K1 cells over expressing the rGCGr or CHL cells over expressing the rGLP-1r by osmotic lysis and differential centrifugation. Membranes (100µg protein) were incubated in siliconised polypropylene tubes with 100pm (500Bq) of either $^{125}$I-GLP-1 or $^{125}$I-glucagon in competition with unlabelled peptide. Total specific binding was calculated as the difference in counts between assays in the presence (nonspecific) and absence (total) of up to 6000nM of peptides. Data was plotted and IC$_{50}$ values calculated.

**Statistical analysis**

cAMP EC$_{50}$’s and binding IC$_{50}$’s were calculated using non-linear regression with Prism V5 (GraphPad Software Inc. San Diego, CA, USA). Differences in cumulative food intake and body weight were analysed using one way ANOVA and post hoc tests with Dunnett or Bonferroni correction (Prism v5, GraphPad Software Inc. San Diego, CA, USA). In all cases $P < 0.05$ was considered statistically significant.

**Results**

**In vivo**

Daily administration of 25nmol/kg OXM14E3 to rats significantly increased bodyweight over 7 days (27.5 ± 2.2g, $p < 0.01$) compared to vehicle controls (11.4 ± 2.6g) and OXM14 (9.2 ± 3.8g) (Figure 2.B). Daily 25nmol/kg OXM15E3 administration significantly increased bodyweight over 7 days (19 ± 3.3g, $p < 0.01$) compared to OXM15 (4.5 ± 2.7g, Figure 2.B). Food intake was not increased after administration of either OXM14E3 or OXM15E3 (Figure 2.A).

Daily administration of 10nmol/kg and 20nmol/kg of OXM15E3 resulted in a significant increase in rat bodyweight over 7 days (15.1 ± 1.9g, $p < 0.05$ and 17.7 ± 1.5g, $p < 0.01$ respectively) compared to vehicle controls (7.9 ± 2.0g) (Figure 2.D), without altering food intake (Figure 2.C).
**In vitro**

At the rGCGr the binding affinity of the Glu-3 substituted peptides OXM14E3 and OXM15E3 was 63 fold and 120 fold lower compared to OXM14 and OXM15 respectively (Figure 3.D). Receptor binding affinity for the Glu-3 analogues at the rGLP-1r was 2.7 - 4.4 fold greater than their Gln-3 counterparts (Figure 3.D).

Substituting glutamate at position 3 decreased peptide efficacy at the rGCGr. The efficacy of OXM14E3, OXM15E3 and OXME3 was 200, 25 and 13 fold lower respectively compared to their Gln-3 equivalents OXM14, OXM15 and OXM. Glu-3 substitution increased rGLP-1r efficacy by 1.2 - 1.6 fold (Figure 3.D).

Concentrations of 0.03-1nM of OXM14E3 (Figure 3.A) and 0.03-0.3nM OXM15E3 (Figure 3.B) decreased the percentage of glucagon mediated cAMP production at the rGCGr by a mean of 18.4% and 27.5% respectively. Concentrations of 3- and 30nM of OXM14E3 and OXM15E3 increased cAMP levels compared to glucagon stimulation alone (OXM14E3 30nM: 155% increase, OXM15E3 30nM; 214% increase) (Figure 3.A and Figure 3.B respectively). Except 30nM, all concentrations of OXME3 tested decreased glucagon mediated cAMP accumulation by a mean of 40.3% (Figure 3.C). 30nM OXME3 increased cAMP levels by 9% compared to glucagon stimulation alone (Figure 3.C).

**Conclusions**

Structurally, oxyntomodulin is the 29 amino acids of glucagon with a C-terminal octapeptide tail [2]. The dual agonist activity of oxyntomodulin at the GCGr and GLP-1r makes it difficult to investigate the mechanisms involved in the weight reducing effects of exogenous oxyntomodulin administration, as receptor antagonists also block the actions of endogenous glucagon and GLP-1. Previously it has been reported that exchanging the position 3 glutamine (Gln/Q) of oxyntomodulin or a related analogue, for glutamate (Glu/E), diminishes mouse GCGr activity without affecting activity at the mouse GLP-1r [6, 7]. These GLP-1r selective Glu-3 peptides offer an alternative method for investigating the contribution of GCGr and GLP-1r activity to the metabolic effects of oxyntomodulin and related analogues. Several previous studies have used Glu-3 analogues to explore the metabolic
effects of oxyntomodulin administration in mice, reporting that GCGr activity was necessary for [6-8], but the effect of Glu-3 peptide administration in rats has not been investigated.

The current study reports the novel observation that substituting glutamate at position 3 of two oxyntomodulin-like dual analogues caused a significant increase in bodyweight in a rat model, without altering food intake. In line with previous findings at the mouse receptor [6, 7], in vitro activity at the rGCGr was diminished by Glu-3 substitution. Interestingly, glucagon mediated activation of the rGCGr in vitro was reduced by the lower concentrations of the Glu-3 analogues tested, including Glu-3 of native oxyntomodulin. This suggests that the Glu-3 OXM analogues may be partial agonists that can also act as competitive antagonists in vitro, despite reduced rGCGr binding affinity.

Glucagon administration increases energy expenditure [12, 13] and in rodents this is postulated to be due to activation of brown adipose tissue (BAT) [14]. Exogenous peripheral administration of native oxyntomodulin or long acting analogues also increases energy expenditure [3, 4, 6] and several murine studies have suggested a GCGr mediated mechanism is responsible [6, 7]. It could be speculated that Glu-3 OXM analogues may antagonise the activity of endogenous glucagon, possibly causing a reduction in energy expenditure and resulting in the observed increase in rat bodyweight. However endogenous glucagon has not currently been shown to play a tonic role in energy expenditure or weight regulation. Investigation of the effects of Glu-3 peptides on energy expenditure in rats is needed.

The increase in bodyweight following Glu-3 administration may be a rat specific phenomenon, as previous results with Glu-3 oxyntomodulin-like peptides were obtained in mice and did not show an increase [6, 7]. However Glu-3 peptides used in these previous studies were derivatised and administered at higher doses than the current study. A recently published study demonstrated that analogues of glucose-dependent insulinotropic polypeptide (GIP) with a Proline substitution at amino acid position 3 were full agonists at the human GIP receptor (GIPr) and partial agonists that could acts as competitive antagonists at the rat and mouse GIPr [15]. It is possible this may also be the case with Glu-3 oxyntomodulin-like analogues. However, the agonist or antagonist properties of Glu-3 analogues at the mouse and human GCG receptors have not been fully
investigated. Additionally, differences in peptide and receptor homology between species may contribute to the differential effects observed with Pro-3 GIP analogues. Human GIP has several different residues compared to rat and mouse GIP [15], whereas the amino acid sequences of glucagon and oxyntomodulin are identical in the 3 species. Therefore the relevance of the findings with Pro-3 GIP analogues to the current study is unclear.

In conclusion, oxyntomodulin-like analogues with a Glu-3 substitution significantly increase bodyweight in a rat model, independently of food intake. Glu-3 analogues appear to be partial agonists at the rGCGr and can also act as competitive antagonists in vitro. Therefore Glu-3 oxyntomodulin-like analogues are unsuitable tools to investigate the effects of oxyntomodulin analogue activity in a rat model.

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Conflict of interest statement

SP, JM and SB have no potential conflicts of interest to report.

References


Figure 1. Schematic of the amino acid structures of the oxyntomodulin-like dual analogues investigated in the current study.

Figure 2. Food intake (A) and bodyweight change (B) after 7 days of daily SC 25nmol/kg OXM14, OXM15, OXM14E3, OXM15E3 or vehicle, n=6-7. Food intake (C) and bodyweight change (D) after 7 days of daily SC administration of various doses of OXM15E3 or vehicle, n= 11-12. Data are presented as mean ± SEM. Statistical analysis was conducted using one-way ANOVA and post hoc tests with Bonferroni (A, B) or Dunnett (C, D) corrections. *P <0.05, **P <0.01 versus vehicle controls; #P <0.01 versus Gln-3 peptide.
**Figure 3.** *In vitro* cAMP levels measured in CHO cells over expressing the rat glucagon receptor following incubation with various concentrations of OXM14E3 (A), OXM15E3 (B) or OXME3 (C) in the presence of 0.1nM glucagon. Mean cAMP levels (± SEM) are shown as a percentage of the levels produced by glucagon stimulation alone, n=3. (D) Mean *in vitro* efficacy (cAMP EC$_{50}$) and affinity (IC$_{50}$) of oxyntomodulin analogues and native peptides at the rat glucagon receptor (rGCGr) and rat GLP-1 receptor (rGLP-1r). cAMP EC$_{50}$ values were calculated using CHO cells over-expressing the rGCGr or CHL cells over-expressing the rGLP-1r. IC$_{50}$ values were calculated using membranes produced from the CHO cells or CHL cells. n=3.