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3 **(D-Ser²)Oxm[mPEG-PAL]: a novel chemically modified analogue of oxyntomodulin**
4
5 **with antihyperglycaemic, insulinotropic and anorexigenic actions**
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59 *Running title:* Novel analogue of oxyntomodulin
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ABSTRACT

Oxyntomodulin (Oxm) is a hormone which has been shown to exhibit a range of potentially beneficial actions for alleviation of obesity-diabetes. However, exploitation of Oxm-based therapies has been severely restricted due to degradation by the enzyme dipeptidylpeptidase-IV (DPP-IV). Thus, the aim of this study was to assess the glucose-lowering, insulin-releasing and anorexigenic actions of chemically modified, enzyme resistant analogues of Oxm. Oxm, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL], were incubated with DPP-IV to assess enzyme stability and pancreatic beta-cells to evaluate insulin secretion. cAMP production was assessed using glucagon-like peptide-1 (GLP-1) and glucagon receptor transfected cells. *In vivo* effects of Oxm analogues on glucose homeostasis, insulin secretion, food intake and bodyweight were examined in obese diabetic (*ob/ob*) mice. (D-Ser²)Oxm[mPEG-PAL] displayed enhanced DPP-IV resistance compared to (D-Ser²)Oxm and Oxm. All peptides demonstrated similar *in vitro* cAMP and insulin-releasing actions, which was associated with dual action at GLP-1 and glucagon receptors. Acute administration of (D-Ser²)Oxm[mPEG-PAL] and (D-Ser²)Oxm reduced plasma glucose and food intake, whilst plasma insulin levels were elevated. Once-daily administration of (D-Ser²)Oxm[mPEG-PAL] for 14 days to *ob/ob* mice decreased food intake, bodyweight, plasma glucose and increased plasma insulin. Furthermore, daily (D-Ser²)Oxm[mPEG-PAL] improved glucose tolerance, increased glucose-mediated insulin secretion, pancreatic insulin content, adiponectin and decreased both visfatin and triglyceride levels. The ability of enzyme resistant (D-Ser²)Oxm[mPEG-PAL] to improve glucose homeostasis, insulin secretion, satiety, bodyweight and markers of fat metabolism suggests significant promise for Oxm-based therapies for obesity-diabetes.

1. Introduction

Oxyntomodulin (Oxm) is a 37 amino acid peptide hormone released from intestinal L-cells into the blood in response to feeding [1]. Oxm is a member of the proglucagon-derived peptide family comprising glucagon, glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) [2]. Despite the well documented biological actions of glucagon, GLP-1 and GLP-2 [3], the precise biological role of Oxm remains unclear. Studies have demonstrated that Oxm increases core body temperature and heart rate in rodents [4] while in humans it has been shown to increase energy expenditure and voluntary physical activity [5]. Oxm reduces bodyweight in animal and human studies due possibly to suppression of pre-prandially released ghrelin [4,6]. Recent evidence has also suggested that Oxm may play a role in glucose homeostasis through long-term bodyweight reduction, most probably due to the causal link between obesity and type 2 diabetes [7] as well as enhancing glucose-dependent insulin secretion [8].

While separate receptors for other proglucagon derived peptides have been characterised, a pharmacologically unique binding site for Oxm has yet to be identified [9]. It has been suggested that Oxm induces catabolic effects that favour weight loss through activation of the glucagon receptor while modulating glucose homeostasis through agonism of the GLP-1 receptor [10]. Indeed, the insulinotropic properties of GLP-1 and the regulation of metabolism in fasting through the glucagon receptor are well established [11,12]. Oxm has been shown to activate both GLP-1 and glucagon receptors, albeit with a 10-100-fold reduced potency relative to GLP-1 and glucagon [13]. This may point to the possibility of a dual action Oxm-based therapy aimed at both obesity and type 2 diabetes [10,14].

Despite their anti-diabetic and anti-obesity potential, Oxm-based therapies have not yet reached the clinic, due to the susceptibility of the native hormone to enzymatic degradation [15].

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3 Proglucagon peptides are rapidly degraded by the ubiquitous enzyme dipeptidylpeptidase-IV
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5 (DPP-IV) [16]. However, many studies have demonstrated greatly increased bioactivity of other
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7 DPP-IV susceptible gut hormones through chemical modifications [17,18]. Notably, substitution
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9 of the naturally-occurring L-Ala at position 8 in GLP-1 or position 2 in GIP results in DPP-IV
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11 resistance [19-22]. Furthermore, C-terminal PEGylation and fatty acid derivatisation of GIP and
12
13 GLP-1 results in longer-acting and more potent analogues, due largely to reduced renal clearance
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15 through binding to plasma proteins [23-27]. Building on these observations and approaches to
16
17 stabilise related gut hormones, we developed a novel Oxm analogue with D-Ser at position 2 and
18
19 C-16 palmitic acid conjugated via a mini-PEG linker at the C-terminus, (D-Ser²)Oxm[mPEG-
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21 PAL], and assessed its antihyperglycaemic, insulinotropic and anorexigenic properties. m-PEG
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23 was used as a linker in view of previous reports that such linkers improve the actions of other
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25 acylated peptides such as GLP-1 [23].
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37 **2. Materials and methods**

38 **2.1. Peptide synthesis**

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40 Oxm, (D-Ser²)Oxm, and (D-Ser²)Oxm[mPEG-PAL] (each >97% purity) were purchased from GL
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42 Biochem Ltd., (Shanghai, China). (D-Ser²)Oxm was created by substitution of Ser at position 2
43
44 with its D-isomer. (D-Ser²)Oxm[mPEG-PAL] was generated similar to (D-Ser²)Oxm with further
45
46 addition of a C-16 fatty acid moiety attached to the C-terminus via a miniPEG linker (Product:
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48 HK(mini-PEG-Pal)-38-NH₂; Molecular formula: C₂₂₀H₃₄₇N₆₄O₆₅S₁). Observed molecular masses
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50 for Oxm, (D-Ser²)Oxm, and (D-Ser²)Oxm[mPEG-PAL] were 4445.7, 4449.8 and 4957.8 Da,
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56 respectively, corresponding closely to theoretical values and confirming structural identities.
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2.2 Degradation of Oxm peptides by DPP-IV

Oxm, (D-Ser²)Oxm, and (D-Ser²)Oxm[mPEG-PAL] were incubated at 37°C in 50 mM triethanolamine-HCl (pH 7.8; Sigma-Aldrich, Dorset, UK) with purified porcine DPP-IV (5 mU; Sigma-Aldrich, Dorset, UK) for 0, 2, 4, 8 and 24 h. Degradation profiles were obtained using HPLC analysis as described previously [24] and HPLC peak area data used to calculate percentage intact peptide remaining at various time points during the incubation. In addition, the established DPP-IV inhibitor Vildagliptin (10 mM; Novartis Pharma AG, Basel, Switzerland) was added to 2 h incubations with Oxm and native GLP-1.

2.3. *In vitro* cAMP production and insulin secretion

Effects of Oxm, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] on cAMP production and insulin secretion were assessed in clonal pancreatic BRIN-BD11 cells [28]. Effects of peptide analogues, as well as native GLP-1 and glucagon on cAMP production were also evaluated using Chinese Hamster Lung (CHL) cells transfected with human GLP-1 receptor [29] and glucagon receptor transfected HEK2935 cells [30]. For cAMP studies, cells were seeded (100,000 cells per well) into 96-well plates (Nunc, Roskilde, Denmark) and washed with Hanks Buffered Saline (HBS) buffer prior to incubation with native or Oxm peptides (10^{-6} to 10^{-12} M) in the absence or presence of 10^{-6} M glucagon or GLP-1 receptor antagonists, DesHis¹DesPhe⁶Glucagon-amide and exendin (9-39), respectively (in the presence of 200 μ M IBMX for 20 min at 37°C). After incubation, medium was removed and cells lysed prior to measurement of cAMP using HTS Immunoassay Kit (Millipore, Watford, UK). Percentage cAMP production was calculated from the maximal response induced by the agonist or antagonist peptides at 10^{-6} M. For insulin-release studies, BRIN-BD11 cells were seeded (150,000 cells per well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37°C. Following 40 min pre-incubation (1.1 mM glucose; 37°C), cells were incubated (20 min; 37°C) in the presence of 5.6 mM glucose

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3 with a range of peptide concentrations. After 20 min incubation, buffer was removed from each
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5 well and aliquots (200 μ l) stored at -20°C for measurement of insulin.
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10 2.4. Animals

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12 The majority of experiments were performed using obese diabetic (*ob/ob*) mice (20-22 weeks of
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14 age) derived from the Aston colony to assess *in vivo* biological properties of Oxm peptides [31].

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17 Animals were age-matched, divided into groups and housed in an air-conditioned room at $22 \pm$
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19 2°C with a 12h light / 12h dark cycle. Animals had free access to drinking water and normal
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21 laboratory chow (Trouw Nutrition, Cheshire, UK). Male NIH Swiss mice (6-8 weeks and 26-30
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23 g; Harlan Ltd, Blackthorne, UK) had free access to drinking water and high fat diet composed of
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25 45% fat, 20% protein and 35% carbohydrate (percent total energy 26.15 KJ/g; Special Diet
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27 Services, Essex, UK) for 140 days prior to commencement of acute *in vivo* studies. This diet
28
29 resulted in progressive body weight gain (54.8 ± 5.2 vs. 38.9 ± 2.9 g, $p < 0.05$) and
30
31 hyperglycaemia (8.8 ± 0.5 vs. 7.2 ± 0.4 mM/L, $p < 0.05$) compared with age-matched controls on
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33 normal laboratory chow (data not shown). All animal experiments were performed in accordance
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35 with the ‘Principles of laboratory animal care’ (NIH publication no. 85-23, revised 1985) as well
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37 as UK Animals (Scientific procedures) Act 1986. No adverse effects were observed following
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39 administration of any of the peptides.
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49 2.5. Acute and persistent effects of Oxm peptides on glucose-lowering, insulin release and 50 51 food intake *in vivo*

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54 In a first series of experiments, *ob/ob* mice and high-fat fed mice received glucose alone (18
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56 mmol/kg bw; ip) or in combination with Oxm, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] (each
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58 at 1400 nmol/kg bw; final volume 8 ml/kg bw). In a second series of experiments using *ob/ob*
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60 mice to assess duration of bioactivity, glucose (18 mmol/kg bw; ip) was administered four, eight
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3 and twelve hours after injection of saline vehicle (0.9% (w/v) NaCl), Oxm, (D-Ser²)Oxm or (D-
4 Ser²)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). In a final series of experiments, accumulated
5 food intake was measured in 12-h fasted *ob/ob* mice and high-fat fed animals at zero hours, and
6 additionally at four, eight and twelve hours in *ob/ob* mice after injection of saline vehicle (0.9%
7 (w/v) NaCl) Oxm, (D-Ser²)Oxm or (D-Ser²)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw).
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10 11 12 13 14 15 16 17 2.6. Sub-chronic effects of once-daily administration of (D-Ser²)Oxm[mPEG-PAL] in *ob/ob* 18 mice 19

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22 Once-daily injections of (D-Ser²)Oxm[mPEG-PAL] (1400 nmol/kg bw; ip) or saline vehicle
23 (0.9% (w/v) NaCl) were administered at 16:00 h over a 14-day period. Food intake, bodyweight,
24 plasma glucose and insulin were monitored at intervals of 2-4 days during the treatment period
25 and for an additional 14 days following cessation of peptide administration. Glucose tolerance
26 (18 mmol/kg bw; ip) and insulin sensitivity (50 U/kg bw; ip) tests were performed on days 14
27 and 28. At termination, blood for measurement of circulating triglycerides, adiponectin and
28 visfatin was taken and pancreatic tissues were excised and processed for measurement of insulin
29 following extraction with ice-cold ethanol (5 ml/g) as described previously [24].
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44 2.7. Biochemical analyses 45

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47 Blood samples were collected from the cut tip on the tail vein of conscious mice into chilled
48 fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) at time points
49 indicated in the Figures. Samples were immediately centrifuged using a Beckman
50 microcentrifuge (Beckman Instruments, Galway, Ireland) for 30 s at 13,000 x *g*. Whole blood
51 samples were used to measure plasma glucose values with an Ascensia[®] CONTOUR[®] Microfill
52 Blood Glucose Meter and Ascensia[®] MICROFILL[®] test strips (Bayer Healthcare, Uxbridge, UK).
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54 Plasma and pancreatic insulin was assayed by a modified dextran-coated charcoal
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3 radioimmunoassay as described previously [32]. Plasma triglyceride levels were measured using
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5 a Hitachi Automatic Analyser 912 (Boehringer Mannheim, Germany). Circulating adiponectin
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7 and visfatin were measured using ELISA kits (Phoenix Pharmaceuticals Inc., Burlingame, CA,
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9 USA). All analyses were carried out according to the manufacturer's instructions.
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15 2.8. Statistical analysis

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17 Results are expressed as means \pm SEM and data compared using the unpaired Student's *t*-test with
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19 Chi Squared analysis for correlations. Where appropriate, data were compared using repeated
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21 measures ANOVA or one-way ANOVA, followed by the Student-Newman-Keuls post-hoc test.
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23 Incremental area under the curve (AUC) analyses for plasma glucose and insulin were calculated
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25 using GraphPad Prism version 3.02. Groups of data were considered to be significantly different
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27 if $p < 0.05$.
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37 3. Results

38 3.1. DPP-IV stability

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40 As depicted in Fig. 1A, Oxm was progressively degraded over 24 h with an estimated *in vitro*
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42 half-life of 9.3 hr. In contrast, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] maintained a
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44 significantly higher percentage of intact peptide ($p < 0.001$) over the 4-24 h period with
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46 biological half-lives >24 hr. A significantly greater percentage of intact (D-Ser²)Oxm[mPEG-
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48 PAL] (60% increase; $p < 0.001$) was observed after 24 h compared with degradation of (D-
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50 Ser²)Oxm (Fig. 1A). Incubation with Vildagliptin for 2 hr completely inhibited DPP-IV mediated
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52 degradation of Oxm and native GLP-1 (data not shown).
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61 3.2. cAMP production and insulin secretion in BRIN-BD11 cells

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3 Oxm, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] stimulated cAMP production in a
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5 concentration-dependent manner with similar potency in BRIN-BD11 cells with EC₅₀ values of
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7 1.84±0.09 nM; 1.81±0.37 nM; and 1.97±1.21 nM, respectively (Fig. 1B). Similarly, all peptides
8
9 stimulated insulin secretion in a concentration-dependent and equipotent manner with maximal
10
11 insulin secretion observed at the highest peptide concentrations (40-150% increase; p < 0.001;
12
13 Fig. 1C).
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20 3.3. cAMP production in GLP-1R and GR transfected cells

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22 Receptor transfected cells were used to assess specificity of effects of Oxm and novel analogues.
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24 Table 1 shows cAMP production stimulated by various concentrations of Oxm, (D-Ser²)Oxm, (D-
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26 Ser²)Oxm[mPEG-PAL] and either native GLP-1 or glucagon. All Oxm peptides stimulated
27
28 cAMP production in a concentration-dependent manner in both GLP-1 and glucagon receptor
29
30 transfected cells with approximately similar potencies. In the presence of GLP-1 and glucagon
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32 receptor antagonists, cAMP production induced by Oxm peptides was antagonised in both GLP-1
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34 (65-70% maximal antagonism; p < 0.01 to p < 0.001) and glucagon receptor (73-78% maximal
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36 antagonism; p < 0.001) transfected cells compared to control (respective natural ligands).
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44 3.4. Acute actions of Oxm, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] on glucose-lowering 45 46 and insulin secretion *in vivo*

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48 All peptides significantly lowered plasma glucose concentrations (35-43% reduction; p < 0.05 to
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50 p < 0.001) compared to glucose alone in *ob/ob* mice (Fig. 2A). This effect was confirmed by
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52 AUC analysis (32-38% reduction; p < 0.001). Decreased glucose concentrations were associated
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54 with significantly increased plasma insulin levels (66-82% increase; p < 0.01 to p < 0.001) (Fig.
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56 2B) which was confirmed by AUC analysis (58-66% increase; p < 0.001). As shown in Fig. 4A,
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58 Oxm peptides also significantly lowered plasma glucose (44 to 53% reduction; p < 0.01 to p <
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3 0.001) following administration of glucose in high-fat fed mice. This effect was corroborated by
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5 plasma glucose AUC (35 to 48% reduction; $p < 0.01$ to $p < 0.001$).
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10 3.5. Time-dependent effects of Oxm, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] on glucose-
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12 lowering *in vivo* in *ob/ob* mice
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15 As can be seen in Fig. 3A, animals treated with Oxm, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-
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17 PAL] four hours prior to a glucose load had significantly decreased plasma glucose (23-57%
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19 reduction; $p < 0.05$ to $p < 0.001$) compared to saline controls. This was corroborated by AUC
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21 analysis (25-57% reduction; $p < 0.05$). Compared with Oxm, both (D-Ser²)Oxm and (D-
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23 Ser²)Oxm[mPEG-PAL] demonstrated significantly improved glucose-lowering effects (15-32%
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25 reduction; $p < 0.05$) at 60 min post-glucose load. Eight hours post-administration, (D-Ser²)Oxm
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27 and (D-Ser²)Oxm[mPEG-PAL] had significantly reduced (25-42% reduction; $p < 0.05$ to $p <$
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29 0.01; Fig. 3B) plasma glucose levels compared to both saline and Oxm (AUC: 25-34% reduction;
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31 $p < 0.05$ to $p < 0.01$). At twelve hours post-administration, no significant differences were
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33 observed between any of the treatment groups (Fig. 3C).
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41 3.6. Time-dependent effects of Oxm, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] on feeding
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43 *in vivo*
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46 Accumulative food intake was significantly reduced (13-56% reduction; $p < 0.05$ to $p < 0.01$) for
47
48 (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] at all time points up to 1400 min post-
49
50 administration (Fig. 5A). Furthermore, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] reduced
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52 food intake, four (3-30% reduction; $p < 0.05$ to $p < 0.01$; Fig. 5B) and eight hours (7-10%
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54 reduction; $p < 0.05$; Fig. 5C) post-administration compared to saline controls. In contrast, Oxm-
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56 treated mice only demonstrated significant reductions in food intake when administered at zero
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58 hours (4-23% reduction; $p < 0.05$). At twelve hours, no significant differences were observed
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3 between treatment groups (Fig. 5D). As shown in Fig. 4B, Oxm peptides also significantly
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5 inhibited food intake in high-fat fed mice relative to saline controls (33 to 53% reduction; $p <$
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7 0.05 to $p < 0.001$). The two Oxm analogues were also more effective at inhibiting food intake
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9 during the latter stages of the experiment ($p < 0.01$ to $p < 0.001$).

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15 3.7. Sub-chronic effects of once-daily administration of (D-Ser²)Oxm[mPEG-PAL] on
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17 bodyweight, food intake, plasma glucose and insulin levels in *ob/ob* mice
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19 Daily administration of (D-Ser²)Oxm[mPEG-PAL] for 14 days significantly reduced bodyweight
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21 (11-15% reduction; $p < 0.05$ to $p < 0.01$; Fig. 6A) and food intake (11-29% reduction; $p < 0.05$ to
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23 $p < 0.001$; Fig. 6B). Similarly, non-fasting plasma glucose concentrations were significantly
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25 decreased (22-27% reduction; $p < 0.05$ to $p < 0.01$; Fig. 6C) and this was accompanied by
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27 significantly increased plasma insulin concentrations (45-54% increase; $p < 0.01$; Fig. 6D). No
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29 significant differences in any of the above parameters were observed 14 days following cessation
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31 of treatment.
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39 3.8. Sub-chronic effects of once-daily administration of (D-Ser²)Oxm[mPEG-PAL] on glucose
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41 tolerance and insulin response to glucose in *ob/ob* mice
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43 Once-daily treatment with (D-Ser²)Oxm[mPEG-PAL] for 14 days resulted in significantly
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45 reduced plasma glucose excursion (24-37% decrease; $p < 0.05$ to $p < 0.001$) following an ip
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47 glucose load (AUC: 26% decrease; $p < 0.01$, Fig. 7A). This improvement in glucose-lowering
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49 was associated with markedly improved glucose-mediated insulin responses (55-65% increase; p
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51 < 0.05 to $p < 0.01$; Fig. 7C), which were confirmed by AUC analysis (57% increase; $p < 0.01$).
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53 Following 14 days cessation of treatment, no significant differences in glucose tolerance or
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55 insulintropic responses were noted (Figs. 7B and 7D).
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3 3.9. Sub-chronic effects of once-daily administration of (D-Ser²)Oxm[mPEG-PAL] on insulin
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5 sensitivity, pancreatic insulin content, circulating adiponectin, visfatin and triglycerides in *ob/ob*
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7 mice
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9
10 Table 2 shows that (D-Ser²)Oxm[mPEG-PAL] treated mice exhibited lower glucose
11
12 concentrations following insulin ($p < 0.05$). Pancreatic insulin content was significantly increased
13
14 (47%; $p < 0.001$). Furthermore, treatment with (D-Ser²)Oxm[mPEG-PAL] significantly increased
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16 circulating adiponectin (37% increase; $p < 0.01$) and decreased circulating visfatin (23%
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18 reduction; $p < 0.01$) and triglycerides (28% reduction; $p < 0.01$). No significant differences were
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20 observed in any of these parameters following cessation of treatment (data not shown).
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29 **4. Discussion**

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32 Although being one of the less well characterised members of the proglucagon family, Oxm has
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34 been shown to exhibit a range of potentially useful actions for the alleviation of obesity and
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36 diabetes [1,7,33]. These include bodyweight loss associated with decreased food intake and
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38 increased energy expenditure and voluntary physical activity [4,6,34]. Recently Oxm has been
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40 suggested to play a role in glucose homeostasis by lowering plasma glucose, most probably by
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42 improving pancreatic beta-cell function [8]. The cellular mechanisms responsible for these
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44 beneficial effects are poorly understood. However, recent evidence suggests a dual mode of
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46 action mediated through activation of both GLP-1 and glucagon receptors [10,14]. This contrasts
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48 with some hybrid GLP-1-glucagon peptides which can have weak glucagon agonism in some
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50 tissues or even block glucagon sufficiently to exert beneficial effects [10].
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59 The possible exploitation of therapies based around Oxm has been severely restricted due to
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61 proteolytic degradation of the peptide by the ubiquitous enzyme DPP-IV [15]. Improved
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3 biological efficacy of other proglucagon-derived peptides has been achieved through chemical
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5 modifications enabling DPP-IV resistance and prolonging biological half-life [17,18]. In the
6
7 current paper, the effects of a novel chemically modified Oxm analogue, (D-Ser²)Oxm[mPEG-
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9 PAL], has been examined on DPP-IV resistance, cellular cAMP production and insulin secretion,
10
11 as well as acute and sub-chronic effects on glucose homeostasis, food intake and bodyweight
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13 regulation in obese diabetic (*ob/ob*) mice.
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20 As expected from previous observations on DPP-IV resistance of N-terminally modified GLP-1,
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22 (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] remained almost completely intact throughout the
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24 24 h incubation [35]. This contrasted sharply with Oxm which was progressively degraded over
25
26 time with an *in vitro* half life of 9.3 hr. The *in vitro* half-life of Oxm is moderately longer than
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28 that of native GLP-1, consistent with previous observations that Oxm is less susceptible to
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30 degradation by DPP-IV. Furthermore, addition of the established DPP-IV inhibitor vildagliptin in
31
32 our assay system completely inhibited degradation of Oxm. (D-Ser²)Oxm showed good but
33
34 intermediate stability indicating that substitution with the D-isomer at Ser² is insufficient to
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36 confer total resistance to DPP-IV. However, the development of a specific assay to directly
37
38 measure Oxm and related analogues in plasma would provide more precise details of
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40 endoprotease-mediated degradation and circulating half-life. Thus, the improved performance of
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42 (D-Ser²)Oxm[mPEG-PAL] suggests that the C-terminal modification with C-16 fatty acid
43
44 attached through a mini-PEG linker may completely mask the DPP-IV binding site. The addition
45
46 of hydrophobic moieties might also have disrupted the hydro-affinity of (D-Ser²)Oxm[mPEG-
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48 PAL] for DPP-IV as previously reported with incretin hormones [24,27]. Moreover, the *in vitro*
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50 DPP-IV assay used in this study does not exactly mimic the protease-laden *in vivo* situation nor
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52 rule out the possible involvement of other endoproteases [15].
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3 (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] displayed similar concentration-dependent cAMP
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5 stimulation to Oxm with broadly similar EC₅₀ values observed for the peptide family in BRIN-
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7 BD11, GLP-1 and glucagon receptor transfected cells. Consistent with dual agonism, all Oxm-
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9 derived peptides activated receptors for GLP-1 and glucagon receptors, but with less favourable
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11 EC₅₀ values compared to native GLP-1 and glucagon. Consistent with this action, cAMP
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13 production by Oxm peptides was markedly reduced in the presence of GLP-1 and glucagon
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15 receptor antagonists. The equi-potency of (D-Ser²)Oxm[mPEG-PAL] to other Oxm peptides
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17 indicates that the analogue retained full receptor affinity for both biochemical pathways. This
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19 view fits with observations that (D-Ser²)Oxm[mPEG-PAL] enhanced insulin stimulation from
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21 BRIN-BD11 cells in a concentration-dependent manner similar to Oxm. Interestingly, other
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23 studies also point to a possible insulinotropic role for Oxm [8]. This maintenance of *in vitro*
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25 biological efficacy for (D-Ser²)Oxm[mPEG-PAL] coupled with a strong resistance to DPP-IV
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27 supports the idea of improved potency of the peptide *in vivo*.
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37 To assess the antihyperglycaemic, insulinotropic and anorexigenic effects of (D-
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39 Ser²)Oxm[mPEG-PAL] *in vivo* we employed obese diabetic (*ob/ob*) mice [31]. These mice
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41 represent a well established model of obesity and type 2 diabetes, presenting with hyperphagia,
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43 obesity, moderate hyperglycaemia and severe hyperinsulinaemia. All Oxm peptides significantly
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45 reduced plasma glucose excursion following a glucose load which was associated with a strong
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47 increase in plasma insulin concentrations. This is consistent with a possible glucose-dependent
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49 stimulation of insulin secretion similar to that observed with other incretin hormones [36]. The
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51 antihyperglycaemic effect of native Oxm was not sustained beyond four hours. In contrast, (D-
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53 Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] elicited a glucose-lowering action for up to eight hours
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55 after initial administration, with (D-Ser²)Oxm[mPEG-PAL] showing a trend towards reduced
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57 plasma glucose even up to twelve hours post-treatment. Similarly, whereas inhibition of feeding
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3 by native Oxm did not persist for more than 60 min, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL]
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5 decreased food intake for much longer periods even when administered four or eight hours before
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7 feeding. This extended glucose-lowering and satiety action suggests a much longer plasma half-
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9 life of the Oxm analogues, similar to the findings of *in vitro* DPP-IV studies, providing a basis
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11 for use of (D-Ser²)Oxm[mPEG-PAL] in longer-term studies. Observations of acute glucose-
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13 lowering and inhibition of feeding in high-fat fed mice with diet-induced obesity suggests that
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15 these effects of Oxm analogues extend to other animal models.
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22 Daily administration of (D-Ser²)Oxm[mPEG-PAL] for 14 days to *ob/ob* mice significantly
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24 reduced bodyweight and food intake. Thus despite probable half-life around 12 hr, administration
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26 of peptide at 16:00 h, immediately before the dark period with associated feeding, induced clear-
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28 cut biological effects. Importantly, sub-chronic (D-Ser²)Oxm[mPEG-PAL] treatment also reduced
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30 plasma glucose concentrations with an associated increase of plasma insulin. This was
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32 accompanied by improvements in glucose tolerance, insulin response to glucose and pancreatic
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34 insulin content, suggesting an important anti-diabetic action in this commonly employed animal
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36 model. Observed up-regulation of glucose homeostasis was also associated with moderate
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38 improvement of insulin sensitivity and lowered glucose concentrations. These observations add
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40 to previous suggestions of enhanced beta-cell function and glucose homeostasis with use of
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42 Oxm-based therapies [8] and the general utility of enzyme-resistant acylated peptides [23,25,27].
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44 Furthermore, sub-chronic (D-Ser²)Oxm[mPEG-PAL] treatment significantly increased circulating
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46 adiponectin levels while decreasing visfatin and triglyceride concentrations, which are supported
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48 by studies using a dual GLP-1/glucagon agonist [10]. Although the precise mechanisms for these
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50 changes in adipokines are unclear, they suggest that the weight loss observed following (D-
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52 Ser²)Oxm[mPEG-PAL] treatment may be at least partly attributable to reduction in adipose
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54 tissue. Further studies incorporating pair-fed groups assessing parameters such as locomotor
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3 activity, energy expenditure and respiratory quotient may prove useful to address this.
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5 Interestingly, all of these effects were reversed following 14 days of cessation of treatment,
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7 indicating that continuing peptide exposure is required for sustained effects. Oxm peptides were
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9 well tolerated and no noticeable changes in general behaviour or activity was observed following
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11 administration.
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17 In conclusion, this study has demonstrated that N-terminal modification by substitution of L to D-
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19 isomer at Ser² and addition of a C-16 palmitic acid conjugated via a mini-PEG linker at the C-
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21 terminus of Oxm substantially improved DPP-IV resistance without compromising cellular
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23 actions including cAMP production, stimulation of insulin secretion and inhibition of feeding. (D-
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25 Ser²)Oxm[mPEG-PAL] clearly exhibited enhanced biological activity over native Oxm and (D-
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27 Ser²)Oxm. Indeed, the positive acute and sub-chronic effects of (D-Ser²)Oxm[mPEG-PAL] on
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29 glucose homeostasis, beta-cell insulin secretion, food intake, bodyweight and circulating markers
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31 of fat metabolism encourage further studies to progress Oxm-based therapies for obesity-
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33 diabetes.
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49
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3 **Tables and table legends**
4
5

6 Table 1 - Effects of Oxm analogues on cAMP production in GLP-1 and glucagon receptor transfected
7 CHL cells
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| Peptide | Agonist cAMP response (EC ₅₀ / nM) | | Maximal cAMP antagonism in presence of 10 ⁻⁶ M antagonist (%) | |
|------------------------------------|--------------------------------------------------|----------------|-----------------------------------------------------------------------------|---------------|
| | GLP-1 R | Glucagon R | GLP-1 R | Glucagon R |
| GLP-1 | 0.05 ± 0.09 | — | 100.0 | — |
| Glucagon | — | 0.56 ± 0.08 | — | 100.0 |
| Oxm | 8.60 ± 0.53*** | 4.82 ± 0.81*** | 69.7 ± 1.2*** | 80.2 ± 3.8*** |
| (D-Ser ²)Oxm | 7.73 ± 0.62*** | 7.33 ± 0.52*** | 63.9 ± 3.2*** | 78.2 ± 1.9*** |
| (D-Ser ²)Oxm[mPEG-PAL] | 8.89 ± 0.43*** | 8.16 ± 1.23*** | 68.4 ± 6.3** | 62.5 ± 2.2*** |

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21 For agonist experiments, GLP-1 and glucagon receptor transfected CHL cells were exposed to
22 various concentrations (10⁻¹² to 10⁻⁶ M) of Oxm peptides or native GLP-1 or glucagon (as
23 appropriate) for 20 min (n=4) and cAMP production measured using ELISA. For antagonist
24 studies, Oxm peptides or native GLP-1 or glucagon (as appropriate) (each at 10⁻⁶ M) were
25 incubated in the presence of exendin-9-39 amide (GLP-1 receptor antagonist) and
26 DesHis¹DesPhe⁶Glucagon-amide (glucagon receptor antagonist) each at 10⁻⁶ M. Data represent
27 means ± SEM. **p < 0.01, ***p < 0.001 compared to natural ligands, GLP-1 and glucagon,
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Table 2 - Effects of once-daily administration of (D-Ser²)Oxm[mPEG-PAL] on insulin sensitivity, pancreatic insulin content, adiponectin, visfatin and triglyceride levels in *ob/ob* mice

| Parameter | Saline vehicle | (D-Ser ²)Oxm[mPEG-PAL] |
|-------------------------------|-----------------|------------------------------------|
| Insulin sensitivity (mM.Min) | 1189.13 ± 14.21 | 1007.44 ± 8.5* |
| Insulin content (µg/g tissue) | 4.31 ± 0.27 | 6.33 ± 0.23*** |
| Adiponectin (ng/ml) | 1.06 ± 0.09 | 1.45 ± 0.06** |
| Visfatin (ng/ml) | 1.35 ± 0.06 | 1.02 ± 0.05** |
| Triglycerides (mmol/l) | 6.39 ± 0.23 | 4.62 ± 0.45** |

Tests were conducted following 14 days intraperitoneal treatment with (D-Ser²)Oxm[mPEG-PAL] (1400 nmol/kg bw) or saline vehicle (0.9% (w/v) NaCl). Data are expressed as means±SEM for 5 mice. **p < 0.01, ***p < 0.001 compared to saline-treated group.

Legends to Figures

Fig. 1 - Effects of Oxm analogues on (A) DPP-IV resistance, (B) cAMP production and (C) insulin secretion. (A) Resistance of Oxm, (D-Ser²)Oxm, (D-Ser²)Oxm[mPEG-PAL] and GLP-1 to degradation by DPP-IV (5 mU) was measured (n=3) following 0, 2, 4, 8 and 24 h incubations. Reaction products were subsequently analyzed by HPLC and degradation expressed as a percentage of intact peptide. ***p < 0.001 compared with Oxm, $\Delta\Delta\Delta$ p < 0.001 compared with (D-Ser²)Oxm. (B) BRIN-BD11 cells were exposed to various peptide concentrations for 20 min (n=4) and cAMP production subsequently measured using ELISA. (C) BRIN-BD11 cells were incubated with a range of peptide concentrations for 20 min (n=8) in the presence of 5.6 mM glucose and insulin-release measured by radioimmunoassay. Data represent means \pm SEM. ***p < 0.001 compared with 5.6 mM glucose control.

Fig. 2 - Acute effects of Oxm analogues on plasma glucose (A) and insulin response (B) in *ob/ob* mice. Plasma glucose and insulin concentrations were measured prior to and after intraperitoneal administration of glucose alone (18 mmol/kg bw) or in combination with Oxm, (D-Ser²)Oxm and (D-Ser²)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). The time of glucose injection is indicated by the arrow (0 min). Plasma glucose and insulin AUC values for 0-30 min are also included. Data are expressed as means \pm SEM for 6 mice. *p < 0.05, **p < 0.01, ***p < 0.001 compared to glucose alone.

Fig. 3 - Persistent effects of Oxm analogues on glucose-lowering in *ob/ob* mice. Plasma glucose concentrations were measured prior to and after intraperitoneal administration of glucose (18 mmol/kg bw) in animals injected four (A), eight (B) and twelve (C) hours previously with saline (0.9% (w/v), NaCl) or either Oxm, (D-Ser²)Oxm or (D-Ser²)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). The time of glucose injection is indicated by the arrow (0 min). Plasma glucose AUC values for 0-60 are included. Data represent means \pm SEM for 6 mice. *p < 0.05, **p < 0.01, ***p < 0.001 compared to saline control. Δ p < 0.05 compared with Oxm-treated mice.

Fig. 4 – Acute effects of Oxm analogues on plasma glucose (A) and food intake (B) in high-fat fed mice. Plasma glucose concentrations were measured prior to and after intraperitoneal administration of glucose alone (18 mmol/kg bw) or in combination with Oxm, (D-Ser²)Oxm or (D-Ser²)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). The time of glucose injection is indicated

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3 by the arrow (0 min). Plasma glucose AUC values for 0-60 min are also included. Food intake
4 was measured in animals injected intraperitoneally at zero hours with saline vehicle or Oxm
5 peptides (each at 1400 nmol/kg bw). Data are expressed as means±SEM for 6 mice. *p < 0.05,
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8 **p < 0.01, ***p < 0.001 compared to control animals. ^Δp < 0.05 compared to Oxm-treated mice.
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12 Fig. 5 - Persistent effects of Oxm analogues on food intake in *ob/ob* mice. Food intake was
13 measured in animals injected intraperitoneally at zero (A), four (B), eight (C) and twelve (D)
14 hours previously with saline (0.9% (w/v), NaCl) or either Oxm, (D-Ser²)Oxm or (D-
15 Ser²)Oxm[mPEG-PAL] (each at 1400 nmol/kg bw). Mice were fasted for 12 h prior to re-
16 feeding. Data represent means±SEM for 6 mice. *p < 0.05, **p < 0.01 compared to saline
17 control. ^Δp < 0.05 compared with Oxm-treated mice.
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25 Fig. 6 - Effects of once-daily administration of (D-Ser²)Oxm[mPEG-PAL] on (A) body weight,
26 (B) food intake, (C) plasma glucose and (D) plasma insulin in *ob/ob* mice. Parameters were
27 measured prior to and 14 days during intraperitoneal treatment with (D-Ser²)Oxm[mPEG-PAL]
28 (1400 nmol/kg bw) or saline vehicle (0.9% (w/v) NaCl) and for 14 days following cessation of
29 treatment. Data are expressed as means±SEM for 9 mice. *p < 0.05, **p < 0.01, ***p < 0.001
30 compared to saline-treated group.
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38 Fig. 7 - Effects of once-daily administration of (D-Ser²)Oxm[mPEG-PAL] on glucose tolerance
39 and insulin response to glucose in *ob/ob* mice. Tests were conducted following 14 days
40 intraperitoneal treatment (A and C) with (D-Ser²)Oxm[mPEG-PAL] (1400 nmol/kg bw) or saline
41 vehicle (0.9% (w/v) NaCl) and 14 days following cessation of treatment (B and D). Data are
42 expressed as means±SEM for 9 mice. *p < 0.05, **p < 0.01, ***p < 0.001 compared to saline-
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Figure 1

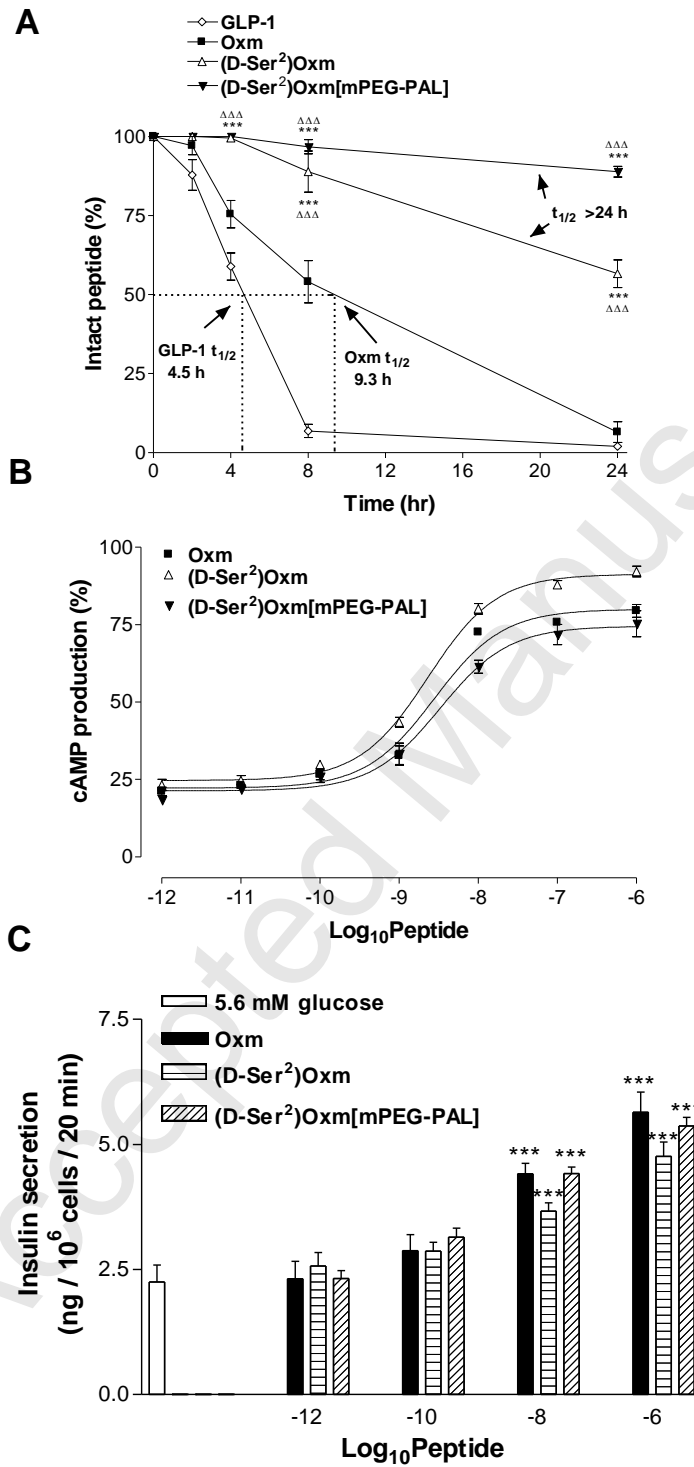


Figure 2

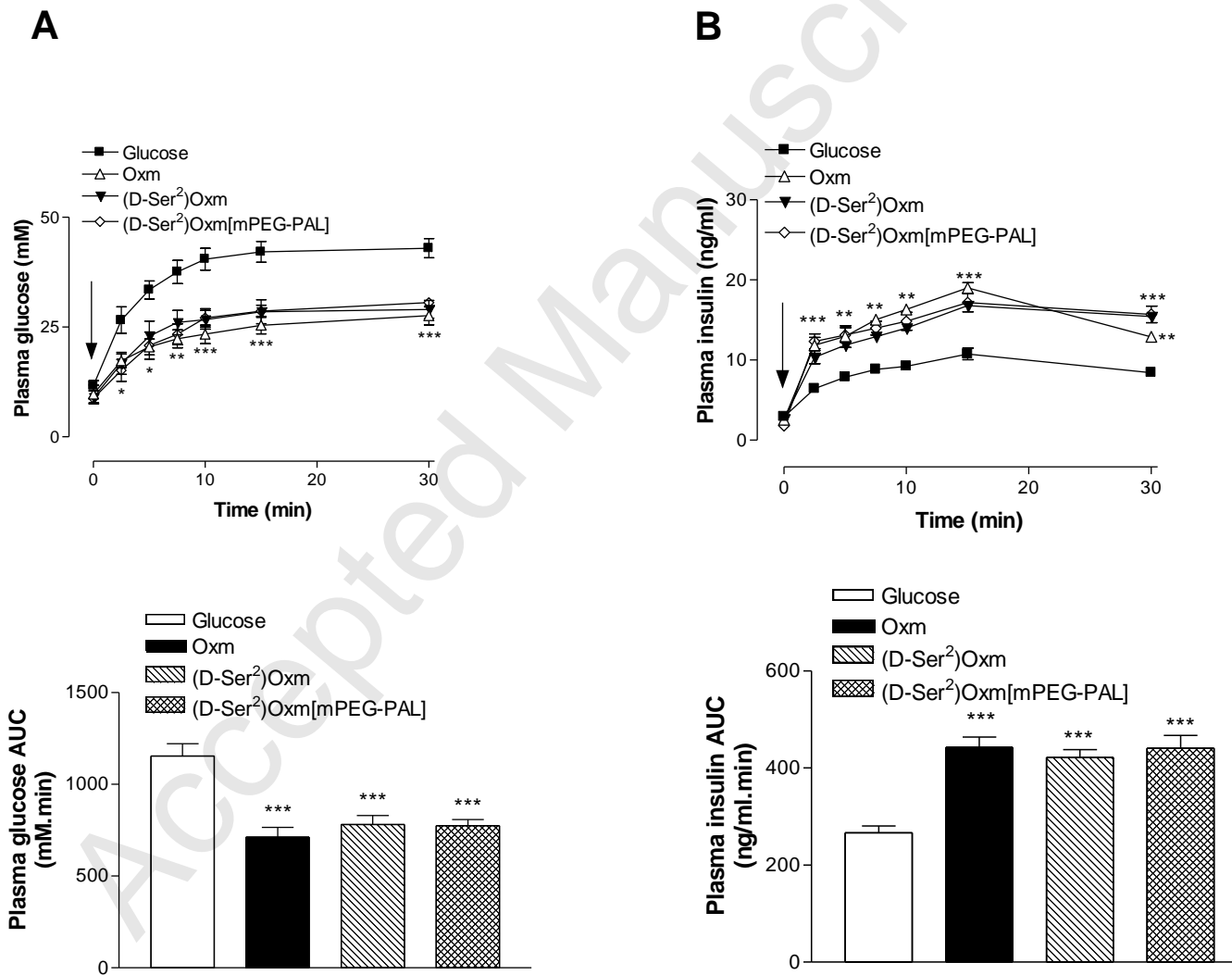


Figure 3

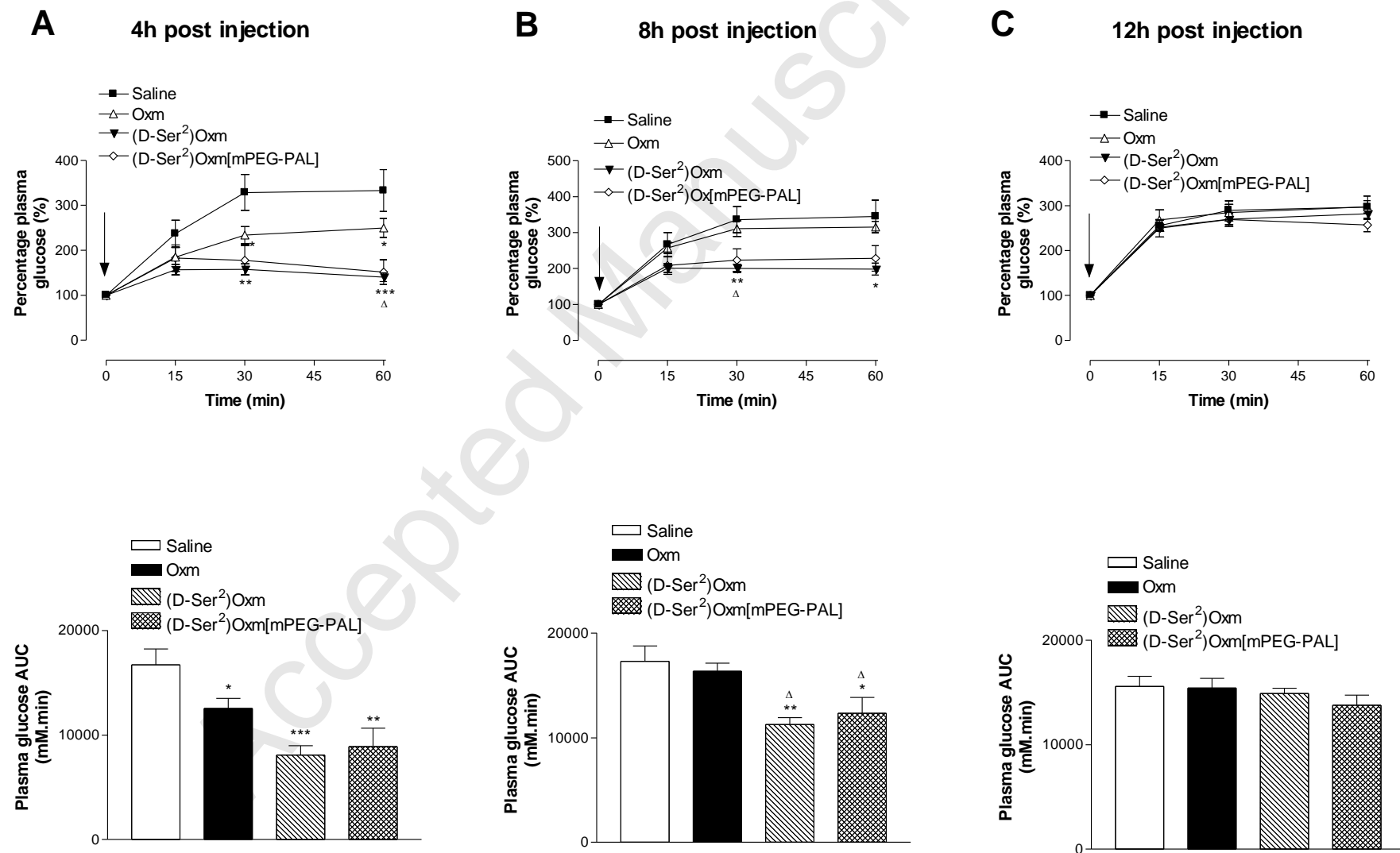
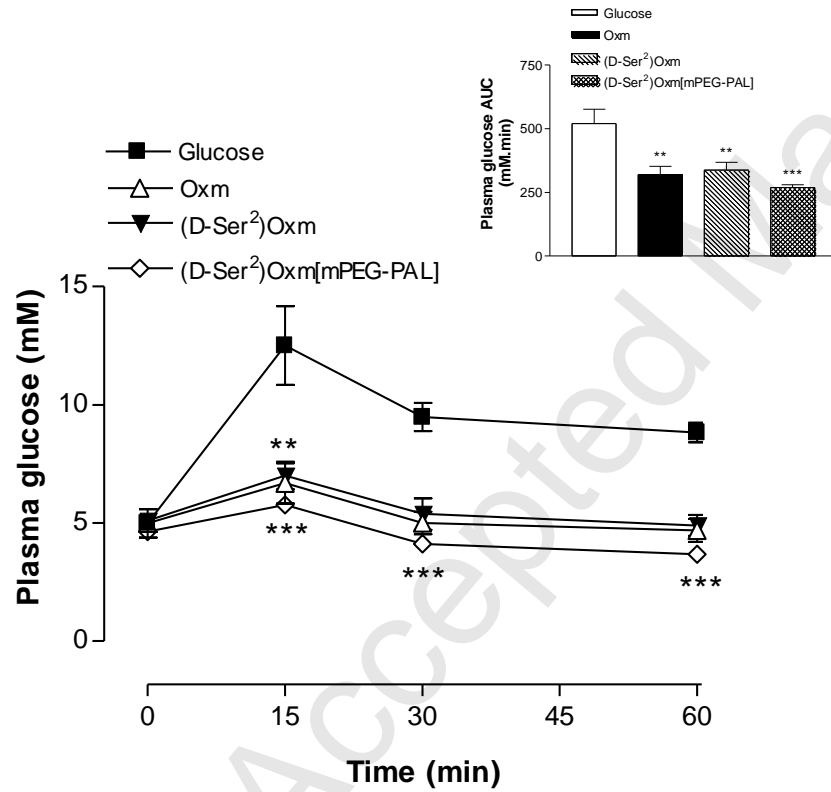


Figure 4

A



B

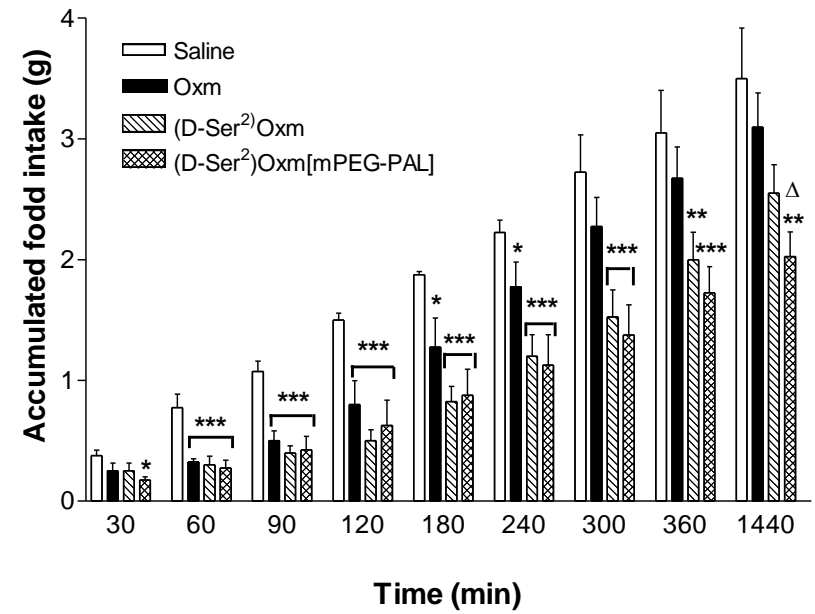


Figure 5

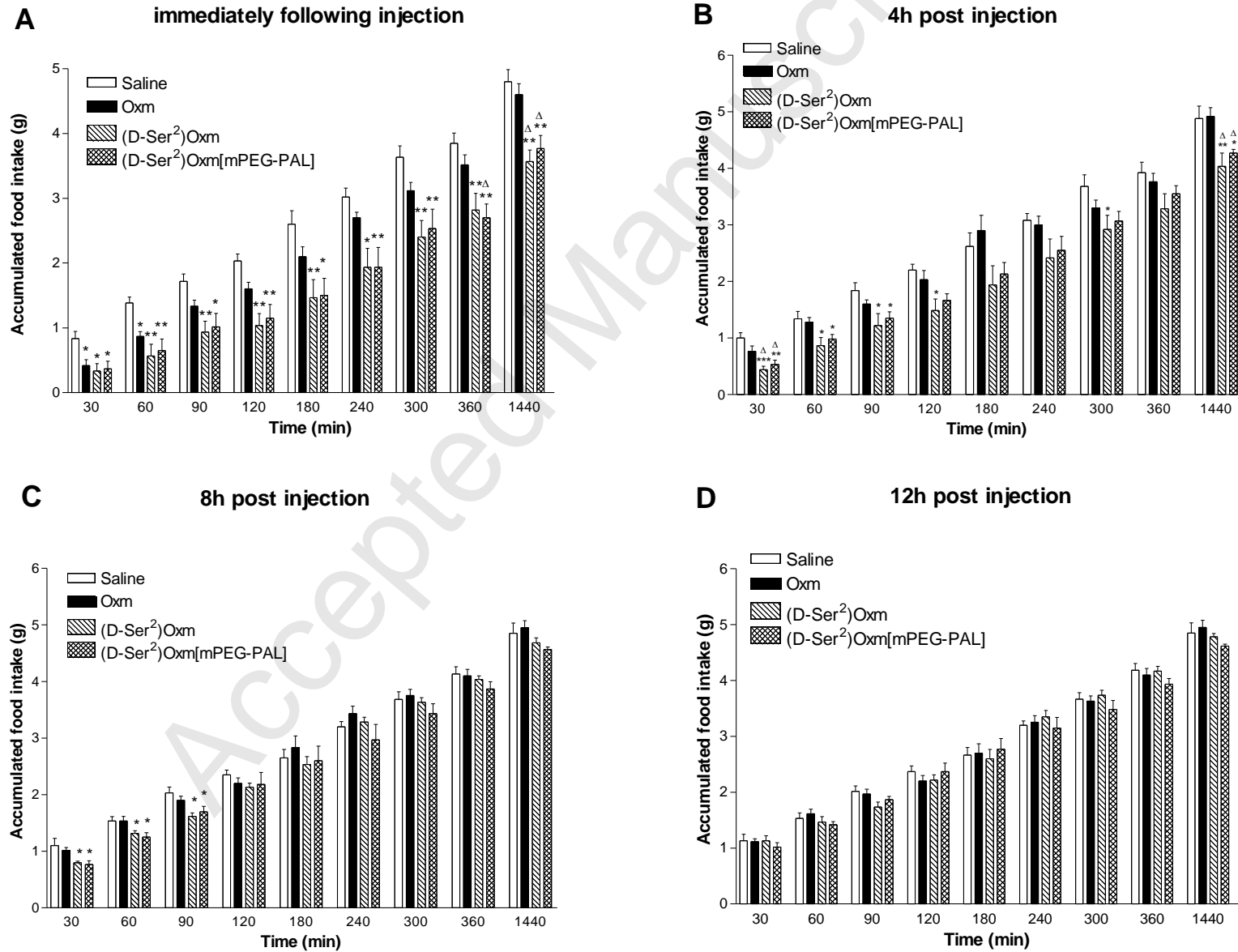
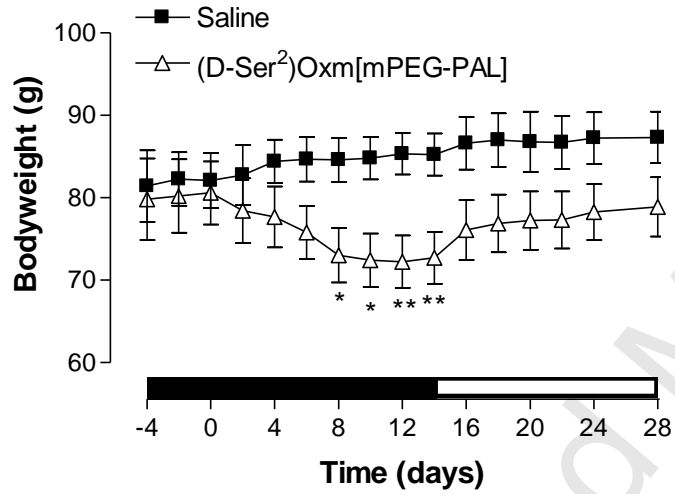
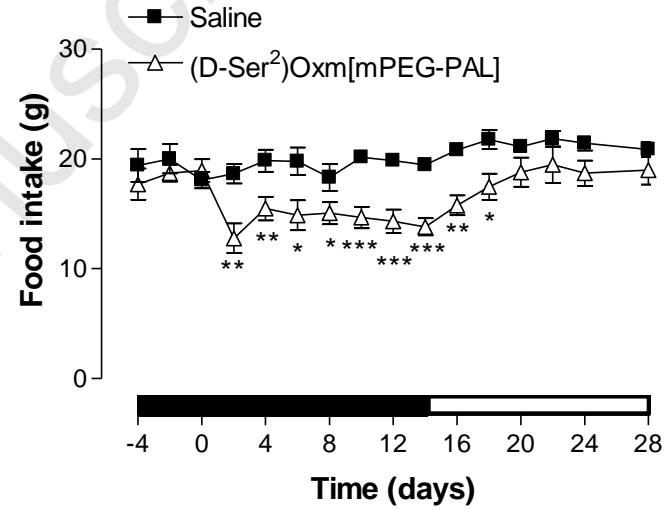


Figure 6

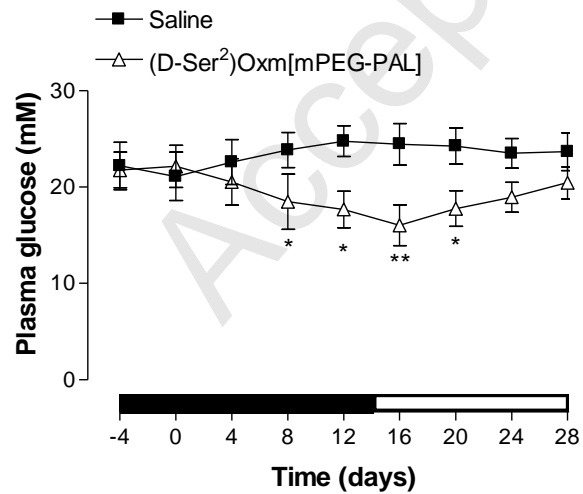
A



B



C



D

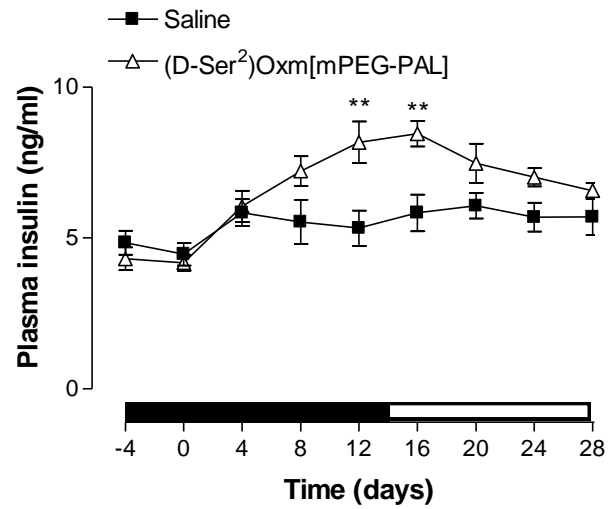
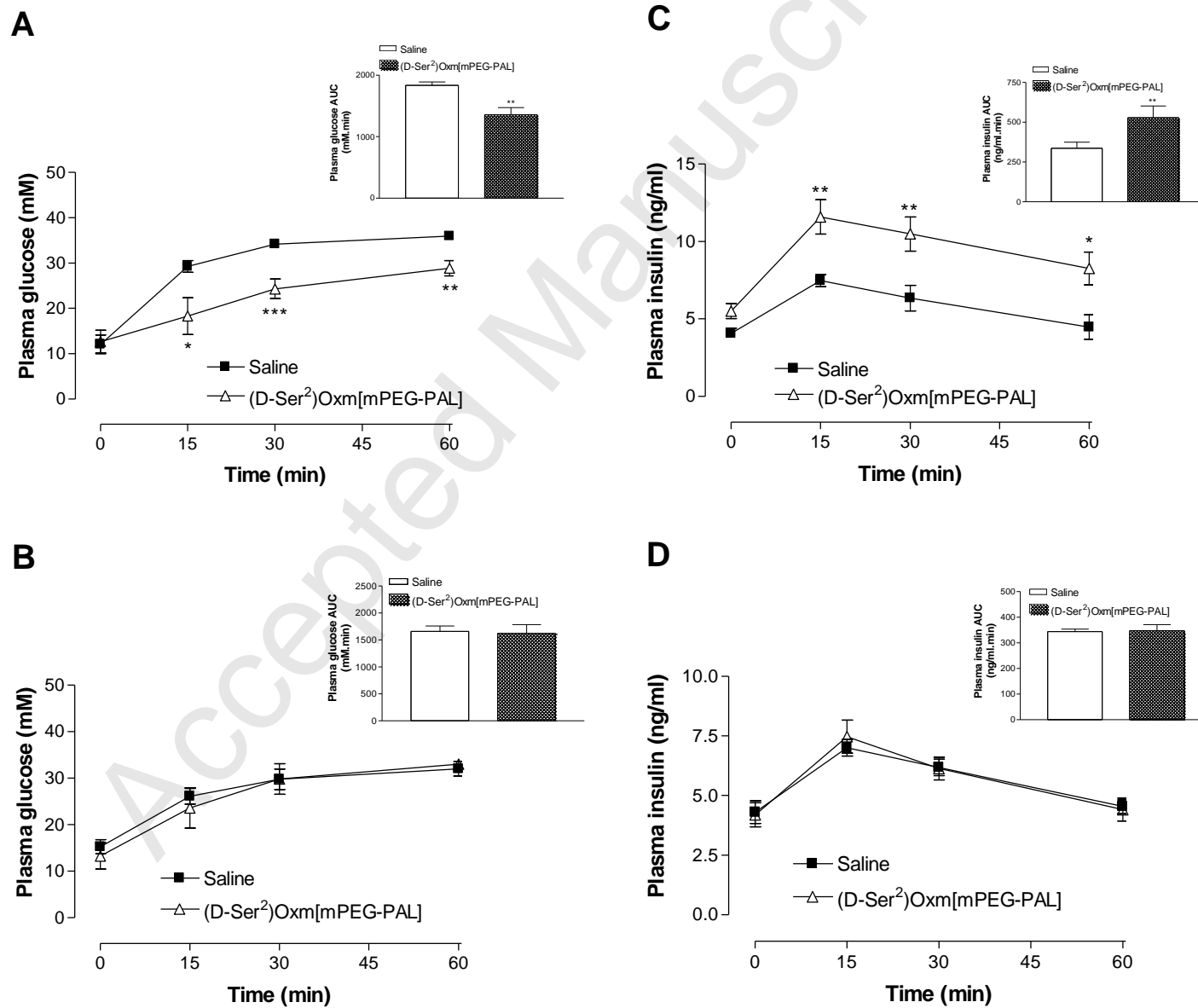
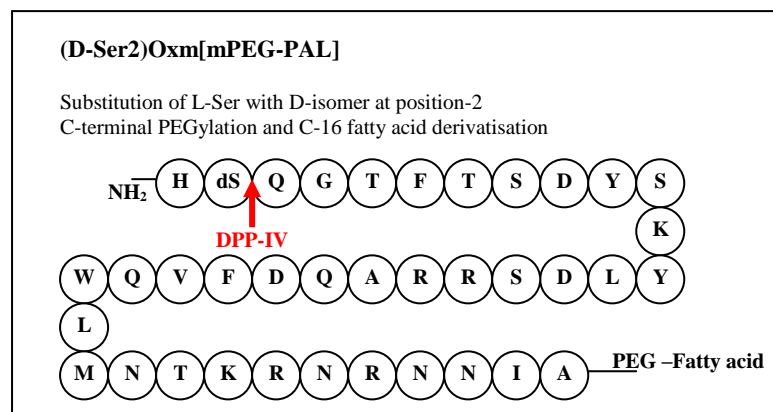


Figure 7



Graphical Abstract



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Table 1 - Effects of Oxm analogues on cAMP production in GLP-1 and glucagon receptor transfected CHL cells

| Peptide | Agonist cAMP response (EC ₅₀ / nM) | | Maximal cAMP antagonism in presence of 10 ⁻⁶ M antagonist (%) | |
|------------------------------------|--------------------------------------------------|----------------|-----------------------------------------------------------------------------|---------------|
| | GLP-1 R | Glucagon R | GLP-1 R | Glucagon R |
| GLP-1 | 0.05 ± 0.09 | — | 100.0 | — |
| Glucagon | — | 0.56 ± 0.08 | — | 100.0 |
| Oxm | 8.60 ± 0.53*** | 4.82 ± 0.81*** | 69.7 ± 1.2*** | 80.2 ± 3.8*** |
| (D-Ser ²)Oxm | 7.73 ± 0.62*** | 7.33 ± 0.52*** | 63.9 ± 3.2*** | 78.2 ± 1.9*** |
| (D-Ser ²)Oxm[mPEG-PAL] | 8.89 ± 0.43*** | 8.16 ± 1.23*** | 68.4 ± 6.3** | 62.5 ± 2.2*** |

For agonist experiments, GLP-1 and glucagon receptor transfected CHL cells were exposed to various concentrations (10⁻¹² to 10⁻⁶ M) of Oxm peptides or native GLP-1 or glucagon (as appropriate) for 20 min (n=4) and cAMP production measured using ELISA. For antagonist studies, Oxm peptides or native GLP-1 or glucagon (as appropriate) (each at 10⁻⁶ M) were incubated in the presence of exendin-9-39 amide (GLP-1 receptor antagonist) and DesHis¹DesPhe⁶Glucagon-amide (glucagon receptor antagonist) each at 10⁻⁶ M. Data represent means±SEM. **p < 0.01, ***p < 0.001 compared to natural ligands, GLP-1 and glucagon, respectively.

Table 2 - Effects of once-daily administration of (D-Ser²)Oxm[mPEG-PAL] on insulin sensitivity, pancreatic insulin content, adiponectin, visfatin and triglyceride levels in *ob/ob* mice

| Parameter | Saline vehicle | (D-Ser ²)Oxm[mPEG-PAL] |
|-------------------------------|-----------------|------------------------------------|
| Insulin sensitivity (mM.Min) | 1189.13 ± 14.21 | 1007.44 ± 8.5* |
| Insulin content (µg/g tissue) | 4.31 ± 0.27 | 6.33 ± 0.23*** |
| Adiponectin (ng/ml) | 1.06 ± 0.09 | 1.45 ± 0.06** |
| Visfatin (ng/ml) | 1.35 ± 0.06 | 1.02 ± 0.05** |
| Triglycerides (mmol/l) | 6.39 ± 0.23 | 4.62 ± 0.45** |

Tests were conducted following 14 days intraperitoneal treatment with (D-Ser²)Oxm[mPEG-PAL] (1400 nmol/kg bw) or saline vehicle (0.9% (w/v) NaCl). Data are expressed as means ± SEM for 5 mice.

p < 0.01, *p < 0.001 compared to saline-treated group.