

Investigation of the role of pharmacokinetics and the development of novel anti-obesity agents

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Abstract

Oxyntomodulin is a gut hormone that promotes satiety by exhibiting an anorectic effect through the action at the GLP-1 receptor while at the same time it has been suggested that it is able to increase energy expenditure through the glucagon receptor, albeit with a less potency than native peptides. This dual mode of action makes it an ideal candidate for an anti-obesity medication. However, oxyntomodulin possesses an inadequate pharmacokinetic profile with a very short half-life and duration of action. These observations promoted the idea that oxyntomodulin could be used as the starting point for developing an anti-obesity drug agent through modifications of its primary amino acid structure.

The exact knowledge of the pharmacokinetic properties of peptides (half-life, metabolic clearance rate, volume of distribution) is crucial in drug development as it allows investigating the effect of amino acid changes in the presence of the peptide in the plasma and correlates this with the exerted biological effect. The first aim of this thesis was to develop a reliable *in vivo* animal model that could be used to measure the pharmacokinetic properties of anti-obesity drug candidates based on oxyntomodulin. Results have shown that the proposed model is robust enough to be used for this scope.

The second aim was to develop an analogue, based on oxyntomodulin, with a higher anorectic effect and a prolonged duration of action with the aim of developing an anti-obesity medication that could be used as a therapeutic agent in humans given once-weekly. Point substitutions in the primary amino acid structure of oxyntomodulin (positions 16, 23, 24, 29 and 30) were done in order to enhance the anorectic effect of oxyntomodulin and 26 analogues were developed and tested in order to investigate the effect of these substitutions. The pharmacokinetic properties of these analogues were measured with the help of the *in vivo* animal model. Other substitutions in the C-terminal of the analogues were made in order to allow the formation of a subcutaneous depot when given in a zinc-containing slow-release formulation with the aim to prolong the presence of the peptide in the circulation.

Data presented in this thesis have identified OXM analogue X26 presents a 8.3– and 45-fold higher potency at the GLP-1R and GCGR respectively when compared to native OXM. When administered in a zinc containing formula, X26 is present in the circulation 7 days post-injection. Furthermore, it has demonstrated a significantly longer half-life than OXM (21.3 vs. 12 min). Its ability to inhibit food intake and reduce body weight has been shown in rodent species; mice and rats. Analogue X26 is a novel and valuable pharmaceutical approach for the treatment of obesity.

Declaration of Contributors

The majority of the work in this thesis was performed by the author. All collaboration and assistance is described below:

Chapter 3:

Animal studies were performed with the assistance of Dr James Minnion and Dr Joyceline Cuenco.

Chapters 4, 5 and 6:

Animal studies were performed with the assistance of Dr James Minnion, Dr Joyceline Cuenco and Dr Elina Akalestou.

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Abbreviations

ATP Adenosine triphosphate
ADME Absorption, Distribution, Metabolism and Excretion
AgRP Agouti-related peptide
ANOVA Analysis of variance
AP Area postrema
ARC Arcuate nucleus
BAT Brown adipose tissue
BDNF Brain-derived neurotrophic factor
BMR Basal metabolic rate
BSA Bovine serum albumin
Ca²⁺ Calcium
cAMP cyclic adenosine monophosphate
CART Cocaine-amphetamine related transcript
CCK Cholecystokinin
CHO Chinese hamster ovary
CNS Central nervous system
DFBS Dialysed fetal bovine serum
DIO Diet Induced Obese
DIT Diet-induced thermogenesis
DMEM Dulbecco's modified Eagle medium
DMN Dorsomedial nucleus
DPP4 Dipeptidyl peptidase 4
DVC Dorsal vagal complex
ECD Extracellular domain
EDTA Ethylene diamine tetra-acetic acid
ELISA Enzyme-linked immunosorbant assay
EE Energy expenditure
EX-4 Exendin-4
FDA Food and drug administration
g grams
GCG Glucagon
GCGR Glucagon receptor
GI Gastrointestinal
GIP Gastric inhibitory polypeptide
GLP-1 Glucagon-like peptide-1
GLP-1R Glucagon-like peptide-1 receptor
GLP-2 Glucagon-like peptide-2
GPCR G-protein coupled receptor
GRPP Glicentin related polypeptide
GTP Guanosine triphosphate
HEPES N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGCGR Human glucagon receptor
hGLP-1R Human glucagon-like peptide-1 receptor
ICV Intracerebroventricular
IP Intraperitoneal
IPGTT Intraperitoneal Glucose Tolerance Test

IV Intravenous
K⁺ Potassium
LHA Lateral hypothalamic area
LLOQ Lower limit of quantification
MC4R Melanocortin receptor 4
MCR Metabolic clearance rate
MEM Minimum Essential Medium
mGCGR Mouse glucagon receptor
mGLP-1R Mouse glucagon-like peptide-1 receptor
Na⁺ Sodium
NEAT Non-exercise activity thermogenesis
NMR Nuclear magnetic resonance
NPY Neuropeptide-Y
NTD N-terminal domain
NTS Nucleus tractus solitarius
OXM Oxyntomodulin
PBS Phosphate buffered saline
PC Prohormone convertase
PD Pharmacodynamics
PK Pharmacokinetics
PMSF Phenylmethylsulphonylfluoride
POMC Pro-opiomelanocortin
PP Pancreatic polypeptide
PVN Paraventricular nucleus
PYY Peptide tyrosine-tyrosine
RIA Radioimmunoassay
RYGB Roux en Y gastric bypass
SC Subcutaneous
SEM Standard error of the mean
SNS Sympathetic nervous system
SPA Spontaneous physical activity
TM Transmembrane
T2DM Type II diabetes mellitus
UCP-1 Uncoupling protein-1
V_d Volume of distribution
VMN Ventromedial nucleus
α-MSH alpha-melanocortin-stimulating hormone

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Chapter 1

General introduction

1.1. Obesity

It is an undeniable fact that Western societies are facing an increasing epidemic of obesity. The definition of obesity is abnormal or excessive fat accumulation that impairs health. The body mass index (BMI) is a very common tool used to categorize the degrees of obesity and is calculated as the ratio of body weight (body weight) in kilograms (kg) divided by the height in meters squared (kg/m^2) (1). The normal range for adults lies between 18.5 and 25 kg/m^2 and overweight is classified as being 25 to 30 kg/m^2 . Type I obesity is defined as having a BMI between 30 and 35 kg/m^2 or exceeding ideal body weight by 20%, type II obesity is classified as having a BMI between 35 and 40 kg/m^2 while morbid (or type III) obesity starts when BMI exceeds 40 kg/m^2 .

Estimates raise the number of obese individuals in the U.K. to 12 million adults and 1 million children (2). The latest World Health Organization report estimates there are 1.2 billion people overweight out of which the obese individuals number 300 million. In an effort to predict the future trends in obesity, Foresight estimated that by 2050, 60% of males and 50% of females would be obese (3). In an inverse relation, the percentage of people with a healthy BMI at 2050 will decline from 30% currently to less than 10% and from 40% to 15%, for men and women respectively.

Obesity without either medical or surgical intervention is rarely reversible and is the result of energy intake exceeding energy expenditure (EE) for a considerable amount of time with numerous factors influencing the pathogenesis of the disease. In summary the causes known so far are (4):

- Genetic: Initially with the discovery of the *ob* gene in 1994 and then with the discovery of the hormone leptin and the melanocortin system.
- Early life: Without knowing yet the exact details, it seems that the gestation period and the early life feeding patterns influence the individual's adult life risk for obesity.
- Activity behavior: It is generally accepted that the modern day western type of society is characterized by a sedentary life, less manual jobs and less need for walking and physical activity joined by the abundance of low cost, low-value, fattening food.
- Other factors such as the modern-day living environment, technological advances and people's belief's and attitudes.

1.1.1. Medical conditions associated with obesity

The obesity pandemic is a risk factor for a broad spectrum of diseases such as type 2 diabetes mellitus (T2DM), hypertension, coronary artery disease and stroke incidents, tumours, sleep apnoea, non-alcoholic fatty liver disease and certain type of cancers (5).

1.1.2. Economic costs of overweight and obesity

Obesity is a huge financial burden for each country due to its direct and indirect financial costs involved. The House of Commons Health Select Committee has estimated that the total annual cost of obesity for England was almost £16 billion for 2007 (2). This sum includes all the aspects that have a financial cost (direct and indirect) as direct costs of treatment, the cost of dependence on state benefits, loss of earnings, reduced productivity, absence from work etc. The total annual cost of the overweight and the obesity to the National Health System is expected to rise to £49.9 billion by 2050 (at today's prices) (3).

1.2. Current treatment options

In overweight and in patients with type II obesity, diet modification and an increased level of physical activity is advisable. Current recommendations for “healthy eating” are based on the instructions by the Committee on the Medical Aspects of Food Policy and subsequently the Scientific Advisory Committee on Nutrition (6-8). In short the guidelines recommend reducing the consumption of total fat and sugars to no more than 35% and 11% of the total food energy received respectively, increasing total carbohydrate intake to more than 50% of food energy and increasing the consumption of dietary fibre, fruits and vegetable. Although such modalities can temporarily decrease body weight, they have failed to show a sustained effect mostly due to poor compliance and weight regain (9).

In patients with type II obesity and in those that present comorbidities (irrespective of the class of obesity in which they belong), in addition to diet and increased physical activity, the use of drugs is being considered (2). Currently, the most widely used medication is Orlistat (Xenical) which is a saturated derivative of lipstatin; a potent natural inhibitor of pancreatic lipases and acts by preventing the absorption of fats from the human diet. The effects of Orlistat are moderate with an average weight loss of 5% of total body weight over the course of 1 year and it is associated with a range of unpleasant gastrointestinal (GI) side-effects (10). Saxenda, a glucagon-like peptide-1 receptor (GLP-1R) agonist was approved in 2014 by the United States Food and Drug Administration (FDA) as a treatment option for chronic weight management at a dose of 3 mg (11).

The current guidelines for the treatment of obesity type III according to National Institute for Health and Excellence recommend bariatric surgery for patients that have a BMI of 40 kg/m² or more, or between 35 kg/m² and 40 kg/m² and other significant disease and when all appropriate non-surgical measures have been tried but have failed to achieve clinically beneficial weight loss for at least 6

months (2). Bariatric surgery is recommended as a first-line option (instead of lifestyle interventions or drug treatment) for adults with a BMI of more than 50 kg/m² in whom surgical intervention is considered appropriate.

Non-pharmacological treatment options for obesity are currently being evaluated but their usefulness remains to be seen, an example being vagus nerve stimulation. The aim of reversible vagal blockade is to stop both ascending and descending neural traffic and preclinical work in animals and preliminary studies in human subjects showed enhanced satiety, decreases in food intake, and weight loss over prolonged follow-up periods (12). A multicentre, prospective trial however revealed that weight loss was not greater in treated subjects compared to controls (13).

1.3. Energy Homeostasis

Energy balance is considered to be paramount in species survival and thus it is presumed that energy homeostasis has evolved to modify the level of adiposity according to each species' ecological niche (14). Maintaining a greater than 10% reduction in body weight by obese or non-obese humans causes a reduction in EE beyond that predicted by the loss of body mass (15-19). When fat mass is decreased a number of body responses are set in motion. Hunger and energy efficiency are increased, circulating leptin, sympathetic nervous system tone and bioactive thyroid hormones are decreased while parasympathetic nervous system tone is increased (15, 16, 20). The major organ responsible for this observed decline in EE is the skeletal muscle (21). The true question however lies in the pathophysiological system that governs the body's defense against an increase in adiposity.

Obesity development is also clearly related to reward/hedonic drives and such reward-related consumption can result in caloric intake exceeding requirements (22, 23). This concept is strengthened by the extensive neuroanatomical connections that are shared between food intake and energy homeostasis mechanisms (24, 25).

Bariatric surgery poses even more challenges in regards to body fat homeostasis. Currently, it is the only known effective treatment for morbid obesity with patients subjected to Roux en Y gastric bypass (RYGB) procedure losing more than 60% of excess body weight (26). Roux en Y gastric bypass causes a reduction in hunger, a decreased craving for energy dense foods with no alteration of circulating thyroid hormones (27, 28). Although the exact mechanisms remain elusive, the gut-brain communication axis has emerged as a plausible explanation. Studies with implantable sleeves that cause a bypass of the duodenum and proximal ileum demonstrated that upper intestinal bypass in an

important contributor to weight loss after RYGB (29). Also, post-RYGB patients exhibit increased post-prandial levels of the anorexigenic hormones peptide tyrosine-tyrosine 3-36 (PYY₃₋₃₆) and glucagon-like peptide-1 (GLP-1) even before substantial fat loss while the orexigenic hormone ghrelin decreases (not in all studies though) (30). Another factor involved is the reward effect by the nutrient sensing occurring in the duodenum; in RYGB patients' duodenum is bypassed thus food reward may decrease (31, 32).

1.3.1. Energy expenditure

There are four main components to total EE: basal metabolic rate (BMR), physical activity, cold-induced thermogenesis (shivering and non-shivering) and the thermic effect of food [diet-induced thermogenesis (DIT)] (33). Basal metabolic rate is produced by cellular and organ functions that are necessary for survival while adaptive thermogenesis is the additional heat produced in response to temperature changes or diet (34, 35). Diet-induced thermogenesis is an increase in heat production, resulting from the action of converting food into components for use or storage via the activity and consumption of energy by enzymes and transporters. Diet-induced thermogenesis is generally a small component of overall EE, around 5-10% and the main determinant of DIT is the energy content of food (36). The hypothalamic-pituitary-thyroid (HPT) axis and the sympathetic nervous system (SNS) are both efferent signals in the regulation of EE.

1.3.1.1. Control of Physical Activity

Spontaneous physical activity accounts for 8-15% of total 24 h EE and in this case thermogenesis is derived from muscular activity (37). There are two components of physical activity; the voluntary one and a seemingly unconscious subcomponent of physical activity that contributes to thermogenesis, Non-Exercise Activity Thermogenesis (NEAT) or Spontaneous Physical Activity (SPA). Non-Exercise Activity Thermogenesis is everything that goes together with voluntary or conscious physical activity such as fidgeting, maintenance of posture and spontaneous muscle contraction (33).

1.3.1.2. Basal Thermogenesis

Basal thermogenesis is the heat produced by maintaining body temperature and cellular integrity, ion gradients, protein turnover and enzyme activity. It constitutes the largest single component of EE reaching approximately 60% of the daily total EE (33). Basal metabolic thermogenesis is by definition the lowest sustained rate of EE and therefore does not can be used therapeutically as a target to increase EE.

1.3.1.3. Brown adipose tissue and adaptive thermogenesis

Adaptive thermogenesis occurs primarily in the brown adipose tissue (BAT). In contrast to white adipose tissue, BAT contains a large population of dark coloured mitochondria that give the distinctive brown colour to this tissue. Brown adipocytes apart from mitochondria also contain triglycerides. Brown adipose tissue deposits are especially abundant in newborns, they can account for 25% of fat mass in human infants and are important for non-shivering thermogenesis and for maintenance of temperature homeostasis (38, 39). Recently, a small quantity of BAT has been identified in adults, 10-20 g (40). However, as little as 50 g of active BAT in humans would have a positive impact on body weight and metabolic health suggesting a role of BAT in energy homeostasis in the adult population (41-43). On the other hand, white adipose tissue has been estimated to account for 7% of total EE through the thermogenesis generated via the enzyme reactions involved with lipid mobilisation (44, 45).

BAT plays a role in the adaptation to conditions of high energy diet and also to periods of cold exposure. Under these conditions BAT in rats is activated and contributes to an increase in EE and heat generation respectively (43). These responses are greatly dependent on the SNS and the HPT axis (34, 35). The SNS innervates BAT, with noradrenaline involved as a neurotransmitter activating α - and β -adrenergic receptors on brown adipocytes after release from SNS nerve endings (38, 46). Brown adipocytes are also activated by the thyroid hormone T3 with thyroid hormone receptors being highly expressed in BAT (47).

The high thermogenic capacity of BAT is achieved by uncoupling oxidative phosphorylation and adenosine triphosphate (ATP) production (33). Activation of brown adipocytes stimulates gene expression of the uncoupling protein-1 (UCP-1), unique to BAT mitochondria and that is responsible for protons influx to the mitochondrial matrix against the proton gradient created by oxidative phosphorylation (38, 48). Uncoupling protein-1 therefore uncouples substrate combustion from ATP synthesis, which results in heat generation and allows a faster rate of mitochondrial combustion of fatty acid substrates (48).

Glucagon and GLP-1 are two gut hormones that have been associated with increased energy expenditure. Glucagon receptors are known to be present in BAT and GCG infusion has been shown to be thermogenic and increase oxygen consumption (49, 50). The two possible mechanisms involved include an increased thermogenesis by BAT (possibly mediated by catecholamines) and/or futile substrate cycling (49).

1.4. Regulation of food intake

The regulation of food intake depends on the release of different hormones both from the periphery and the gastro-intestinal tract. These hormones, depending on their exact function, act at a wide range of receptors located at the peripheral tissues and the brain to initiate or to terminate the process of food intake. Termination of food intake once a meal has been initiated depends on the perception of satiation which refers to the perception of fullness while satiety refers to the reduced interest in food after a meal (14).

1.5. Central control of food intake

1.5.1. Hypothalamus

The hypothalamus plays a critical role in the control of appetite. Initially it was believed that the lateral hypothalamic area (LHA) and the ventromedial hypothalamic nucleus (VMN) controlled appetite. It was later on demonstrated that many more hypothalamic nuclei and neuronal circuits are actually involved. The hypothalamic nuclei communicate with the brainstem and higher cortical centres. Peripheral signals from the GI system and the adipose tissue are able to penetrate the blood-brain barrier at the area of the median eminence of the hypothalamus and the area postrema (AP) of the brainstem (51).

Within the arcuate nucleus (ARC) of the hypothalamus there are two distinct populations of neurons that exhibit opposing effects on food intake. Neurons that express the neuropeptide Y (NPY) and the agouti related peptide (AgRP) stimulate food intake, whereas neurons co-expressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) suppress feeding (52). Both populations of neurons project to the paraventricular nucleus (PVN), although the ARC also communicates with other hypothalamic nuclei such as the dorsomedial nucleus (DMN), LHA and the VMN (51).

The POMC neurons produce α -melanocyte stimulating hormone (α -MSH) which binds to the melanocortin-4 (MC4R) receptors in the PVN to inhibit food intake (53). Studies in MC4R knock-out mice demonstrated these animals exhibit hyperphagia and obesity (54). In humans the mutations of MC4R account for 6% of severe early-onset obesity and more than 70 different mutations have been recognised (55).

NPY/AgRP neurons have extensive projections within the hypothalamus, including the PVN, DMN and LHA. NPY given intracerebroventricularly (ICV) causes increase in food intake in rats through stimulation

of Y1 and Y5 receptors and by inhibiting POMC neurons in the ARC (56). Other orexigenic hormones are melanin-concentrating hormone and orexin which are found in the LHA.

PVN receives NPY/AgRP and POMC/CART projections from the ARC, and contains the anorectic thyrotropin-releasing hormone and corticotrophin-releasing hormone. Destruction of the PVN causes hyperphagia and obesity (57). The VMN expresses the brain-derived neurotrophic factor (BDNF) that was shown to suppress food intake through MC4R signalling (58). Selective deletion of BDNF results in obesity (59).

1.5.2. Brainstem

The dorsal vagal complex (DVC) of the brainstem consists of the dorsal motor nucleus of vagus (DVN), AP, and the nucleus of the tractus solitarius (NTS). The DVC is important because it receives vagal afferents from the gut regarding gastric distension and gut hormone levels. It has been shown that transection of all gut sensory vagal fibres results in an increase in meal size and meal duration (60, 61). Within the brainstem, vagal afferent neurons have been shown to express a variety of receptors for peptides such as PYY, GLP-1 and glucagon-like peptide-2 (GLP-2) (62-64).

The brainstem mimics the hypothalamus in regards to the expression of leptin and insulin receptors, and of glucose sensing mechanisms (65). Other neuronal populations known to regulate appetite, such as POMC neurons also exist within the NTS. These neurons demonstrate signal transducer and activator of transcription 3 activation in response to leptin administration (66). Furthermore, administration of leptin into the DVC suppresses food intake (65). Therefore, signals from the periphery have pivotal roles in transmitting information via afferent vagal fibres to the caudal brainstem or directly to the hypothalamus to modify appetite.

1.6. Peripheral adipose-derived signals

1.6.1. Leptin

Both insulin and leptin are implicated in the long-term regulation of energy balance. Leptin production is the result of the *ob* gene transcription and it is predominantly secreted by adipocytes. The circulating levels of insulin and leptin are related to the adipose tissue mass within the body and leptin has a diurnal and pulsatile pattern, with peak levels at night (67, 68). Leptin exerts its anorectic effect via the ARC nucleus, where both NPY/AgRP and POMC/CART neurons express leptin receptors (69). Leptin

reduces food intake and increases EE by inhibiting NPY/AgRP neurons and activating POMC/CART neurons (53, 70, 71).

The Ob-Rb receptor, which is highly expressed in the hypothalamus is thought to act as the main leptin receptor involved in the control of the appetite (72). The *db/db* mouse which has an inactivating mutation in the Ob-Rb receptor has an obese phenotype while leptin-deficient *ob/ob* mice exhibit hyperphagia and obesity, which can be reversed by leptin administration (73-75). High levels of leptin are often and as a result there is a failure to respond to exogenous leptin. This leptin resistance is likely to result from reduced leptin receptor signal transduction or an impaired ability of the blood-brain barrier to transport leptin (76, 77).

1.6.2. Adiponectin

Adiponectin is a hormone that was first discovered in the mid-1990s and is uniquely produced and secreted by mature adipocytes (78). It is present in the circulation in different forms; ranging from homotrimers to larger aggregates, known as high molecular weight multimers (79). Adiponectin is able to increase AMP-kinase and peroxisome proliferator-activated receptors activities as well as fatty acid oxidation and glucose uptake through the action on two adiponectin receptors belonging to the class of 7-transmembrane [7-(TM)] cell surface receptors (80). It is believed to have anti-inflammatory and anti-diabetic effects (81, 82).

Paradoxically, with increasing obesity the concentration of adiponectin in the blood is decreased, that was attributed to the influence of inflammatory cytokines (83). In a human study, Cnop et al. measured intra-abdominal and subcutaneous (SC) fat areas by computer tomography scan and showed that intra-abdominal fat is strongly correlated to adiponectin when compared to SC fat (84). Thus, it could be argued that it is the distribution of the fat mass and not the total amount per se that is the most important determinant of circulating adiponectin levels, inflammatory status and insulin resistance in obese subjects.

1.6.3. Insulin

Insulin is synthesized by the pancreatic β cells and is rapidly secreted post-prandially. Apart from insulin's well characterised hypoglycaemic effects, it also possesses an anorectic role (85). Intracerebroventricular administration of insulin has been shown to cause a dose-dependent inhibition of food intake and body weight gain in rodents while intrahypothalamic insulin injection to the PVN also results in decreased food intake (86). Hypothalamic nuclei involved in the control of energy intake, such as the ARC, DMN, and PVN, express insulin receptors (87). Although the

mechanism of insulin-mediated anorexia has not been yet elucidated, hypothalamic NPY seems to be involved.

1.7. Peripheral hunger and satiety signals

1.7.1. Ghrelin

Ghrelin is a 28 amino acid peptide discovered by Dr.Cummings in 1995. Ghrelin is principally secreted from X/A-like cells within gastric oxyntic glands in the fundus of the stomach (88). When the gastric fundus is disconnected during metabolic surgery there is an 80% reduction of plasma ghrelin levels; the remainder is secreted from the intestine, pancreas, pituitary, and colon (89). To date it is the only GI hormone which has been shown up to now to be orexigenic; it is able to stimulate food intake and when administered chronically it can cause obesity (90). Negative correlations between circulating ghrelin levels and body mass index are found in humans. Obese individuals have lower levels of ghrelin in comparison to normal weight people while subjects with diet induced weight loss have higher levels of circulating ghrelin (91, 92).

Ghrelin levels rise preprandially and fall rapidly in the postprandial period but they are not decreased by water ingestion (93). In humans, ghrelin levels have a diurnal rhythm which is identical to the diurnal rhythm of leptin, with both hormones rising throughout the day to a zenith at 0100 h, then falling overnight to a nadir at 0900 h (93). Both central and peripheral administration of ghrelin in rodents cause an increase food intake and body weight along with a reduction in fat utilisation (93, 94).

Ghrelin mediates its orexigenic action via stimulation of NPY/AgRP coexpressing neurons within the ARC of hypothalamus. Peripheral administration of ghrelin increases c-fos expression in the ARC NPY/AgRP neurons. Furthermore, ablation of both AgRP and NPY neurons completely abolishes the orexigenic effect of ghrelin (95, 96). Intracerebroventricular injection of ghrelin induces c-fos expression in the NTS and AP (97). Ghrelin also increases gastric motility, upstimulates the hypothalamo-pituitary-adrenal axis, and possesses cardiovascular effects such as vasodilatation and enhanced cardiac contractility (89).

1.7.2. Cholecystokinin

Cholecystokinin (CCK) was the first gut hormone found to be involved in appetite control as early as 1973 (98). Cholecystokinin is secreted postprandially (within 15 minutes after meal ingestion) by the L cells of the duodenal and jejunal mucosa and has a short plasma half-life of only a few minutes (99). Cholecystokinin decreases food intake rapidly but transiently via the activation of vagal afferents (100). The effects of appetite inhibition by CCK can be amplified by leptin (101). Other physiological functions of CCK include stimulating the release of enzymes from the pancreas and gall bladder, promoting intestinal motility, and delaying gastric emptying (100).

There are two CCK receptor subtypes known; CCK1 and CCK2 receptors and the anorectic action of CCK appears to be mostly mediated via CCK1 receptors on the vagal nerve (102, 103). Cholecystokinin 1 and 2 receptors are widely distributed in brain and some studies suggest that leptin and CCK may interact synergistically in inducing short-term inhibition of food intake and long-term reduction of body weight (104, 105).

1.7.3. Pancreatic polypeptide-fold peptides

The pancreatic-polypeptide (PP)-fold family comprises of neuropeptide Y (NPY), PYY, and PP which are composed of a chain of 36 amino acids and share amino acid homology. PYY and PP are secreted from GI tract, whereas NPY is predominantly distributed in the central nervous system (CNS) (106). This family acts via G protein coupled receptors (GPCR); Y1, Y2, Y4, Y5, and Y6 (107).

1.7.3.1. Peptide tyrosine-tyrosine

Peptide tyrosine-tyrosine is an anorexigenic hormone and it owes its name to the tyrosine (Tyr) residues at both the C and N terminals. Peptide tyrosine-tyrosine is co-secreted from the L cells of the distal gut together with oxyntomodulin (OXM) and GLP-1 in response to ingested nutrients. The concentration of PYY is highest in the rectum, and decreases proximally in the GI tract. It is also found in CNS regions such as the hypothalamus, medulla, pons, and spinal cord (108). There are two circulating forms, PYY₁₋₃₆ and PYY₃₋₃₆; PYY₃₋₃₆ is the biologically active major circulating form and is produced by cleavage of the N-terminal Tyr-proline (Pro) residues from PYY by the enzyme dipeptidyl-peptidase 4 (DPP4) (109). PYY₁₋₃₆ has affinity to all Y receptors, while PYY₃₋₃₆ acts mainly through the hypothalamic Y2 receptor.

The secretion of PYY is important for satiety. Circulating PYY concentrations are low during fasting and increase rapidly post-prandially. They peak 1-2 hours following a meal and remain elevated for several hours; PYY release is increased proportionally to the calorie intake [12].

1.7.3.2. Pancreatic polypeptide

Pancreatic polypeptide is secreted post-prandially from the PP cells in the pancreatic islets of Langerhans. Pancreatic polypeptide exerts its anorectic effects through the Y4 receptor in the brainstem (AP and NTS) and hypothalamus (DVN, ARC, and PVN) (110). In addition, it may also act through the vagus nerve (111). Similar to PYY, paradoxical effects on food intake are observed following PP injection, depending on the administration route; peripheral PP administration has an anorectic effect while central PP administration has the opposite effect on food intake (56). The exact mechanism of this phenomenon is yet unclear but activation of different populations of receptors might be the cause. Other physiological effects of PP include delaying gastric emptying, attenuating pancreatic exocrine secretion, and inhibiting gallbladder contraction (112).

Plasma PP levels show diurnal variations; lowest levels are observed in the early morning and highest in the evening. Circulating PP concentrations increase after a meal in proportion to the caloric intake, and remain increased for up to 6 hours postprandially (113). Circulating PP levels seem to be inversely proportional to adiposity; higher levels are reported in subjects with anorexia nervosa (114). Some, but not all studies have demonstrated significant reductions in circulating levels of PP in obese subjects (115-118).

1.8. Proglucagon gene

The glucagon (GCG) gene is mainly expressed in the intestine, pancreas and CNS. The posttranslational processing of the proglucagon gene by prohormone convertases (PC) 1, 2 and 3 is tissue-specific and results in the production of a variety of molecules (119). More specifically it contains residues for glicentin related pancreatic peptide (GRPP) (residues 1-30), GCG (residues 33-61), GLP-1 (78-108) as well as GLP-2 (126-158) (120-124). If the pro-glucagon cleavage takes place in pancreas then the end-products are GCG, GRPP, a major pro-glucagon fragment and intervening peptide-1 (120). In the brain and the gut the cleavage products are glicentin (residues 1-69), which is a GCG containing peptide, GLP-1, GLP-2 and an intervening peptide-24 (120, 122). Finally it should be noted that in the intestine glicentin can be subdivided to two products, GRPP and OXM (124).

1.8.1. The incretin effect

The incretin effect is namely the concept that when an oral nutrient (ex. glucose) is administered, it causes a greater degree of insulin secretion in comparison to the one caused by the administration of a parenteral isoglycemic glucose infusion. The incretin hormones are peptides that are released from the gut as a response to ingestion of nutrients and stimulate the release of insulin (125-128). The

underlying mechanism is that there are gut derived factors that increase the insulin secretion from the β -cells of the pancreas.

The confirmation of this entero-pancreatic communication came in 1964 with the help of advanced peptide chemistry and the development of highly specific and sensitive radioimmunoassay (RIA) (129-131). The two hormones that today fulfill the criteria of an incretin effect are gastric inhibitory peptide (GIP) and GLP-1.

The signaling mechanisms of the incretin hormones GLP-1 and GIP involve different receptors (7-TM GPCR receptors) for each one of them. The locations in which they are found include the pancreas, the stomach, the heart, the lung, the brain and the skeletal muscles. The mechanism through which they exert their function is based on an increase in the intracellular cyclic adenosine monophosphate (cAMP) levels with a subsequent activation of protein kinase A.

1.8.2. GLP-1

The GLP-1 is a naturally occurring peptide consisting of 30 amino acids and is synthesized in the endocrine L cells of the terminal gut. It is the product of the pro-glucagon peptide which itself is encoded by the pro-glucagon gene (120). The predominant form of GLP-1 circulating in the plasma is the 7-36 amide while the C-terminally non-amidated 7-37 form is also produced (132). Both of these peptides are functional and are able to release insulin. Two other forms that also exist, the 1-37 and the N-terminally extended form are not biologically active (32, 133). Glucagon-like peptide-1 is released from the gut into the circulation shortly after ingestion of a meal, i.e. in a few minutes and carbohydrate rich meals are potent stimulators of GLP-1's secretion (134-136).

1.8.2.1. Peripheral functions of GLP-1

Glucagon-like peptide 1 has a well-known role in regulating glucose homeostasis and it has been shown to increase β -cell mass by stimulating β -cell proliferation and by inhibiting β -cell apoptosis (137-139). In addition, it works together with glucose in the pancreas to stimulate insulin synthesis by pancreatic β -cells including increasing cAMP, replenishing β -cells insulin stores by promoting insulin gene transcription as well as by restoring glucose sensitivity in glucose resistant β -cells (32, 140-142). In addition, GLP-1 up-regulates somatostatin secretion by pancreatic δ -cells and inhibits GCG secretion by pancreatic α -cells (143, 144).

If the GLP-1R gene is disrupted by a targeted mutation, glucose tolerance is significantly reduced, and fasting hyperglycaemia occurs (145). Moreover, GLP-1R antagonism by the antagonist exendin-(9-39) results in a decrease of the incretin effect of endogenous GLP-1 in rats (146). On the other hand,

exendin-4 (EX-4) administration, a long acting GLP-1 agonist, causes an increase in β -cell mass and inhibits β -cell apoptosis (147, 148).

Glucagon-like peptide 1 actions include a satiety effect when released post-prandially by inhibiting gastric emptying, acetylated ghrelin production and secretion of gastric juices (149-151). However the decrease of these effects post-vagotomy suggest that these effects are regulated in a higher centre (152).

1.8.2.2. Functions of GLP-1 in the central nervous system

Glucagon-like peptide 1 receptor is known to be expressed in the caudal part of the NTS of the brainstem in the DMN and the PVN of the hypothalamus (153). Intracerebroventricular injections of GLP-1 inhibit food intake in fasted rats and a lesion in the ARC nucleus causes an abolition of the anorectic effect of GLP-1 in animals (154, 155). The mechanism of control of GLP-1 on food intake most likely is regulated by the brainstem and the hypothalamus (156). Moreover, rodents that have undergone vagotomy or transection of brainstem-hypothalamic pathway exhibit no anorectic effects after a peripheral administration of GLP-1 which suggests that the effects of peripheral GLP-1 may be dependent on GLP-1 binding to receptors on the subdiaphragmatic vagus nerve (157).

Glucagon-like peptide 1 has also been implicated in energy homeostasis and has been associated with increases in EE (158). Furthermore, ICV administration of GLP-1 causes an alteration in the proportion of carbohydrate to fat that is utilised as energy substrate (159). Studies investigating glucose turnover in GLP-1R null mice and also in wild-type mice with hyperinsulinemic clamps suggested an incretin-independent increase in hepatic glucose uptake (160).

1.8.3. Exendin peptides

Exendins (exendin-3 and EX-4) belong to a family of peptides that have originally been isolated from the saliva of the Gila monster lizard *Heloderma suspectum*. Exendin-4 has a similar action to GLP-1 with regard to glucose homeostasis, the delay of gastric emptying and food intake inhibition (161, 162). In addition, EX-4 stimulates glucose-dependent insulin secretion and supports β -cell function and proliferation, as well as islet neogenesis from the precursor cells both *in vitro* and *in vivo* (148, 163-165). Exendin-4 mediates its actions through coupling to GLP-1R and in fact is a more potent agonist to the receptor than GLP-1 (166, 167).

1.8.4. Glucagon

Glucagon is a peptide hormone consisting of 29 amino acids and is released from α cells of the islets of Langerhans of the pancreas. In general, GCG is a hormone that is involved in the regulation of the metabolic pathways involved in glucose homeostasis and counteracts the actions of insulin in peripheral tissues and mainly in the liver.

The cleavage of proglucagon gives different by-products in different tissue while the liberation of proglucagon itself is controlled by cell-specific expression of PC enzyme. The essential role of PC enzyme for processing of proglucagon was revealed in studies where knockout mice did not possess the ability to synthesize PC (168, 169). In these studies, the PC-2 knockout mice presented mild hypoglycemia, elevated proinsulin and an important defect in converting proglucagon to mature GCG.

The trigger for GCG release is hypoglycemia and its secretion, like insulin, is well regulated (170). Glucagon secretion is promoted with the action of sodium (Na^+) and calcium (Ca^{2+}) channels which preserve action potentials in times of low blood glucose levels. When depolarization occurs, there is significant Ca^{2+} influx which together with the ATP-sensitive potassium (K^+) channels promote GCG secretion (171, 172). The climbing levels of glucose eventually lead to normoglycemia which inhibits the further rise of GCG through the elevated cytosolic ATP, the blockade of K^+ channels and the termination of the Na^+ and the Ca^{2+} -caused action potentials. All these result in the inhibition of Ca^{2+} influx and the termination of GCG secretion.

Studies in rats point out that an important role in the GCG inhibition is possibly played by paracrine signaling from the β -cells (173, 174). However, other studies in mice and humans demonstrated that it is glucose that inhibits GCG secretion at concentrations that are not sufficient to stimulate insulin secretion (172). This inhibiting action of glucose has been shown *in vitro* studies in both isolated α cells and intact pancreatic cells (175). Other factors that are known to control the GCG secretion are GLP-1 and GLP-2, fatty acids, circulating amino acids and the autonomic nervous system (176-180).

The glucagon receptor (GCGR) is found in liver, kidney, heart, intestinal smooth muscle, brain, adipose tissue and on islet β cells and exhibits 42% a.a. sequence similarity with the GLP-1R (181-185). Glucagon activates multiple G protein-mediated signal transduction pathways in liver cells, leading to stimulation of adenylyl cyclase and phosphoinositol turnover (186, 187).

1.8.5. Oxyntomodulin

Oxyntomodulin is a 37-amino acid peptide that is derived from the proglucagon gene. Its presence was first reported in 1981 and it was isolated from porcine jejuno-ileum (188, 189). It contains the entire 29-amino acid sequence of GCG, extended by an 8-amino acid C-terminal octapeptide: Lys-

Arg-Asn-Lys-Asn-Asn-Ile-Ala-Lys (where Lys is Lysine, Arg is Arginine and Asn is Asparagine) (188). Its primary structure is identical in all mammals except for Lys³³ that is present in pigs and cattle instead of the Arg³³ that exists in rats and humans (190). However, this small change has no effect on OXM's biological properties (191).

Post-translation processing of the proglucagon prohormone in brain and intestine yields mainly GLP-1, GLP-2 and glicentin. Glicentin is then further cleaved to produce OXM and GRPP (192). The regions in the brain that contain OXM or the parent peptide glicentin include the medulla oblongata, olfactory bulbs, cerebellum, cortex and the hypothalamus (193). Oxyntomodulin in the GI tract is synthesized by specialized enteroendocrine cells, named L cells, that co-secrete GLP-1 and glicentin (194). Oxyntomodulin's presence has been reported in significant amounts in the human distal intestine while in rodents, the GI tissue concentration of OXM gradually increases from the duodenum towards ileum, thereafter decreasing in caecum and colon (124, 195).

Oxyntomodulin is secreted by the L-cells in proportion to nutrient ingestion (196). Oxyntomodulin's release begins 5-10 minute postprandially peaking at around 30 minutes (196, 197). There is a diurnal variation in the circulating levels of OXM, independently of food intake, with highest levels in the evening and lowest in the early morning (198).

Oxyntomodulin's mechanism of action involves the activation of the expression of the FOS gene in the ARC and PVN nuclei, AP and the NTS (199-201). Within the hypothalamus, the anorectic effect of peripherally administered OXM may be mediated by an increase in α -MSH, a product of POMC that acts via melanocortin receptors to inhibit feeding (199). It is yet unknown how OXM crosses the blood-brain barrier. Lipophilicity and a neutral charge are two factors that could be beneficial in the transport of OXM through the tight endothelial junctions of the blood-brain barrier. Leptin, a hormone also involved in regulating body weight, crosses the blood-brain barrier with the help of a saturable transport system and possibly through the choroid plexus and ventricular system (202).

The actions of OXM can be largely thought to be mediated through the interaction with both the GLP-1R and the GCGR albeit with a much lower affinity in comparison to GLP-1 and GCG respectively. More specifically, OXM's affinity to the GCGR is only about 2% of that of GCG and its affinity to the GLP-1R is two orders of magnitude lower than that of GLP-1 itself (192). Interestingly, GLP-1 and OXM both potently inhibit food intake at equimolar doses in rats, despite their difference in affinity to the GLP-1R (203-205). As a result it is hypothesised that OXM acts through a separate, unknown yet, receptor.

Oxyntomodulin is ineffective in inhibiting food intake in mice deficient in the GLP-1R while it retains its anorectic effect when administered in GCGR null mice (201). Administration of the GLP-1

antagonist, exendin-(9-39), into the cerebral ventricles inhibits the anorectic actions of both GLP-1 and OXM (154, 201, 204). An alternative theory is that OXM acts through a receptor that has not yet been identified, but that is also antagonized by exendin-(9-39) (203). The effect of peripherally administered OXM is also blocked when exendin-(9-39) is administered directly into the ARC nucleus, whereas the effect of peripheral GLP-1 is retained, regardless of the antagonism of hypothalamic receptors in this region (199, 203).

Oxyntomodulin's actions affect a variety of systems such as the GI, pancreatic, cardiovascular, renal and the nervous system. Oxyntomodulin has been shown to regulate the gastric acid secretion. It has been shown to inhibit histamine-, pentagastrin-, and meal-stimulated gastric acid secretion in conscious rats and pentagastrin and meal-stimulated acid secretion in man (196, 206). The effects of OXM on gastric emptying are still under dispute; acute administration of OXM does not decrease gastric emptying in mice while intravenous (IV) infusion of OXM inhibits gastric emptying in humans (207, 208). Oxyntomodulin decreases pancreatic exocrine secretion while there is some *in vitro* evidence that similar to GLP-1, it promotes insulin release (52, 195, 209).

The administration of OXM both centrally and peripherally has been observed to reduce short-term food intake in rats (204). Dakin et al. demonstrated that dark-phase food intake and fast-induced feeding are decreased after central administration of OXM (administration in the cerebral ventricles, PVN or ARC nuclei of the hypothalamus) or following an intraperitoneal (IP) injection (199). Repeated administration of OXM over a period of 7 days reduced body weight gain and adiposity, when given IP or into the cerebral ventricles of male Wistar rats (210). Interestingly, OXM-treated animals lose more weight than control animals that consume the same amount of calories, which suggests that OXM increases EE (199, 210, 211).

In humans, OXM reduces food intake by $19 \pm 6\%$ in normal-weight volunteers when given IV (3.0 pmol/kg/min) before a single study meal (212). In the overweight or obese group, OXM retains its inhibitory effect on appetite as shown in a study where OXM administered SC over a 4-week period resulted in a significant weight loss of 2.3 ± 0.4 kg ($2.4 \pm 0.4\%$ body weight), in 14 overweight or obese study participants (213).

Body weight loss is a result of decreased energy intake and/or increased EE. The question as to whether there is increased EE following OXM administration, remains unanswered. Animals injected peripherally or ICV with OXM lose more weight than pair-fed animals, suggestive of an increase in EE, postulated to be mediated by the thyroid axis (199, 210). However, analysis of EE in mice did not confirm an acute effect of ICV OXM on metabolic rate (201). On the other hand, a study of EE in

humans following repeated SC administration of OXM showed an increase in activity-specific EE, although no change in BMR was observed (203).

1.9. Drug discovery in the obesity field

Due to the increased incidence of the obesity worldwide, pharmaceutical companies have invested time and money in producing an effective anti-obesity drug. However there haven't been many successes up to now have mainly due to serious side effects that have eventually caused the drugs' withdrawal or prevented it from obtaining FDA approval. The anti-obesity drugs may act through different mechanisms and can affect appetite, metabolic rate, and/or inhibit caloric absorption. They generally fall into 3 broad classes: 1) peripherally acting, 2) centrally acting, and 3) combination (i.e., central and peripheral acting) (214).

Peripherally acting drugs exert their effects by reducing the calorie absorption in the GI system or by affecting metabolic and/or control systems outside the CNS. Currently, the only peripherally acting anti-obesity drug globally approved for long-term use is Orlistat, a lipase inhibitor.

With drugs that act centrally on the CNS satiety and EE may be increased directly by thermogenesis and lipolysis or through the stimulation of the sympathetic nervous system. Due to the action of this class of drugs in the SNS there is a major possibility for the occurrence of serious adverse effects; Sibutramine and methamphetamine have both been withdrawn from the market due to adverse cardiovascular effects.

Drugs that act both on the CNS and the periphery aim to take advantage of a suppression of food intake centrally and by regulating metabolic functions at diverse peripheral organs, including gut, liver, adipose tissue, and skeletal muscle. Rimonabant was marketed as a promising agent that worked by blocking endocannabinoid binding to neuronal CB1 receptors and increasing EE through an action at the periphery (215). Despite initial use in Europe, it failed to secure FDA approval mainly due to concerns for an increased incidence of neurologic and psychiatric problems, including a twofold increase in suicide (216).

Oxyntomodulin and GLP-1 have been shown to cause weight loss in humans over a short time scale, and in the absence of the typical unwanted side effects that have limited previous obesity therapies. Gut peptides are therefore likely to be an ongoing area of exciting development in the quest to develop safe and efficacious treatments for obesity, as their nature offers potential advantages in efficaciously exploiting the natural regulatory circuits while minimizing unwanted side effects (217).

1.10. Designing a new anti-obesity drug candidate

In order to investigate the biological effect of drug candidates developed in an anti-obesity drug discovery program, a series of experiments to test their *in vivo* and *in vitro* characteristics must be performed. An important feature of peptide drug candidates that needs to be investigated is their interaction with the receptors that mediate their action. Precise ligand-receptor interaction is crucial as it allows peptides to trigger or block downstream signalling within the cell. Binding of agonist peptides to the receptor does not always translate into triggering the highly amplified intracellular messaging pathways, but is an essential requisite, in combination with ligand efficacy, in determining drug potency.

A number of established techniques can be employed to investigate ligand-receptor interaction. The sites in the primary structure of a peptide which are important for its bioactivity can be determined by sequential truncation from either side of the molecule (151, 218). Additionally, alanine (Ala) scanning can be used to determine whether a particular residue possesses a distinct characteristic, e.g. enabling better receptor binding, offering enhanced enzymatic stability etc. (219, 220). Other techniques such as crystallography and computer modelling can be useful in identifying individual interactions between the ligand and receptor (221, 222).

The development of peptide analogues based on a native molecule allows evaluation of the effect of the changes in the primary structure of the molecule with regard to receptor binding, cell signalling, enzymatic stability and bioactivity. With increased understanding of the residues that are important, more focused changes can be made and more potent analogues developed.

Structure-function studies of GCG, GLP-1, EX-4 and OXM, a family of related peptides, have identified the residues of importance for receptor binding and activation (219, 222-224). Unfortunately, despite promising preliminary results of drug candidates based on these peptides, there are currently available only two anti-obesity medications with moderate efficacy.

The reasons behind the scarcity of such anti-obesity medications in the market include among others the occurrence of significant side effects such as nausea and vomiting and only modest efficacy when compared to placebo treatment, frequently with weight regain after cessation of drug administration (10, 225, 226). In rodent studies, GLP-1R agonists administration has been shown to cause significant weight loss. However, this has not been fully translated in humans due to tolerability issues; the attained pharmacological plasma levels in humans may cause nausea and vomiting which was not evident previously in the rodent studies due to the lack of the physiological reflex to vomit from the rodents.

Oxyntomodulin is a proglucagon-derived peptide that has been shown to bind to the GCG receptor (GCGR) and GLP-1R, inhibiting appetite and resulting in weight loss in humans (211-213). These features make it an attractive target for developing a drug to treat obesity. However, limitations in using the native OXM peptide include a short half-life and rapid clearance from blood plasma. The development of peptide analogues that can generate better PD and PK profiles may serve as long-acting drugs to treat obesity.

1.10.1. G-protein coupled receptors and G-proteins

Both the GCGR and GLP-1R are GPCR's which is a large protein family of receptors whose role is to transmit chemical signals from the outside of the cell to the inside. They include receptors for a wide variety of ligands such as peptides, amino acids, phospholipids, fatty acids and nucleotides. About 80% of known hormones and neurotransmitters activate cellular signal transduction mechanisms by GPCR's (227). Abnormalities in GPCR's are known to cause many diseases such as diabetes mellitus, blindness, allergies, depression and cardiovascular defects. It is estimated that more than half of the modern drugs' cellular targets are GPCR's (228).

G-protein coupled receptors share a common membrane topology of 7-TM helices connected by alternative extracellular loops and intracellular loops, with an extra-cellular N-terminus and a cytoplasmic C-terminus (229). Binding of a ligand to the GPCR causes a conformational change that result in the GPCR activating a G-protein.

G-proteins are a family of proteins that are also called "guanine-nucleotide binding proteins" due to their ability to bind and hydrolyse guanosine triphosphate (GTP) to guanosine diphosphate. Following GTP's hydrolysis, signal transduction occurs within the cell through two main pathways, the cAMP signal pathway and the phosphatidylinositol signal pathway (230).

There are several amino acids within a receptor that are crucial for the ability of the ligand to bind to the receptor. In many cases, the binding sites of antagonists are different to those of an agonist. In general, small ligands are thought to bind in the central cavity formed by the TM helices, while larger peptide ligands interact with the extra-cellular regions of the receptors.

1.10.1.1. Family B G-protein coupled receptors

The receptor for the gut hormone secretin was the first of family B GPCR's to be cloned (231). Within this family of GPCR's, there is 30-50% amino acid homology. Family B GPCR's do not share significant homology with other GPCR's families, such as family A and family C, with less than 20% similarity. Both the GCGR and GLP-1R are members of the secretin-GPCR family, subfamily B1 of family B (232-234).

The members of this subfamily are expressed in the GI tract and/or brain. They signal predominantly through the cAMP-dependent pathway after receptor binding via the Gs subunit, which stimulates adenylate cyclase and causes a subsequent increase in the levels of the second messenger cAMP (235).

Family B receptors comprise of a moderately sized extracellular N-terminal domain (NTD), of 100–160 amino acid residues, containing several cysteines (Cys) that form disulphide bridges (236). The NTD is connected to a juxtamembrane domain of seven membrane-spanning α -helices with intervening loops and a C-terminal tail. Intracellular loops interact with G-proteins to stimulate intracellular signalling (235). The NTD is thought to contain the ligand-binding site and contains six conserved Cys residues, as well as two conserved tryptophans (Trp) and an aspartate, which are important for the correct folding of the domain (232, 237).

1.10.1.1.1. Two domain model of family B G-protein coupled receptors-ligand interaction

In 2005, Hoare described a two domain model for the ligand-receptor binding complex in family B GPCR's, which would subsequently trigger intracellular signalling. They proposed that the C-terminal end of the ligand tethers to the NTD, leaving the ligand N-terminus free to interact with the 7-TM domain of the receptor (235). The ligand adopts an α -helical conformation, sandwiched between the two β -sheets of the NTD, with the ligand C-terminus fixed by intermolecular hydrogen bonds to NTD side chains. The residues of the ligand that interact with the NTD form an amphipathic α -helix occupying a complementary ligand binding groove on the surface of the NTD. Interestingly, the above-mentioned model of family B GPCR activation has been challenged recently. The alternative hypothesis is that the binding of the ligand to the receptor causes a conformational change that exposes an 'endogenous agonist', which then interacts with the receptor core domain to initiate downstream signalling inside the cell (238, 239).

1.10.1.1.2. Structure-function relationship of GLP-1

The potential use of GLP-1 analogues as an anti-obesity treatment has sparked further interest in understanding its mechanism of action. High-affinity binding sites for GLP-1 were found in insulinoma cell lines, which suggested the hormone could mediate its actions through specific receptors located on the surface of pancreatic β -cells (141). Subsequently, GLP-1R were identified in rodent insulinoma cell lines, rat and human pancreatic β -cells and somatostatin secreting cells (144, 240, 241). Rat GLP-1R and hGLP-1R are 463 amino acids in length and are 90% identical, differing only at 42 amino acid positions (166, 242, 243). The mouse GLP-1R (mGLP-1R) is 489 amino acids (244).

Exendin-4 is a potent agonist displaying a similar binding affinity to the GLP-1R as GLP-1, while the amino terminally truncated form exendin-(9-39) is an antagonist of GLP-1, inhibiting GLP-1 binding and the resultant cAMP formation (166, 167). Recently the crystal structure of hGLP-1R-NTD in complex with exendin-(9-39) has been resolved (245). The hGLP-1R-NTD is similar to those of other published structures of family B members with six conserved Cys residues forming disulphide bonds and two regions of anti-parallel β -sheets. More specifically, hGLP-1R-NTD contains an α -helix and the tertiary structure is stabilized by the disulphide bonds and by multiple intra-molecular interactions between the secondary structure elements.

The crystal structure of GLP-1 in complex with the extracellular domain (ECD) of GLP-1R was resolved in 2010 (246). The GLP-1-bound structure revealed similar key features to the exendin-(9-39)-bound structure with little differences specific for GLP-1. The C-terminal segment [Ala¹⁸-Valine at position 27 (Val²⁷)] of GLP-1 interacts with the NTD of the receptor. This particular segment of GLP-1 contains 5 amino-acids that are conserved between GLP-1 and EX-4 in humans. The structure of the GLP-1 molecule, when bound to the GLP-1R, is described as being two regular α -helical segments separated by a kink around Gly²². The structure of His¹-Gly⁴ has not yet been resolved, possibly due to the inherent flexibility in this part of GLP-1 and other peptide ligands for family B receptors (247).

Ligand-binding analyses of recombinant GLP-1R expressed on the surface of β -cells showed that the affinity for the binding of GLP-1 is approximately 1 nM. All other peptides of the GCG superfamily bind poorly or not at all, with the exception of GCG, which is a weak, full agonist with a binding affinity 100- to 1,000-fold lower than that of GLP-1 (248, 249).

1.10.2. Structure-function relationship of GLP-1

The sequence of GLP-1 is highly conserved in all animal species, including, man and rodents. This probably reflects both its physiological importance and suggests the entire amino acid sequence of GLP-1 is required for its full biological activity. Structure-function relationship studies of GLP-1 have summarised that the N-terminal region of GLP-1 is important for receptor activation while the C-terminal part is involved to receptor binding (250). Studies involving substitution of individual amino acids demonstrated that histidine in position 1 (His¹), Gly⁴, phenylalanine in position 6 (Phe⁶), threonine in position 7 (Thr⁷), aspartic acid in position 9 (Asp⁹), Phe²² and isoleucine in position 23 (Ile²³) are important for the binding affinity and biological activity of GLP-1 (223, 251). Replacement of the amino acids in these positions with Ala decreased the binding to the GLP-1R and the bioactivity of the analogues in comparison to native GLP-1 (219).

Analysis of two-dimensional nuclear magnetic resonance (NMR) spectroscopy demonstrated that GLP-1 in a membrane-like environment consists of an N-terminal random coil segment (residues 1-7), a linker region (15-17) and two helical segments (7-14 and 18-29) (252). Once the N-terminal segment of GLP-1 binds to the GLP-1R, both the receptor and GLP-1 are likely to undergo mutual structural changes, which means that the receptor-bound conformation of GLP-1 can be different from its conformation in free state (253).

There is still uncertainty around the importance of the linker region of GLP-1 with regard to interaction with its receptor, particularly Gly at position 16. Some studies suggest that Gly¹⁶ offers high flexibility within GLP-1, and its removal has been shown to reduce GLP-1 binding affinity (222, 252, 253). In contrast, substitution of Gly¹⁶ by Ala¹⁶ had no effect on either GLP-1 binding affinity or biological activity, and furthermore it was proposed that the linker region weakens GLP-1 binding affinity compared to EX-4 (223, 245, 247).

Removal of the N-terminal His (to yield GLP-1 (8-37)) results in a 90% loss of receptor binding and insulinotropic activity (254, 255). Additionally, an N-terminal truncation of GLP-1 by two residues reduces binding affinity to approximately 1% that of full-length peptide (256). A reduction in the biological activity can also be seen by the addition of an amino acid to the N-terminus of GLP-1(6-37) (254). The C-terminal of the GLP-1 molecule has been shown to be important for its biological activity as the truncation of the C-terminus results in a reduction of biological activity and was therefore hypothesised that the C-terminal end of the molecule is important for receptor specificity (254-257).

1.10.3. Structure-function relationship of exendin-4

Exendin-4 is a 39 amino acid peptide with an overall 53% identity with GLP-1. In the N-terminus region (a.a. 1-9), the identity between EX-4 and GLP-1 is 80%, differing only at position 2, which highlights the importance of this region in GLP-1R activation (258). In 1993, Goke et al. demonstrated that the substitution of the Ala² in GLP-1 to a Gly² in EX-4 protects EX-4 from rapid degradation in plasma by DPP4 resulting in a longer half-life (167, 259).

The structure of EX-4 consists of three distinct regions: the N-terminal region comprising residues 1–8, the central helical region comprising residues 9–30, and the C-terminal region comprising residues 31–39, which may form a Trp-cage motif in conjunction with part of the central helix (260). The central region of EX-4 shares only eight identical residues with GLP-1; these conserved residues lie on the same face of an ideal α -helix which suggests that this conserved face of the helix contacts the binding pocket on the GLP-1R (218). Both GLP-1 and EX-4 share an α -helix from residues 18-27, which has been shown, by X-ray crystallography, to fit into the ECD pocket. However, the affinity of GLP-1 and

EX-4 to the ECD differs with the weaker affinity of GLP-1 thought to be caused by Gly at position 16 compared to glutamic acid (Glu) in EX-4, which lowers the propensity for α -helical formation (247).

The crystal structure of the N-terminal truncated form exendin-(9–39) bound to the hGLP-1R showed it had very limited interaction between the C-terminal extension and the receptor (245). In addition, receptor binding data comparing native EX-4 with the truncated form of EX-4 demonstrated that they bound with similar affinity to the GLP-1R. This suggests that the most critical interactions with the hGLP-1R lie between Glu¹⁵-Lys²⁷ (261).

1.10.4. Structure-function relationship of glucagon

Glucagon is a well-characterised hormone known for its ability to increase plasma glucose through glycogenolysis and gluconeogenesis. The investigation of GCGR antagonists as potential diabetes treatments has provided a detailed knowledge of the receptor-ligand structure and function (221, 251, 262). Glucagon receptors belong to the family B of GPCR's receptors.

Glucagon has 3 domains; the N-terminus, a mid-section and a C-terminus. In the N-terminus, amino acids 1-9 are important for receptor activation. In particular, residues 1-5 and residue 9 are intimately involved in the transduction of the biological message and the bioactivity of GCG (221). Phenylalanine at position 6 is a key residue for GCG binding. The mid-section, also known as a hinge region (a.a. 10-15), allows the molecule to correctly align itself to fit and activate the GCGR. Residues of the hinge region, along with Phe⁶, Tyr¹⁰ and Leu¹⁴, form a hydrophobic patch which is important for determining the shape of the molecule. The C-terminal (residues 19-29) has an α -helical conformation with amphipathic properties and appears to be primarily important for receptor recognition and binding (221).

1.10.5. Structure-function relationship of oxyntomodulin

Oxyntomodulin's primary structure is 37 amino acids, comprising of the entire GCG sequence with an 8 amino-acid octapeptide extension at the C-terminus. It was initially classified as an enteroglucagon, due to its secretion from the terminal ileum of the small intestine and its glucagon-like action, but it is also highly expressed in the brain (192). Oxyntomodulin like GLP-1, GLP-2 and GCG is derived from the proglucagon gene. Oxyntomodulin is most highly expressed in the brain and the gut and its physiological functions include the regulation of food intake, gastric emptying and gastric secretion.

1.10.5.1. Interaction of oxyntomodulin with GLP-1 receptor and glucagon receptor

Despite the fact that OXM was discovered more than 30 years ago, it still remains unclear whether it possesses a distinct receptor or if all its actions are mediated by the GLP-1R and GCGR (263). Oxyntomodulin is a full agonist of the hGLP-1R and hGCGR albeit with a reduced affinity when compared to the native molecules; the affinities of OXM for the GCGR and GLP-1R are 2% and 1% of the respective affinities of the native ligands (195, 264).

Recently, OXM was shown to be a full agonist in recruiting β -arrestin 2 to the GCGR, but only a partial agonist in recruiting β -arrestin (β -arrestin 1 and β -arrestin 2) and GPCR kinase 2 to the GLP-1R. It has been suggested that OXM is a GLP-1-R-biased agonist relative to GLP-1 having less preference toward cAMP signalling relative to phosphorylation of extracellular-signal-regulated kinases 1 and 2, but similar preference for cAMP relative to Ca^{2+} (265). Such differences in the intracellular signalling following receptor activation could imply that the GLP-1R mediated *in vivo* effects of OXM could be different from those of GLP-1 (266). It is difficult to predict the difference in biological effects between biased and non-biased ligands and extensive work is required to define how β -arrestin bias can aid in drug development efforts.

Oxyntomodulin and GLP-1 have also been shown to activate different hypothalamic pathways in the brain; peripheral administration of OXM causes an increase in c-Fos in the ARC, but not in the brainstem region (199, 267, 268). The exact receptor activation and intracellular signalling caused by OXM in these brain regions is still unclear (269).

1.10.5.2. Oxyntomodulin effect on food intake and body weight

Oxyntomodulin's actions affect a variety of systems such as the GI, pancreatic, cardiovascular, and renal functions. In humans, OXM was seen to reduce food intake by $19 \pm 6\%$ in normal-weight volunteers when given IV (3.0 pmol/kg/min) before a single study meal (212). When administered in an overweight or obese group SC over a 4-week period, OXM retained its inhibitory effect on appetite. This resulted in a significant weight loss of $2.3 \pm 0.4 \text{ kg}$ ($2.4 \pm 0.4\%$ body weight) (213).

The administration of OXM both centrally and peripherally has been observed to reduce short-term food intake in rats (204). Dakin et al. in 2004 demonstrated that dark-phase food intake and fast-induced feeding decreased after central administration of OXM (specifically, in the cerebral ventricles, PVN or ARC nuclei of the hypothalamus), or following an IP injection of OXM (199). In addition,

repeated administration of OXM over a period of 7 days, reduced body weight gain and adiposity when given IP or ICV of male Wistar rats (210).

OXM is ineffective in inhibiting food intake in mice deficient in the GLP-1R while it retains its anorectic effect when administered in GCGR null mice (201). Intracerebroventricular administration of the GLP-1 antagonist exendin-(9-39), inhibits the anorectic actions of both GLP-1 and OXM (154, 201, 204). It was proposed that OXM acts through a receptor that has not yet been identified, but that is also antagonized by exendin-(9-39) (203). The effect of peripherally administered OXM is also blocked when exendin-(9-39) is administered directly into the ARC nucleus, whereas the effect of peripheral GLP-1 is retained regardless of the antagonism of hypothalamic receptors in this region (199, 203). These findings indicate that our understanding of the exact mechanism of action of OXM is still incomplete.

Interestingly, OXM-treated animals lose more weight than control animals that consume the same amount of calories, which suggests that OXM increases EE (199, 210, 211). Rats injected peripherally or ICV with OXM lost more weight than pair-fed animals, suggestive of an increase in EE, which is thought to be mediated by the thyroid axis (199, 210). However, analysis of EE in wild-type mice did not confirm an acute effect of ICV administered OXM on metabolic rate (201). Conversely, a study of EE in humans following repeated SC administration of OXM showed an increase in activity-specific EE, although no change in BMR was observed (203).

Recently, two studies expanded the initial findings on the actions of OXM on EE and showed that OXM has glycogenolytic properties in perfused mice by functionally activating the GCGR (270, 271). These data correlate with previous published literature where GCG was shown to be able to inhibit food intake in man and to increase thermogenesis, satiety, lipolysis, fatty acid oxidation, and ketogenesis (272-276).

OXM has been shown to regulate gastric acid secretion. In particular, it has been shown to inhibit histamine-, pentagastrin-, and meal-stimulated gastric acid secretion in conscious rats, and pentagastrin and meal-stimulated acid secretion in man (196, 206). The effects of OXM on gastric emptying are still in dispute; acute administration of OXM does not decrease gastric emptying in mice, while IV infusion of OXM inhibits gastric emptying in humans (207, 208).

Oxyntomodulin decreases pancreatic exocrine secretion (277), while there is some *in vitro* evidence that, similar to GLP-1, it promotes insulin release (195, 209). *In vivo* studies demonstrated that OXM can improve glucose metabolism during a glucose tolerance test in mice (207, 278). The acute improvement of glucose metabolism demonstrated by OXM in mice was also confirmed in T2DM

volunteers where OXM significantly increased insulin secretion and glucose lowering versus placebo (279).

1.10.5.3. Oxyntomodulin as a therapeutic anti-obesity medication

Oxyntomodulin, being a dual agonist of the GLP-1R and the GCGR, is potentially a useful therapeutic for obesity as combines the food intake inhibition of GLP-1 with the enhanced EE effects of GCG. Further evidence that OXM may be an important regulator of body weight is provided by bariatric surgery patients who have elevated levels of OXM post-surgery (280, 281).

Wynne et al demonstrated that SC administration of OXM caused substantial weight loss over a period of one month (211). However, due to the short circulatory half-life of OXM this study required administration of OXM three times a day. Also as OXM is relatively weak (it has a bioactivity which is 13-fold lower than GLP-1 in the GLP-1R and 9-fold lower than GCG in the GCGR) relatively large doses of peptide were required making the use of native OXM cost prohibitive as a treatment of obesity.

Oxyntomodulin has been the focus of interest in our Department as a potential starting point for developing an anti-obesity medication. Point substitutions in the primary structure of OXM could help develop an agent with enhanced characteristics than the native molecule; greater body weight reduction and longer duration of action. Experimentation from within our Department has identified substitutions in positions 20, 21 and 27 of native OXM as being beneficial in developing an anti-obesity drug based on OXM.

1.10.5.3.1. Position 20

Oxyntomodulin and GCG both have Glutamine (Gln) in position 20. Histidine was introduced in this position in an effort to improve the zinc binding of the molecule and consequently its PK profile. Histidine has the ability to chelate zinc and form an insoluble complex in the SC depot, thus allowing for a slower release in the circulation.

1.10.5.3.2. Position 21

Published studies suggested the importance of position 21 for the binding and activation of the GCGR; the Lys^{17,18}Glu²¹GCG is a super-agonist with 5-7 times the receptor affinity and adenylase cyclase activity of similarly substituted compounds (282). This increased potency was due to a significant increase in overall α -helical content as observed by circular dichroism spectroscopy (283). The cause of the increased helicity was revealed by crystallography studies that proposed the formation of a lactam bridge between Lys¹⁸ and Glu²¹ of GCG in aqueous solution, which is probably more optimal

than the salt bridge between Arg¹⁸ and Glu²¹ (221). Oxyntomodulin possesses Arg in position 18, an amino-acid similar to Lys, and Asp in position 21. When Asp²¹ was substituted for Glu²¹ an enhancement in the GCGR binding affinity and potency was seen (unpublished data from our group).

1.10.5.3.3. Position 27

The designed OXM analogues have the methionine in position 27 (Met²⁷) substituted by Leu²⁷ in an effort to simulate the binding of GLP-1 to the ECD. The motive behind this substitution is because native Met²⁷ is non-polar, sulphur containing proteinogenic amino acid that makes the molecule oxygen-sensitive. More specifically, the thiol ester bond within Met is known to be prone to oxidation which results in the formation of a reactive and potentially damaging sulfoxide group (284). A previous study demonstrated that substituting Met with Leu at position 27 (which has a similar size and charge to Met) resulted in the prolongation of the half-life and increases the safety of the molecule (285).

Position 27 of GLP-1 is occupied by Val which is the final residue in the α -helix of GLP-1 and it is the final residue in the C-terminus of GLP-1 which interacts with the ECD (286). Val²⁷ of GLP-1 is located on the hydrophobic face of the α -helix and behaves similarly to Leucine (Leu) and Isoleucine (Ile).

Substitutions in the C-terminal part of GLP-1 with the corresponding GCG residues [Met²⁷, Asn²⁸, Thr²⁹, Arg³⁰], found also in OXM, has decreased the affinity to the GLP-1R several hundred-fold without an increase in the affinity to the GCGR (219). Furthermore, the introduction of GLP-1 residues, [Val²⁷, Lys²⁸ Gly²⁹, Arg³⁰] into the C-terminal part of the GCG improved the affinity to the GLP-1R up to 200-fold (251). Previous work (unpublished data) in the Department demonstrated that Leu²⁷ in OXM increases the affinity for GLP-1R.

1.10.6. Zinc-containing formulations

The effort to design OXM analogues with improved PK characteristics than native OXM (with a longer half-life and lower MCR) was centred on the administration of the analogue in a preparation that contains zinc. Zinc is the second most abundant trace metal in the human body, second only to iron; an average 70-kg adult human contains 2.3 g of zinc (287). There are several hundred zinc-enzymes and more than 1000 zinc binding domains in the mammalian proteome (288). Zinc can act as an electron pair acceptor with His, glutamate (Glu), Asp and tyrosine acting as electron pair donors (289). Zinc molecules may not bind to their ligand in the order in which they occur in the sequence, and the pattern of the zinc/ligand interaction can be linear, interleaved, intertwined or clustered (288).

Zinc-containing formulations have been previously used with successful results to deliver drugs after SC administration (290). Experimentation with insulin has shown that the PK stability includes the imidazole ring and charge of His (291). Histidine becomes protonated when pH reaches a value of 6 as it gains protons and changes its amino group from NH to NH^{2+} . Positively charged His molecules repel when they are still diluted in the acidic environment of zinc (pH 6). However, once the formulation is injected into the tissue, the body fluids buffer the acidic medium of the injection solution and bring the isoelectric point of His to a pH of 7.4. At this stage His is half-negatively and half-positively charged; the negative part will attract positively charged zinc forming bonds between the two in a process known as chelation which induces a slower release (292).

1.11. Peptide therapeutics

Proteins and peptides have been used extensively in the treatment of human diseases with the earliest examples being insulin, thyroid hormones and coagulation factors. The technological progress with recombinant DNA technology and solid-phase synthesis provided a renewed interest on the field. Currently there are more than 200 proteins and peptides that have been approved by the FDA and that are used for the treatment of human diseases (293). However, the rate limiting step in the increased use of proteins as treatment modalities is the difficulty in designing an efficient delivery system that will be site-specific. Thus, very often candidate proteins are adequately active in the molecular/cellular level but fail to confirm these characteristics when tested *in vivo* due to their inadequate pharmacokinetic (PK) profile. The reasons for such an unsatisfactory therapeutic profile include poor oral bioavailability, inadequate stability, immunogenicity, short half-life and inability to reach the target organ/tissue (294).

1.12. Proteins and peptide inactivation

Proteins that are used as drug candidates have to face many challenges before being able to exert their biologic effect. Any physical or chemical alterations of their native structure may result in a significant change of its *in vivo* activity. Proteins can undergo aggregation with other protein molecules or form aggregates. While travelling in the circulation, proteins are subjected to factors such as circulation pressure, proteolysis in the plasma, low pH etc.

Proteins can provoke an unwanted immunogenic response in the host that results in the formation of antibodies against the protein. This in turn causes an accelerated drug clearance from the blood and a decrease in drug efficacy. The ways to tackle protein immunogenicity is through humanization of murine monoclonal antibodies, introducing minor structural changes or by using polymers to hide immunoreactive sites (295, 296).

The two main pathways of inactivation of small peptides are through renal filtration and through proteolytic degradation. The proteolytic pathway uses a group of peptidases or oligopeptidases that act preferentially on oligopeptides with up to 100 residues (297, 298). These peptidases can be found in a freely soluble form or attached to cell surfaces. The soluble forms found in the plasma usually originate from a membrane-anchored site. The peptidases that cleave hormones are either exo- or endo-peptidases, being specific in regards to their cleavage site target. The end result of the action of a peptidase is the activation or the inactivation of a peptide.

1.13. Aims of investigation

1. To investigate the effects of rational changes to the domain structure of OXM on receptor binding, secondary messenger activation and food intake
2. To develop an *in vivo* model for assessing the pharmacokinetic properties of analogues of OXM
3. To design an analogue of OXM that is more potent and causes longer lasting inhibition of food intake and body weight loss than native OXM

Chapter 2

Materials and methods for chapters 3, 4, 5 and 6

2.1. Peptides

All peptides used in this thesis were purchased from Bachem, Ltd (Merseyside, UK) and are the human sequence. In humans, it has been demonstrated that the circulating active form of GLP-1 is GLP-1(7-36) amide and it is this form which shall be referred to as GLP-1 in this document (132).

2.2. Custom synthesis of analogues

All OXM analogues were synthesised by and purchased from Insight, Ltd (Wembley, UK). Peptides were synthesised using an automated fluorenylmethyloxycarbonyl (FMOC) solid phase peptide synthesis method with each amino acid sequentially added from the C to the N terminus. Peptides were cleaved from the resin then purified using reverse phase preparative high performance liquid chromatography (HPLC) followed by lyophilisation. All peptides supplied had a purity of >95% evaluated by HPLC. All amino acids are presented in Appendix A.

2.3. Animal studies

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licence 70/7236).

2.3.1. Animals

2.3.1.1. C57BL/6J mice

Adult male mild (Diet Induced Obese) DIO C57BL/6J mice (Charles River, Margate, Kent, UK) weighing 30-45 grams (g) were maintained in individual cages under controlled temperature (21-23 °C) and lights (12:12 hr light-dark cycle lights on at 0700). Animals were allowed *ad libitum* access to water at all times, and *ad libitum* access to 60% high fat diet (Research Diets Inc, New Brunswick, USA) unless otherwise stated. To minimise any non-specific stress effects animals were regularly handled and acclimatised to SC injections.

2.3.1.2. Wistar rats

Adult male Wistar rats (Charles River Ltd., Margate, Kent, UK) weighing 300-500 g were maintained in individual cages under controlled temperature (21-23 °C) and lights (12:12 hr light-dark schedule, lights on at 0700). Animals were allowed *ad libitum* access to water at all times, and *ad libitum* access to RM1 diet (Special Diet Services, UK) unless otherwise stated. To minimise any non-specific stress effects animals were regularly handled and acclimatised to SC injections.

2.4. Acute feeding studies using mice

Experiments were started in the early light-phase (0800-0900). In order to better demonstrate the anorectic effect of the analogues, SC injections were administered to mice fasted from 1600 on the previous day. Fasted mice were used as the greater food intake after a fast is more likely to successfully demonstrate anorectic effects.

Mice received a single SC injection of vehicle (0.9% weight/volume NaCl) or peptide, maximum volume 0.1 ml (volume injected by body weight), using a 0.5 ml insulin syringe with a 29 gauge needle. After injection, the mice were returned to their home cage and supplied with a known weight of food. Remaining food was reweighed at pre-determined regular intervals over a 48 hour period by using a balance accurate to 0.01 g and a visual inspection of the cage made for any small pieces of food. All studies were conducted under conditions in which all external disturbances were minimised.

2.5. Pharmacokinetics

2.5.1. Investigation of the pharmacokinetic properties following single subcutaneous bolus injection in rats

Studies were carried out to determine the plasma clearance of peptides following SC administration. Male Wistar rats were stratified by body weight and placed into groups of 4 rats of equal mean body weight. Each rat was administered 1mg of peptide dissolved in 20 µl of vehicle (0.9% weight/volume NaCl) at time 0. Prior to injection at 0 hours and at 0.5, 1.5, 3, 6 and 24 hours following administration, blood was collected by superficial venesection of the lateral tail vein. Collection tubes, microtubes flushed with 1:10 heparin:saline prior to sample collection, were used to prevent clotting. No more

than 300 µl of whole blood was collected into a microtube and placed on ice. Samples were centrifuged at 4 °C for 10 minutes at 10000 x g (3-18K SciQuip, Sigma, USA) and the upper plasma layer was isolated and stored at -20 °C. Plasma peptide concentrations were determined by RIA (section 2.8).

2.5.2. Measurement of *in vivo* pharmacokinetics properties with the use of the constant infusion model in rats

Studies were carried out to determine the *in vivo* PK properties [MCR, half-life, Volume of distribution (V_D)] of OXM analogues with the use of the constant infusion model after IV administration in male Wistar rats (section 3.2.2).

Animals were anaesthetised with gas anesthetic (isoflurane via cone mask at 2%) with an appropriate gas scavenging system. The operating procedure began with the catheterization of the right external jugular vein. Once this was completed, a baseline sample was taken, the peptide was reconstituted with 1 ml of Gelofusine at a concentration of 30 nmol/ml and the left femoral vein was subsequently dissected and catheterized. The infusion pump ran for 1 hour at a rate of 0.3 ml/hr. Once the 60 minutes infusion period elapsed, the catheter was removed from the femoral vein. Once all the necessary samples had been collected the animals were culled with a Schedule 1 method.

All blood samples were taken from the right jugular vein at a volume of 100 µl volume except for the baseline sample which was 500 µl. Timing of blood sampling can be seen in Figure 1 in section 3.2.2. Blood samples were dispensed into 1 ml heparinized microtubes containing 20 µl aprotinin (Nordic Pharma, UK). The tubes were then spun in a centrifuge (3-18K SciQuip, Sigma, USA) for 10 minutes at 10000 x g at room temperature. Plasma supernatant was collected and stored in a microtube at -20 °C until assay. Plasma peptide concentrations were determined by RIA (section 2.8).

2.6. Receptor Binding Assays

The binding studies detailed below tested the specific binding of peptides to the receptors on these membranes. Human GLP-1R (hGLP-1R) and human GCGR (hGCGR) overexpressing Chinese hamster ovary (CHO) cell lines were kindly provided by Dr. James Minnion. Cell membrane suspensions were prepared from both hGLP-1R overexpressing and hGCGR overexpressing CHO cell lines.

2.6.1. Preparation of membranes from tissues

Membranes were prepared by the method of homogenisation and differential centrifugation. Tissues were collected from mice which were maintained in conditions as described in section 2.3.1.1, except they were either singularly housed or in groups of ten mice.

Mice were killed by cervical dislocation in the early light phase between 0800 and 1200, the tissues snap frozen in liquid nitrogen and stored at -80 °C. Tissues from a minimum of 40 mice were required for a membrane preparation of each tissue. Tissues were homogenised (Ultra-Turrax T25, IKA Labortechnik, Staufen, Germany) in ice cold homogenisation buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.4 containing 0.25 M sucrose, 10 µg/ml soybean trypsin inhibitor (Sigma-Aldrich, Dorset, UK), 0.5 µg/ml pepstatin (Sigma-Aldrich, Dorset, UK), 0.5 µg/ml antipain (Sigma-Aldrich, Dorset, UK), 0.5 µg/ml leupeptin (Sigma-Aldrich, Dorset, UK), 0.1 mg/ml benzamidine (Sigma-Aldrich, Dorset, UK) 1ml 100 KIU/ml aprotinin (Nordic Pharma, UK)] at 4 °C.

The homogenates were centrifuged at 1,000 x g (Beckman J2-21, rotor JS-13.1) for 15 minutes at 4 °C, and supernatants were then centrifuged at 100000 x g (Sorvall Ultracentrifuge OTD55B, rotor A-841, DuPont, ST.Netos, Cambridgeshire, UK) for 60 minutes at 4 °C. Pellets were removed and resuspended in 10 volumes of the above buffer without sucrose using hand-held glass/Teflon homogenisers (Jencons, East Grinstead, West Sussex, UK), and re-centrifuged at 100000 x g for 60 minutes at 4 °C as before. Finally, pellets were resuspended to a final protein concentration of 2-3 mg/ml and stored at -80 °C. Protein concentration was measured by Bradford assay (section 2.6.2.3).

2.6.2. Preparation of membranes from cells

2.6.2.1. Maintenance of cells

Chinese hamster ovary cells overexpressing the GLP-1R or GCGR (LGC Prochem, Middlesex, UK) were maintained in Dulbecco's modified eagle medium (DMEM) without sodium pyruvate containing 4.5 g/l glucose (Invitrogen). This was supplemented with 10% dialysed fetal bovine serum (DFBS) (Sigma-Aldrich, Dorset, UK), 2% Minimum Essential Medium (MEM) Non-Essential Amino Acids Solution 10 mM (Sigma-Aldrich, Dorset, UK), 5% HEPES and 1% antibiotic v/v [(5000 Units/ml Penicillin; 5000 µg/ml Streptomycin), Sigma-Aldrich, Dorset, UK]. Cells were passaged by first removing the culture media, adding 5ml cell detachment buffer containing ethylene diamine tetra-acetic acid (EDTA) (0.02 M) and incubating at 37 °C until all cells detached from the surface of the flask and from

each other. Five millilitres of culture media was added to the flask and the cells were transferred to a 30 ml sterilin before being centrifuged at 100 x g for 5 minutes. The cell pellet was resuspended in fresh culture media and seeded at a desired dilution into new flasks.

2.6.2.2. Receptor purification

The culture medium was removed and the cells detached from the flasks using cell ice cold 0.02 M phosphate buffered saline (PBS) (BDH®) and scrapping, a minimum of 30 T175 cm² flasks used for a membrane preparation. Cells were centrifuged at 200 x g for 5 minutes (3-18K SciQuip, Sigma, USA), the supernatants discarded and the pellets put onto ice. Pellets resuspended in 20 ml of ice-cold homogenisation buffer (as for tissue membrane preparation, section 2.6.1) and cells homogenised for 1 minute with an Ultra Turrax homogeniser (Ultra-Turrax T25, IKA Labortechnik, Staufen, Germany). Homogenates were further centrifuged for 30 minutes at 4 °C 100000 x g (Sorvall Ultracentrifuge OTD55B, rotor A-841, DuPont, ST.Netos, Cambridgeshire, UK). Supernatants were discarded and pellets resuspended in homogenisation buffer without sucrose using a hand-held/Teflon homogeniser (Jencons, East Grinstead, West Sussex, UK). Finally, pellets were resuspended to a final protein concentration of 1-2 mg/ml and stored at -80 °C. Protein concentration was measured by Bradford assay (section 2.6.2.3).

2.6.2.3. Bradford Assay

0.1 ml cell membrane solution (purified from cells overexpressing GLP-1R or GCGR) was added to 3ml of Bradford reagent (Sigma-Aldrich, Dorset, UK). A standard curve was also determined using known concentrations of bovine serum albumin (BSA) (Sigma-Aldrich, Dorset, UK) (0, 0.25, 0.5, 1.0, 1.5 mg/ml solution) was added to 3 ml of Bradford reagent. All solutions were incubated at room temperature for 45 minutes. A sample of each solution was added to a cuvette and absorbance was measured at 595 nm using a spectrophotometer (WPA UV 1101). The standard curve was used to determine the unknown concentration of protein in the membrane samples.

2.6.3. Iodination of peptides

Iodination of peptides attaches ¹²⁵I molecules to the peptide, enabling them to be used as tracers for displacement assays. Iodination technique used was dependent on the physical properties of the peptides being iodinated.

2.6.3.1. The direct iodogen method

The direct iodination method used was as previously described by Bloom et al. and used to iodinate GCG (146). Three nanomoles (15 µg) of peptide in 10 µl of 0.2 M phosphate buffer pH 7.2 was reacted with 37 MBq of Na ¹²⁵I (Amersham) and 23 nM (10 µg) of 1,3,4,6,-tetrachloro-3α, 6α-diphenylglycoluril (iodogen reagent, Pierce Chemical Co, Perbio Science UK Ltd., Cramlington, UK) for 4 minutes at 22 °C. The reaction products were separated by reversed-phase high performance liquid chromatography using an 80 min 25-45% acetonitrile/H₂O/0.05% TFA gradient.

2.6.4. GLP-1 receptor binding studies

Cell membranes from either animal tissue (lung) or from hGLP-1R overexpressing CHO cell lines were used for these studies. The assays were performed in 1.5 ml siliconised microtubes, with a total volume of 500 µl. The assay buffer [20 mM HEPES (pH 7.4), 1 mM MgCl₂, 5 mM CaCl₂, 1% BSA, 0.05% Tween20, 0.1 mM diprotin A and 0.2 mM phenylmethylsulphonylfluoride (PMSF)]. Each assay tube contained competing unlabelled and radio-labelled ligands (1000 counts per second) and a dose response of unlabelled peptide was tested (2-3 tubes per concentration). The final reagent added was 10 µl of the membrane suspension (at protein concentration 1-2 µg/ml) and microtubes were then immediately vortexed and incubated at room temperature for 90 minutes. To separate free and bound radiolabel microtubes were centrifuged at 15781 x g at 4 °C for 3 minutes, the supernatant removed and discarded, another 500 µl of assay buffer added to wash the pellet of unbound radio-ligand, and then centrifuged as before. The supernatant was again discarded and the pellets measured for γ radiation for 240 seconds (Gamma counter, NE 1600, NE Technology Ltd, Reading, UK).

The half-maximal inhibition concentrations (IC₅₀) were then calculated and compared for each peptide tested. IC₅₀ values were calculated using the Prism 5.01 program (GraphPad Software Inc. San Diego, USA) using the following regression fit line:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X))})$$

2.6.5. Glucagon receptor binding studies

The assay was completed as above except for the following differences. The assay buffer used was 25 mM HEPES (pH 7.4), 2 mM MgCl₂, 1% BSA, 0.05% Tween20, 0.1 mM diprotin A and 0.2 mM PMSF. The radiolabel used was I¹²⁵-GCG. Cell membranes from either animal tissue (liver) or from hGCGR overexpressing CHO cell lines were used for these studies.

2.7. cAMP Accumulation Assay

This assay measured cAMP accumulation in cells in response to incubation with putative receptor agonists. Chinese hamster ovary cells overexpressing the hGLP-1R or the hGCGR were plated onto 48 well plates one day prior to assay at 100000 cells/ml in 250 µl of standard media [(DMEM 6429, with L-glutamine, 4.5 g glucose, sodium pyruvate), Sigma-Aldrich, Dorset, UK] supplemented with 10% DFBS, 2% MEM Non-Essential Amino Acids Solution 10 mM (Sigma-Aldrich, Dorset, UK), 5% HEPES and 1% antibiotic v/v [(5000 Units/ml Penicillin; 5000 µg/ml Streptomycin), Sigma-Aldrich, Dorset, UK]. On the following day, the media was removed and replaced with DFBS-free DMEM media and cells incubated for 1 hour. An appropriate range of concentrations of a peptide to be tested were made in serum free DMEM. The serum free media was aspirated carefully from each well and replaced with incubation media containing the test peptide. The cells were incubated for exactly 30 minutes from this point. After the incubation, media was removed and replaced with 110 µl lysis buffer (0.1 M HCl with 0.5% Triton-X) and incubated for 10 minutes to allow sufficient cell lysis. The lysate, now containing accumulated cAMP, was stored at -20 °C before assay by an enzyme-linked immunosorbant assay (ELISA).

The lysate was diluted at a ratio of 1:2 and assayed using a “Direct cAMP ELISA” kit (ADI-900-066, EnzoLifeSciences, UK) as described in the assay manual. Briefly, 50 µL of Neutralising Reagent was added to each well except the total and blank wells. Provided standards (100 µl) and samples (100 µl) were added to appropriate wells before adding 50 µl of conjugate and 50 µl of antibody into each well. The plate was then sealed and left to incubate at room temperature for 2 hours on a plate shaker (~500 rpm). Contents were emptied from wells and washed three times with 400 µl of wash buffer. ‘Substrate solution’ (200 µl) was added to each well and left to incubate on bench top for 1 hour. After this incubation, 50 µl of ‘stop solution’ was added into each well, and optical density was read at 405 nm on a plate reader (Multiskan RC 381, Labsystems, MA, USA). Concentrations of cAMP were calculated from a standard curve and plotted using Prism 5.01 program (GraphPad Software Inc. San Diego, USA). The half-maximal effective concentrations (EC₅₀) were then calculated and compared for each peptide tested. EC₅₀ values were calculated using the following regression fit line:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X))})$$

2.8. Radioimmunoassay

All samples were assayed using an established in house RIA as described in Appendix B.

2.8.1. Exendin-4 radioimmunoassay

A RIA was used to determine the levels of EX-4 or peptide analogue in plasma samples. Exendin-4-like immunoreactivity was measured with a specific and sensitive RIA, as described in Appendix B. The assay was performed in a total volume of 0.7 ml of 0.06 M phosphate EDTA buffer (0.05 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.0006 M KH_2PO_4 , 0.01 M disodium-EDTA $\cdot 2\text{H}_2\text{O}$, 0.008 M NaN_3), at pH 7.4, containing 0.3% BSA. The antiserum (R4) was produced in rabbits against purified EX-4 coupled to BSA by glutaraldehyde and used at a final dilution of 1:3500. This antibody cross reacts to EX-4 and does not cross-react with other known GI hormones. A radio-labelled form of the EX-4 analogue, EX-4(1-30)-Tyr³¹, was prepared by the direct iodination method and purified by reverse phase high-pressure liquid chromatography. All samples were assayed in duplicate. Standard curves prepared for each assay used the purified peptide to be tested and prepared in assay buffer at 2 pmol/ml and added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 μl . The assay was incubated for 3 days at 4 °C before charcoal separation of the free and antibody-bound label. Both the pellet and supernatant counted for 180 seconds in a γ -counter (model NE1600, Thermo Electron Corporation). EX-4 concentrations in the samples were calculated using a non-linear plot (GraphPad Prism Software Inc. San Diego Version 5.01, USA) and results calculated in terms of the standard.

2.8.2. Glucagon radioimmunoassay

Glucagon N-terminal-like immunoreactivity was measured by RIA, as described in Appendix B. The assay was performed in a total volume of 0.7 ml of 0.06 M phosphate EDTA buffer (0.05 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.0006 M KH_2PO_4 , 0.01 M disodium-EDTA $\cdot 2\text{H}_2\text{O}$, 0.008 M NaN_3), at pH 7.4, containing 0.3% BSA. The antiserum (R2641) was produced in rabbits against the purified GCG (1-16) fragment, coupled to BSA by glutaraldehyde and used at a final dilution of 1:1000. This antibody cross reacts to the N-terminal sequence of GCG present in peptide analogues and does not cross-react with other known GI hormones. ¹²⁵I-GCG was prepared by the direct iodination method and purified by reverse phase high-pressure liquid chromatography. All samples were assayed in duplicate. Standard curves prepared for each assay used the purified peptide to be tested and prepared in assay buffer at 2 pmol/ml and added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 μl . The assay was incubated for 3 days at 4 °C before charcoal separation of the free and antibody-bound label. Both the pellet and supernatant counted for 180 seconds in a γ -counter (model NE1600, Thermo Electron Corporation). Peptide concentrations in the samples were calculated using a non-linear plot (GraphPad Prism Software Inc. San Diego Version 5.01, USA) and results calculated in terms of the standard.

2.8.3. GLP-1 radioimmunoassay

The immunoreactivity of GLP-1 was measured by an in-house developed RIA (142). The antibody (Af1) was produced in rabbits against GLP-1 coupled to BSA. The antibody cross reacts 100% with all amidated forms of GLP-1, but does not cross react with glycine (Gly) extended forms [GLP (1-37) and GLP (7-37)] or any other known pancreatic or GI peptide. ¹²⁵I-GLP-1 was prepared by the iodogen method and purified by HPLC (299). The assay was performed in a total volume of 700 µl of 0.06 M sodium barbitone buffer (pH 8) containing 0.3 % BSA. All samples were assayed in duplicate. Standard curves prepared for each assay used the purified peptide to be tested and prepared in assay buffer at 0.25 pmol/ml and added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 µl. The assay was incubated for 3 days at 4 °C before charcoal separation of the free and antibody-bound label. Both the pellet and supernatant counted for 180 seconds in a γ-counter (model NE1600, Thermo Electron Corporation). Peptide concentrations in the samples were calculated using a non-linear plot (GraphPad Prism Software Inc. San Diego Version 5.01, USA) and results calculated in terms of the standard. The assay was incubated for three days at 4 °C before separation of the free from antibody bound label by charcoal adsorption. The limit of detection was 7.5 pmol/l. Intra and inter assay variation was 5.4% and 11.5% respectively.

2.8.4. Oxyntomodulin radioimmunoassay

Currently it is not possible to directly measure levels of OXM and glicentin as distinct peptides by RIA, as, despite many attempts there are no antibodies that are specific for either peptide, due to the high level of sequence homology of the two peptides. The structure of glicentin encompasses the entire sequence of OXM, plus an N-terminal extension called the glicentin-related pancreatic polypeptide which contains 30 amino-acids. Therefore, when quantifying glicentin and OXM, it is only possible to measure them collectively, and this is referred to as “glucagon-like immunoreactivity” (GLI). A means of estimating human enteroglucagon (glucagon-like immunoreactivity of intestinal origin) in tissues and plasma is based on the subtraction of RIA values obtained with the C-terminal-directed glucagon antiserum RCS5 from the total glucagon-like immunoreactivity determined with the N-terminal- to midmolecule-directed glucagon antiserum R59 (196). It has been demonstrated that the major fragment of GLI in rat small intestine is OXM (40 % glicentin and 60 % OXM) (300). Therefore, applying this relationship to GLI levels derived from a RIA, it is possible to calculate the approximate levels of OXM.

2.9. Statistical Analysis

All data are expressed as the mean value $\pm$ S.E.M. Interval food intake and peptide hormone levels were compared by one-way analysis of variance (ANOVA) followed by post-hoc Dunnet's or Tukey's correction. Continuous *in vivo* data was analysed using a two-way ANOVA followed by a post-hoc Tukey's correction. Insulin release was compared by one-way ANOVA followed by post-hoc Tukey's correction. Prism Version 5.01 (GraphPad Software Inc. San Diego, USA) was used for statistical analysis. In all cases, values of $p < 0.05$ were considered statistically significant.

Chapter 3

The development of a surgical rat model for investigating the *in vivo* pharmacokinetic properties of gut hormones

3.1. Introduction

3.1.1. Pharmacokinetics basic principles

The development of high throughput approaches to drug development studies has been driven by the advances in high-speed chemistry and pharmacological screening (301). There is a constant need for screening more and more compounds as the safety precautions for new drugs and the market competition increase (302, 303). In such a setting the use of animal models for investigating the PK properties of drug candidates is invaluable.

Pharmacokinetics is a branch of pharmacology dedicated to the determination of the fate of substances administered externally to a living organism. It can be more simply defined as what the body does to the drug, as opposed to pharmacodynamics (PD) which may be defined as what the drug does to the body (304). Pharmacokinetics is often studied together with the PD. Pharmacokinetics involves the study of the mechanism by which a substance is absorbed and distributed within the body, the metabolic changes that occur to the substance and finally the routes of excretion of the substance and its metabolites from the body. In short these processes are commonly referred as ADME (Absorption, Distribution, Metabolism and Excretion). Recently a fifth process has been added to ADME, a process termed “liberation”, referring to the process of release of the drug from its formulation which is now considered a vital part of PK (305, 306).

In the drug development setting, PK is important because it allows characterising of the relationship between drug dosage regimens and drug concentration-time profiles (307). The appropriate dosage regimen is fundamental in achieving the best result as low doses may be ineffective and high doses may result in toxic side effects; the plasma concentration of the drug needs to lie within the therapeutic window. As well as the concentration range, the shape of the concentration-time profile can be important for some drugs. Certain drugs require “flat” profiles with little fluctuation in concentration while others such as some antibiotics require high peak concentrations for optimum antimicrobial activity (aminoglycosides). Knowledge of the ADME properties of a drug allows predictions of the therapeutic outcome, its toxicity and the drug’s dosage regimen to be made. Thus, over the last two decades, the use of PK modelling techniques to aid investigating a drug actions and its development has become standard practice (308, 309).

3.1.1.1. Absorption

Absorption of a drug is thought to be 100% only when given by an IV route. In any other mode of administration i.e. oral, SC, intramuscularly, a percentage of the drug is not absorbed. The fraction of

the administered dose that is absorbed is the one that will reach the systemic circulation and is termed F, i.e. bioavailability (310). The factors that influence the bioavailability include the physicochemical characteristics of the drug itself, its formulation, other concomitant use of drugs, blood flow, gut motility and the presence of pathology such as vomiting or diarrhea. Characteristics of drugs which favor a high percentage of absorption are high lipid solubility of the compound and low ionization.

3.1.1.2. Volume of distribution

Volume of distribution is defined as the apparent volume into which the drug should theoretically be distributed in order to achieve the measured concentration (310). Different substances present different distribution patterns in the body, some distribute equally between all compartments, other remains preferentially in the vascular space while others pass to the tissues and remain there. If the V_D is known, the loading dose required to reach a target concentration can be calculated by the formula:

$$D_L = V_D \times (\text{Target conc.} - \text{Measured conc}) / F_{sm}$$

where D_L is the loading dose, V_D is the Volume of distribution, F is the bioavailability if the drug is not given IV (if drug is administered IV, then F is equal to 1), S is the salt factor and M is the molar correction factor if the drug is in a salt form and if concentrations are reported in molar units respectively.

3.1.1.3. Clearance

Clearance is the removal of the drug from the body and is defined as the volume of fluid that is cleared of the drug per unit of time by the process of metabolism and excretion (311). Clearance can be divided in 2 parts; the renal clearance and the non-renal clearance.

In cases where there is a continuous infusion of the drug, the levels of the substance in the body will rise until the rate of administration, infusion rate, equals the rate of excretion. In that case, a steady state is said to be reached. If the clearance rate has previously been calculated, it is possible to calculate the dose rate required to maintain a steady state concentration (C_{ss}) because:

$$\text{Dosage rate (mg/hr)} = \text{Target } C_{ss}(\text{mg/l}) \times \text{Clearance (litre/hr)}$$

In all routes of administration except IV, the dosage rate is replaced by Dose/ Dosing interval.

3.1.1.4. Elimination rate constant and half-life

For most drugs the rate of elimination is proportional to the amount of the drug given, i.e. there is a constant fraction of the drug that is removed per unit of time and the decline of concentration is exponential. The proportion of the substance that is eliminated per unit of time is known as the elimination rate constant (k) and it depends on the clearance and the V_D .

$$K = CL/V_D$$

The half-life is defined as the time required for the concentration of the substance to drop by 50% from its original value and consequently $t_{1/2} = 0.693/k$. If a flat PK profile is desirable, then the required dosage interval must be less than the half-life to avoid big fluctuations of the high peak and the low trough concentrations.

3.1.2. Animal models of investigating the *in vivo* pharmacokinetic properties of peptides

Rodents and in particular rats, are extensively used as experimental models for safety pharmacology and PK studies of compounds prior to human trials (312). As the field of drug development keeps expanding and the importance of PK studies has become more widely accepted, reports of serial blood sampling and analysis for drug and/or drug metabolites have appeared in the literature with increasing frequency (313). Regulatory requirements for accurate PK profiles of novel compounds is driving forward the development and refinement of methodology for determining plasma time course data for drugs and/or drug metabolites in rodents.

The optimisation of an animal model for investigating the PK properties of drug candidates in an anti-obesity drug development program is a complex task and requires the following decisions to be made:

- Selecting a species/strain of animals that is representative of human
- Selection of the IV routes that best serve the PK properties that are investigated
- The number and time-points of obtaining the blood samples
- Accurate and adequate selection of the compound dose and the duration/rate of the infusion
- The use of a reliable delivery system
- Validation of the model

Pharmacokinetic studies utilise the most common laboratory species (rat, mouse, rabbit and dog), and doses are preferably within the therapeutic dose ranges (if known) rather than approaching toxicological doses. In general, rodents are used because of their short lifespan, allowing for the growth of a large number of animals in a short period of time and, consequently, the feasibility of

many studies. In contrast, large animals live longer, thus allowing for longitudinal studies but their use requires special ethical considerations (314).

It is generally believed that using the appropriate PK principles, PK data can be extrapolated to humans with adequate accuracy (315). This was formalised in the concept of allometric scaling, which states that anatomical, physiological and biochemical variables in mammals (such as tissue volumes, blood flow and process rates) can be scaled across species as a power function of the body weight (316, 317). The methodology was applied to the prediction of plasma concentration–time profiles and the main PK parameters (318, 319).

One of the basic concepts underlying the *in vivo* rodent model is that there have to be two different IV routes (320, 321). The first IV route is used for infusing the drug and the second one is required for blood sampling. Previously a variety of IV access routes have been used; Kervran et al. used the right saphenous vein for infusing and the carotid artery for sampling while Parkes et al. have used the saphenous vein for infusion and the saphenous artery for sampling (322, 323). Ohsima et al. in 1988 used the femoral vein as the entry point for administering the peptide and the jugular vein for taking blood samples (322).

Every IV route has its own advantages and disadvantages. Techniques such as orbital sinus puncture and tail vein sampling allow for serial blood collection; however, the sample volume obtained is very small and the procedure can induce stress to the animal. Moreover, drawing of blood from peripheral sites such as the tail may result in a different PK outcome than using blood taken from central sites (324). The decision of which available IV routes to choose is not simple and depends on numerous factors such as time constraints, surgical expertise and available instruments.

The total amount of blood removed should allow the accurate measurement of plasma concentrations of the drug while retaining the circulatory status of the animals unaffected. It has been shown that total blood volume can generally be estimated as 55 - 70 ml/kg body weight. Current guidelines state that up to 10% of the total blood volume can be taken on a single occasion from a normal, healthy animal on an adequate plane of nutrition with minimal adverse effects.

A critical component of the constant infusion technique is the use of a delivery system in order to infuse the peptides in an accurate and reproducible manner. Most of the infusion pumps that are commercially available require a significant cost for their purchase which is prohibitively expensive for occasional users or small research groups.

When the cost of the specialist equipment is added to the cost of the consumables, purchasing and maintenance of animals, manpower and samples analysis cost, a significant amount of financing is

needed in order to perform PK experiments. These cost implications prevent many groups working on translational research from conducting PK studies during the discovery stages of research, hindering effective drug discovery. However, if an affordable pump system was available this would be a significant help in making PK studies more widely available.

3.1.3. Summary

Detailed knowledge of the ADME properties of compounds is an important aspect during the early phases of drug discovery, and animal models are used to guide compound design and selection. Pharmacokinetic studies that focus on the measurement of plasma concentrations of gut hormones or of analogues that have been developed based on the primary structure of gut hormones, can provide invaluable information with regards to the required frequency of administration of the peptide. Furthermore, such PK studies can monitor the period that the plasma concentration of the drug remains within the therapeutic range (drug plasma concentration at higher levels are likely to cause adverse effects).

Pharmacokinetic experiments usually require a dedicated team of highly trained individuals with a significant cost to be taken into account. Therefore, I aim to develop a surgical animal model for the investigation of the PK properties of gut hormones and drug candidates that would produce accurate and reproducible results with easily accessible and affordable equipment.

Aims

- To develop a surgical animal model for the accurate investigation of the PK properties of gut hormones and drug candidates.
- To optimise the animal model to require the least amount of financial resources so as to be readily available.
- To validate the surgical animal model by testing gut hormones (GCG, GLP-1, OXM) and synthetic EX-4.

3.2. Methods

3.2.1. Peptides

All peptides used in this chapter were purchased from Bachem, Ltd (Merseyside, UK). All peptides used in the studies described in this thesis are the human sequence.

3.2.2. Measurement of *in vivo* pharmacokinetic properties (Metabolic clearance rate, Half-life and Volume of distribution) with the use of the constant infusion model in rats

All animal procedures were approved by the British Home Office, under the United Kingdom Animal (Scientific Procedures) Act 1986 (Project License 70/7236). Adult Male Wistar rats weighing 400-600 g were maintained double housed cages under controlled temperature (21–23 °C) and lights (12-h light, 12-h dark cycle, lights on at 0700 h). Animals had ad libitum access to water and standard RM1 chow diet (Special Diet Services, Devon, UK) unless stated otherwise. A graphical representation of the study design can be seen in Figure 1. Animals were anesthetized and the right external jugular vein and the left femoral vein were catheterized. Chosen dose and infusion rate had previously been shown to induce a steady state of peptide concentration within the first 40 minutes of infusion. After the 60 minutes infusion period, the femoral catheter was removed and the infusate was collected for another 40 minutes. Blood samples were taken from the jugular catheter during regular intervals throughout the entire procedure. Once all the necessary samples had been collected the animals were culled with a Schedule 1 method.

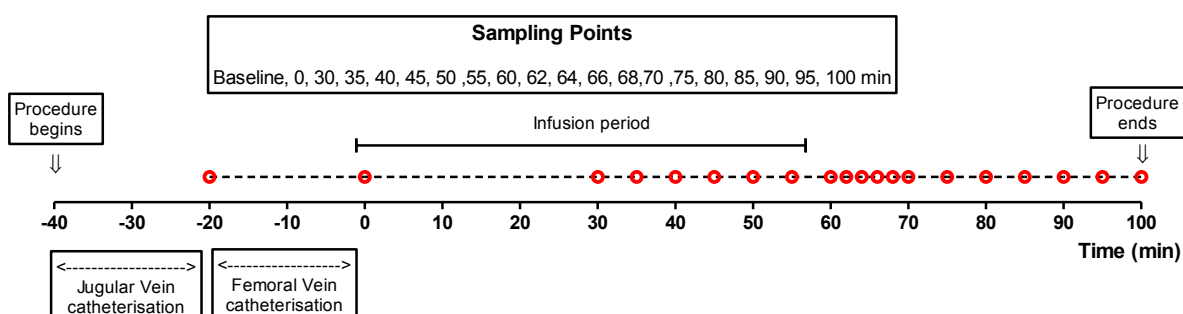


Figure 1: Graphical representation of the study design of continuous IV infusion of peptide to anesthetised male Wistar rats. Once the animal was anesthetised, the right jugular vein was cannulated and a baseline

blood sample taken. The left femoral vein was then cannulated and connected to the infusion pump. Infusion of the analogue lasted for 60 minutes. Sampling points are displayed in red circles over time in minutes.

3.2.2.1. Anaesthesia

Animals were anaesthetised with gas anesthetic via cone mask with an appropriate gas scavenging system. Induction was done with oxygen flow at 0.8 to 1.5 L/min and the isoflurane vaporizer to 3% to 5% while in maintenance the face mask was connected to the Bain circuit and the O₂ flow adjusted to 2-3% and the isoflurane vaporizer to 2 to 2.5%. Animals were monitored regularly throughout the procedure to ensure the correct level of anaesthesia was maintained.

3.2.2.2. Surgical technique

3.2.2.2.1. Jugular vein catheterisation

Once animals were anaesthetised they were placed on a heat mat covered with a sterile drape with the forepaws slightly extended and taped to the heat mat. Care was taken so as to prevent overextension of the forepaws that would result in difficulty in respiratory movements. The orientation of the animal was with its back on the mat and its head facing the person performing the procedure. The skin from the right side of the neck extending from the jaw up to the right clavicle was lifted carefully with angular forceps and a 2 cm vertical incision was performed with a surgical blade. Due to the tension, once the incision is performed the skin at the area separates and there is adequate exposure to the tissues underneath without the need for skin removal. The platysma muscle was dissected and the right clavicle was used as a guide to locate the point where the jugular vein passes under the clavicle. Once the jugular vein was identified, upwards dissection was performed in order to free the jugular from its medial and lateral branches. At the superior part of the incision, the three main branches of the jugular vein were identified and individually ligated. Blunt dissection was then used to clean the right jugular vein of fat and excess tissue until the vein was easily visible. Generally, in young rats the jugular vein is visible under a thin layer of tissue cover while in older rats, fat and platysma needed to be separated before the jugular vein is visible. The salivary gland was held out of the area of the vein using hemostats (Fine Science Tools, Heidelberg, Germany) and with careful lifting of the vein, the area under the vein is dissected.

When possible a 2 cm section of vein is made visible. Three separate pieces of 4-0 Vicryl suture were passed under the vein. One of these threads was moved to the rostral end while the other two pieces were moved caudally towards the heart and a loose knot was prepared for each of them. The vein was put under slight tension by pulling the thread superiorly and with extreme caution a small incision (v-shaped cut) is made with the spring-scissors on the upper surface of the vein at about midway the

distance between the superior and inferior ties. The pre-prepared tubing was inserted into the cut, and temporarily secured in place with masking tape. About 100 µl of heparinized saline is infused through the catheter and the syringe was gently drawn back to allow approximately 100µl of blood to fill the tube and to ensure that the vein had been correctly cannulated. After confirmation of correct positioning of the catheter, the three loosely knotted threads were tightened to hold the cannula in place.

3.2.2.2.2. Femoral vein catheterisation

The left femoral vein was used as an access route for IV infusion of the peptide. The skin above the region was clipped and a horizontal 2 cm incision was made. As for the jugular area, once incised, the skin is retracted, allowing for a good visualization of the structures underneath. The SC fat was dissected with blunt scissors and the left saphenous vein tied off and cut. The small lateral branches of the femoral vein were cauterised and by gentle traction of the saphenous vein, the femoral vein was lifted and the dissected from its bed. The dissection extended from 1 cm laterally to the point of insertion of the saphenous vein up to as medial as possible. The communicating branches of the femoral vein to the deep venous system were carefully ligated and cut or cauterised. The femoral artery and the femoral nerve were then dissected free from the femoral vein and held as far away as possible. The most lateral edge of the femoral vein was tied off with the help of a 4-0 Vicryl ligature and the ligature was held with a small haemostat to provide lateral traction. Four individual ligatures of Vicryl 4-0 were passed under the femoral vein and the three of them were used to secure the catheter in place once positioned. The fourth one which should be the most medial one was used to control the proximal side of the femoral vein while trying to insert the catheter and as the tie that occluded the femoral vein once the catheter was removed from the vein. The technique of inserting the catheter is the same as described previously in the jugular vein catheterisation. The only difference is that after insertion of the catheter and checking its patency, the catheter was connected to the pump and the infusion began.

3.2.2.2.3. Catheter preparation

All cannula tubing required was prepared before the start of the procedure. Polyethylene tubing (internal diameter (ID) 0.45 mm, outside diameter (OD) 0.91 mm) was cut to 10 - 12 cm length for the jugular and the femoral vein respectively. One end had a 1ml syringe with a 25 gauge needle attached to it. A 1ml syringe was filled with heparinized saline (100 I.U/ml) and attached to the needle. The other end of the tube was cut with a scalpel blade to create a beveled edge. Catheter implantation was performed with the help of surgical loupes with a magnifying force of 2.5-3.5x.

3.2.3. Pump and peptide preparation

Peptide was prepared immediately prior to use. Peptides were resuspended in gelofusine, to a final concentration of 0.03 nM. The pump used in this study was a Medtronic 407c insulin pump with a 1.0 ml reservoir (MMT-103A). The pump was programmed at a rate of 300 μ l/hour. The femoral catheter was primed by running the pump for 20 minutes prior to catheter placement. A bolus dose of 1.5 nmol was administered only for EX-4. At the end of the infusion period, the femoral catheter was carefully removed from the femoral vein and allowed to continue working with the infusate collected in a microtube.

3.2.4. Pump infusate concentration measurement

After the removal of the femoral catheter from the femoral vein, the pump was allowed to run inside a microtube for 20 minutes and the infusate was collected and assayed by RIA.

3.2.5. Sampling

All blood samples were taken from the right jugular vein. Whenever blood samples were to be taken, a new empty 1ml syringe was connected to the tubing. This was gently drawn back to remove 100 μ l of blood. Once this was collected another empty 1 ml syringe was reattached, ensuring there were no air bubbles in the tubing. All blood samples were of 100 μ l volume except for the baseline which was 500 μ l. Timing of blood sampling can be seen in Figure 1. One baseline sample after insertion of jugular vein and then 19 samples in total taken on the following time points from the beginning of the infusion: 0, 30, 35, 40, 45, 50, 55, 60, 62, 64, 66, 68, 70, 75, 80, 85, 90, 95 and 100 min.

Blood samples were collected in 1.5 ml heparinized microtubes containing a protease inhibitor cocktail [4 μ l of a stock solution [1 Tablet of S8820 SIGMAFAST™ protease inhibitor Tablets in 10ml distilled water (dH₂O) – 10x solution, (Sigma-Aldrich, Dorset, UK)]. The tubes were then spun in a centrifuge (3-18K SciQuip, Sigma, USA) for 10 minutes at 10000 x g at 4 °C. Plasma supernatant was collected and stored at -20 °C until assayed.

Total blood volume (TBV) taken from each rat was within the recognized guidelines (325).

3.2.6. Radioimmunoassay

All samples were assayed using established in house RIA. Radioimmunoassays for EX-4, GCG GLP-1 and OXM were performed as described in the respective sections in section 2.8.

3.2.7. Calculations

Peptide concentrations were plotted on a logarithmic scale versus time and computed to yield the slope from which the half-life ($t_{1/2}$) was determined. All peptides were infused at a theoretical concentration of 0.03 nM and at an infusion rate of 0.3 ml/hr, the theoretical amount of peptide infused was 9 nmol. In this study we assumed that endogenous production of GCG, GLP-1 or OXM remained constant during the exogenous infusion experiments. This assumption is supported by the steady level of GCG exhibited in similar experiments performed by Kervran in 1990.

The Half-life is defined as the time required for the concentration of the substance to drop by 50% from its original value and can be calculated as follows (326):

$$t_{1/2} \text{ (min)} = \frac{\ln(2)}{\text{gradient}}$$

where $\ln(2)$ is the logarithm of 2 and equals 0.693

Clearance is the removal of the drug from the body and is defined as the volume of fluid that is cleared of the drug per unit of time by the process of metabolism and excretion. The total clearance is the sum of the renal clearance and the non-renal clearance and also equals to the extraction ratio multiplied by the flow rate. The unit of clearance is volume/time (e.g. litre/hr). The metabolic clearance rate (MCR), expressed in ml/min/kg, was calculated by dividing the infused dose (pmol/kg/min) by the plateau increment in plasma peptide (pmol/l) using Tait's formula (327):

$$\text{MCR (ml kg}^{-1} \text{ min}^{-1}) = \text{infusion rate/steady state concentration}$$

Volume of distribution is a theoretical number that assumes the drug is at equal concentration in the tissue and in the circulation and represents the volume (or mass) of tissue is required to result in that circulating concentration. The apparent V_D (in ml/kg) was obtained by dividing the MCR by the slope of the regression line for disappearance. Volume of distribution was calculated using the following formula (328):

$$V_D = t_{1/2} \times \text{MCR} / \ln(2)$$

where $\ln(2)$ is the logarithm of 2 and equals 0.693

The steady state concentration represents the time points at which the amount of the drug administered is equal to the amount of the drug eliminated during the same period (329). At steady state the plasma concentrations of the drug at any time during any dosing interval, as well as the peak and trough, are similar. The time to reach the steady-state concentration is dependent on the half-life of the agent under consideration (330). Plasma steady state concentrations of peptides were taken as the mean of the four values obtained at 40, 45, 50, 55 and 60 minutes. All values shown represent the mean $\pm$ S.E.M.

3.2.8. Statistical analysis

All data are expressed as the mean value $\pm$ standard error of the mean (SEM).

3.3. Results

The time-courses of the mean plasma concentrations of GCG, OXM, EX-4 and GLP-1 during the IV primed-continuous peptide infusion were measured by RIA (Figure 2, Figure 3, Figure 4, Figure 5). The preinfusion values of GCG, OXM and GLP-1, were approximately 43, 67 and 35 pmol/l respectively. Steady-state concentration levels were observed between 30 and 60 minutes after the start of the infusion of 9 nmol/hr of the peptide. The steady-state concentration levels of EX-4 and OXM were between 2- and 8-fold higher in comparison to the ones attained by GLP-1 and GCG (16.8±1.7, 19.7±5.8, 2.6±0.3 and 8.3±0.3 pmol/ml for EX-4, OXM, GLP-1 and GCG respectively) (Table 1). A summary of the steady-state concentrations and the PK properties achieved by the peptides can be seen in Table 1.

	Metabolic clearance rate (ml/min/kg)	Half-life (min)	Volume of distribution (ml/kg)	Steady state concentration (pmol/ml)
EX-4	10.1±1.0	35.0±4.2	430.0±67.3	16.8±1.7
GLP-1	83.9±9.9	1.2±0.3	149.7±53.5	2.6±0.3
GCG	40.7±10.1	3.2±0.9	147.0±29.0	8.3±0.3
OXM	12.8±3.2	12.0±2.1	231.6±94.0	19.7±5.8

Table 1: Comparison of PK properties (MCR, Half-life and Volume of distribution) for EX-4, GLP-1, GCG and OXM measured during continuous IV infusion in anaesthetised male Wistar rats. Steady state concentration represents the mean of the five values obtained at 40, 45, 50, 55 and 60 minutes of the infusion phase. Values are shown as mean ± S.E.M.

The MCR of GCG was 40.7±10.1 ml/min/kg while OXM had an almost 3-fold lower MCR (12.8±3.2 ml/min/kg). Exendin-4 presented the lowest MCR of all peptides and GLP-1 the highest one (10.1±1.0 and 83.9±9.9 ml/min/kg respectively).

Exendin-4 presented the highest half-life with 35.0±4.2 minutes while GLP-1 had the lowest half-life with 1.2±0.3 minutes. Glucagon presented a half-life of 3.2±0.9 minutes and OXM had a half-life of 12.0±2.1 minutes.

Glucagon and GLP-1 presented a similar apparent V_D (147.0±29.0 and 149.7±53.5 ml/kg respectively). Exendin-4 had a 2-fold higher V_D in comparison to OXM (430.0±67.3 versus 231.6±94.0 ml/kg).

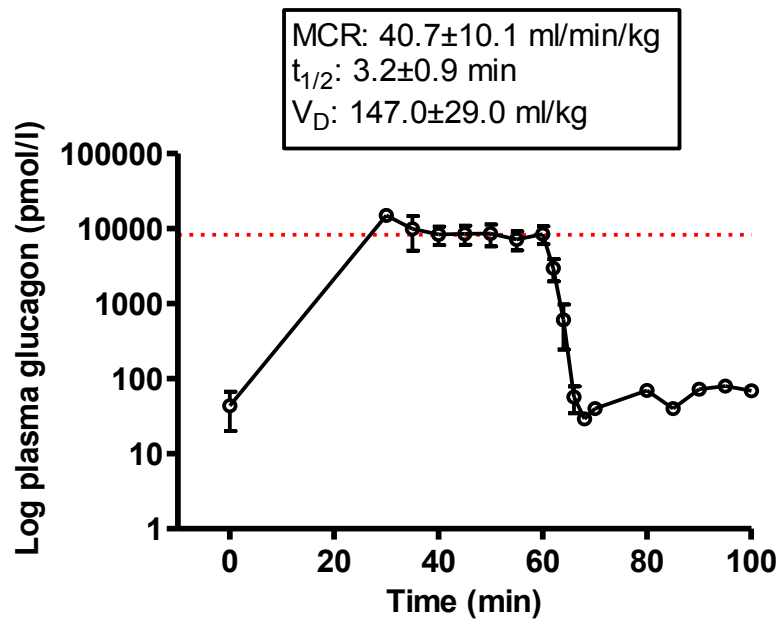


Figure 2: Plasma concentration of GCG during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Infusion of the analogue is performed through the left femoral vein while blood sampling occurs through the right jugular vein. Infusion begins at “0” time-point and lasts until “60” minute at which point the infusion is stopped. Values are shown as mean $\pm$ S.E.M. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

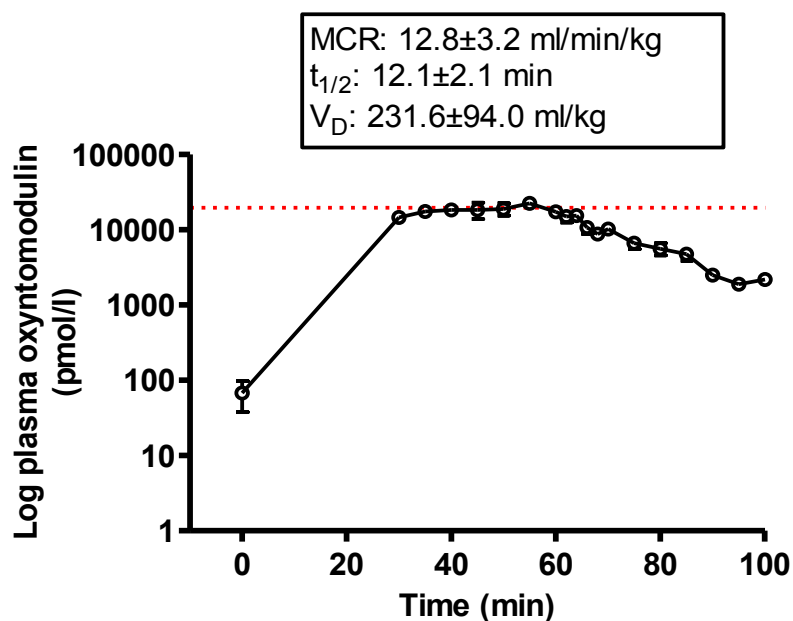


Figure 3: Plasma concentration of OXM during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Infusion of the analogue is performed through the left femoral vein while blood sampling occurs through the right jugular vein. Infusion begins at “0” time-point and lasts until “60” minute at which point the infusion is stopped. Values are shown as mean $\pm$ S.E.M. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

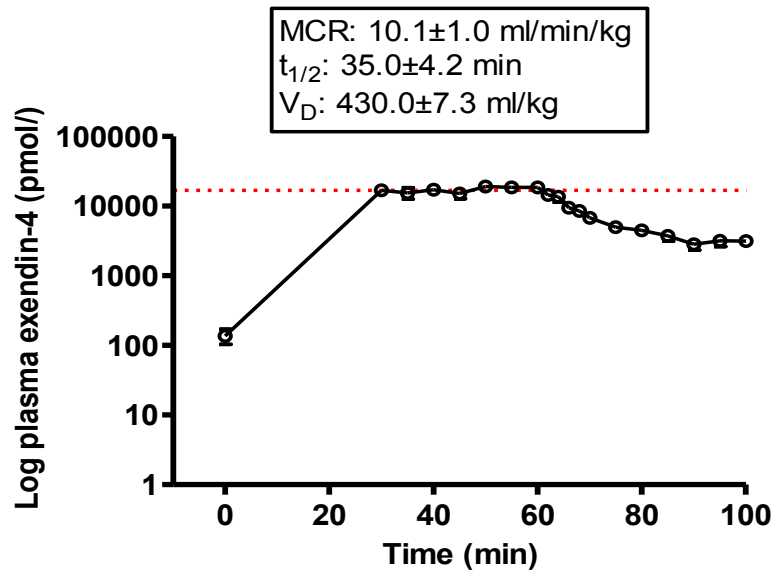


Figure 4: Plasma concentration of EX-4 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Infusion of the analogue is performed through the left femoral vein while blood sampling occurs through the right jugular vein. Infusion begins at “0” time-point and lasts until “60” minute at which point the infusion is stopped. Values are shown as mean $\pm$ S.E.M. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

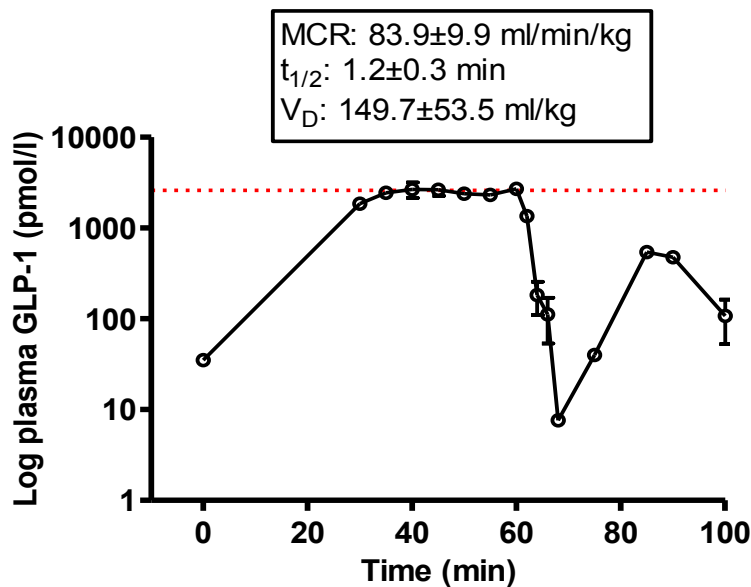


Figure 5: Plasma concentration of GLP-1 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Infusion of the analogue is performed through the left femoral vein while blood sampling occurs through the right jugular vein. Infusion begins at “0” time-point and lasts until “60” minute at which point the infusion is stopped. Values are shown as mean $\pm$ S.E.M. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

3.4. Discussion

For a drug, in particular a peptide drug, to be effective it has to have appropriate PK properties. To help predict the PK characteristics of novel drugs in man, animal models have long been utilised providing valuable insight into these parameters (322, 323, 331-334). However, many of these PK models are expensive, highly technical and requiring specialist equipment, putting them beyond the accessibility of small academic groups. Lack of reliable PK models is a barrier for these groups to exploit their intellectual property and undertake drug discovery work. The aim of the work described in this chapter was to develop an inexpensive and readily accessible animal model for completing PK analysis of compound libraries and potential drugs. Having designed the PK model, it then had to be validated to confirm it produced reproducible and accurate results.

The most important PK parameters to determine in the evaluation of novel drug compounds are the MCR, the half-life and the V_D ; their results can be translated to valuable information when it comes to selecting the appropriate dosage parameters. The MCR is one of the most important PK parameters as it allows the calculation of the steady-state concentration of a substance for a given dosage rate (305). Determination of a compounds half-life allows the calculation of how long it will take for its plasma levels to be reduced by half the original concentration and can be used to estimate the appropriate dosage regime and for how long a drug should be stopped if a patient has toxic drug levels. The importance of V_D lies in influencing the duration of the drug action. Since half-life ($0.693/k_{el}$ where k is the elimination rate constant) is determined by the V_D and the clearance, manipulation of V_D is an important tool for changing the duration of action of the drug. The amount of the free drug in the circulation is important, since this is the compound actually passing through the membranes and into the tissues where it is expected to exert its effect. A low V_D means that the drug remains mostly in the circulation with limited tissue penetration while the opposite guarantees a widespread tissue diffusion and access to the target organ. The V_D cannot be measured directly as it is a theoretical value but instead it is calculated by measuring the MCR and the half-life. In the anti-obesity drug discovery field the goal is to produce a drug with long half-life and low MCR allowing for a long time period between doses and with high V_D as the drug is required to penetrate the blood-brain barrier and act centrally in the brain.

The aim of this thesis was to develop an anti-obesity medication that would be administered subcutaneously in the clinical practise. The selection of the IV route to test the PK properties of the drug candidates with the above described PK model was done because the IV route eliminated the confounder factor of drug absorption from the tissues and into the systemic circulation. Although it is

still possible to calculate the PK properties when is given in a non-IV route, I used the IV route for measuring the PK properties of my analogues and a different method to assess plasma concentration over time when given subcutaneously.

Frequent plasma sampling is required to achieve accurate MCR and PK values. Therefore, in the development of a PK model I first focused on obtaining a reliable access point to minimise the occurrence of issues such as catheter dislodgement or blockage and inadequate blood sample volume. A further IV access point is also required to infuse the drug. The IV access point would ideally be quick to isolate, minimising the length of surgery prior to start of plasma sampling. In current methodologies, the jugular vein is often referred to as the most appropriate vessel for collecting blood samples. It is recommended due to its proximity to the surface and the long distance from any other major vessels. However, a significant disadvantage of using the jugular vein is its small diameter which makes the catheterisation process a difficult, time consuming task and requires adequate surgical skill. The saphenous vein is also widely recommended as a good access point to the venous system. Like the jugular vein, it has the advantage of being close to the surface, thus reducing the amount of dissection to locate it, but also has a narrow diameter which requires a highly skilled surgeon for the catheterisation process. Another recommended access point to the venous system for sampling is the carotid arteries. Both the right and left side of the carotid arteries can be cannulated. The left carotid artery presents a longer section for intervention, and thus is the vessel of choice for many researchers (335). As the right carotid artery branches off the innominate (brachiocephalic) artery after bifurcation from the aortic root, only a short vessel segment is available for intervention (335). Furthermore, passage of a catheter along the right carotid artery may end up in the left ventricle causing fatal arrhythmias. However, cannulation of the carotid artery is inherently more challenging and in the event of encountering technical difficulties during catheter insertion, high blood loss is difficult to avoid due to the increased pressure in the arterial system in comparison to the venous one.

The femoral vein has several advantages as an access point to the venous systems; it is easy to locate, the diameter of the vessel is adequately large to allow insertion of catheters and it can be exposed along its course allowing several attempts to venotomies should the initial attempt fail. A disadvantage of using the femoral vein is dissecting free the femoral vein from the femoral artery without damaging the artery is a moderately difficult task. For the reasons mentioned above, in this series of studies the left femoral vein was used as the infusion site and the right jugular vein as the vascular access for blood sampling.

Exposing a significant length of both routes and isolating every collateral branch present reduces blood loss and to allow another venotomy to be performed if the first attempt is unusable. The use of a

single tie to stabilise the catheter, as recommended by several protocols often proves inadequate, resulting in the dislodgement of the catheter and inability to continue taking samples. One or two extra ties are not time-consuming and provide more robust catheterisation for uninterrupted blood sampling (336). The method described above refined the process by placing two ligatures in the proximal (central) part of the catheter and one distally (peripheral).

The total amount of blood volume removed during the sampling period (2.4 ml in total; 19 samples of 100 µl each and a single sample of 500 µl) was well below the recommended limit of sampling volume (7.4% of the TBV for a 500 g rat compared to a recommended limit of 10% of TBV). Thus the circulatory status of the animal during the experiment was considered to remain unaffected by the sampling process and renal and hepatic vascular supply remained steady throughout the procedure. It is of particular importance in PK studies to maintain a stable vascular supply as the kidney and the liver have been shown to be important for the metabolism of gut hormones and altering vascular supply to these organs could directly affect clearance rate.

The specialist equipment required by many described PK models is expensive and thus inaccessible for many researchers. The PK model described in this chapter utilised an insulin pump. Insulin pumps and the necessary reservoir are cheap and are widely available. The insulin pump is also easy-to-use, with an integrated alarm whenever infusion is stopped unexpectedly. Other equipment routinely used in these experiments included a reusable cautery pen and magnifying loupes. The use of the cautery pen allows for easy and rapid haemostasis, minimising blood loss. In experienced hands the cautery pen can also be used for dissection, minimising operating time. Surgical loupes are particularly valuable for femoral vein catheterisation when relatively inexperienced, but are also useful for experienced operators, and they are vital should the first catheterisation attempt fail and the vessel collapse, making the localisation of the venotomy site difficult. Vascular mini-clamps are widely used to minimize bleeding during catheter insertion, but the clamps are expensive and easily misplaced due to their small size. Alternatively, a suture folded in half, passed around the vessel and put under tension by slight traction with the help of a normal size haemostat grasping the suture can be used.

The animal model used in this thesis was developed as a platform for testing the PK properties of drug candidates that were designed based on the primary structure of OXM. In the present study, model validation was performed by measuring the *in vivo* PK properties of OXM, GCG, EX-4 and GLP-1 using primed continuous infusion of the peptides. The PK properties of these peptides are known to be very different thus testing the model across all likely PK parameters. Furthermore, the PK parameters of the control agents would serve as a point of reference for the evaluation of the drug candidates' developed later on.

The rapid degradation of GLP-1 in blood by proteases, specifically DPP4, has been well characterized and contributes significantly to its short half-life (256, 337, 338). The half-life and MCR of GLP-1 in my study were 1.2 minutes and 83.9 ml/min/kg respectively closely resembling the results of previous studies. The reported half-life and MCR of GLP-1 from Parkes et al. in 350 g rats was 0.9 minutes and 34 ± 4 ml/min respectively (323, 339).

On the other hand, EX-4 has been shown to be much more stable in plasma and more resistant to degradation by DPP4 *in vitro*. This factor contributes to the lower plasma clearance rate and long half-life for EX-4 (versus GLP-1 and GCG) reported in this study and in the literature. Parks et al. reported a half-life of 40 ± 4 min and an MCR of 4.8 ± 0.4 ml/min for EX-4 in 350 g rats (339).

The PK properties of GCG as measured in this study matched the values reported in the published literature. More specifically, the MCR of GCG obtained in this study (40.7 ± 10.1 ml/min/kg) is similar to the MCR reported by Kervran et al. in 1990 (36 ± 5 ml/min/kg), by Oshima et al. in 1988 (36 ± 5 ml/min/kg) and by Katz et al. in 1978 (31.8 ± 1.2 ml/min/kg) (322, 323, 340).

The MCR of OXM calculated in my study matched the results published by Kervran et al. in 1990 (mean MCR for porcine and rat OXM were 11.3 ± 0.7 and 11.9 ± 0.5 ml/min/kg respectively) (322). When tested in species with a higher volume to surface area, the MCR of OXM was lower as expected; 5.2 ± 0.7 ml/min/kg in humans and 6.96 ± 0.99 ml/min/kg in pigs (195, 341). The kidney has been previously shown to be important for the degradation of OXM and GCG and acute bilateral nephrectomy in rats has reduced their MCR by approximately 35% (322, 340).

The half-life of OXM in my study was 12.1 minutes and approximates the half-life reported by Kervran et al. (rat OXM: 6.4 ± 0.5 min and porcine OXM: 8.2 ± 0.5 min) (322). The difference observed may be attributed to differences in protocol (Kervran et al. used a much smaller dose of 0.56 nmol). The V_D of OXM observed in this study (0.24 l/kg) was also similar to the findings of Kervran et al. (0.1 l/kg).

The technique used to measure the plasma concentrations of the agents largely determines the dosages used for the agents tested. As the PK of a test agent is not dependent on its plasma level, experiments with different plasma concentrations can be compared. More specifically, the calculation of the half-life is not dependent on the amount of drug administered and thus its value is not affected by differences in the infusate concentration. Furthermore, It has been shown that under steady-state conditions, the MCR of peptides is independent of the number of compartments into which they are distributed and remains constant over a wide peptide concentration range (327). The above-mentioned make it possible to compare the PK characteristics of the test agents used in this chapter with the published literature regardless of differences in dosages and methodology.

3.5. Conclusions

The PK properties of drug candidates are important for the designing of new and improved agents and they can only be calculated by *in vivo* experiments in animal models.

The advantages presented by the use of the proposed animal model for evaluating the PK properties of peptides include the affordability of the pump setting (pump and reservoir) due to its low cost which bypasses the need for an expensive dedicated pump system. The rest of the consumables used are easily accessible and the setting of the procedure can be relatively easily reproduced.

At the same time the design of the study has been optimised to measure the PK properties of gut hormones or analogues developed based on gut hormones, producing consistent and reproducible results while minimising animal use.

Chapter 4

The design of oxyntomodulin analogues as an anti-obesity treatment

Investigation of amino acid substitutions at positions 29, 30 and at the C-terminal

4.1. Anti-obesity drug development based on an oxyntomodulin analogue. Modification of positions 29, 30 and of the C-terminal poly-Histidine tail.

The work presented in this thesis is centred around developing an anti-obesity agent based on the native OXM with several modifications that were the result of previous work done as part of the drug discovery program in the Department. The molecule used as a starting point for developing new analogues in this thesis was OXM(1-30) with point modifications in positions 20, 21 and 27 (as described in sections 1.10.5.3.1 to 1.10.5.3.3). Novel point modifications of this thesis in the primary structure of OXM aimed to enhance the bioactivity and the PK profile of the analogue. The novel point substitutions that were examined in this chapter are at positions 29, 30 and the C-terminal poly-His tail (Table 2).

4.1.1. Position 29

A further change in the primary structure of OXM to enhance GLP-1R binding was the substitution of Thr²⁹ with Gly²⁹. Adelhorst in 1994, published the results of Ala scanning of GLP-1, which revealed that Gly²⁹ is critical for the structure-function of the molecule as it allows it to adapt the conformation recognized by the receptor (219). In this series of OXM analogues, the Thr found in native OXM was substituted by Gly in an effort to improve binding to the GLP-1R.

Furthermore, Gly²⁹ was also shown to be important for GLP-1R binding in EX-4. Nuclear magnetic resonance analysis of the central region of EX-4, residues 10-30, demonstrated a highly stabilized helical structure (247). This region is highly conserved with GLP-1, with eight residues being the same in both molecules. The eight identical amino acids in both EX-4 and GLP-1 lie on the same face of an ideal α -helix of their sequence, suggesting the face of the helix is likely to mediate the critical contact with the receptor (218). Glycine in position 29 is one of the 8 conserved amino-acids; it was hypothesised that the substitution of Thr²⁹ with Gly²⁹ in OXM would enhance GLP-1R binding affinity.

4.1.2. Position 30

In the analogues X3 to X5 developed in this chapter, position 30 is occupied by Gly in contrast to Lys in the native OXM molecule. Glycine³⁰ is also found in EX-4 and is well-known for offering conformation flexibility in the amino acid structure. This substitution was made to examine whether

it can increase GLP-1R and zinc binding, and thus improve the bioactivity and PK profile of the molecules.

4.1.3. C-terminal poly-Histidine tail

The peptides discussed in this thesis were designed with the aim of being able to bind zinc ions present in the formulation with which they were administered. For this reason a poly His-tail in the C-terminal of the molecule was introduced in this series of analogues. When analogues are administered in a zinc-containing formulation, the His residues are able to chelate to zinc molecules (342). The resulting zinc-peptide complex forms a precipitate at neutral pH, forming an insoluble SC depot that allows a slow release of the peptide in the circulation and protects it from the actions of aminopeptidases. This approach presents dual benefits, firstly the early post-administration peak in plasma peptide concentration is avoided (which has been linked to the occurrence of nausea in GLP-1 mimetics), and secondly there is a prolongation of the PK profile of the peptide.

All analogues designed in this chapter have a variable His C-terminal tail whose aim it is to further improve the PK profile by increasing the binding with the zinc molecules and subsequently prolong the half-life of the molecules. The manner that the poly-His tail is connected to the rest of the peptide varies between the analogues; in X1 and X2 a single Gly at position 29 is present, while in X3-X5 there is a Gly²⁹-Gly³⁰ present.

4.1.4. Aims

Hypothesis:

Rational modifications to the amino acid sequence of OXM will result in an OXM analogue more efficacious at inhibiting food intake and promoting weight loss than native OXM.

Aims:

- To investigate effects of rational point substitutions to the sequence of OXM with regard to properties at the GLP-1R and GCGR.
- To examine the effect of specific changes to the structure of OXM on its *in vivo* PK parameters.
- To investigate the effect of SC administration of OXM analogues to mice on food intake and body weight following acute peripheral administration.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
GCG	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T								
GLP1	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R	NH2						
EX4	H	G	E	G	T	F	T	S	D	L	S	K	Q	M	E	E	E	A	V	R	L	F	I	E	W	L	K	N	G	G	P	S	S	G	A	P	P
OXM	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	K	R	N	R	N	N	I	A
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
X1	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	H	E	F	V	Q	W	L	L	N	G	H							
X2	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	H	E	F	V	Q	W	L	L	N	G	H	H						
X3	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	H	E	F	V	Q	W	L	L	N	G	G	H						
X4	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	H	E	F	V	Q	W	L	L	N	G	G	H	H					
X5	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	H	E	F	V	Q	W	L	L	N	G	G	H	H	H				

Table 2: Amino acid sequences of peptides used in chapter 4. Blue filled residues are conserved residues across the GLP-1 and GCG peptides; yellow letters are amino acids derived from the GLP-1 sequence; red letters are amino acids derived from the EX-4 sequence; grey filled residues represent point substitutions.

4.2. Materials and Methods

4.2.1. Custom design of peptide analogues

Five analogues of OXM were designed and synthesised as described in section 2.2. The amino acid sequences of these analogues as well as OXM, EX-4, GLP-1 and GCG are shown in Table 2. In order to simplify the comparison of GLP-1 and EX-4, the residue-numbering scheme of EX-4 was applied to GLP-1 such that the first residue of GLP-1, His⁷, was renamed as His¹, Ala⁸ of GLP-1 was renamed Ala² etc.

4.2.2. Competitive receptor binding assays

4.2.2.1. Glucagon receptor binding assays

4.2.2.1.1. Receptor binding of ligands to the human glucagon receptor

Binding studies were completed as described in section 2.6.5, using CHO cell membrane (cells overexpressing the hGCGR) as the source of GCGR (section 2.6.2). ¹²⁵I-GCG was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean ± SEM.

4.2.2.1.2. Receptor binding of ligands to the mouse glucagon receptor

Binding studies were completed as described in section 2.6.5, using mouse liver cell membranes as the source of mouse GCGR (mGCGR) (section 2.6.1). ¹²⁵I-GCG was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean ± SEM.

4.2.2.2. GLP-1 receptor binding assays

4.2.2.2.1. Receptor binding of ligands to the human GLP-1 receptor

Binding studies were completed as described in section 2.6.4, using CHO cell membrane (cells overexpressing the hGLP-1R) as the source of GLP-1R (section 2.6.2). ¹²⁵I-GLP-1 was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean ± SEM.

4.2.2.2.2. Receptor binding of ligands to the mouse GLP-1 receptor

Binding studies were completed as described in section 2.6.4, using mouse lung cell membrane as the source of mouse GLP-1R (mGP-1R) (section 2.6.1). ^{125}I -GLP-1 was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

4.2.3. cAMP accumulation studies

4.2.3.1. *In vitro* bioactivity studies at the human glucagon receptor

The ability of ligands to stimulate cAMP production was investigated, as described in section 2.7, using CHO cells overexpressing the hGCGR (section 2.6.2) as the source of GCGR. EC₅₀ values were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

4.2.3.2. *In vitro* bioactivity studies at the human GLP-1 receptor

The ability of ligands to stimulate cAMP production was investigated, as described in section 2.7, using CHO cells overexpressing the hGLP-1R (section 2.6.2) as the source of GLP-1R. EC₅₀ values were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

4.2.4. Acute feeding studies in mice

The effect of OXM analogues on food intake were investigated in fasted mice, as described in section 2.4. Peptides were administered in early light phase via SC injection and food intake was measured at 1, 2, 4, 8 and 24 and 48 hours post-injection, unless otherwise stated. Statistical analysis of the results was completed as described in section 2.9, results are shown as mean $\pm$ SEM (n=7-8 per group). Doses administered were chosen based on the observations from previous feeding studies (not shown). Exendin-4 (10 nmol/kg) was used as a positive control and OXM analogues were administered at 50 nmol/kg.

4.2.5. Pharmacokinetics

4.2.5.1. Investigation of the pharmacokinetic properties in an *in vivo* constant infusion model

Studies were carried out to determine the *in vivo* PK properties (MCR, half-life, V_D) of OXM analogues with the use of the constant infusion model after IV administration in male Wistar rats (section 3.2.2). Plasma peptide concentrations were determined by RIA (section 2.8).

4.2.5.2. Investigation of the pharmacokinetic properties following single subcutaneous bolus injection in male Wistar rats

Studies were carried out to investigate the formation of a slow release depot and measure plasma clearance following a SC administration (section 2.5.1). Male Wistar rats were stratified by body weight and placed into groups of 5 rats of equal mean body weight. Each rat was administered 1 mg of peptide (50 mg/ml) reconstituted in 1:1 molar ratio Zn:NaCl (saline) at time 0. Plasma peptide concentrations were determined by RIA (section 2.8).

4.3. Results

4.3.1. First generation of oxyntomodulin analogues

4.3.1.1. Binding affinities of oxyntomodulin, glucagon, GLP-1, exendin-4 and oxyntomodulin analogues to the glucagon receptor

4.3.1.1.1. Binding to membrane preparation of CHO cells overexpressing human glucagon receptor

Binding assays were completed as described in section 4.2.2.1.1. Binding studies using ^{125}I -GCG routinely resulted in greater than 90% specific binding to a cell membrane preparation of CHO cells overexpressing the hGCGR. The binding affinities of OXM and GCG to the hGCGR are shown graphically in Figure 6 and summarised in Table 3. Oxyntomodulin was shown to bind to the hGCGR with approximately 17-fold lower affinity than GCG (OXM $\text{IC}_{50} = 8.70 \pm 0.64$ nM, GCG $\text{IC}_{50} = 0.50 \pm 0.02$ nM). Glucagon-like peptide-1 and EX-4 did not displace ^{125}I -GCG at the concentration tested (EX-4 $\text{IC}_{50} > 10000$ nM, GLP-1 $\text{IC}_{50} > 10000$ nM).

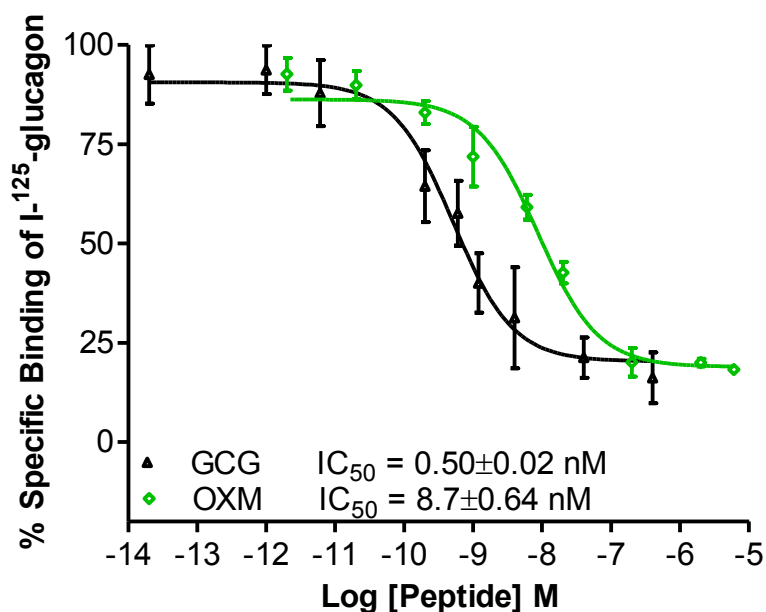


Figure 6: Competitive receptor binding affinity of GCG and OXM to the hGCGR. Purified cell membranes of CHO cells overexpressing the hGCGR were the source of the receptors. ^{125}I -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 5 OXM analogues (X1-X5) to the hGCGR were tested and compared with endogenous ligands (Figure 7, Table 3).

Analogues X1 and X3 had a 1.8-fold lower affinity than GCG to the hGCGR respectively (X1 $IC_{50} = 0.90 \pm 0.09$ nM, X3 $IC_{50} = 0.89 \pm 0.24$ nM) (Figure 7). Analogues X2 and X4 had a 1.1-fold lower affinity than GCG respectively (X2 $IC_{50} = 0.55 \pm 0.03$ nM, X4 $IC_{50} = 0.45 \pm 0.07$ nM). Analogue X5 bound to the hGCGR with a 1.2-fold lower affinity than GCG (X5 $IC_{50} = 0.58 \pm 0.19$ nM).

Oxyntomodulin presented a 9.7- and 9.8-fold lower affinity to the hGCGR when compared to analogues X1 and X3 respectively. Affinity of oxyntomodulin to the hGCGR was 15-, 15.8 and 19.3-fold weaker than analogues X5, X2 and X4 respectively.

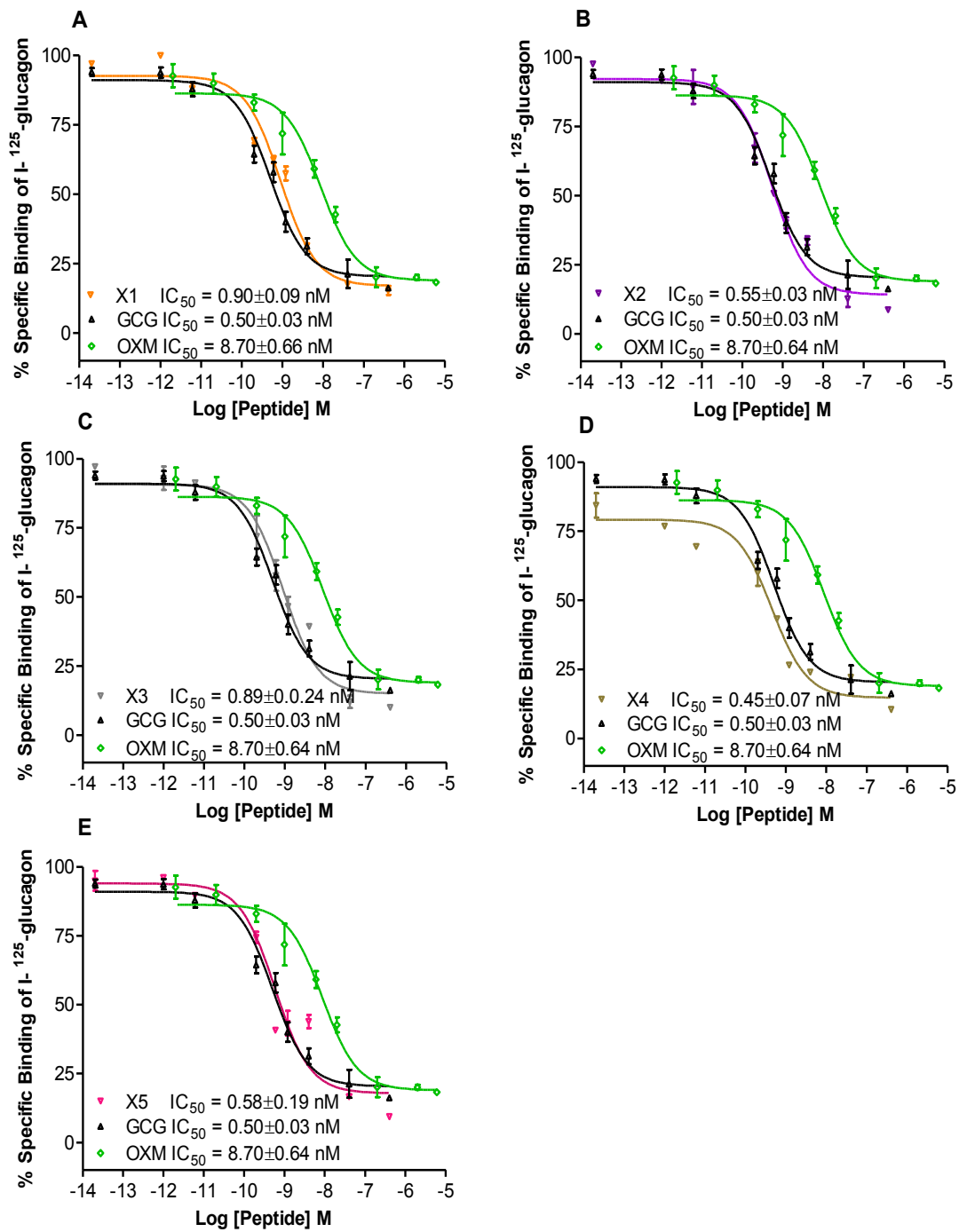


Figure 7: Competitive receptor binding affinity of GCG, OXM and 5 OXM analogues A: X1, B: X2, C: X3, D: X4, E: X5 to the hGCR. Purified cell membranes of CHO cells overexpressing the hGCR were the source of the receptors. I^{125} -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

4.3.1.1.2. Binding to membrane preparation of mouse liver cells overexpressing mouse glucagon receptor

Binding assays were completed as described in section 4.2.2.1.2. Binding studies with I^{125} -GCG routinely demonstrated greater than 90% specific binding to membranes prepared from mouse liver tissue. The binding affinities of GCG and OXM to the mGCGR are shown graphically in Figure 8 and summarised in Table 3. The affinity of OXM for the mGCGR was 4-fold lower than GCG (OXM IC_{50} = 5.73 ± 0.89 nM, GCG IC_{50} = 1.42 ± 0.06 nM). Exendin-4 and GLP-1 did not displace I^{125} -GCG at the concentration tested (EX-4 IC_{50} = >10000 nM, GLP-1 IC_{50} >10000 nM).

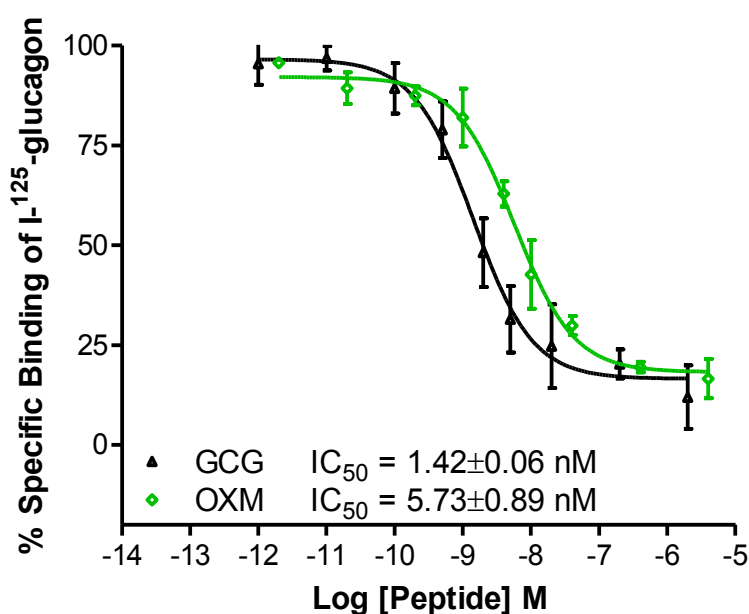


Figure 8: Competitive receptor binding affinity of GCG and OXM to the mGCGR. Cell membrane preparations from mouse liver overexpressing the mGCGR were the source of the receptors. I^{125} -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 5 OXM analogues (X1-X5) to the mGCGR were tested and compared with endogenous ligands (Figure 9, Table 3).

Analogues X1, X2 and X3 presented a 4-, 3.2- and 3.6-fold lower affinity to the mGCGR than GCG respectively (X1 IC_{50} = 5.73 ± 0.89 nM, X2 IC_{50} = 4.51 ± 0.26 nM, X3 IC_{50} = 5.06 ± 1.38 nM) (Figure 9). Analogues X4 and X5 presented a 2.9- and 2.6-fold lower affinity to the mGCGR than GCG respectively (X4 IC_{50} = 4.09 ± 0.85 nM, X5 IC_{50} = 3.75 ± 0.28 nM). When compared to OXM, analogue X1 which did not differ in its binding affinity to the mGCGR from OXM. Analogues X2 to X5 presented a higher affinity to the mGCGR than OXM that ranged from 1.1-fold for X3, 1.3-fold for X2, 1.4-fold for X4 and 1.5-fold for X5.

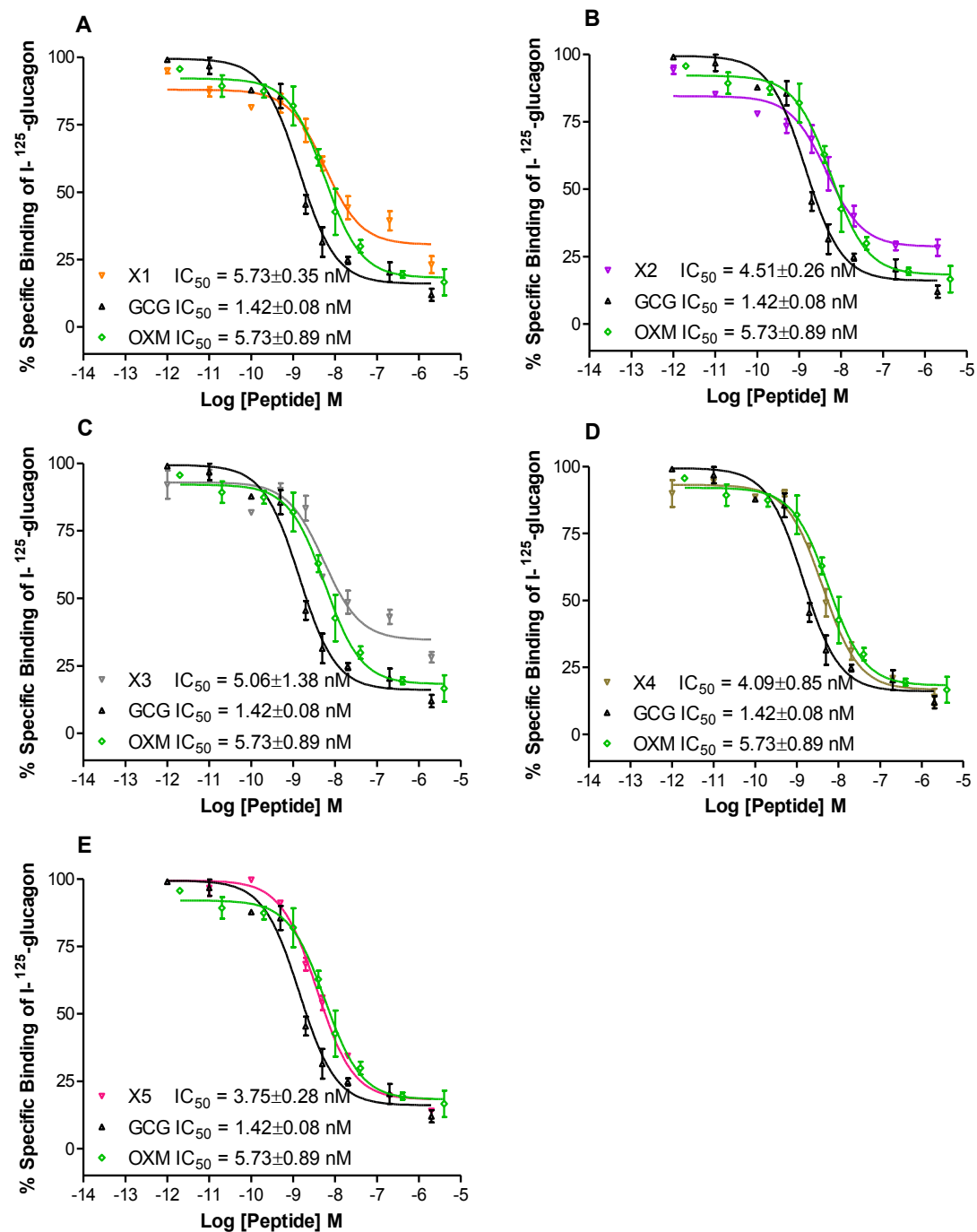


Figure 9: Competitive receptor binding affinity of GCG, OXM and 5 OXM analogues A: X1, B: X2, C: X3, D: X4, E: X5 to the mGCGR. Cell membrane preparations from mouse liver overexpressing the mGCGR were the source of the receptors. I^{125} -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

4.3.1.2. Binding affinities of oxyntomodulin, glucagon, GLP-1, exendin-4 and oxyntomodulin analogues to the GLP-1 receptor

4.3.1.2.1. Binding to membrane preparation of CHO cells overexpressing human GLP-1 receptor

Binding assays were completed as described in section 4.2.2.1.1. Binding studies using ^{125}I -GLP-1 routinely resulted in greater than 90% specific binding to a cell membrane preparation of CHO cells overexpressing the hGLP-1R. The binding affinities of EX-4, GLP-1 and OXM to the hGLP-1R are shown graphically in Figure 10 and summarised in Table 3. Oxyntomodulin was shown to bind to the hGLP-1R with approximately 431-fold lower affinity than GLP-1 (OXM $\text{IC}_{50} = 129.30 \pm 13.70$ nM, GLP-1 $\text{IC}_{50} = 0.30 \pm 0.02$ nM). Exendin-4 bound to the hGLP-1R with a similar affinity to GLP-1 (EX-4 $\text{IC}_{50} = 0.38 \pm 0.01$ nM). Glucagon did not displace ^{125}I -GLP-1 from the membrane at concentrations tested (GCG $\text{IC}_{50} > 10000$ nM).

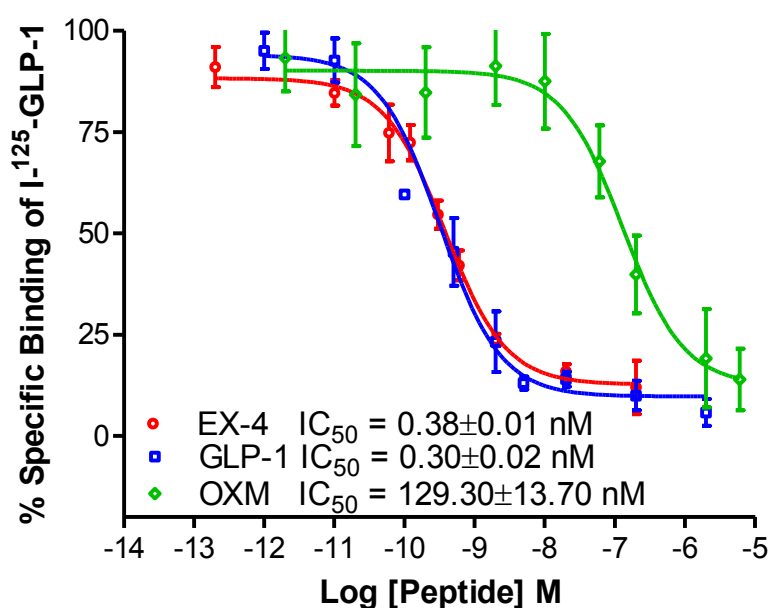


Figure 10: Competitive receptor binding affinity of EX-4, GLP-1 and OXM to the hGLP-1R. Purified cell membranes of CHO cells overexpressing the hGLP-1R were the source of the receptors. ^{125}I -GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 5 OXM analogues (X1-X5) to the hGLP-1R were tested and compared with the endogenous ligands (Figure 11, Table 3).

Analogues X1 and X2 had approximately a 24.3-fold and 15.5-fold lower affinity respectively, than GLP-1 (X1 IC_{50} = 7.28±0.72 nM, X2 IC_{50} = 4.66±0.01 nM) (Figure 11). Analogues X3 to X5 presented a 36-, 32.3- and 25.8-fold lower affinity to the hGLP-1R respectively when compared to GLP-1 (X3 IC_{50} = 10.80±3.49 nM, X4 IC_{50} = 9.70±0.74 nM, X5 IC_{50} = 7.73±0.96 nM).

Analogues X1 to X5 presented a higher binding affinity to the hGLP-1R when compared to OXM that ranged from 12-fold for X3, 13.3-fold for X4, 16.7-fold for X5, 17.8-fold for X1 and 27.7-fold for X2.

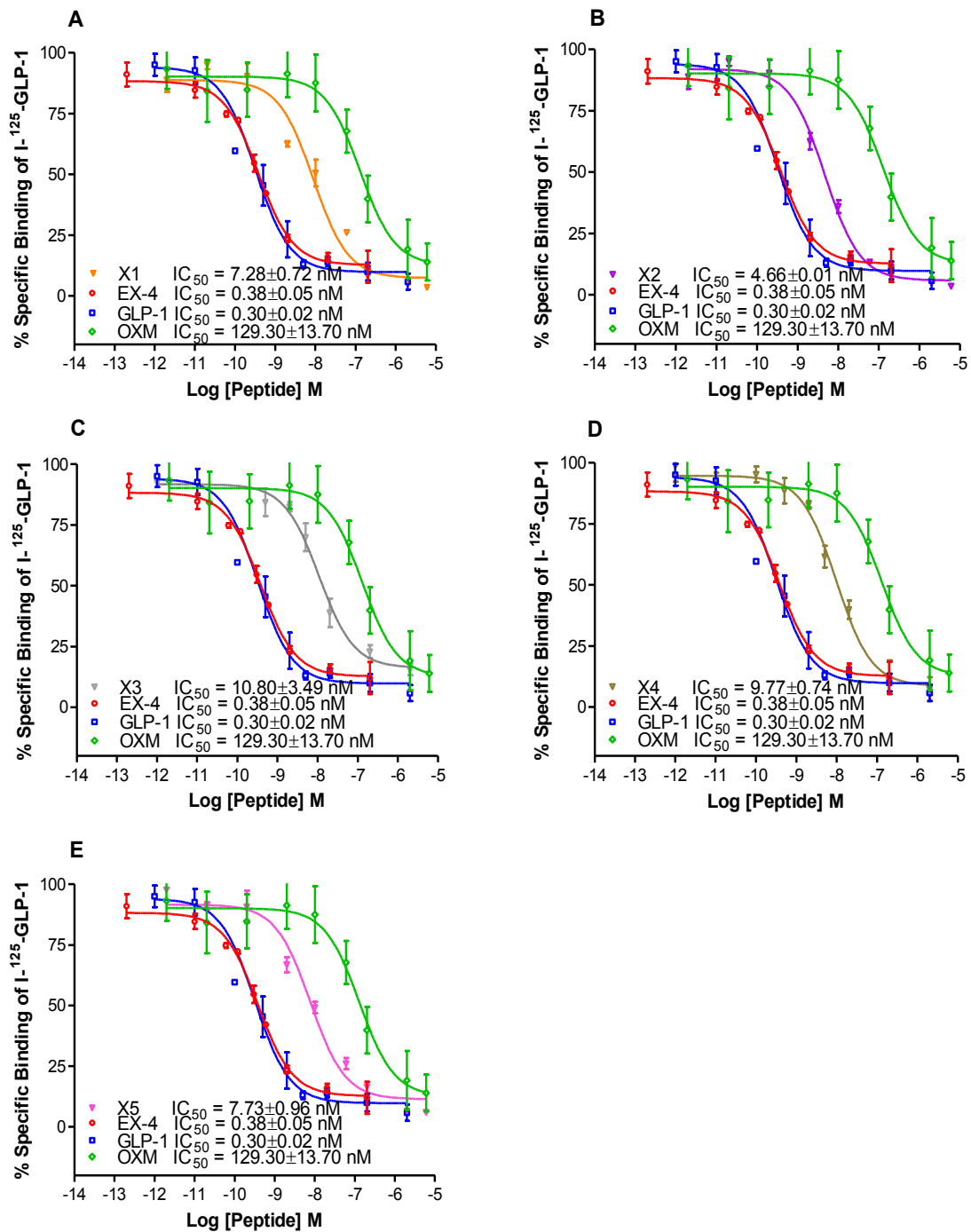


Figure 11: Competitive receptor binding affinity of EX-4, GLP-1, OXM and 5 OXM analogues A: X1, B: X2, C: X3, D: X4, E: X5 to the hGLP-1R. Purified cell membranes of CHO cells overexpressing the hGLP-1R were the source of receptors. ^{125}I -GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

4.3.1.2.2. Binding to membrane preparation of mouse lung cells overexpressing mouse GLP1 receptor

Binding assays were completed as described in section 4.2.2.1.2. Binding studies with I^{125} -GLP-1 have routinely shown greater than 90% specific binding to membranes prepared from mouse lung tissue. The binding affinities of EX-4, GLP-1, GCG and OXM to the mGLP-1R are shown graphically in Figure 12 and summarised in Table 3. The affinity of EX-4 to the mGLP-1R was 2-fold lower approximately than GLP-1 (EX-4 IC_{50} = 4.94 ± 0.13 nM, GLP-1 IC_{50} = 2.32 ± 0.25 nM). Oxyntomodulin bound to the mGLP-1R with an 18-fold lower affinity in comparison to GLP-1, while GCG was 95-fold less efficient in binding to the mGLP-1R (OXM IC_{50} = 41.36 ± 16.53 nM, GCG IC_{50} = 220.0 ± 17.90 nM). Oxyntomodulin had an 8.4-fold lower affinity to the mGLP-1R when compared with EX-4.

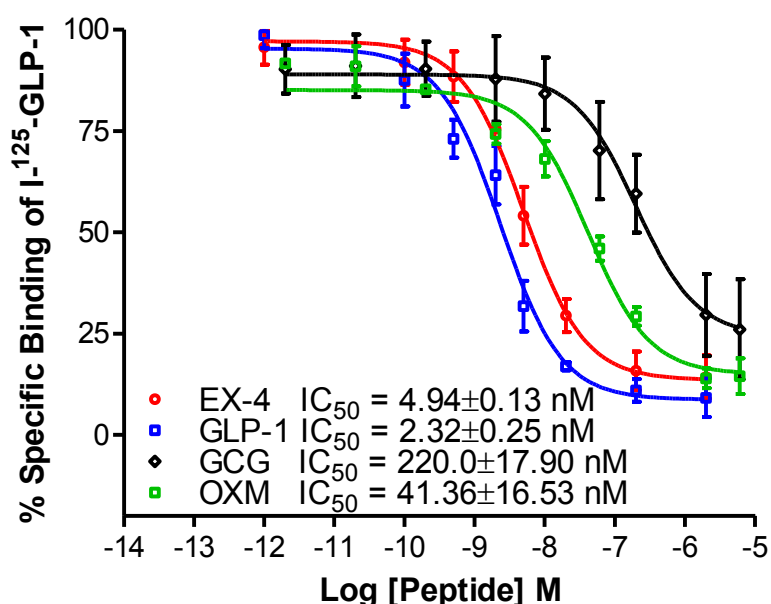


Figure 12: Competitive receptor binding affinity of EX-4, GLP-1, GCG and OXM to the mGLP-1R. Cell membrane preparations from mouse lung overexpressing the mGLP-1R were the source of the receptors. I^{125} -GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 5 OXM analogues (X1-X5) to the mGLP-1R were tested and compared with the endogenous ligands (Figure 13, Table 3).

Analogues X1, X3 and X4 presented a 4.3- to 5.1-fold lower affinity to the mGLP-1R than GLP-1 (X1 IC_{50} = 9.88 ± 2.32 nM, X3 IC_{50} = 11.87 ± 0.87 nM, X4 IC_{50} = 10.59 ± 2.20 nM) (Figure 13). Additionally, analogues X2 and X5 were shown to have a 2.7- and 3-fold fold lower affinity respectively to the mGLP-1R than GLP-1 (X2 IC_{50} = 6.32 ± 0.69 nM, X5 IC_{50} = 6.89 ± 0.44 nM).

When compared to OXM, analogues X1 to X5 bound to the mGLP-1R with a higher affinity that ranged from 3.5-fold for X3, 3.9-fold for X4, 4.2-fold for X1, 6-fold for X5 and 6.5-fold for X2.

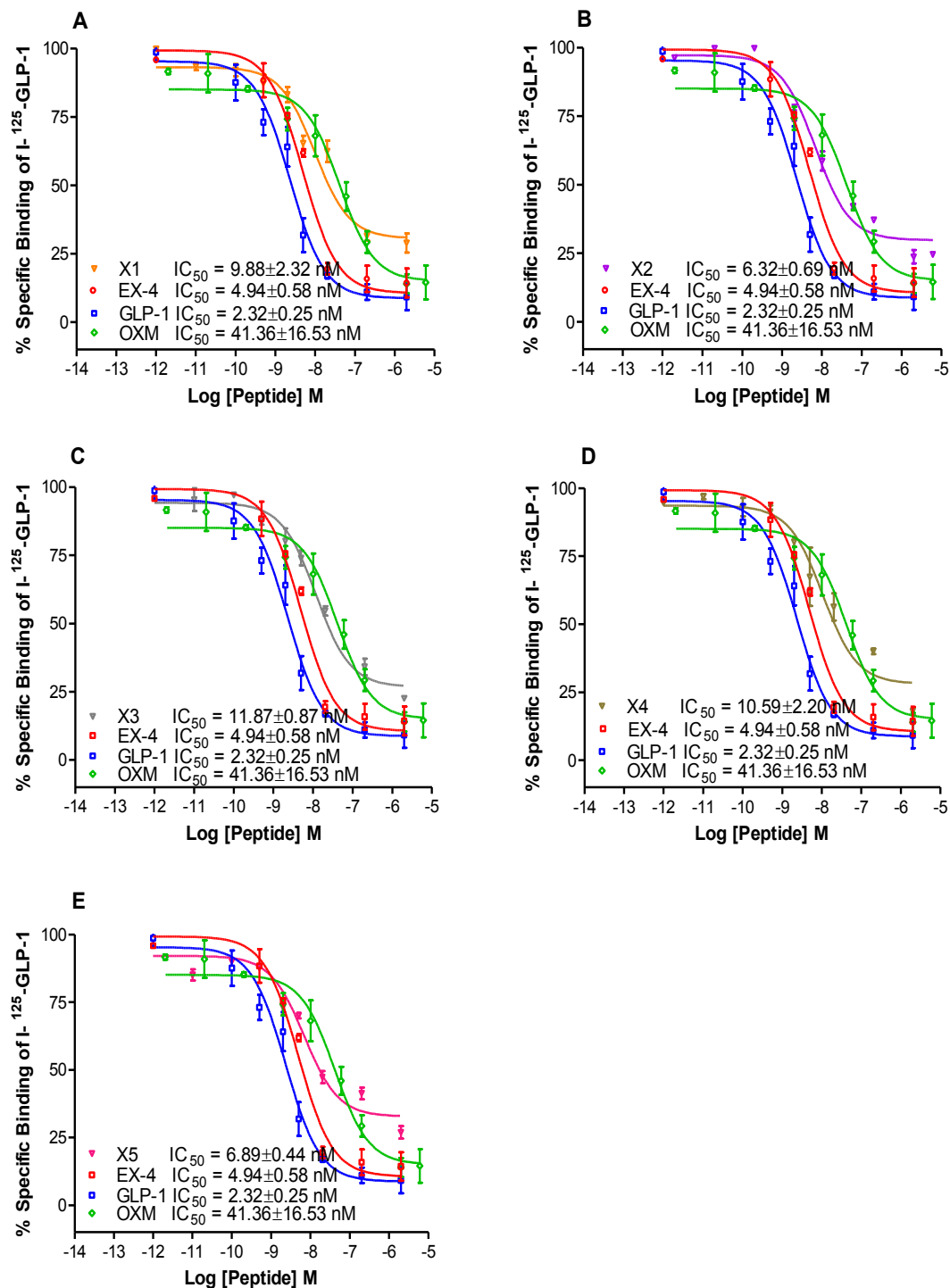


Figure 13: Competitive receptor binding affinity of EX-4, GLP-1, OXM and 5 OXM analogues A: X1, B: X2, C: X3, D: X4, E: X5 to the mGLP-1R. Cell membrane preparations from mouse lung overexpressing the mGLP-1R were the source of the receptors. I^{125} -GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

	GCGR		GLP-1R	
	hGCGR binding affinity IC ₅₀ (nM) ± SEM	mGCGR binding affinity IC ₅₀ (nM) ± SEM	hGLP-1R binding affinity IC ₅₀ (nM) ± SEM	mGLP-1R binding affinity IC ₅₀ (nM) ± SEM
EX-4	>10000	>10000	0.38±0.01	4.94±0.13
GLP-1	>10000	>10000	0.30±0.02	2.32±0.25
GCG	0.50±0.02	1.42±0.06	>10000	220.0±17.90
OXM	8.70±0.64	5.73±0.89	129.30±13.70	41.36±16.53
X1	0.90±0.09	5.73±0.35	7.28±0.72	9.88±2.32
X2	0.55±0.03	4.51±0.26	4.66±0.01	6.32±0.69
X3	0.89±0.24	5.06±1.38	10.80±3.49	11.87±0.87
X4	0.45±0.07	4.09±0.85	9.70±0.74	10.59±2.20
X5	0.58±0.19	3.75±0.28	7.73±0.96	6.89±0.44

Table 3: A summary of IC₅₀ values for EX-4, GLP-1, GCG, OXM and OXM analogues X1-X5 at both the GCGR and GLP-1R (human and mouse receptors). Half-maximal concentrations values are shown as mean ± SEM.

4.3.2. Effect of oxyntomodulin, glucagon, GLP-1, exendin-4 and oxyntomodulin analogues on the human glucagon receptor-mediated cAMP accumulation

The ability of OXM, GCG, GLP-1 and EX-4 to stimulate cAMP accumulation in CHO cells overexpressing the hGCGR was investigated. The potencies of OXM, GCG, GLP-1 and EX-4 are shown graphically in Figure 14 and summarised in Table 4. The bioactivity of OXM at the hGCGR was 8.5-fold lower than GCG (OXM EC_{50} = 0.441 ± 0.319 nM, GCG EC_{50} = 0.052 ± 0.010 nM). Glucagon-like peptide-1 presented a >2200-fold lower bioactivity at the hGCGR when compared with GCG, while EX-4 did not activate the hGCGR (GLP-1 EC_{50} = 114.50 ± 17.30 nM, EX-4 EC_{50} = >10000 nM).

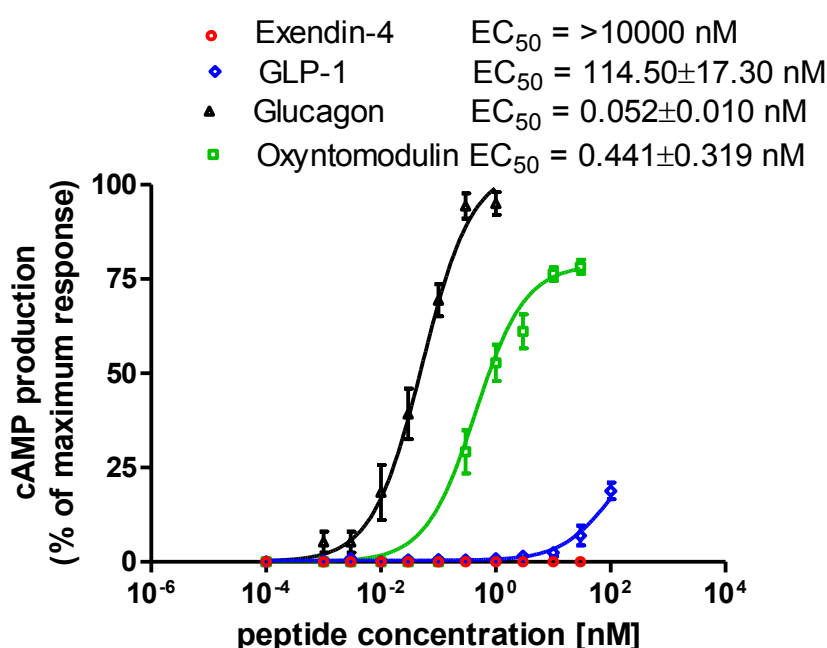


Figure 14: hGCGR-mediated cAMP accumulation by EX-4, GLP-1, GCG and OXM. hGCGR overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

The *in vitro* potencies of 5 OXM analogues (X1 to X5) at stimulating cAMP accumulation at the hGCGR were compared with GLP-1, GCG and OXM (Figure 15, Table 4).

The bioactivity of analogues X1 and X3 at the hGCGR was 2.5- and 1.4-fold lower than GCG (X1 EC_{50} = 0.129 ± 0.020 nM, X3 EC_{50} = 0.0705 ± 0.017 nM) (Figure 15). Analogue X2 demonstrated an approximately 4.7-fold higher bioactivity than GCG (X2 EC_{50} = 0.011 ± 0.002 nM). Analogues X4 and X5 presented a 5.2- and 6.5-fold higher bioactivity respectively when stimulating the hGCGR compared to GCG (X4 EC_{50} = 0.008 ± 0.002 nM, X5 EC_{50} = 0.010 ± 0.001 nM). Analogues X1 to X5 demonstrated a

higher bioactivity at the hGCGR compared to OXM that ranged from 3.4-fold for X1, 6.2-fold for X3, 40.1-fold for X2, 44.1-fold for X5 to 55.1-fold for X4.

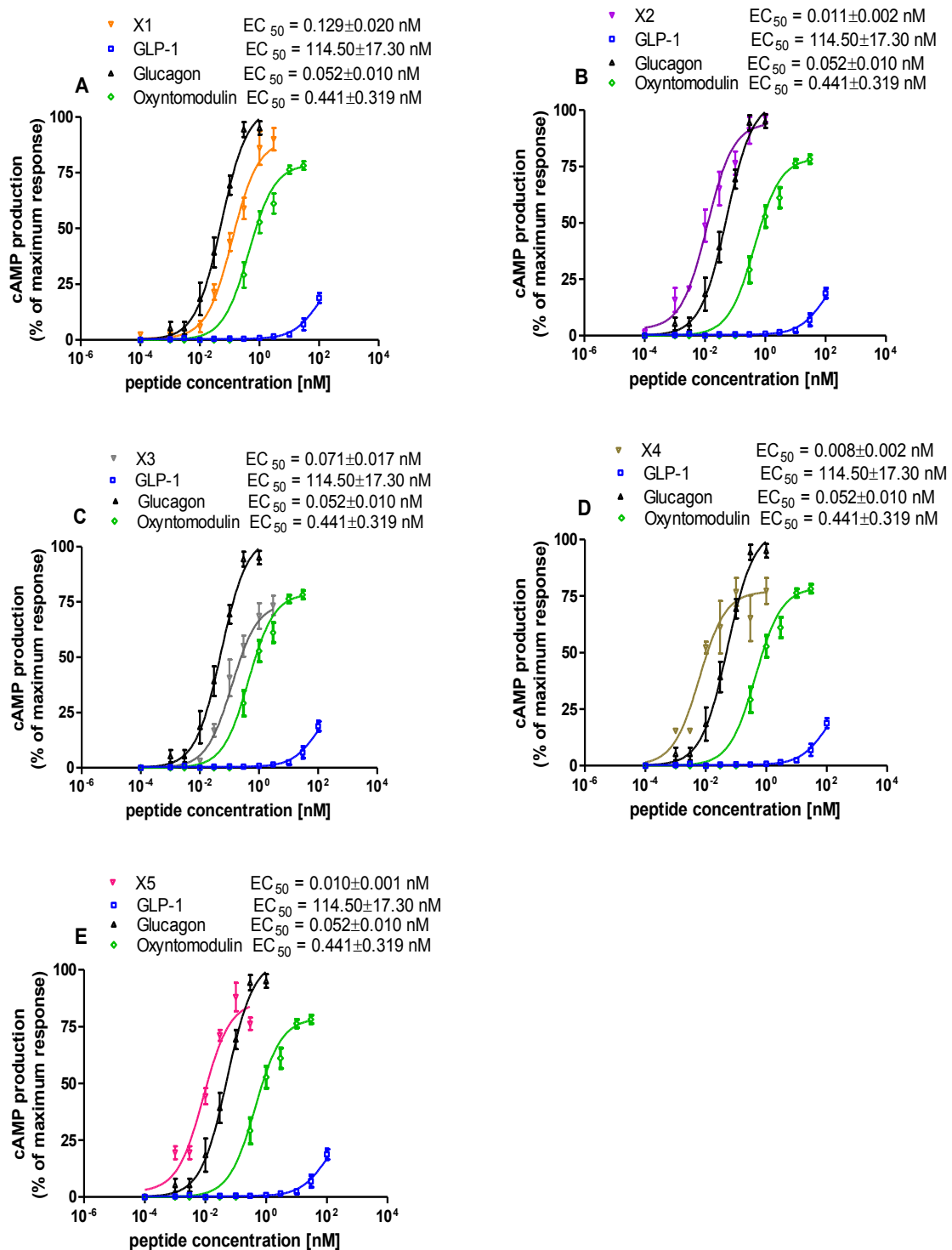


Figure 15: hGCGR-mediated cAMP accumulation by GLP-1, GCG and OXM and 5 OXM analogues A: X1, B: X2, C: X3, D: X4, E: X5. hGCGR overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

4.3.3. Effect of oxyntomodulin, glucagon, GLP-1, exendin-4 and oxyntomodulin analogues on the human GLP-1 receptor mediated cAMP accumulation

The ability of OXM, GCG, GLP-1 and EX-4 to stimulate cAMP accumulation in CHO cells overexpressing the hGLP-1R was investigated. The potencies of OXM, GCG, GLP-1 and EX-4 are shown graphically in Figure 16 and summarised in Table 4. The bioactivity of OXM at the hGLP-1R was 13-fold lower approximately than GLP-1 (OXM EC_{50} = 6.858 ± 1.620 nM, GLP-1 EC_{50} = 0.536 ± 0.010 nM). Glucagon presented a 337-fold lower bioactivity at the hGLP-1R (GCG EC_{50} = 180.70 ± 14.510 nM) when compared to GLP-1, while EX-4 had an almost 1.7-fold higher bioactivity than GLP-1 (EX-4 EC_{50} = 0.313 ± 0.030 nM).

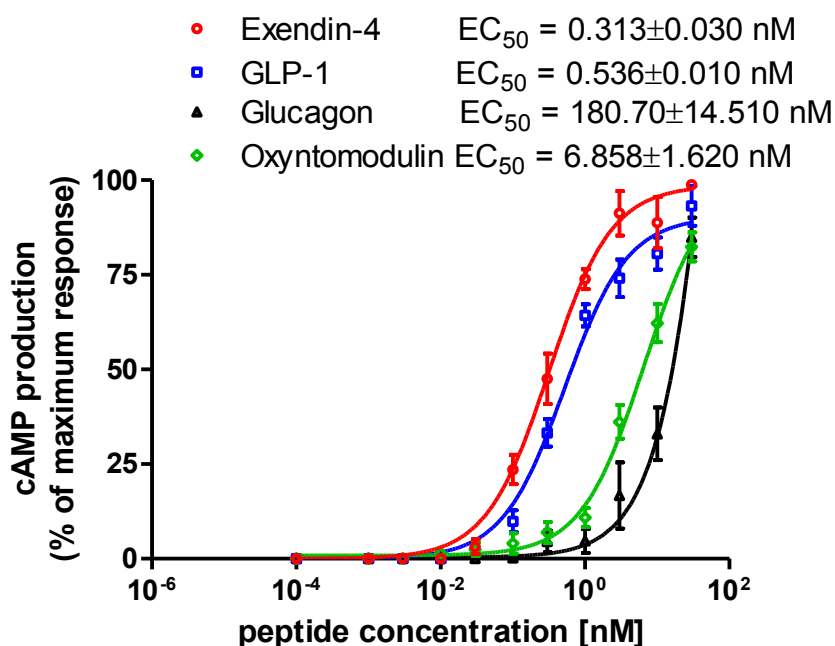


Figure 16: hGLP-1R-mediated cAMP accumulation by EX-4, GLP-1, GCG and OXM. hGLP-1R overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

The *in vitro* potencies of 5 OXM analogues (X1 to X5) at stimulating cAMP accumulation at the hGLP-1R were compared with GLP-1, GCG and OXM (Figure 17, Table 4).

The bioactivity of analogues X1 and X2 at the hGLP-1R were 6.7- and 2.7-fold lower than GLP-1 respectively (X1 EC_{50} = 3.591±0.330 nM, X2 EC_{50} = 1.450±0.180 nM) (Figure 17). Analogue X3 stimulated a cAMP response at the hGLP-1R with a potency approximately 8.2-fold lower than GLP-1 (X3 EC_{50} = 4.420±0.380 nM), whereas analogues X4 and X5 presented a 5.3- and 4.1-fold lower bioactivity at the hGLP-1R respectively compared to GLP-1 (X4 EC_{50} = 2.862±0.690 nM, X5 EC_{50} = 2.224±0.70 nM).

Analogues X1 to X5 demonstrated a higher bioactivity at the hGLP-1R when compared to OXM that ranged from 1.6 fold for X3, 1.9-fold for X1, 2.4-fold for X4, 3.1-fold for X5 and 4.7-fold for X2.

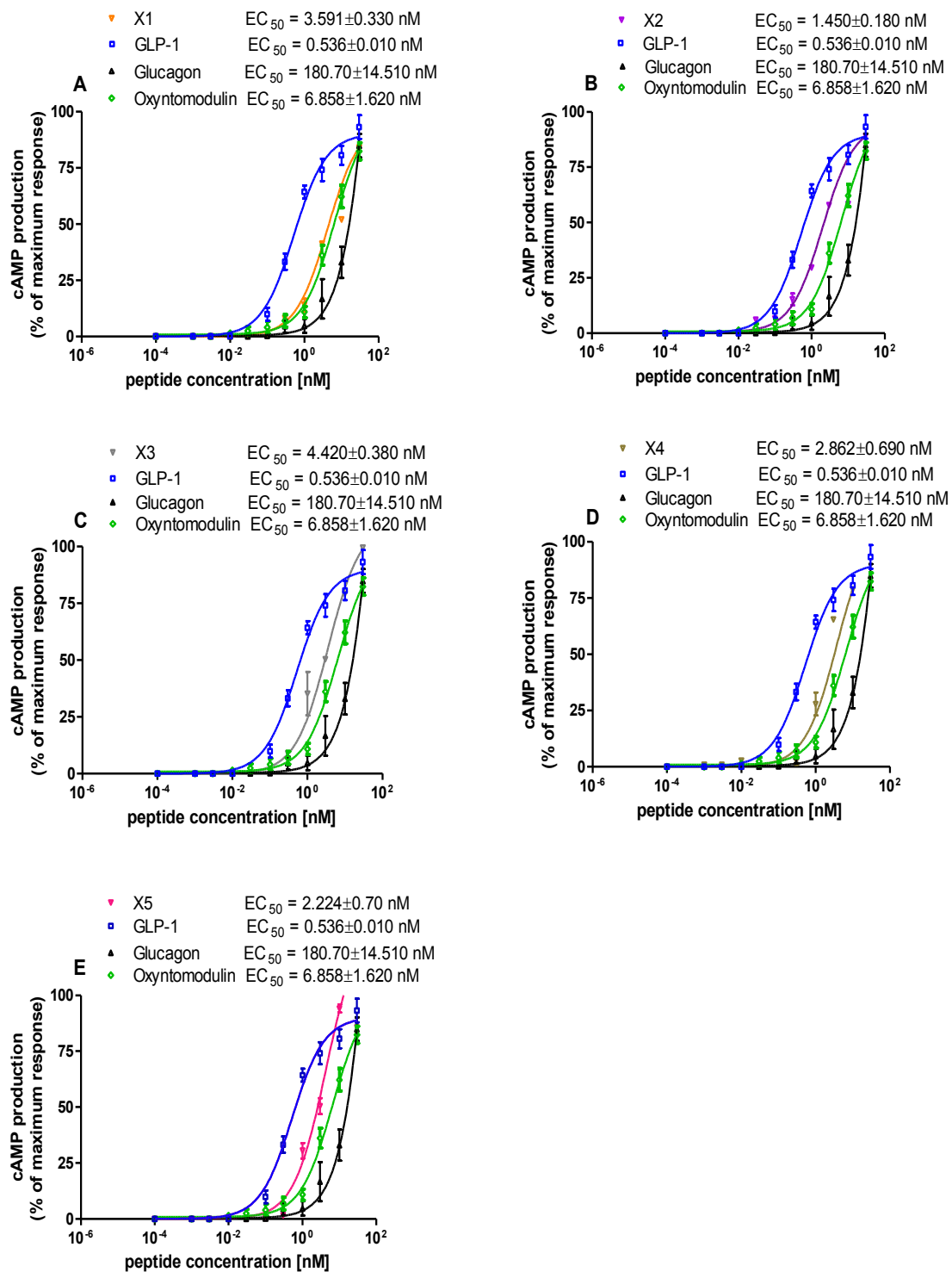


Figure 17: hGLP-1R-mediated cAMP accumulation by GLP-1, GCG and OXM and 5 OXM analogues A: X1, B: X2, C: X3, D: X4, E: X5. hGLP-1R overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

	hGCGR	hGLP-1R
	cAMP bioactivity EC ₅₀ (nM) ± SEM	cAMP bioactivity EC ₅₀ (nM) ± SEM
EX-4	>10000	0.313±0.030
GLP-1	114.50±17.30	0.536±0.010
GCG	0.052±0.010	180.70±14.510
OXM	0.441±0.319	6.858±1.620
X1	0.129±0.020	3.591±0.330
X2	0.011±0.002	1.450±0.180
X3	0.071±0.017	4.420±0.380
X4	0.008±0.002	2.862±0.690
X5	0.010±0.001	2.224±0.70

Table 4: Summary of EC₅₀ values for EX-4, GLP-1, GCG, OXM and OXM analogues X1-X5 at both the hGCGR and hGLP-1R. Half-maximal concentrations values are shown as mean ± SEM.

4.3.4. Effect of exendin-4 and oxyntomodulin analogues on food intake in fasted mice

Subcutaneous administration of EX-4 (10 nmol/kg) and 5 OXM analogues (X1 to X5) at 50 nmol/kg showed that all tested agents significantly reduced food intake to fasted DIO C57BL/6J mice over the first hour post-injection compared with the vehicle control group (Figure 18). At the period of 1-2 hours, EX-4 and all OXM analogues significantly reduced food intake in comparison to the vehicle control group. At the 2-4 hour interval, EX-4, X2, X4 and X5 continued to significantly inhibit food intake compared to the vehicle control group ($p<0.01$, $p<0.05$, $p<0.01$, $p<0.05$ respectively vs. control), while analogues X1 and X3 did not significantly differ from the vehicle control group. In the 4-8hr interval, EX-4 group consumed significantly less food than the vehicle control group ($p<0.05$ vs. control), and no significant difference was noted between the vehicle control group and other treatment groups. There were no significant differences in food intake between the vehicle control group and all other treatment groups in the 8-24 hour and 24-48 hour intervals.

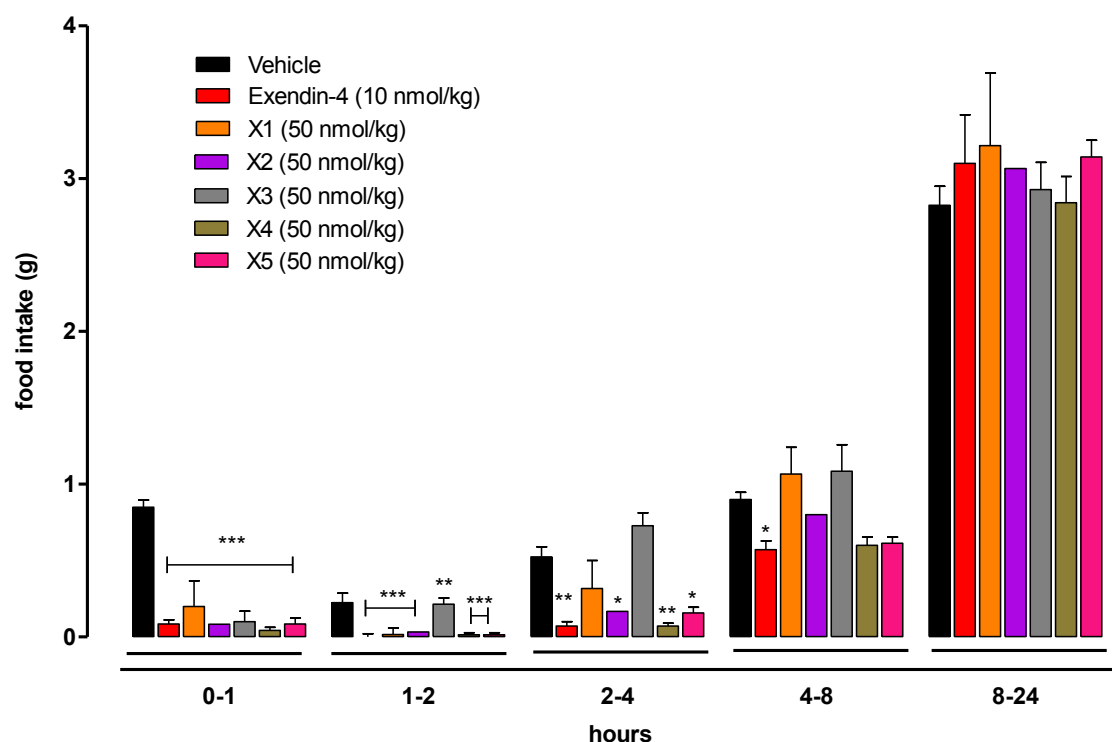


Figure 18: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X1-X5) (50 nmol/kg), on acute food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour and 8-24 hour intervals ($n=7-8$ per group). Statistics: One way ANOVA with Dunnett's post-hoc test $*=p<0.05$, $**=p<0.01$, $***=p<0.001$ vs. vehicle.

Analysis of cumulative food intake data showed that both EX-4 (10 nmol/kg) and all OXM analogues (50 nmol/kg), significantly reduced food intake 0-2 hours post-injection compared with the vehicle control group (Figure 19). The groups did not present statistically significant differences in the inhibition of food intake when they were compared against each other.

Similarly, cumulative food intake at 0-4 hours post-injection was significantly reduced in all OXM analogues groups and EX-4 when compared to the vehicle control group (Figure 20). Test groups X1 and X3 ate significantly more at 0-4 hours post-injection compared with EX-4 ($p<0.01$ and $p<0.001$ respectively). X3 test group ate significantly more, and test group X4 ate significantly less at 0-4 hours post-injection compared to analogue X1 ($p<0.05$ and $p<0.01$ respectively). Cumulative food intake of test groups X2, X4 and X5 during the 0-4 hour period was significantly less than the X3 test group ($p<0.001$ for all three groups).

Cumulative food intake data over 48 hours failed to demonstrate a significant reduction of food intake for OXM analogues and EX-4 compared to the vehicle control group (Figure 21). When the OXM analogues were compared to EX-4, X3 group consumed significantly more food over 48 hours while the rest of the analogues did not differ.

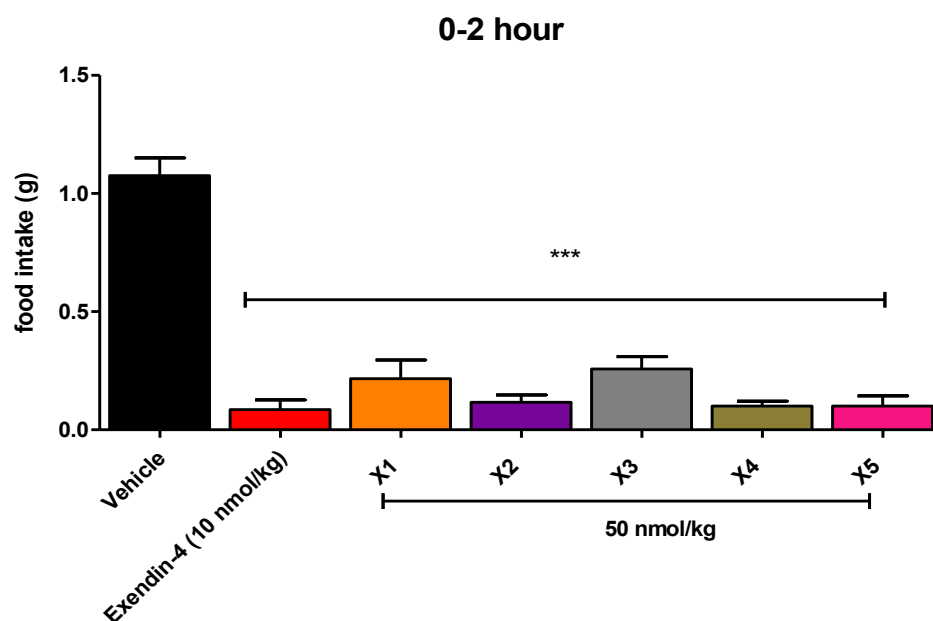


Figure 19: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X1-X5) (50 nmol/kg) on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-2 hours ($n=7-8$ mice per group). Statistics: One way ANOVA with Tukey's post-hoc test ***= $p<0.001$ vs. vehicle.

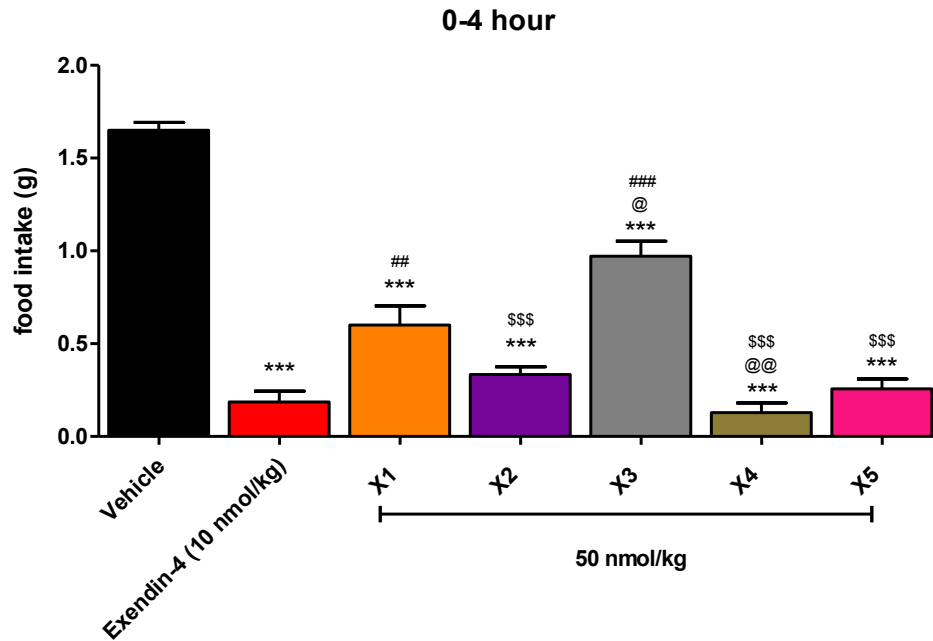


Figure 20: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X1-X5) (50 nmol/kg) on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-4 hours (n=7-8 mice per group). Statistics: One way ANOVA with Tukey's post-hoc test ***=p<0.001 vs. vehicle; ###=p<0.01, ###=p<0.001 vs. EX-4; @=p<0.05, @@=p<0.01 vs. X1; \$\$\$=p<0.001 vs. X3.

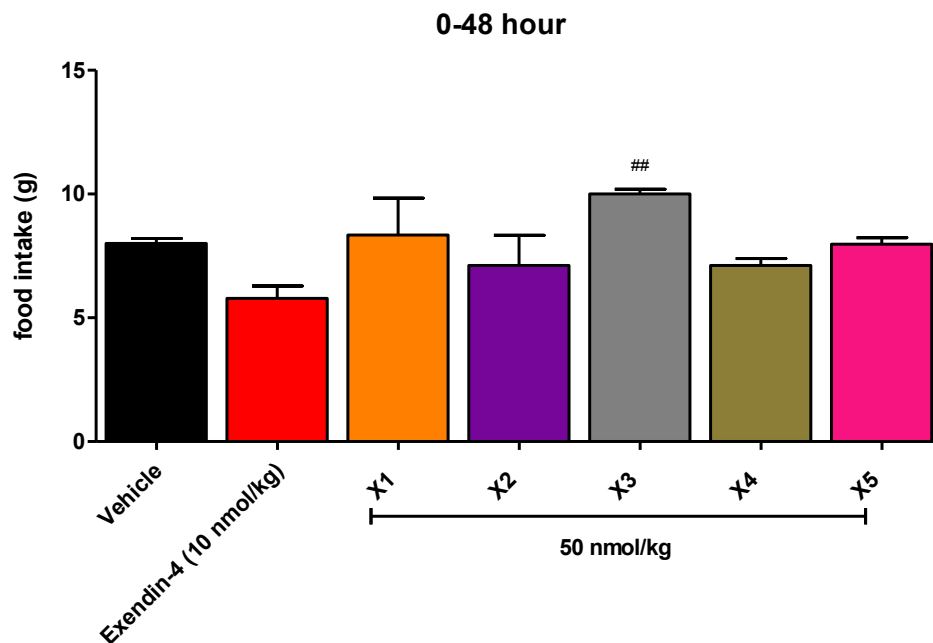


Figure 21: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X1-X5) (50 nmol/kg) on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-48 hours (n=7-8 mice per group). Statistics: One way ANOVA with Tukey's post-hoc test ##=p<0.01 vs. EX-4.

Investigation of the body weight changes that occurred between the period of -16 to 48 hours demonstrated that mice treated with EX-4 and with analogue X2 had a significant reduction in body weight when compared to the vehicle control group ($p<0.001$ and $p<0.01$ respectively) (Figure 22). The average body weight reduction over the 2 day period of the EX-4 group was 2.50 ± 0.50 g compared to vehicle-treated controls; X2 group lost 1.70 ± 0.50 g.

When the body weight change of the OXM analogues treated groups of mice was compared to the respective change of the EX-4 group, it was shown that test groups X2, X4 and X5 did not present a significantly lower body weight change ($p>0.05$ for all 3 groups) over the 2 day period. Mice treated with analogues X1 and X3 caused the least amount of body weight change when compared to the EX-4 group ($p<0.05$ for both groups) over the 2 day period.

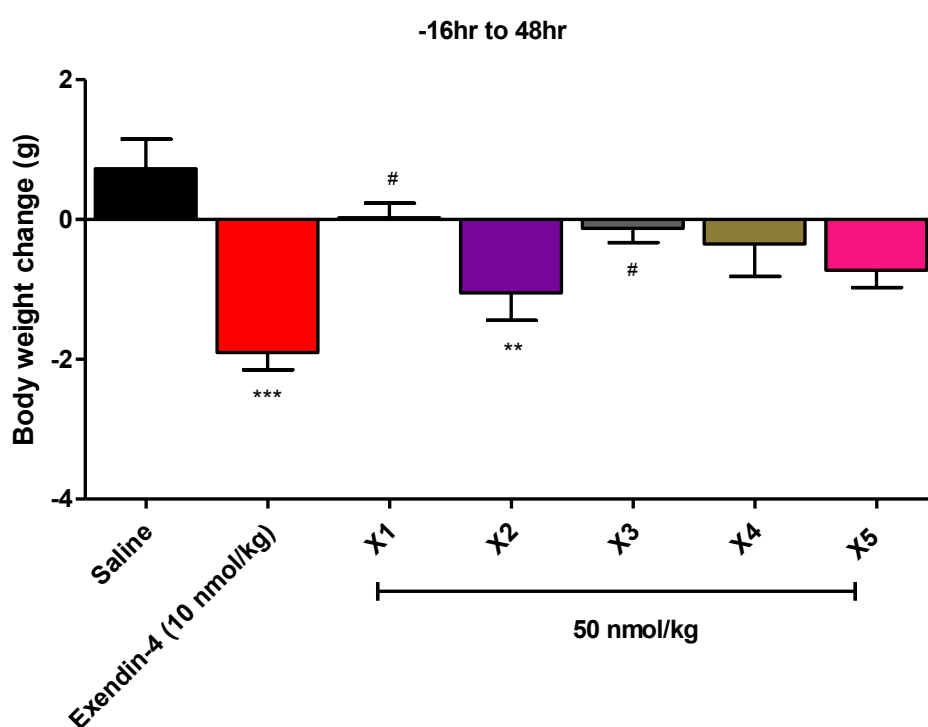


Figure 22: Body weight change over a 2 day period following a single subcutaneous dose of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X1-X5) (50 nmol/kg). Body weight change measured from pre-fasting (-16 hr) to 48 hr post dosing. Error bars are $\pm$ SEM. Statistics: One way ANOVA with Bonferroni's post-hoc test **= $p<0.01$, ***= $p<0.001$ vs. vehicle; #= $p<0.05$ vs. EX-4.

4.3.5. Investigation of the pharmacokinetic properties of oxyntomodulin and oxyntomodulin analogues X1 to X5 in an *in vivo* constant infusion model

The time-courses of the mean plasma concentrations of analogues X1 to X5 during IV primed-continuous peptide infusion were measured by RIA (section 2.8). Steady-state concentration levels were observed between 30 and 60 minutes after the start of the infusion of 9 nmol/hr of peptide. The PK parameters and the steady-state concentration of analogues X1 to X5 are shown graphically in Figure 26 and presented in Table 5.

	Metabolic clearance rate (ml/min/kg)	Half-life (min)	Volume of distribution (ml/kg)	Steady state concentration (pmol/ml)
OXM	12.8±3.2	12.0±2.1	231.6±94.0	19.7±5.8
X1	8.8±2.5	3.7±0.3	48.8±17.0	34.3±2.2
X2	3.2±0.9	6.5±0.6	28.0±7.4	41.1±2.2
X3	6.5±0.8	3.8±0.2	33.8±2.1	19.5±1.3
X4	13.9±2.8	3.9±0.4	57.7±10.3	28.7±5.1
X5	13.6±4.7	5.2±0.8	107.7±51.1	23.7±10.6

Table 5: Pharmacokinetic parameters (MCR, Half-life and Volume of distribution) and the steady-state concentration of OXM and OXM analogues (X1-X5) during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean ± S.E.M.

Analogue X2 presented the lowest MCR, and analogue X4 the highest (3.2±0.9 ml/min/kg and 13.9±2.8 ml/min/kg, respectively) (Figure 23). The MCR of the OXM analogues was not significantly different to the MCR of OXM (12.8±3.2 ml/min/kg), however, analogue X2 had a significantly lower MCR in comparison to X4 (p<0.01).

Analogue X2 presented the highest half-life of the OXM analogues tested (6.5±0.6 min), which was significantly higher when compared to the half-lives of analogues X1 (3.7±0.3 min), X3 (3.8±0.2 min) and X4 (3.9±0.4 min) (p<0.05, p<0.01 and p<0.01 respectively) (Figure 24). Analogue X5 presented a half-life of 5.2±0.8 min. All OXM analogues had a significantly lower half-life when compared to OXM (p<0.001).

The V_D of the OXM analogues ranged between 28.0±7.4 ml/kg (analogue X2) to 107.7±51.1 ml/kg (analogue X5), with no statistical significant differences between the OXM analogues groups (Figure 25). The V_D of all OXM analogues tested, was significantly lower than the V_D of OXM (p<0.001 for analogues X1 to X4 and p<0.05 for analogue X5).

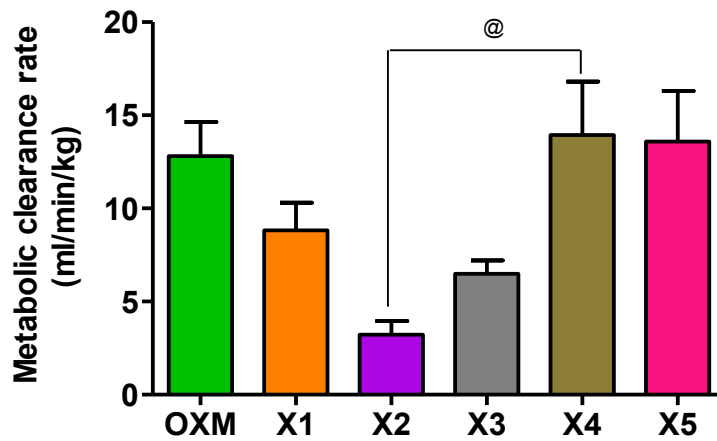


Figure 23: Metabolic clearance rate in ml/min/kg of OXM and OXM analogues X1-X5 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test, while comparisons between the OXM analogues were performed by one-way ANOVA with Bonferroni's correction test. @= $p < 0.05$ between OXM analogues.

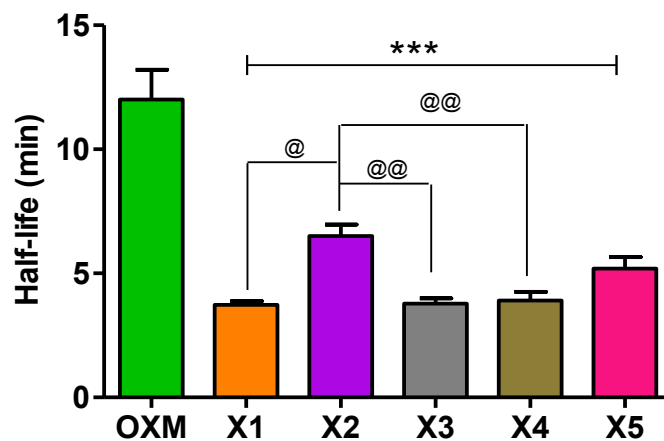


Figure 24: Half-life in minutes of OXM and OXM analogues X1-X5 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test, while comparisons between the OXM analogues were performed by one-way ANOVA with Bonferroni's correction test. ***= $p < 0.001$ vs. OXM; @= $p < 0.05$, @@= $p < 0.01$, @@@= $p < 0.001$ between OXM analogues.

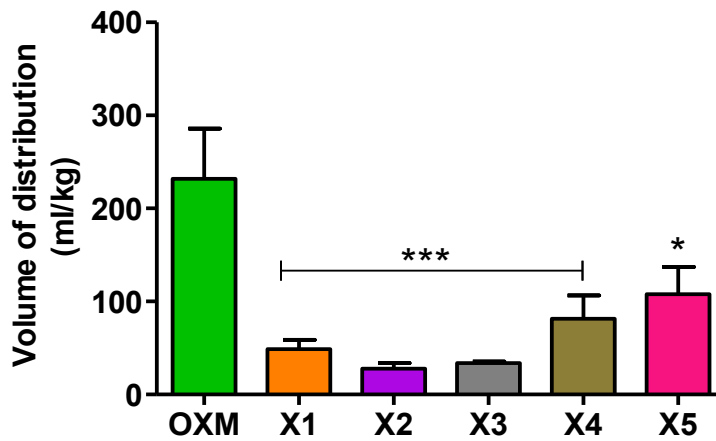


Figure 25: Volume of distribution in ml/kg of OXM and OXM analogues X1-X5 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dennett's correction test, while comparisons between the OXM analogues were performed by one-way ANOVA with Bonferroni's correction test. *=p<0.05, ***=p<0.001 vs. OXM.

The steady-state concentration levels of the analogues ranged between 19.5 ± 1.3 pmol/ml (analogue X3) to 41.1 ± 2.2 pmol/ml (analogue X2) (Figure 26). Analogue X1 reached a steady-state concentration of 34.3 ± 2.2 pmol/ml, while analogues X4 and X5 demonstrated a steady-state concentration level of 28.7 ± 5.1 pmol/ml and 23.7 ± 10.6 pmol/ml, respectively.

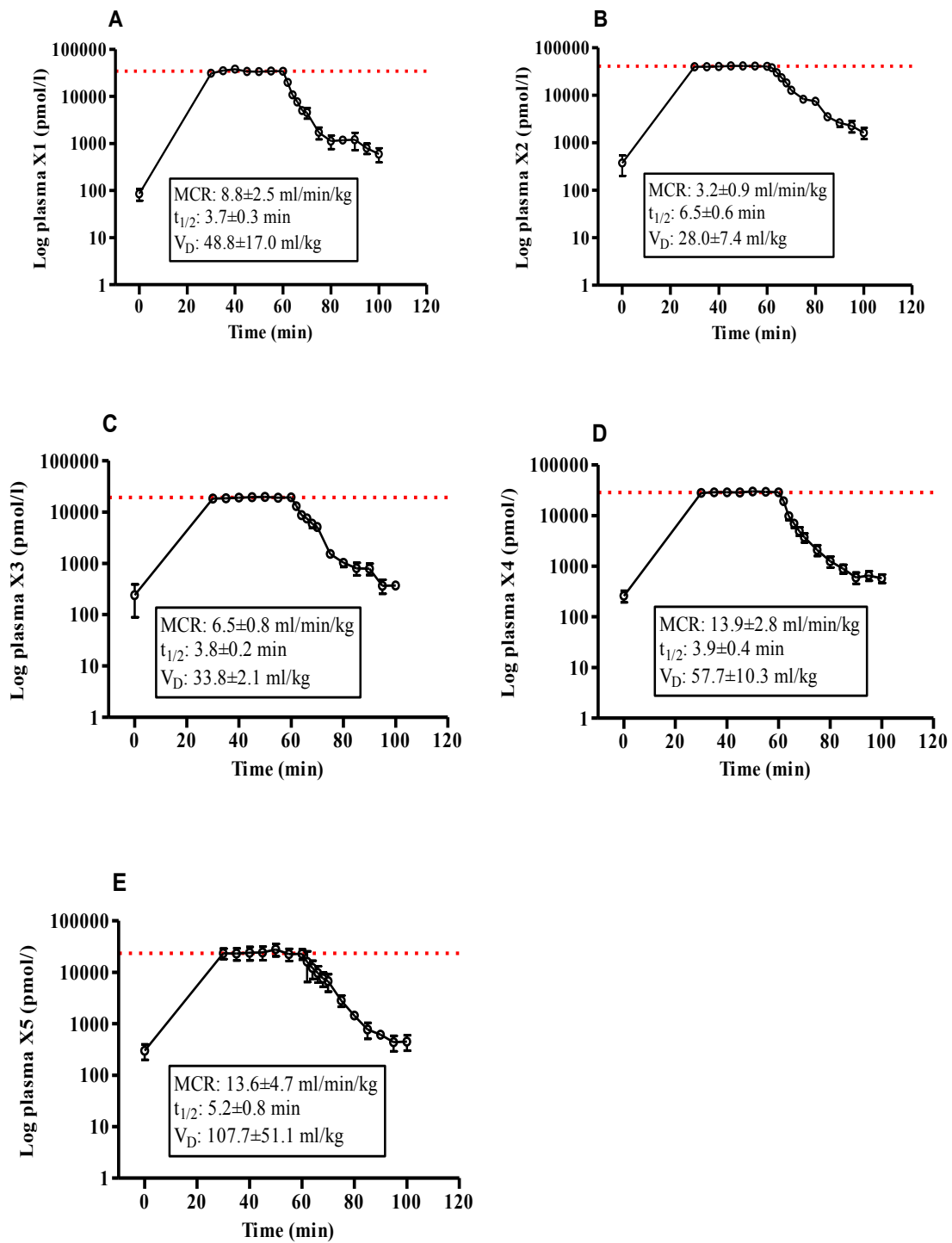


Figure 26: Plasma concentration of analogues X1-X5 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes ($n=4$). A: X1, B: X2, C: X3, D: X4, E: X5. Infusion begins at time-point “0” and lasts until “60” minutes, at which point the infusion is stopped. Values are shown as mean $\pm$ S.E.M. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

4.3.6. Investigation of the pharmacokinetic properties of exendin-4 and oxyntomodulin analogues X1 to X5 following single subcutaneous bolus injection in male Wistar rats

The PK profiles of EX-4 and OXM analogues X1 to X5, when administered as a 1 mg 1:1 molar ratio Zn:NaCl (saline) SC injection to male Wistar rats, were investigated (Figure 27, Figure 28). Time of maximal plasma levels (T_{max}) was 30 minutes post-injection for all peptides. Plasma levels of EX-4 and analogues X1 to X5 were 79.2%, 54%, 73.9%, 39%, 54.6% and 66% of peak recorded plasma level (C_{max}) respectively at 3 hours post-injection and had dropped further to 15.2%, 45%, 69.1%, 51.6%, 74.9% and 61.6% of C_{max} respectively by 24 hours post-injection. Exendin-4 and OXM analogues X1 to X5 were undetectable in plasma after 48 hours (LLOQ was 156 ± 22 , 248 ± 33 , 150 ± 11 , 342 ± 26 , 532 ± 57 , 223 ± 22 pmol/L respectively).

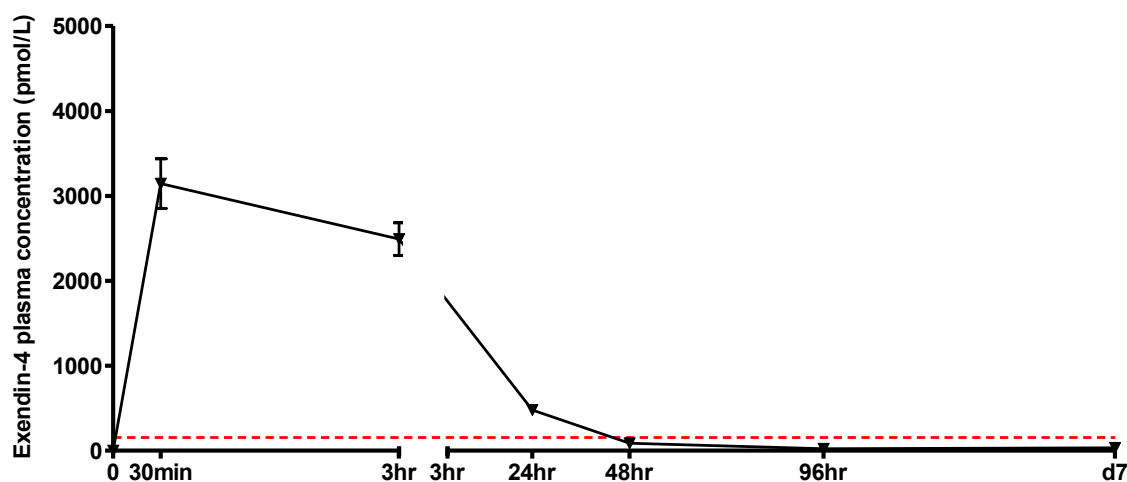


Figure 27: Line graphs illustrating the pharmacokinetic profile of exendin-4 after a single 1 mg SC injection of peptide. Peptide concentration measured in rat plasma using RIA and expressed as mean concentration $\pm$ SEM pmol/L ($n=4$). The lower limits of quantification (LLOQ) for the RIA are shown as horizontal dashed lines. Interpolated values which fall below this level are plotted.

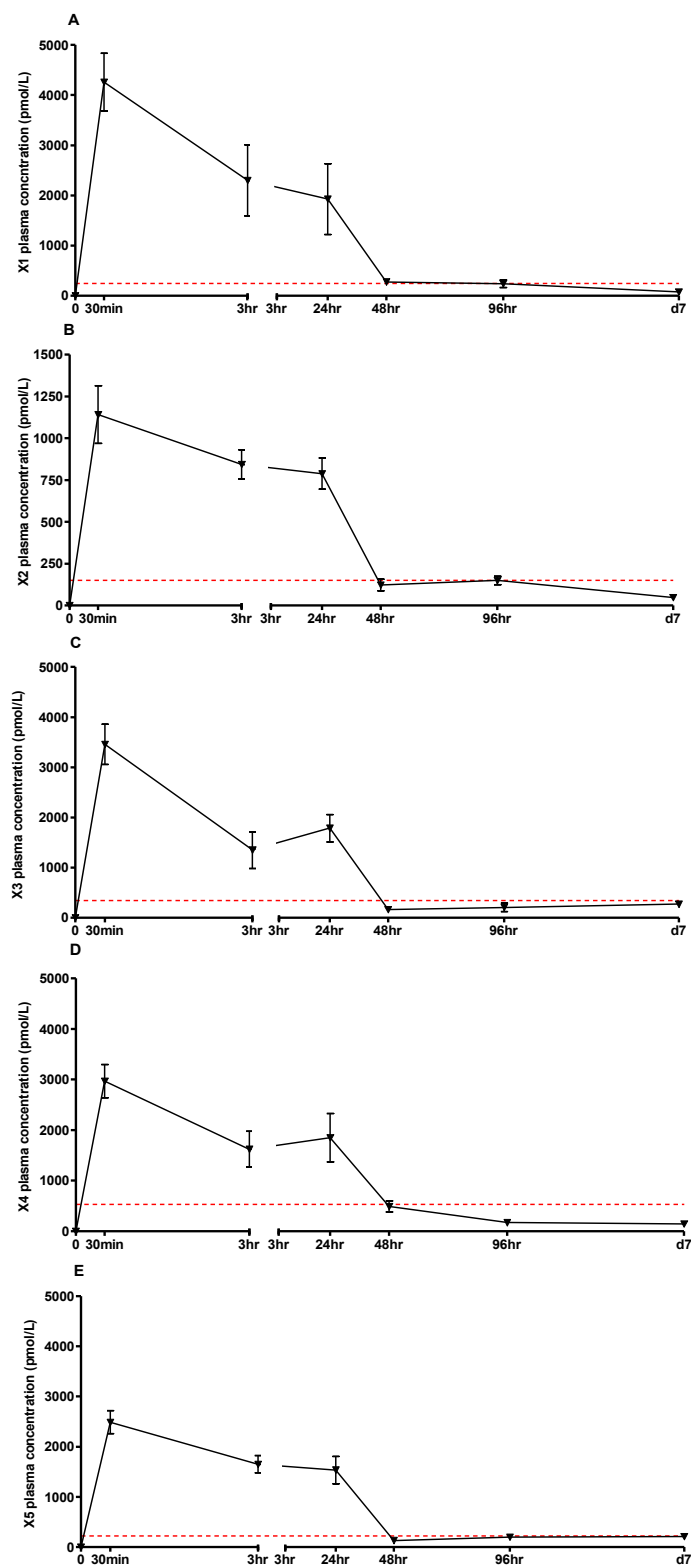


Figure 28: Line graphs illustrating the pharmacokinetic profile of A: X1, B: X2, C: X3, D: X4 and E: X5 after a single 1 mg SC injection of peptide (formulated in 1:1 Zn:NaCl slow release diluent). Peptide concentration measured in rat plasma using RIA and expressed as mean concentration $\pm$ SEM pmol/L (n=4). The lower limits of quantification (LLOQ) for each RIA are shown as horizontal dashed lines. Interpolated values which fall below this level are plotted.

4.4. Discussion

The aim of the work described in this chapter was to design an OXM analogue that would exhibit higher potency and prolonged period of action when compared to the native OXM. The strategy employed to achieve this goal included making point substitutions and C-terminal modifications in the primary structure of OXM. Overall, the analogues developed in this chapter demonstrated higher GLP-1R and GCGR affinity and bioactivity *in vitro* than native OXM, but not as good as native peptides. The PK characteristics of OXM analogues X1-X3 demonstrated a decrease in the MCR when compared to native OXM and a plasma concentration profile (when given subcutaneously) that mimicked the one of EX-4. The *in vivo* effects of analogues X1-X5 in an acute feeding study in mice showed a higher body weight loss when compared to the vehicle control group (only analogue X2 group reached significance levels in body weight loss).

As the overall goal of the project is to develop a long-lasting anti-obesity agent, one of the main endpoints is to assess its efficacy in inducing weight loss. Feeding studies to investigate these biological actions of the analogues were conducted in mice in order to confirm the translation of these effects in humans.

Receptor affinity studies were conducted at both the human and mouse GLP-1R and GCGR in order to ascertain translation of the animal data to humans. Competitive receptor binding assays are useful in investigating the affinity of a ligand for a specific receptor. However, receptor binding assays are unable to distinguish agonists from antagonists and thus cannot provide any information regarding the bioactivity of the ligand. Consequently, the results of receptor binding and bioactivity assays do not always correlate. It has been shown that different ligands with similar affinities for the same receptor can induce cell signalling cascades with different potencies (343). For that reason all OXM analogues were tested in both receptor binding and bioactivity assays and the results were compared. The EC_{50} of a molecule is related to its IC_{50} but does not reflect its magnitude as the receptor occupancy required for a maximal signalling response is generally well below 100% and will vary from receptor to receptor (343).

It is known that the major effector of GLP-1R-mediated action is the second messenger system of cAMP (269). Oxyntomodulin exerts its anorectic effect mainly through the GLP-1R while being a weak agonist of the GCGR, thus all OXM analogues designed in this study were tested for their potency at inducing intracellular accumulation of cAMP both at the GLP-1R and the GCGR. However, OXM was recently shown to be a full agonist in recruiting other second messenger pathways such as β -arrestin to the GCGR and GPCR kinase 2 to the GLP-1R (265). Thus, the cAMP presented here need to be

analysed with caution and in parallel to the *in vivo* mouse feeding study. It will be worthwhile to explore the actions of our analogues in these pathways once their role has been elucidated.

The OXM analogues tested in this chapter demonstrated a good correlation between the IC_{50} and EC_{50} both at the hGLP-1R and hGCGR; analogue X4 demonstrated the higher affinity and potency at the hGCGR and analogue X2 was the most potent and with the higher affinity at the hGLP-1R. Analogue X2 also presented the highest anorectic effect among analogues X1-X5 in the acute feeding in mice; interestingly it presented the highest binding affinity among OXM analogues in the mGLP-1R but not in the mGCGR. The lack of data concerning potency at stimulating cAMP accumulation at the mGLP-1R and mGCGR makes it challenging to directly link *in vivo* effects with binding assay results. Furthermore, there is another pillar which needs to be taken into account when trying to interpret *in vivo* effects and that is the PK properties of the analogues.

The above mentioned studies were designed to test the *in vitro* effects of the OXM analogues; at the same time of equal importance was the effort to produce an analogue with enhanced PK characteristics in comparison to OXM. The PK characteristics of each analogue were measured in a dual way; initially an *in vivo* constant infusion model in rats was used to assess parameters such as half-life, MCR and V_D . This particular model employs an IV injection of analogues in order to exclude confounding factors in the calculation of the PK parameters such as absorption from site of administration and first-pass effect. Secondly, analogues were given in a slow-release zinc-containing formulation SC in rats and the peptide plasma concentration over a period of one week was measured. The latter study mimics the way that drug compounds will be administered in humans and examines the influence of the SC depot on the PK properties of the analogues. The selection of rats for the above-mentioned PK studies was done taking into account the need for repeated blood sampling which cannot be satisfied adequately in mice without causing cardiovascular compromise and thus haemodynamic instability which can confound the PK results.

We hypothesised that by adding a His tail to the OXM analogues, it would enhance binding to the zinc molecules in the solution, resulting in the formation of a SC depot that would cause a slow release of the peptide in the circulation. Such a slow release from the SC depot theoretically causes a gradual increase in the plasma concentration of the peptide over time.

The peptide used as a starting point in this thesis was the native OXM (1-30), but with three point substitutions that were introduced in previous experiments from within our group (positions 20, 21 and 27). The novel point substitutions subsequently implemented in this generation of analogues included the substitution of Thr²⁹ for Gly²⁹, Lys³⁰ for either His³⁰ or Gly³⁰, and the introduction of a variable His-tail at the C-terminal of the molecule.

Out of the five analogues (X1-X5) developed in this chapter, the *in vivo* studies revealed that analogue X2 caused the highest body weight loss in mice at 48 hours in comparison to the other analogues. Although all OXM analogues were effective in reducing food intake for the first 2 hours post-administration in mice, only analogues X2, X4 and X5 continued to suppress food intake at the 2-4 hours period. The hypothesis is that X2 is present for a longer time in the circulation due to its PK characteristics and/or it is more effective in binding and activating the GLP-1R when compared to other analogues. To examine this observed difference in the biological effect of the analogues a number of studies were performed in this chapter each aiming at a specific component of the PK/PD relationship.

In regards to the PK characteristics of analogue X2 it was shown that it presented the more optimal PK profile among the OXM analogues when tested in the *in vivo* SC PK model; X2 demonstrated the slowest decline in its plasma concentration in the 30 min- 3hr period. It also demonstrated the longest half-life when compared to the other OXM analogues (6.5 ± 0.6 min) and the lowest MCR (3.2 ± 0.9 ml/min/kg). Interestingly it presented the lowest V_D of all OXM analogues. Since analogues X1-X5 differ only at the C-terminal tail it seems that the Gly-His-His tail of X2 may offer more protection against degradation from ectopeptidases in comparison to the other tails thus allowing X2 to remain for a longer time in the circulation exerting its biological effect. These results are very interesting and will be valuable in designing the next generation of analogues; however it is uncertain if such subtle differences in the PK properties between analogues X1-X5 can fully explain the observed superiority of X2 in causing body weight loss. Thus, it seems reasonable to assume that the PD characteristics of X2 are the ones mostly responsible for its enhanced bioactivity.

In order to better understand the PD effects of the analogues we have to evaluate their ability to bind to the GLP-1R and GCGR and then activate the second messenger pathway of cAMP as stated previously. As *in vivo* efficacy studies were conducted in mice, peptides were tested for their binding affinity to the mGCGR and mGLP-1R while a cell line (CHO) overexpressing the hGLP-1R and hGCGR was used as an aid to predict their likely effectiveness in humans.

Initial experiments confirmed previous reports describing the selectivity of the GLP-1R and GCGR for their respective endogenous ligands (344). Competitive receptor binding assays demonstrated that GLP-1's binding affinity to the hGCGR was >10000-fold lower than GCG, while conversely, GCG's affinity to the hGLP-1R was >10000-fold lower than GLP-1. A similar picture was also seen at the mGCGR, whereas at the mGLP-1R, GCG had a 95-fold lower binding affinity than GLP-1 approximately. Such a difference could be explained by the sequence variations between the mGLP-1R and hGLP-1R which are 90% similar (244).

In regards to the experiments performed to evaluate the efficacy of the analogues in the hGCGR and the hGLP-1R, it was shown that the bioactivity of EX-4 in the hGLP-1R was 1.7-fold higher than that of GLP-1, whereas OXM was 13-fold less potent than GLP-1 as expected. In the hGCGR, OXM was approximately 8.5-fold less potent than GCG. Exendin-4, as expected and in agreement with the receptor binding results, did not induce a response at the hGCGR while GLP-1 was >2200-fold less potent than GCG.

Analogues X2 and X5 caused the highest body weight loss in the acute feeding study in mice among OXM analogues. Analogues X2 and X5 presented the highest affinity to the mGLP-1R and the mGCGR respectively. In a similar fashion, analogues X2 and X5 presented the highest efficacy in the hGLP-1R, thus providing some preliminary evidence of adequate translation of these results in humans. Taken together, the combination of the PK and the PD characteristics of X2 (longer half-life, higher binding to the mGLP-1R and higher efficacy at the hGLP-1R) provide a plausible explanation as to the superiority of X2 over the rest of the analogues when examining its biological effect and its ability to induce weight loss in rodents.

There a number of other observations that are the result of the studies performed in this chapter. The improvement in the binding affinity of analogues to the GLP-1R compared to OXM seems to be more accentuated in the hGLP-1R rather than the mGLP-1R; this will need to be taken into account when translating rodent results to humans and designing drug dosages. This phenomenon could be attributed to the primary sequence between the mGLP-1R and hGLP-1R which differs at 42 amino acids. In any case, the results are different only in the improvement ratio of the analogues and receptor binding studies are only one part of the puzzle, with bioactivity studies at the hGLP-1R and hGCGR confirming the translation of the results at the level of the human receptors.

In regards to the role of the agonism at the level of the GCGR in interpreting the biological effects of the analogues it must be emphasised that it remains particularly difficult to evaluate precisely. The increased EE caused by the activation of the GCGR can be assessed by measurement of the CO₂ production and O₂ consumption by indirect calorimetry; such studies are not always readily available and are not suitable for a high throughput analogue screening process. All OXM analogues tested in this study had a substitution of Asp²¹ to Glu²¹ in an effort to increase the GCGR binding affinity. Indeed, all OXM analogues displayed a 10- to 19-fold higher affinity to the hGCGR in comparison to OXM while having only a 1-to 2- fold lower affinity when compared to GCG. However, in the mGCGR, the OXM analogues presented only a 1-to 1.5-fold higher affinity than OXM and had a 3-to 4-fold lower affinity than native GCG. Exendin-4, which also has Glu²¹ in its primary structure, does not bind to the GCGR which highlights the importance of the presence of the other residues involved in GCGR binding.

A possible mechanism to explain the increased receptor binding affinity of the OXM analogues to the GCGR involves the formation of a lactam bridge between Arg¹⁷ and Glu²¹. There is evidence that lactam bridges generated between Lys and Glu residues, separated by 3 or 4 residues preferentially, stabilise α -helical conformations (221). Peptides with one or more lactam bridges have a high propensity to form α -helices in aqueous solutions, and it was found that the C-terminal portion of GGC recognises its receptor in an α -helical conformation (221). We can therefore hypothesise that Glu²¹ of OXM analogues increases the α -helix conformation of the C-terminal by forming a lactam bridge with Arg¹⁷. Arginine behaves similar to Lys and also possesses a positively charged side chain resulting in enhanced GCGR binding and bioactivity at this receptor. The increased binding affinity and efficacy at the GCGR by the OXM analogues is an important feature and these modifications will be preserved in future analogues.

Another important conclusion from these studies that may prove valuable in future generations of peptides is that the introduction of the His tail has resulted in a detectable plasma level of the analogues up to 48 hours, which is similar to the PK profile of EX-4. Peak recorded plasma levels were at 30 minutes post-injection for all peptides, while analogue X2 presented the highest 24-hours to 3-hours ratio. Interestingly, analogue X2 had the longest half-life when compared to other OXM analogues when tested in the constant infusion PK study where it was administered without zinc. Interpreting the two above-mentioned studies together, we see that there is a feasible mechanism to improve the PK of analogues; both by point substitutions in the primary sequence and by injecting the analogue in a formulation that offer an increased resistance to degradation. As a result, the usefulness of both pathways will continue to be investigated in future analogue generations.

The importance of the plasma concentration levels that GLP-1R agonists attain is highlighted by the fact that it has been linked to one of their most important side-effects when given in humans; nausea. The occurrence of nausea in humans has been shown to be related to high levels of plasma concentrations of the therapeutic agent (345). Thus, a formulation that prevents the rapid entry of the peptide into the blood stream post-administration could prevent the occurrence of such phenomena. Furthermore, such a formulation would prolong the duration of action of the agent, leading to a decrease in the frequency of administration.

The improved PK characteristics in this series of OXM analogues, which mimic the PK profile of EX-4 when administered SC, are attributed to changes made in position 20, 29 and to changes at the C-terminal tail of the molecule. Position 20 of the native OXM molecule is occupied by Gln which was substituted in these OXM analogues by His²⁰. Both His and Gln have a polar side-chain but only His possesses an imidazole side chain which can bind to zinc.

4.5. Conclusions

Oxyntomodulin analogues with Glu residue at position 21 have demonstrated a higher binding affinity and bioactivity at the GCGR, while positions Leu²⁷ and Gly²⁹ have increased the binding affinity and the bioactivity of the OXM analogues to the GLP-1R.

The addition of the His-tail in this generation of OXM analogues has improved the PK profile of the peptides although their plasma concentration was only detectable for up to 2 days post-administration.

The *in vivo* anorectic effect of this series of OXM analogues was significant for up to 4 hours post-administration in mice. By comparison, EX-4's anorectic effect was present for up to 8 hours. Further work is needed in order to produce an agent that would have a greater food intake inhibition and greater body weight loss than EX-4, while at the same time presenting PK parameters that would allow the drug to be given once weekly.

In summary, the body of the work presented in this chapter has demonstrated that the introduction of a poly-His tail at the C-terminal can prolong the peptide presence in plasma in combination with a slow-release formulation. At the same time Gly29 and Gly30 may enhance the affinity and bioactivity at the GLP-1R. These results are valuable for the development of an anti-obesity agent and will be tested further, in conjunction with other point substitutions, in the next generation of OXM analogues in chapter 5.

Chapter 5

Investigation of amino acid substitutions at positions 16, 23 and at the C-terminal

5.1. Modification of positions 16, 23, 29, 30 and of the C-terminal poly-Histidine tail

A number of novel peptide analogues (X6-X21) were designed and synthesised in this chapter (Table 6) taking into account the observed effects of Gly²⁹, Gly³⁰ and of the poly-His tail as documented in chapter 4. It is important to highlight that point substitutions in drug discovery most often aim at a single goal such as improving GLP-1R or GCGR binding and/or potency or improving the PK profile of the analogues. As the process of drug development continues, some amino acid positions can be revisited and changed again to serve a different function, if there has been another change somewhere else in the molecule that satisfies the original task; a position can initially be aimed to increase GLP-1R binding and then switched to increasing the PK profile if increased GLP-1R binding was achieved by a different way.

Overall the analogues developed in this chapter fall into four different subcategories, each looking into investigating a specific area of anti-obesity drug development. The 1st group of analogues was X6-X9 that aimed at improving the binding and potency at the GLP-1R while also improving the PK profile. The 2nd group was analogues X10-X13 trying to improve the binding and potency at the GLP-1R, improve the PK profile and to investigate whether the helix-capping can improve stability of the α -helix. The 3rd group, X14-X16, focused on improving the binding and potency at the GLP-1R and further investigating helix-capping residues. The 4th group, X17-X21, focused on improving the binding and potency at the GLP-1R and characterising the role of position 29.

Each of these analogues was tested at several functional parameters including *in vitro* and *in vivo* experiments. The *in vitro* tests included testing the analogues' binding affinity to both the GLP-1R and GCGR and their ability to induce a cAMP response at the human GLP-1 and GCG overexpressing cell lines. The *in vivo* experiments aimed at testing the analogues' ability to inhibit rodent food intake and evaluating their PK properties. The overall aim of the analogues was to cause a high and long-lasting anorectic effect when tested *in vivo*.

5.1.1. Position 16

The primary amino acid structure of the OXM analogues synthesized in the previous chapter had Serine (Ser) in position 16 of the molecule. In the analogues tested in this chapter, position 16 is occupied by Glu instead of Ser. This substitution was made in an effort to increase the binding affinity of the OXM analogues to the GLP-1R. Glutamic acid at position 16 is present in EX-4, which binds to the GLP-1R with a 1.5-fold higher affinity than native GLP-1, and it has been shown to lie in the

hydrophilic face of the α -helix. Position 16 of GLP-1 is occupied by Gly; substitution of Gly¹⁶ by Ala has previously been shown not to affect the binding affinity to the GLP-1R which suggests that flexibility around Gly¹⁶ is not necessary for binding to the GLP-1R. Nuclear magnetic resonance analysis of EX-4 and GLP-1 demonstrated that the α -helix in EX-4 is more regular than GLP-1 possibly due to the presence of a helix-stabilising Glu¹⁶-Arg²⁰ interaction found in EX-4 compared to a helix-destabilising Gly¹⁶ in GLP-1 (247). A higher stability of the α -helix could translate in binding to the receptor with a higher affinity.

5.1.2. Position 23

The primary amino acid structure of the OXM analogues synthesized in the previous chapter had Val in position 23 of the molecule. In this chapter, Val²³ has been substituted by Ile. Isoleucine in position 23 can be found in both GLP-1 and EX-4 and it has been shown to be located on the hydrophobic face of the α -helix and to be important for the binding of the molecule to the GLP-1R (219). Both Ile and Val are similar sized amino acids with a same non polar side chain. The introduction of Ile at position 23 of OXM analogues would theoretically increase the binding and bioactivity of the analogues at the GLP-1R.

5.1.3. Position 29

Position 29 in native OXM and GCG is occupied by Thr. On the other hand, in both EX-4 and GLP-1, position 29 is occupied by Gly. There is a significant difference in the size of the molecules as Gly is very small in comparison to Thr. The role of Gly²⁹ on GLP-1R binding has been discussed in chapter 4. Thanks to its flexibility, Gly is able to offer an increased flexibility to the C-terminal poly-His tail. The substitution of Thr²⁹ from Gly²⁹ in analogues X6 to X16 was made in an effort to increase the flexibility of the C-terminal poly-His tail and its binding to the zinc molecules. Analogues X17 to X21 maintained Thr at position 29 to investigate whether a less flexible poly-His tail results in a better PK profile.

5.1.4. Position 30

Analogues X10 to X16 presented in this chapter had position 30 occupied by Gly in contrast to the rest of the analogues which had His at the same position. The reason for this substitution was to investigate whether an increased flexibility of the C-terminal poly-His tail increases the zinc binding and thus improves the PK profile of the analogues.

5.1.5. Positions 32 to 37 (Helix-capping)

The α -helix is characterised by consecutive hydrogen bonds between the amide hydrogen of one amino acid and a carbonyl oxygen of another amino acid spaced at 4 residues further down the helix (346). These hydrogen bonds stabilise the helix and are interrupted at the helix termini due to the lack of the formation of available bond partners (347). Helix “capping” reported in 1988 by Richardson and Richardson described using alternative hydrogen bond patterns that could be used to satisfy backbone hydrogen bonds at the initial and final turns of the helix (348). Numerous studies have confirmed the ability of capping to stabilise α -helices since then (347, 349, 350).

Alanine is a non-polar amino acid has been previously shown to be a strong helix former and can be used for helix-capping interactions (351). Alanine has a small side chain that cannot interact significantly with other side chains, hence helix formation by Ala is stabilized predominantly by the backbone peptide hydrogen-bonds (351). Analogues X10 to X13 have an Ala amino acid introduced after each terminal His in an effort to decrease the molecule’s degradation by increasing the stability of the α -helix. Such effects should be beneficial, in theory, for the PK profile of the analogues.

Alternative methods to Ala helix capping (as seen in X14) were investigated in analogues X15 and X16. Analogues X15 and X16 had the same length poly-His tail with X14, but with a substitution of Ala³⁶ for Glu³⁶ and Gln³⁶ respectively and a C-terminal terminal amino group. Glutamine has a polar uncharged side chain while Glu has a polar positively charged side chain; both have a high tendency for C-terminal helix capping (352).

5.1.6. C-terminal poly-Histidine tail

As demonstrated from the studies in the previous chapter, a poly His-tail in the C-terminal of the molecule can improve the PK profile of the analogues which is likely to be due to its binding to the zinc molecules in the preparation resulting in a slow release from the SC depot. All analogues designed in this chapter have a variable His C-terminal tail whose aim is to further improve the PK profile by increasing the binding with the zinc and prolonging the half-life of the molecules.

The way that the poly-His tail is connected to the rest of the peptide varies between analogues in this chapter; in X16-X9 a single Gly is present, in X10 to X16 there is a Gly-Gly present and finally in analogues X17 to X21 native Thr at position 29 is preserved.

5.1.7. Aims

Hypothesis:

Rational modifications to the amino acid sequence of OXM will result in an OXM analogue more efficacious at inhibiting food intake and promoting higher weight loss than analogues developed in chapter 4.

Aims:

To determine:

- Investigate effects of rational point substitutions to the sequence of OXM on the agonist properties at the GLP-1R and GCGR.
- The effect of specific changes to the structure of OXM on its *in vivo* PK parameters.
- Investigate the effect of SC administration of OXM analogues to mice on food intake and body weight following peripheral administration in an acute and chronic feeding study.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
GCG	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T								
GLP1	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R	NH2						
EX4	H	G	E	G	T	F	T	S	D	L	S	K	Q	M	E	E	E	A	V	R	L	F	I	E	W	L	K	N	G	P	S	S	G	A	P	P	
OXM	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	K	R	N	R	N	N	I	A
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
X6	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	H							
X7	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	H	H						
X8	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	H	H	H					
X9	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	H	H	H	H				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
X10	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	G	H	A					
X11	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	G	H	H	A				
X12	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	G	H	H	H	A			
X13	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	G	H	H	H	H	A		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
X14	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	G	H	H	H	H	H	A	
X15	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	G	H	H	H	H	H	E	NH2
X16	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	G	G	H	H	H	H	H	Q	NH2
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
X17	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	T	H							
X18	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	T	H	H						
X19	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	T	H	H	H					
X20	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	T	H	H	H	H				
X21	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	Q	W	L	L	N	T	H	H	H	H	H			

Table 6: Amino acid sequences of peptides used in chapter 5. Blue filled residues are conserved residues across the GLP-1 and GCG peptides; yellow letters are amino acids derived from the GLP-1 sequence; red letters are amino acids derived from the EX-4 sequence; grey filled residues represent point substitutions.

5.2. Materials and Methods

5.2.1. Custom design of peptide analogues

Five analogues of OXM were designed and synthesised as described in section 2.2. The amino acid sequences of these analogues as well as EX-4 and the naturally occurring OXM, GLP-1 and GCG are shown in Table 6.

5.2.2. Competitive receptor binding assays

5.2.2.1. Glucagon receptor binding assays

5.2.2.1.1. Receptor binding of ligands to the human glucagon receptor

Binding studies were completed as described in section 2.6.5, using CHO cell membrane (cells overexpressing the hGCGR as the source of GCGR (section 2.6.2). ^{125}I -GCG was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

5.2.2.1.2. Receptor binding of ligands to the mouse glucagon receptor

Binding studies were completed as described in section 2.6.5, using mouse liver cell membranes as the source of GCGR (section 2.6.1). ^{125}I -GCG was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

5.2.2.2. GLP-1 receptor binding assays

5.2.2.2.1. Receptor binding of ligands to the human GLP-1 receptor

Binding studies were completed as described in section 2.6.4, using CHO cell membrane (cells overexpressing the hGLP-1R as the source of GLP-1R (section 2.6.2). ^{125}I -GLP-1 was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

5.2.2.2.2. Receptor binding of ligands to the mouse GLP-1 receptor

Binding studies were completed as described in section 2.6.4, using mouse lung cell membrane as the source of GLP-1R (section 2.6.1). ^{125}I -GLP-1 was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

5.2.3. cAMP accumulation studies

5.2.3.1. *In vitro* bioactivity studies at the human glucagon receptor

The ability of ligands to stimulate cAMP production was investigated as described in section 2.7 using CHO cells overexpressing the hGCGR (section 2.6.2) as the source of GCGR. EC₅₀ values were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

5.2.3.2. *In vitro* bioactivity studies at the human GLP-1 receptor

The ability of ligands to stimulate cAMP production was investigated as described in section 2.7 using CHO cells overexpressing the hGLP-1R (section 2.6.2) as the source of GLP-1R. EC₅₀ values were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

5.2.4. Acute feeding studies in mice

The effect of OXM analogues on food intake were investigated in fasted mice, as described in section 2.4. Peptides were administered via SC injection and food intake measured at 1, 2, 4, 8 and 24 and 48 hours post-injection, unless otherwise stated. Statistical analysis of results was completed as described in section 2.9, results are shown as mean $\pm$ SEM (n=7-8 per group). Doses administered were chosen based on the observations from previous feeding studies (not shown). Exendin-4 (10 nmol/kg) was used as a positive control and OXM analogues were administered at 50 nmol/kg.

5.2.5. Chronic feeding studies in mice

The effect of OXM analogues on food intake was investigated in a 6 day chronic study in mice. Analogues were dissolved in ZnCl₂ solution prepared in saline at a zinc to peptide ratio of 1:1. Peptides were administered via SC injection at a dosage of 50 nmol/kg at 0900 to overnight fasted DIO C57BL/6J mice (av. body weight 40.2 g). Exendin-4 was administered at a dosage of 10 nmol/kg without zinc. Food intake and body weight were measured at times -16h, 48h and 6 days.

5.2.6. Pharmacokinetics

5.2.6.1. Investigation of the pharmacokinetic properties in an *in vivo* constant infusion model

Studies were carried out to determine the *in vivo* PK properties (MCR, half-life, V_D) of OXM analogues with the use of the IV constant infusion model in male Wistar rats (section 3.2.2). Plasma peptide concentrations were determined by RIA (section 2.8).

5.2.6.2. Investigation of the pharmacokinetic properties following single subcutaneous bolus injection in male Wistar rats

Studies were carried out to investigate the formation of a slow release depot and measure plasma clearance following a SC administration (section 2.5.1). Male Wistar rats were stratified by body weight and placed into groups of 5 rats of equal mean body weight. Each rat was administered 1 mg of peptide (50 mg/ml) reconstituted in 1:1 molar ratio Zn:NaCl (saline) at time 0. Plasma peptide concentrations were determined by RIA (section 2.8).

5.3. Results

5.3.1. Second generation of oxyntomodulin analogues

5.3.1.1. Binding affinities of oxyntomodulin, glucagon and oxyntomodulin analogues to the glucagon receptor

5.3.1.1.1. Binding to membrane preparation of CHO cells overexpressing human glucagon receptor

Binding assays were completed as described in section 5.2.2.1.1. Binding studies using ^{125}I -GCG routinely resulted in greater than 90% specific binding to a cell membrane preparation of CHO cells overexpressing the hGCGR. The binding affinities of GCG and OXM to the hGCGR are shown graphically in Figure 29 and summarised in Table 7. Oxyntomodulin was shown to bind to the hGCGR with approximately 17-fold lower affinity than GCG (OXM $\text{IC}_{50} = 8.84 \pm 0.47$ nM, GCG $\text{IC}_{50} = 0.52 \pm 0.05$ nM).

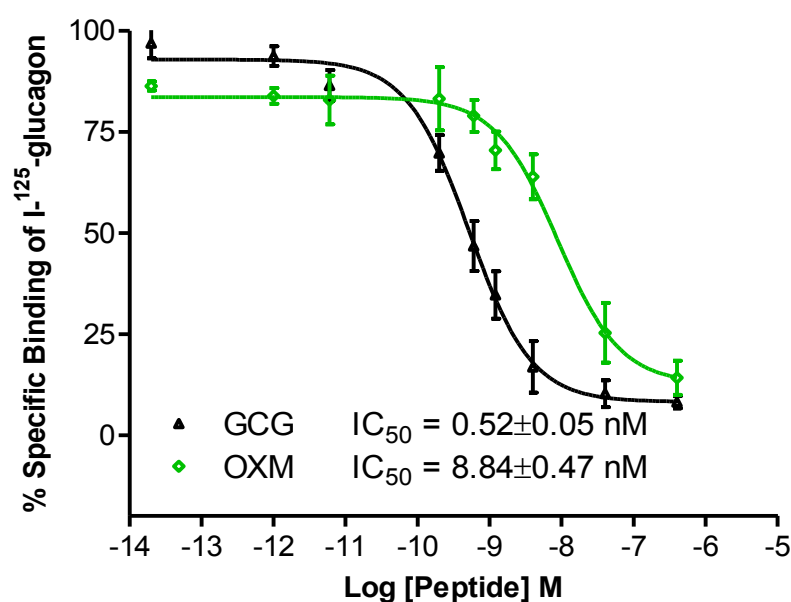


Figure 29: Competitive receptor binding affinity of GCG and OXM to the hGCGR. Purified cell membranes of CHO cells overexpressing the hGCGR were the source of the receptors. ^{125}I -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 16 OXM analogues (X6-X21) to the hGCGR were tested and compared with endogenous ligands (Figure 30, Figure 31, Figure 33, Table 7).

Analogue X6-X9

Analogue X6 had approximately a 1.6-fold lower affinity respectively to the hGCGR than GCG (X6 $IC_{50} = 0.82 \pm 0.02$ nM) (Figure 30). Analogues X7, X8 and X9 had a 1.2- fold lower affinity to the hGCGR than GCG (X7 $IC_{50} = 0.63 \pm 0.16$ nM, X8 $IC_{50} = 0.60 \pm 0.04$ nM, X9 $IC_{50} = 0.62 \pm 0.04$ nM). Oxyntomodulin presented a 10.8- and 14.7-fold lower affinity to the hGCGR when compared to analogues X6 and X8 respectively. Affinity of oxyntomodulin to the hGCGR was 14- and 14.3-fold weaker than analogues X7 and X9 respectively.

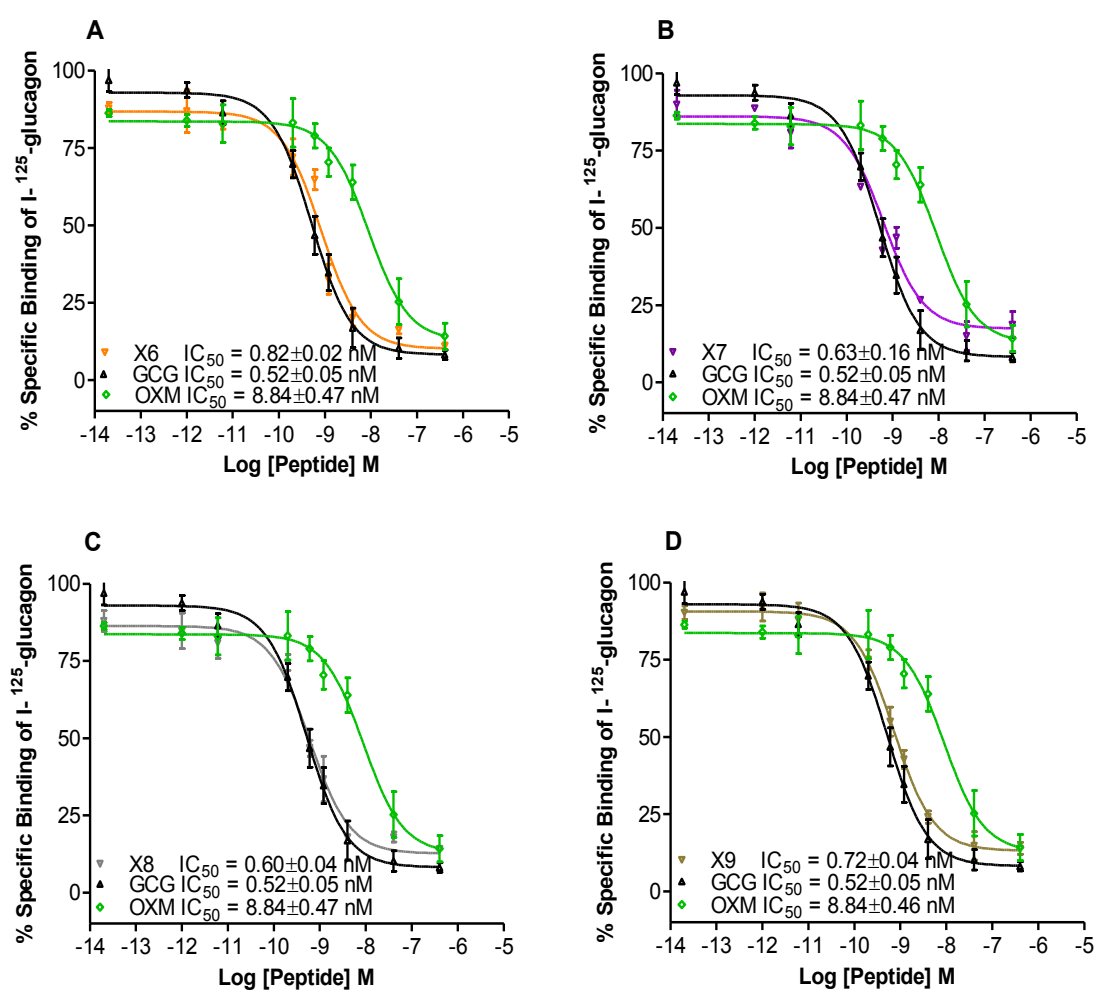


Figure 30: Competitive receptor binding affinity of GCG, OXM and 4 OXM analogues A: X6, B: X7, C: X8, D:X9 to the hGCGR. Purified cell membranes of CHO cells overexpressing the hGCGR were the source of the receptors. I^{125} -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X10-X13

Analogue X10 and X11 had approximately a 1.6- and 1.1-fold lower affinity respectively to the hGCGR than GCG (X10 $IC_{50} = 0.85 \pm 0.32$ nM, X11 $IC_{50} = 0.55 \pm 0.03$ nM) (Figure 31). Analogue X12 and X13 had a 1.4- and 1.2-fold lower affinity respectively to the hGCGR than GCG (X12 $IC_{50} = 0.73 \pm 0.01$ nM, X13 $IC_{50} = 0.62 \pm 0.06$ nM). Oxyntomodulin presented a 10.4- and 16.1-fold lower affinity to the hGCGR when compared to analogues X10 and X11 respectively. Oxyntomodulin had a 12.1- and 14.3-fold lower binding affinity to the hGCGR when compared to analogues X12 and X13 respectively.

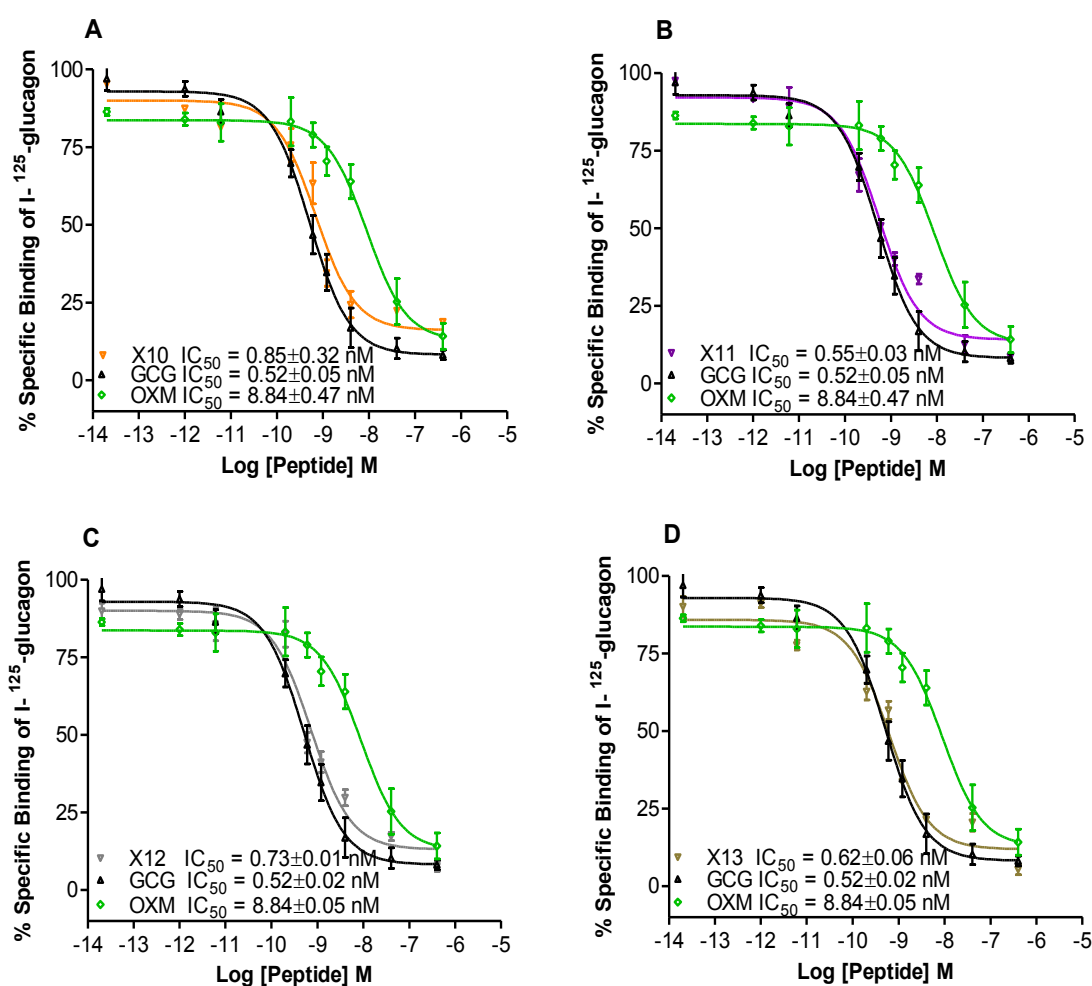


Figure 31: Competitive receptor binding affinity of GCG, OXM and 4 OXM analogues A: X10, B: X11, C: X12, D: X13 to the hGCGR. Purified cell membranes of CHO cells overexpressing the hGCGR were the source of the receptors. I¹²⁵-GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X14-X16

Analogue X14 and X16 had approximately a 1.5-fold lower affinity than GCG to the hGCGR (X14 $IC_{50} = 0.77 \pm 0.06$ nM, X16 $IC_{50} = 0.76 \pm 0.15$ nM) (Figure 32). Analogue X15 had a 1.4-fold lower affinity than GCG to the hGCGR (X15 $IC_{50} = 0.75 \pm 0.06$ nM).

Oxyntomodulin presented approximately a lower affinity to the hGCGR when compared to analogues X14, X15 and X16 that ranged from 11.5-fold for X14, 11.6-fold for X16 and 11.8-fold for X15.

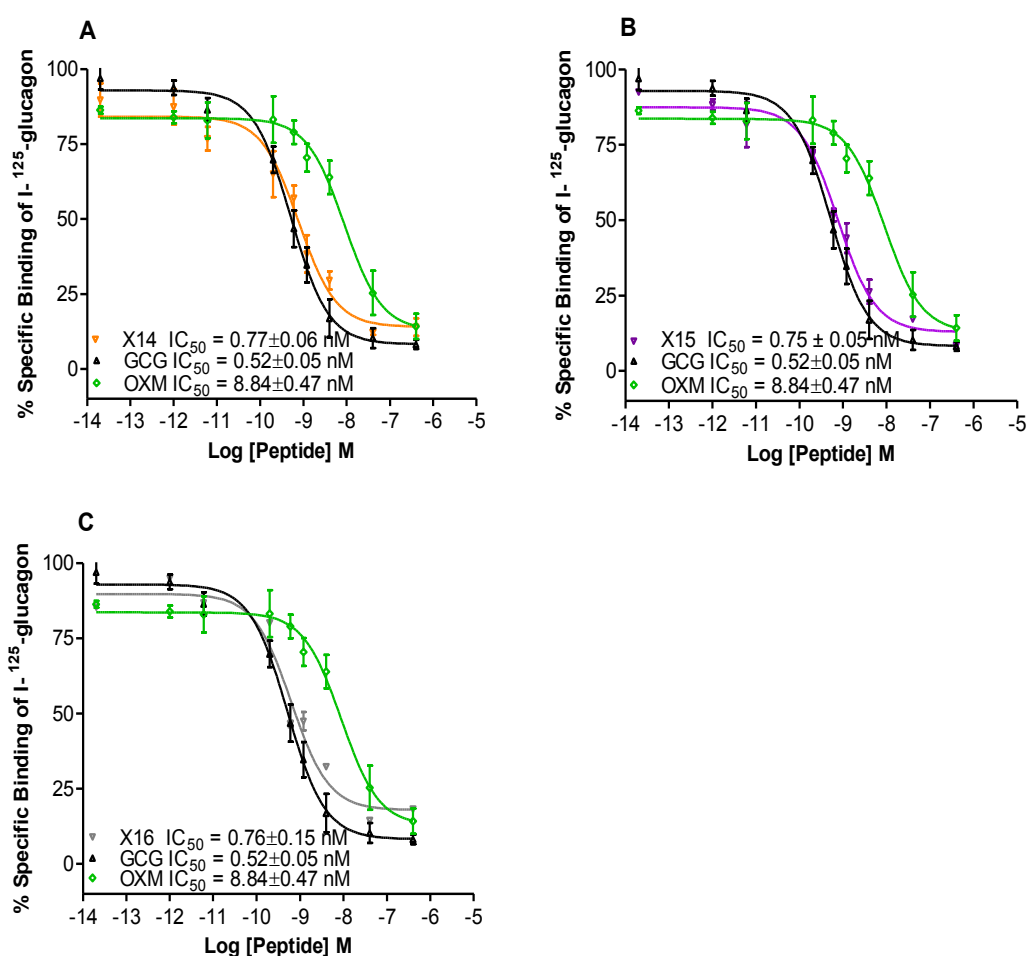


Figure 32: Competitive receptor binding affinity of GCG, OXM and 3 OXM analogues A: X14, B: X15, C: X16 to the hGCGR. Purified cell membranes of CHO cells overexpressing the hGCGR were the source of the receptors. I^{125} -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogues X17-X21

Analogues X17 and X18 had approximately a 1.8- and 1.6-fold lower affinity respectively to the hGCGR than GCG (X17 $IC_{50} = 0.94 \pm 0.10$ nM, X18 $IC_{50} = 0.85 \pm 0.03$ nM) (Figure 33). Analogues X19, X20 and X21 had a 1.5-, 1.6- and 1.4-fold lower affinity respectively to the hGCGR than GCG (X19 $IC_{50} = 0.77 \pm 0.12$ nM, X20 $IC_{50} = 0.83 \pm 0.15$ nM, X21 $IC_{50} = 0.75 \pm 0.01$ nM).

Oxyntomodulin presented a lower affinity to the hGCGR when compared to analogues X17 to X21 that ranged from 9.4-fold for X17, 10.4-fold for X18, 10.7 fold for X20, 11.5-fold for X19 and 11.8-fold for X21.

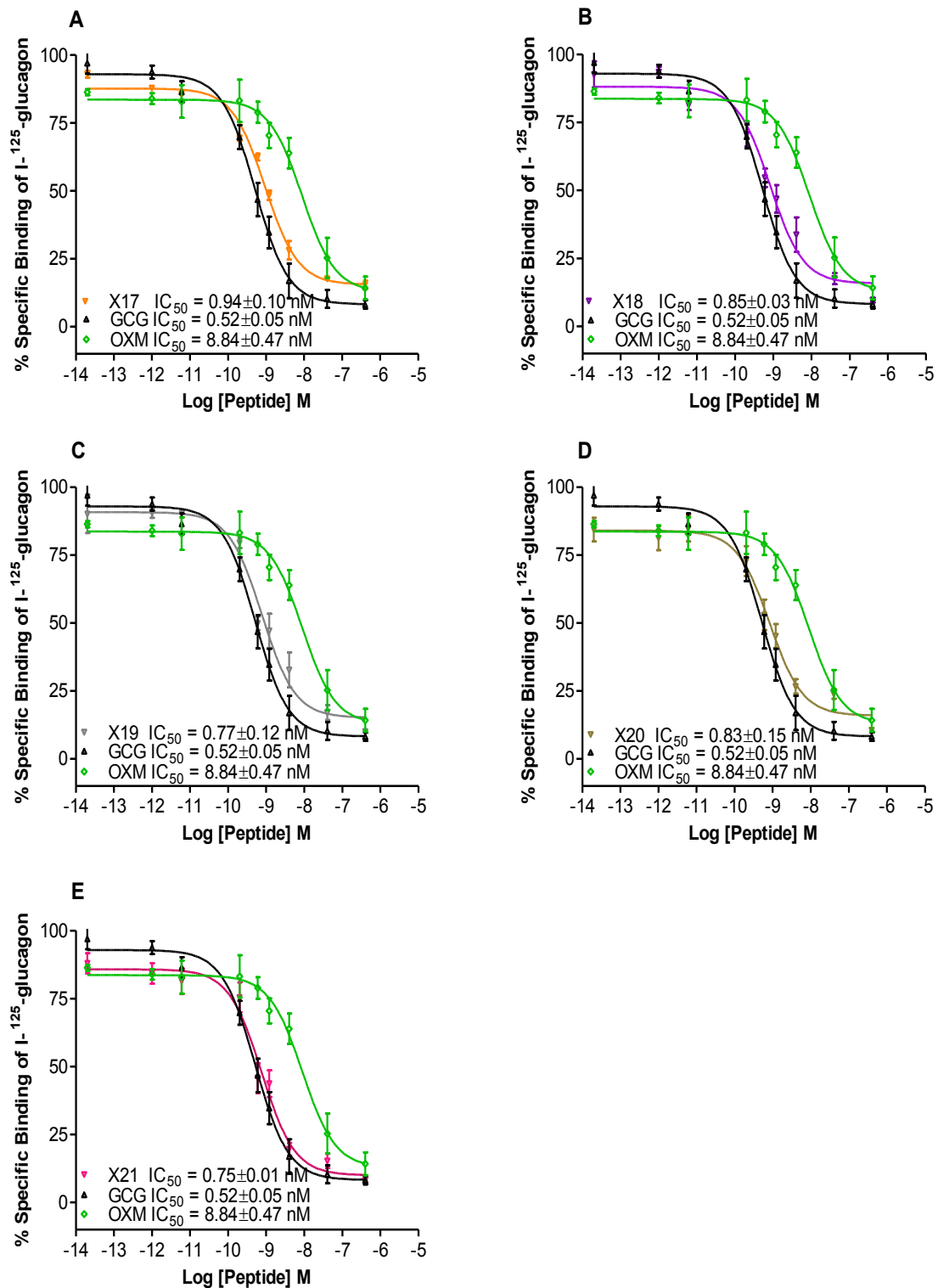


Figure 33: Competitive receptor binding affinity of GCG, OXM and 5 OXM analogues A: X17, B: X18, C: X19, D: X20, E: X21 to the hGCGR. Purified cell membranes of CHO cells overexpressing the hGCGR were the source of the receptors. I¹²⁵-GCG was used as the competing peptide. IC₅₀ values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

5.3.1.1.2. Binding to membrane preparation of mouse liver cells overexpressing mouse glucagon receptor

Binding assays were completed as described in section 5.2.2.1.2. Binding studies with I^{125} -GCG routinely shown greater than 90% specific binding to membranes prepared from mouse liver tissue. The binding affinities of GCG and OXM to the mGCGR are shown graphically in Figure 34 and summarised in Table 7. The affinity of OXM to the mGCGR was 4.4-fold lower than GCG (OXM IC_{50} = 5.87 ± 0.31 nM, GCG IC_{50} = 1.35 ± 0.17 nM).

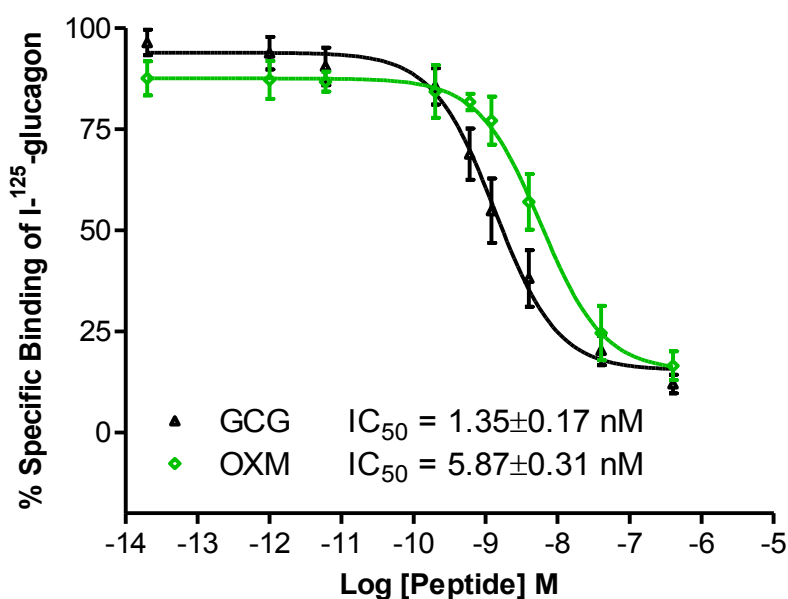


Figure 34: Competitive receptor binding affinity of GCG and OXM to the mGCGR. Cell membrane preparations from mouse liver overexpressing the mGCGR were the source of the receptors. I^{125} -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 16 OXM analogues (X6-X21) to the mGCGR were tested and compared with endogenous ligands (Figure 35, Figure 36, Figure 37, Figure 38, Table 7).

Analogue X6-X9

Analogue X6 and X7 presented a 4.9- and 5.3-fold lower affinity respectively to the mGCGR than GCG approximately (X6 IC_{50} = 6.66 ± 0.80 nM, X7 IC_{50} = 7.11 ± 0.34 nM) (Figure 35). Analogue X8 had a 4.1-fold lower affinity to the mGCGR than GCG while X9 analogue bound to the mGCGR with a 4.9-fold lower affinity than GCG (X8 IC_{50} = 5.54 ± 0.55 nM, X9 IC_{50} = 6.65 ± 1.04 nM).

When compared to OXM, analogues X6, X7 and X9 presented a 1.1- to 1.2-fold lower affinity while analogue X8 had a 1.1-fold higher binding affinity to the mGCGR when compared to OXM.

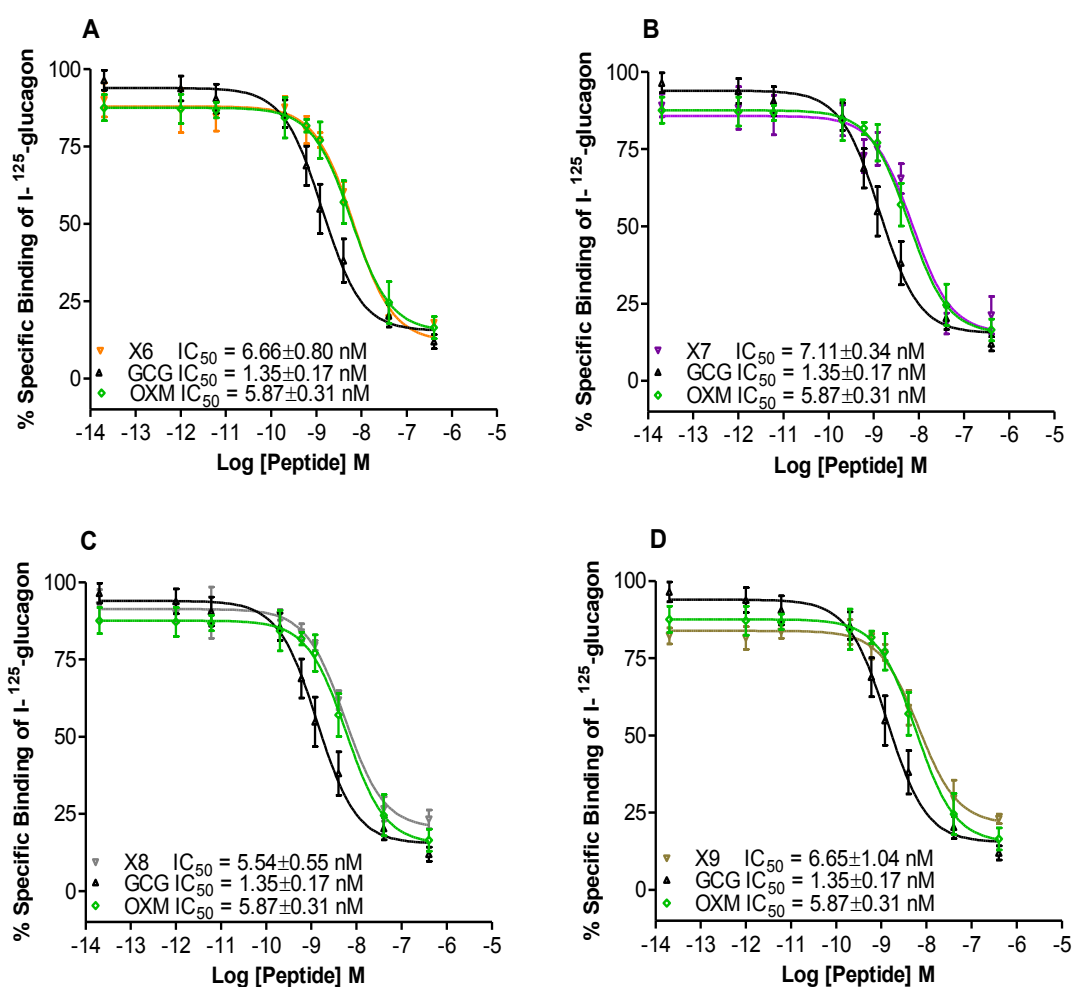


Figure 35: Competitive receptor binding affinity of GCG, OXM and 4 OXM analogues A: X6, B: X7, C: X8, D: X9 to the mGCGR. Cell membrane preparations from mouse liver were the source of the receptors. I^{125} -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X10-X13

Analogue X10 and X12 presented a 3.5-fold lower affinity approximately than GCG to the mGCGR (X10 $IC_{50} = 4.79 \pm 0.66$ nM, X12 $IC_{50} = 4.74 \pm 0.11$ nM) (Figure 36). Analogue X11 and X13 had a 4- and 3.9-fold lower affinity respectively than GCG to the mGCGR (X11 $IC_{50} = 5.46 \pm 0.79$ nM, X13 $IC_{50} = 5.32 \pm 1.05$ nM).

When compared to OXM, analogue X10 and X12 presented a 1.2-fold higher affinity to the mGCGR while analogue X11 and X13 had a 1.1-fold higher affinity to the mGCGR than OXM.

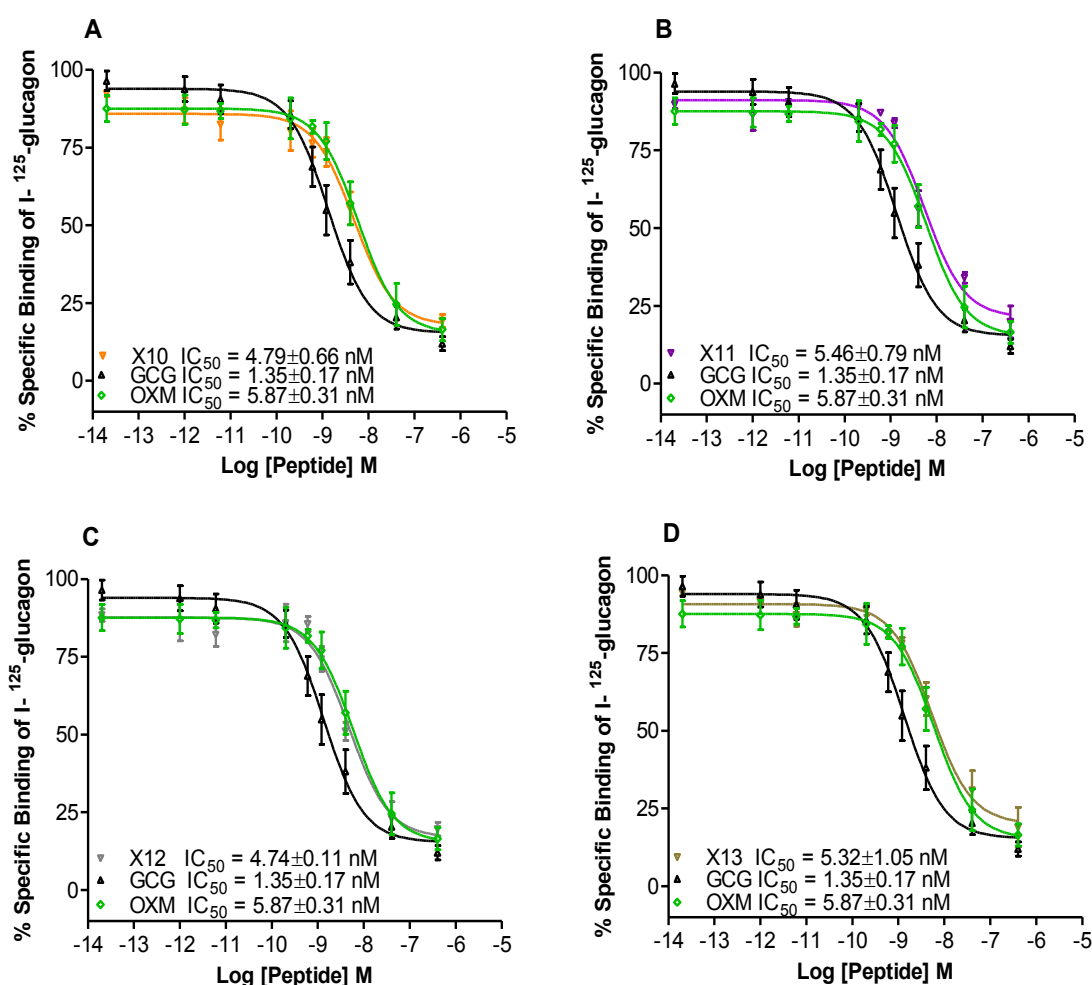


Figure 36: Competitive receptor binding affinity of GCG, OXM and 4 OXM analogues A: X10, B: X11, C: X12, D: X13 to the mGCGR. Cell membrane preparations from mouse liver overexpressing the mGCGR were the source of the receptors. I^{125} -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogues X14-X16

Analogues X14, X15 and X16 presented a 4.4-, 4.6- and 5-fold lower affinity respectively to the mGCGR than GCG (X14 IC_{50} = 5.97 ± 0.45 nM, X15 IC_{50} = 6.16 ± 1.05 nM, X16 IC_{50} = 6.74 ± 1.20 nM) (Figure 37).

When compared to OXM, analogue X16 presented a 1.1-fold higher affinity to the mGCGR. Analogues X14 and X15 did not differ in their binding affinity to the mGCGR from OXM.

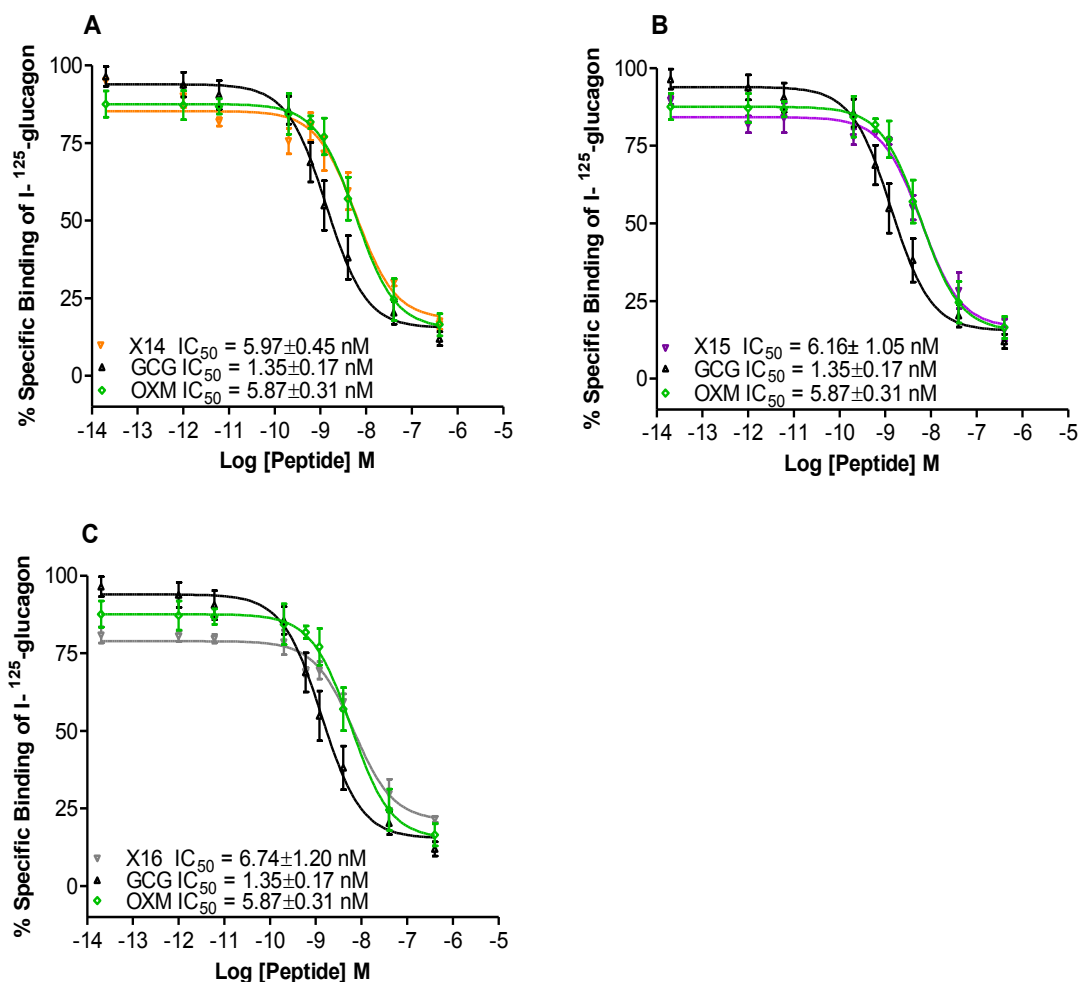


Figure 37: Competitive receptor binding affinity of GCG, OXM and 3 OXM analogues A: X14, B: X15, C: X16 to the mGCGR. Cell membrane preparations from mouse liver overexpressing the mGCGR were the source of the receptors. I¹²⁵-GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogues X17-X21

Analogues X17 and X18 presented a 5.2- and 5-fold lower affinity respectively than GCG to the mGCGR (X17 IC_{50} = 6.99 ± 1.20 nM, X18 IC_{50} = 6.78 ± 0.35 nM) (Figure 38). Analogues X19, X20 and X21 had a 5.4-, 5.8- and 5.1-fold lower affinity respectively to the mGCGR than GCG (X19 IC_{50} = 7.27 ± 1.07 nM, X20 IC_{50} = 7.84 ± 1.40 nM, X21 IC_{50} = 6.92 ± 0.40 nM).

When compared to OXM, analogues X17, X18, X19 and X21 presented a 1.2-fold lower affinity to the mGCGR. Analogue X20 had a 1.3-fold lower binding affinity to the mGCGR than OXM.

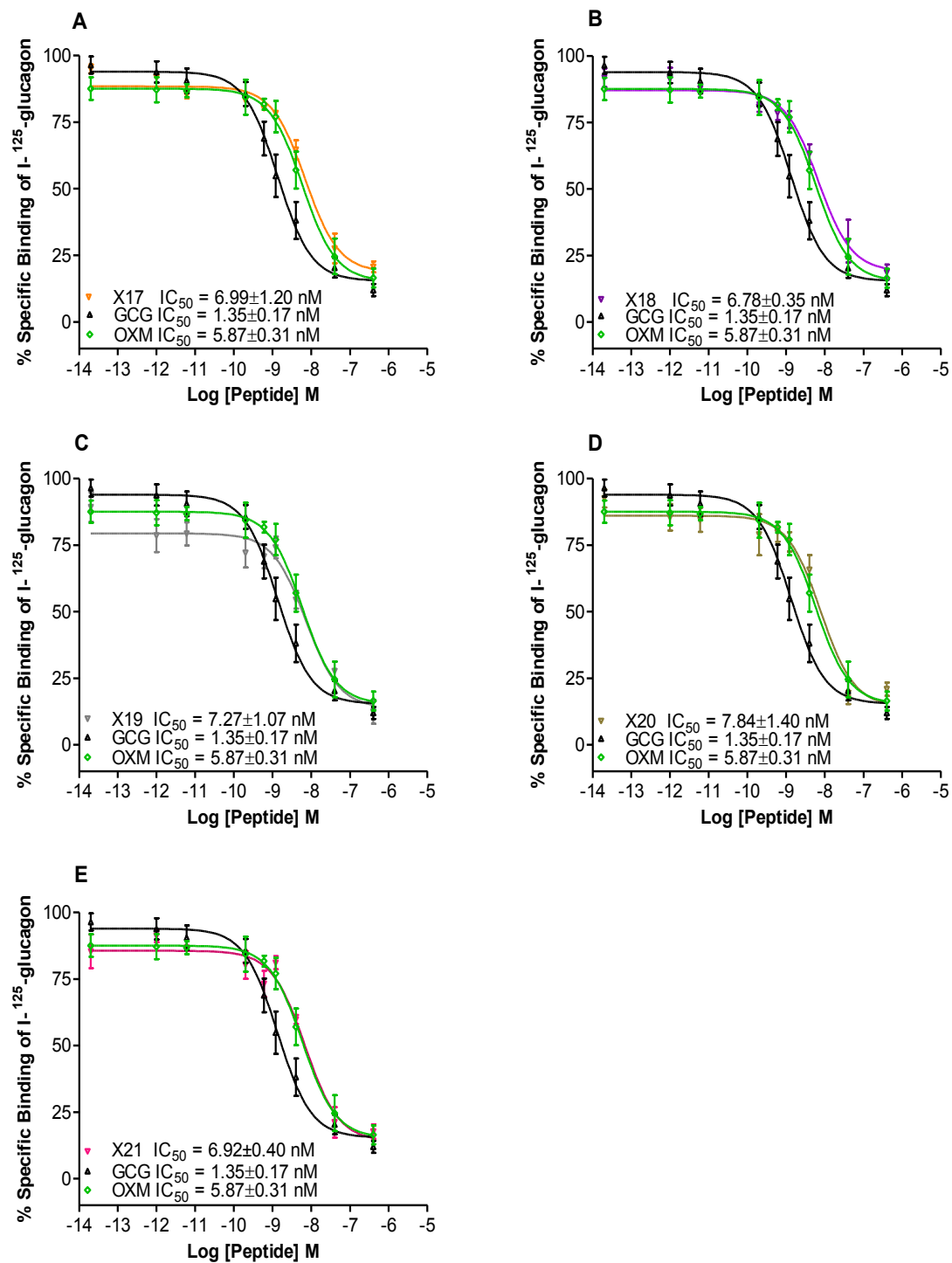


Figure 38: Competitive receptor binding affinity of GCG, OXM and 5 OXM analogues A: X17, B: X18, C: X19, D: X20, E: X21 to the mGCGR. Cell membrane preparations from mouse liver overexpressing the mGCGR were the source of the receptors. I¹²⁵-GCG was used as the competing peptide. IC₅₀ values calculated from a minimum of three separate experiments. Values shown are mean ± SEM.

5.3.1.2. Binding affinities of oxyntomodulin, GLP-1 and oxyntomodulin analogues to the GLP-1 receptor

5.3.1.2.1. Binding to membrane preparation of CHO cells overexpressing human GLP-1 receptor

Binding assays to the GLP-1R were completed as described in section 5.2.2.2.1. Binding studies using ^{125}I -GLP-1 routinely resulted in greater than 90% specific binding to a cell membrane preparation of CHO cells overexpressing the hGLP-1R. The binding affinities of GLP-1 and OXM to the hGLP-1R are shown graphically in Figure 39 and summarised in Table 7. Oxyntomodulin was shown to bind to the hGLP-1R with approximately 390-fold lower affinity than GLP-1 (OXM $\text{IC}_{50} = 121.0 \pm 3.40 \text{ nM}$, GLP-1 $\text{IC}_{50} = 0.31 \pm 0.07 \text{ nM}$).

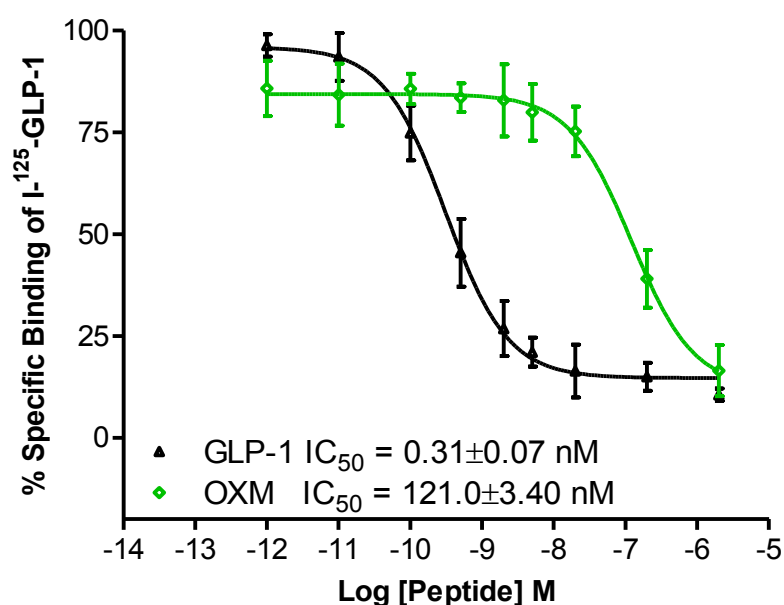


Figure 39: Competitive receptor binding affinity of GLP-1 and OXM to the hGLP-1R. Purified cell membranes of CHO cells overexpressing the hGLP-1R were the source of the receptors. ^{125}I -GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 16 OXM (X6-X21) analogues at the hGLP-1R were tested and compared with endogenous ligands (Figure 40, Figure 41, Figure 42, Figure 43, Table 7).

Analogue X6-X9

Analogue X6 and X7 had approximately a 5.9- and 5.4-fold lower affinity respectively than GLP-1 to the hGLP-1R (X6 IC_{50} = 1.83 ± 0.03 nM, X7 IC_{50} = 1.66 ± 0.15 nM) (Figure 40). Analogues X8 and X9 presented an 8.2- and 8.7-fold lower binding affinity respectively to the hGLP-1R than GLP-1 (X8 IC_{50} = 2.54 ± 0.01 nM, X9 IC_{50} = 2.69 ± 0.50 nM).

When OXM analogues X6 to X9 were compared to OXM, they presented a higher affinity to the hGLP-1R that ranged from 45-fold for X9, 47.6-fold for X8, 66.1-fold for X6 and 72.9-fold for X7.

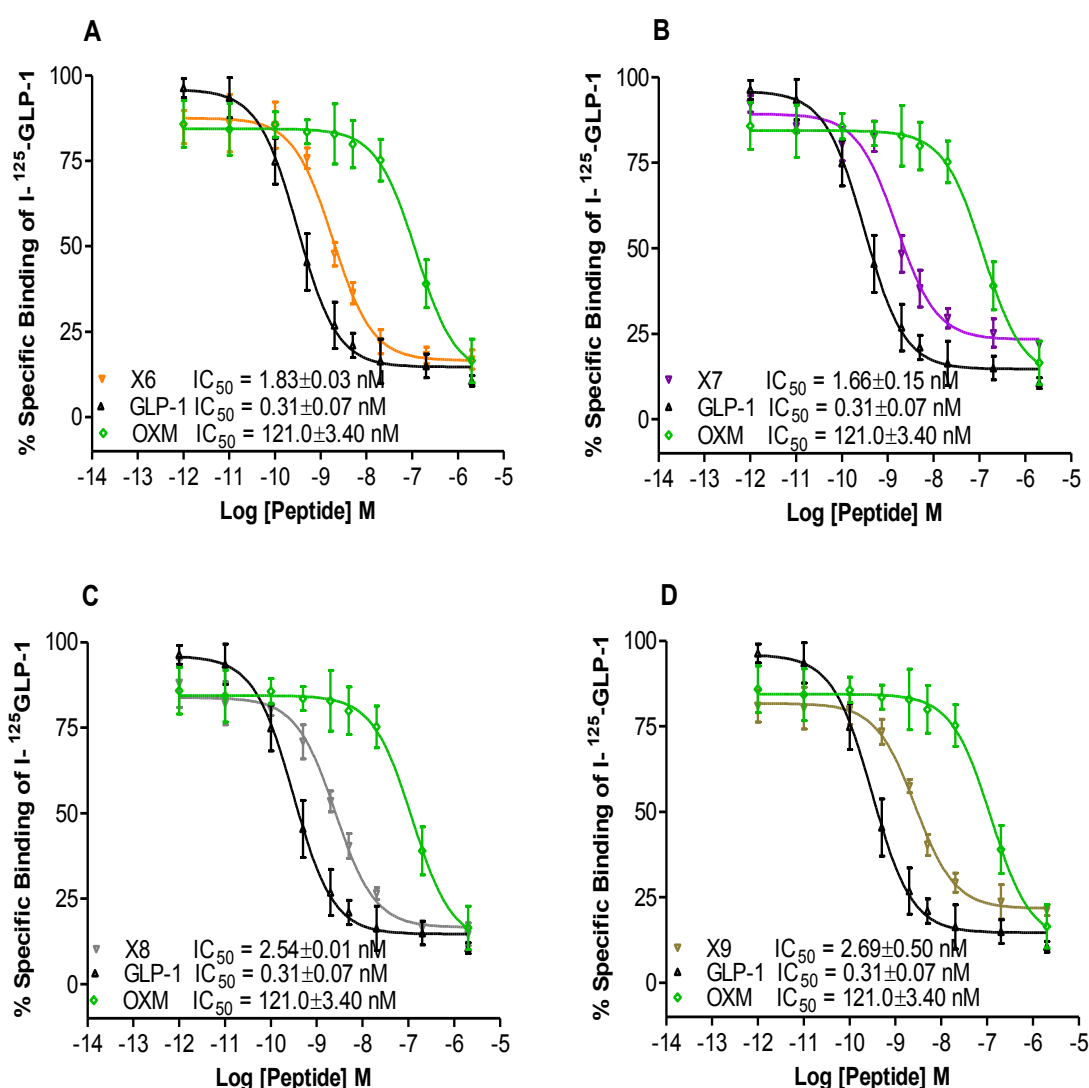


Figure 40: Competitive receptor binding affinity of GLP-1, OXM and 4 OXM analogues A: X6, B: X7, C: X8, D: X9 to the hGLP-1R. Purified cell membranes of CHO cells overexpressing the hGLP-1R were the source of the receptors. I¹²⁵-GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X10-X13

Analogue X10 and X11 had approximately an 8.3- and 9.5-fold lower affinity respectively than GLP-1 to the hGLP-1R (X10 IC_{50} = 2.57 ± 0.35 nM, X11 IC_{50} = 2.93 ± 0.36 nM) (Figure 41). Analogue X12 and X13 presented a 10.6- and 11-fold lower binding affinity to the hGLP-1R than GLP-1 respectively (X12 IC_{50} = 3.28 ± 0.50 nM, X13 IC_{50} = 3.40 ± 1.20 nM).

When OXM analogues X10 to X13 were compared to OXM, they presented a higher affinity to the hGLP-1R that ranged from 35.6-fold for X13, 36.9-fold for X12, 41.3-fold for X11 and 47.1-fold for X10.

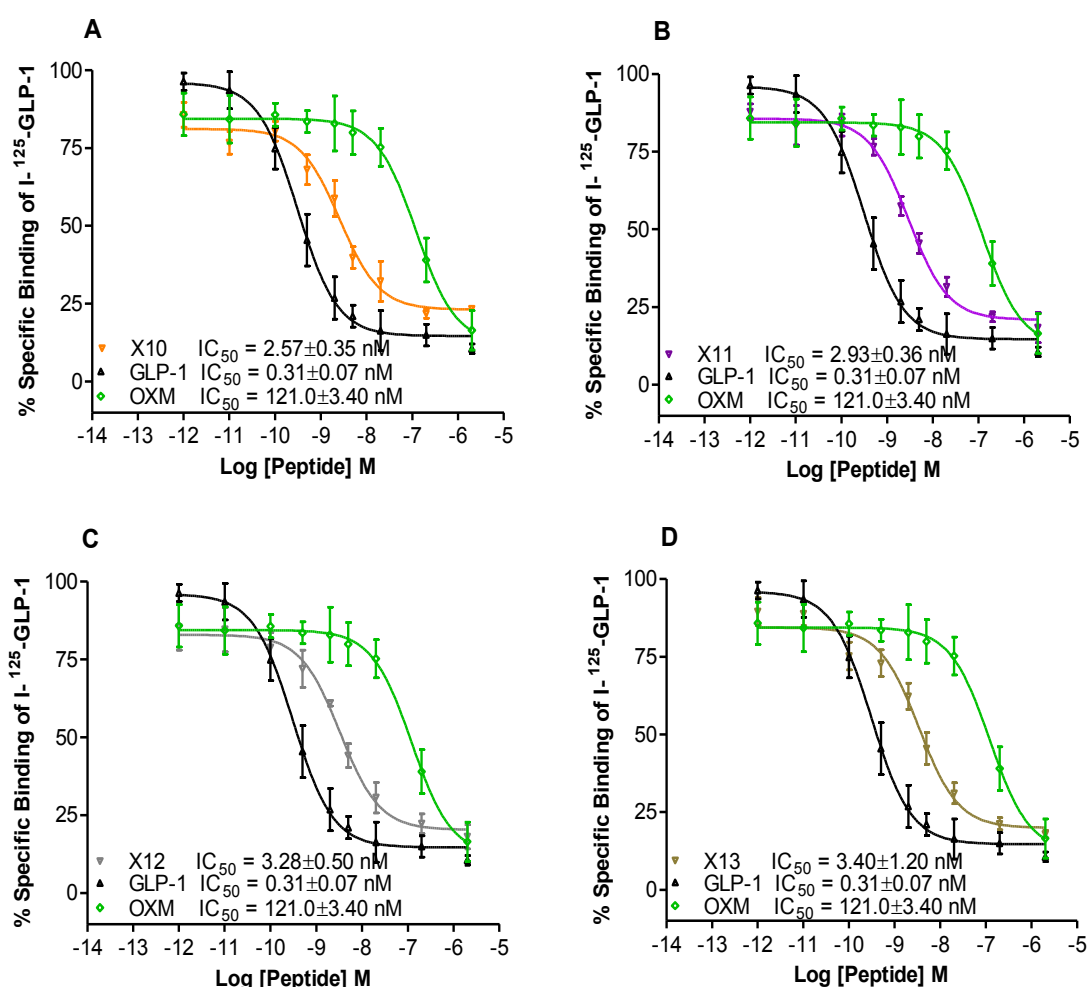


Figure 41: Competitive receptor binding affinity of GLP-1, OXM and 4 OXM analogues A: X10, B: X11, C: X12, D: X13 to the hGLP-1R. Purified cell membranes of CHO cells overexpressing the hGLP-1R were the source of the receptors. I^{125} -GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X14-X16

Analogue X14 and X16 had an 11.8-fold-fold lower affinity than GLP-1 to the hGLP-1R (X14 $IC_{50} = 3.66 \pm 0.93$ nM, X16 $IC_{50} = 3.65 \pm 1.45$ nM) (Figure 42). Analogue X15 presented a 12.4-fold lower affinity than GLP-1 to the hGLP-1R (X15 $IC_{50} = 3.84 \pm 1.27$ nM).

When OXM analogues X14 to X16 were compared to OXM, they presented a higher affinity to the hGLP-1R that ranged from 31.5-fold for X15, 33.1-fold for X14 and 33.2-fold for X16.

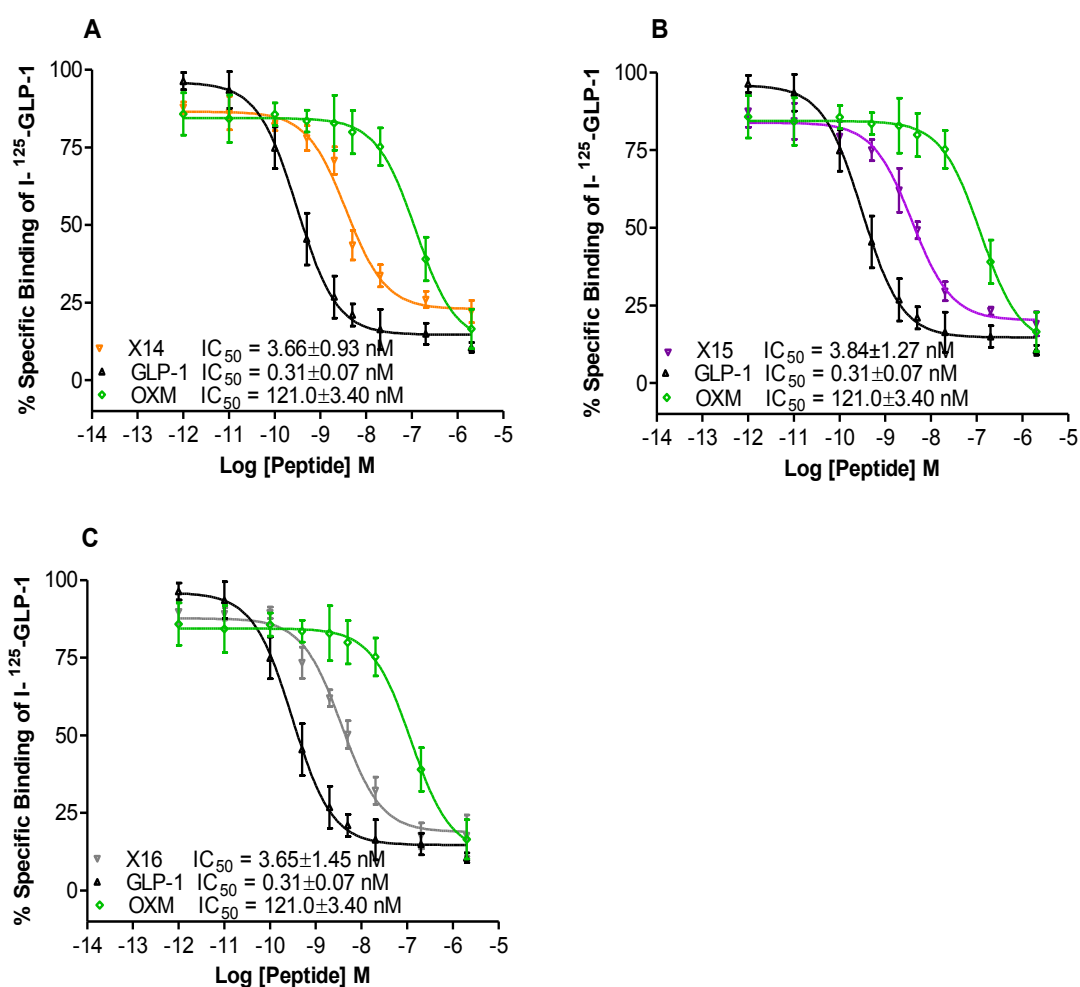


Figure 42: Competitive receptor binding affinity of GLP-1, OXM and 3 OXM analogues A: X14, B: X15, C: X16 to the hGLP-1R. Purified cell membranes of CHO cells overexpressing the hGLP-1R were the source of the receptors. I-¹²⁵-GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogues X17-X21

Analogues X17 and X18 had approximately an 8.2- and 9.5-fold lower affinity respectively than GLP-1 to the hGLP-1R (X17 IC_{50} = 2.54 ± 0.18 nM, X18 IC_{50} = 2.93 ± 0.76 nM) (Figure 43). Analogues X19 and X20 presented a 10.6- and 10.5-fold lower binding affinity to the hGLP-1R than GLP-1 respectively (X19 IC_{50} = 3.29 ± 0.50 nM, X20 IC_{50} = 3.26 ± 0.90 nM). Analogue X21 presented an 11.2-fold lower binding affinity to the hGLP-1R than GLP-1 (X21 IC_{50} = 3.48 ± 0.50 nM).

When OXM analogues X17 to X21 were compared to OXM, they presented a higher binding affinity to the hGLP-1R that ranged from 34.8-fold for X21, 36.8-fold for X19, 37.1-fold for X20, 41.3-fold for X18 and 47.6-fold for X17.

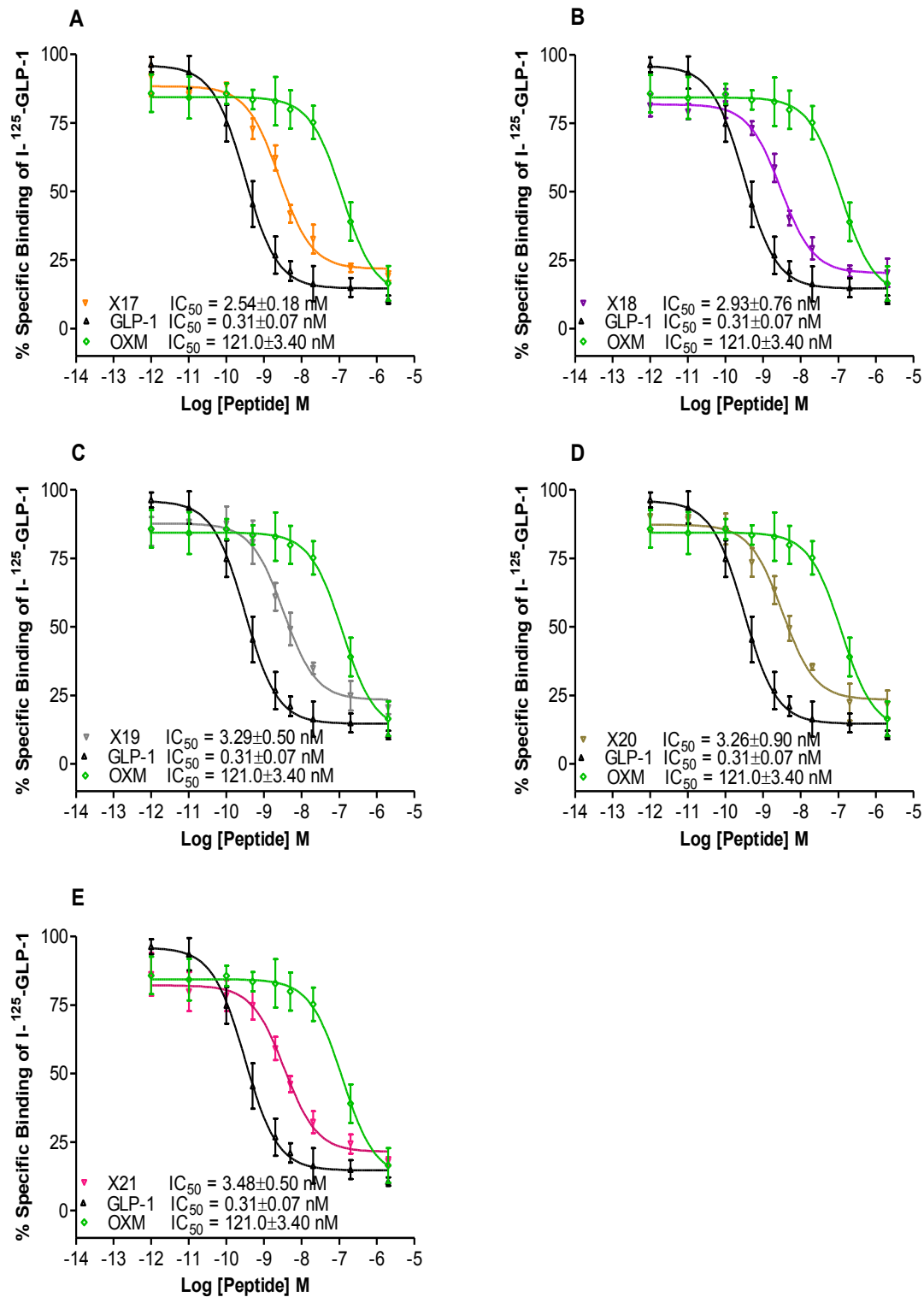


Figure 43: Competitive receptor binding affinity of GLP-1, OXM and 5 OXM analogues A: X17, B: X18, C: X19, D: X20, E: X21 to the hGLP-1R. Purified cell membranes of CHO cells overexpressing the hGLP-1R were the source of the receptors. I¹²⁵-GLP-1 was used as the competing peptide. IC₅₀ values calculated from a minimum of three separate experiments. Values shown are mean ± SEM.

5.3.1.2.2. Binding to membrane preparation of mouse lung cells overexpressing mouse GLP-1 receptor

Binding assays were completed as described in section 5.2.2.2.2. Binding studies with I^{125} -GLP-1 routinely shown greater than 90% specific binding to membranes prepared from mouse lung tissue. The binding affinities of GLP-1 and OXM to the mGLP-1R are shown graphically in Figure 44 and summarised in Table 7. Oxyntomodulin bound to the mGLP-1R with a 17-fold lower affinity approximately in comparison to GLP-1 (OXM IC_{50} = 40.23 ± 3.45 nM, GLP-1 IC_{50} = 2.40 ± 0.07 nM).

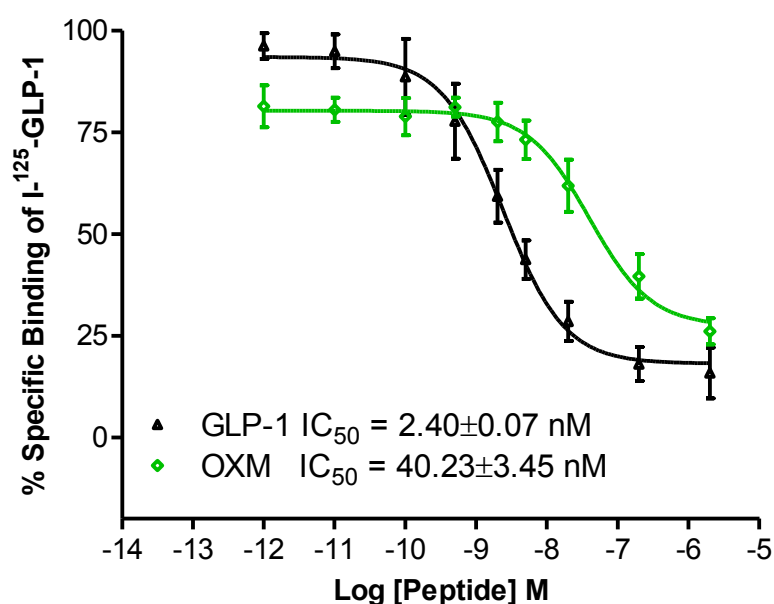


Figure 44: Competitive receptor binding affinity of GLP-1 and OXM to the mGLP-1R. Cell membrane preparations from mouse lung were the source of the mGLP-1R. I^{125} -GLP-1 used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 16 OXM analogues (X6-X21) to the mGLP-1R were tested and compared with endogenous ligands (Figure 45, Figure 46, Figure 47, Figure 48, Table 7).

Analogue X6-X9

Analogue X6 and X7 had approximately a 1.6- and 1.4-fold lower affinity respectively than GLP-1 to the mGLP-1R (X6 $IC_{50} = 3.95 \pm 1.63$ nM, X7 $IC_{50} = 3.41 \pm 0.07$ nM) (Figure 45). Analogue X8 and X9 presented a 1.8- and 2.3-fold lower binding affinity respectively to the mGLP-1R than GLP-1 (X8 $IC_{50} = 4.32 \pm 0.49$ nM, X9 $IC_{50} = 5.49 \pm 0.50$ nM).

When OXM analogues X6 to X9 were compared to OXM, they presented a higher affinity to the mGLP-1R that ranged from 7.3-fold for X9, 9.3-fold for X8, 10.2-fold for X6 and 11.8-fold for X7).

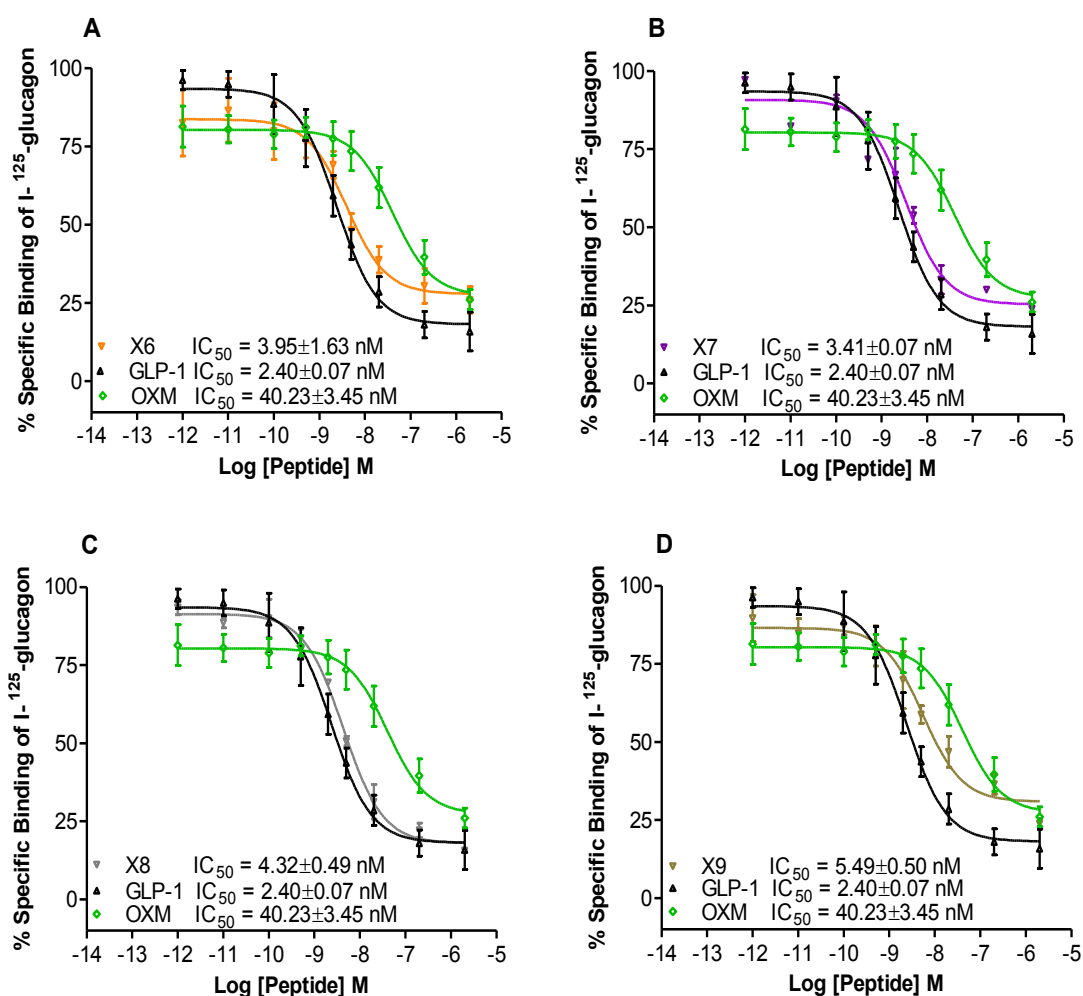


Figure 45: Competitive receptor binding affinity of GLP-1, OXM and 4 OXM analogues A: X6, B: X7, C: X8, D: X9 to the mGLP-1R. Cell membrane preparations from mouse lung overexpressing the mGLP-1R were the source of the receptors. I^{125} -GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogues X10-X13

Analogues X10 and X11 had approximately a 1.8- and 2.3-fold lower affinity respectively than GLP-1 to the mGLP-1R (X10 $IC_{50} = 4.20 \pm 0.25$ nM, X11 $IC_{50} = 5.42 \pm 2.90$ nM) (Figure 50). Analogues X12 and X13 presented a 2- and 1.9-fold lower binding affinity respectively than GLP-1 to the mGLP-1R (X12 $IC_{50} = 4.88 \pm 1.95$ nM, X13 $IC_{50} = 4.48 \pm 0.24$ nM).

When OXM analogues X10 to X13 were compared to OXM, they presented a higher affinity to the mGLP-1R that ranged from 7.4-fold for X11, 8.2-fold for X12, 9-fold for X13 and 9.6-fold for X10.

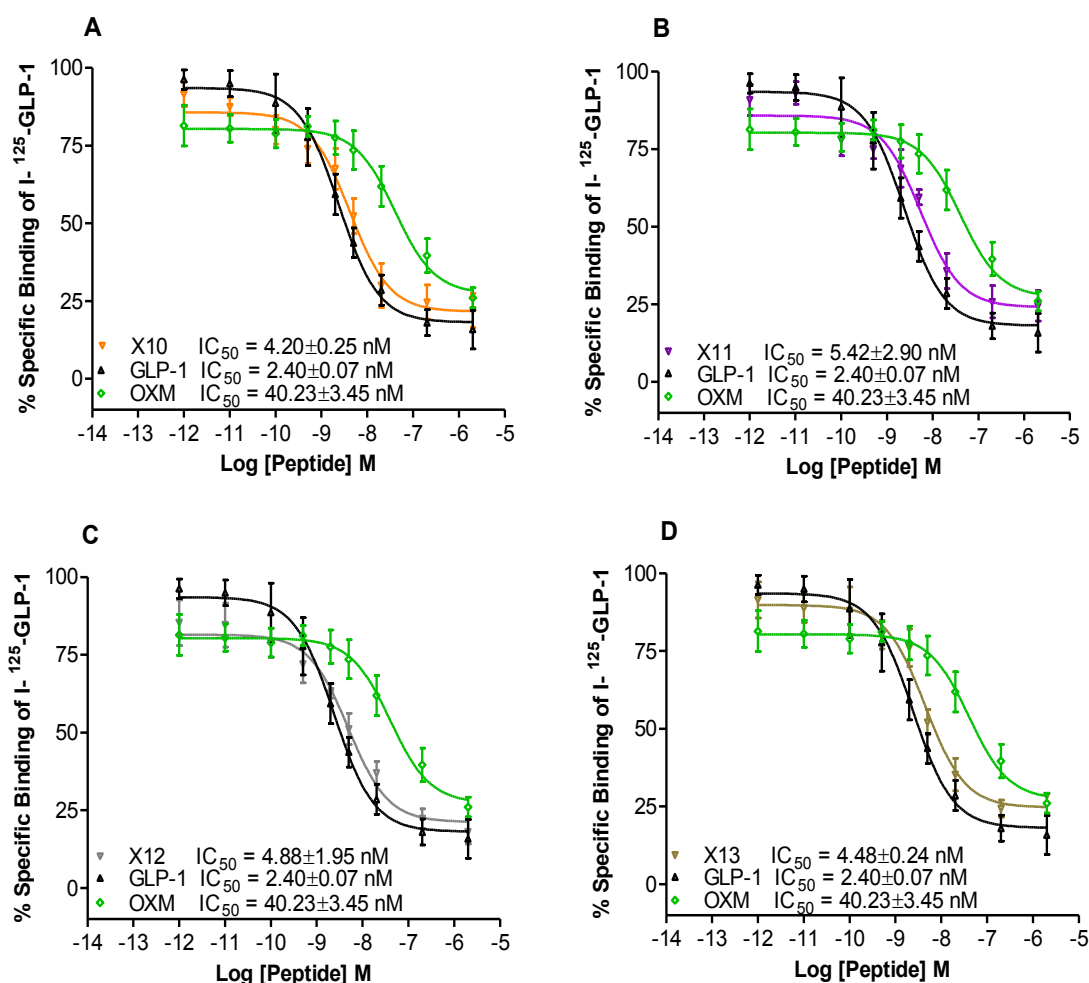


Figure 46: Competitive receptor binding affinity of GLP-1, OXM and 4 OXM analogues A: X10, B: X11, C: X12, D: X13 to the mGLP-1R. Cell membrane preparations from mouse lung overexpressing the mGLP-1R were the source of the receptors. I¹²⁵-GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X14-X16

Analogue X14 and X16 had approximately a 4.3-fold and 3.3-fold lower affinity respectively to the mGLP-1R than GLP-1 (X14 $IC_{50} = 10.21 \pm 2.30$ nM, X16 $IC_{50} = 7.99 \pm 0.30$ nM) (Figure 47). Analogue X15 presented a similar binding affinity to the mGLP-1R when compared to GLP-1 ($IC_{50} = 2.45 \pm 0.05$ nM).

When OXM analogues X14 to X16 were compared to OXM, they presented a higher binding affinity to the mGLP-1R that ranged from 3.9-fold for X14, 5-fold for X16 and 16.4-fold for X15.

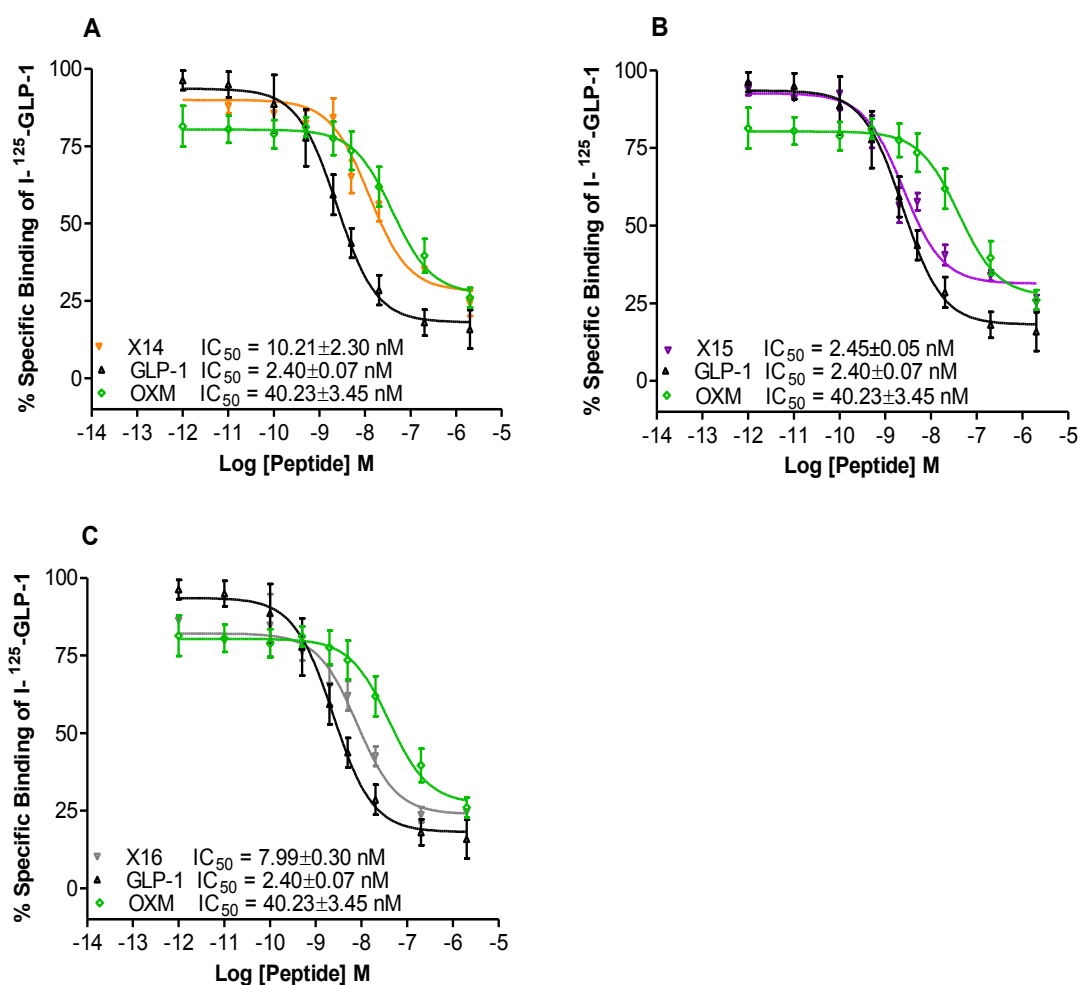


Figure 47: Competitive receptor binding affinity of GLP-1, OXM and 3 OXM analogues A: X14, B: X15, C: X16 to the mGLP-1R. Cell membrane preparations from mouse lung overexpressing the mGLP-1R were the source of the receptors. I¹²⁵-GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogues X17-X21

Analogue X17 had approximately a 2.3-fold lower affinity than GLP-1 to the mGLP-1R (X17 $IC_{50} = 5.50 \pm 0.25$ nM) (Figure 48). Analogues X18 and X19 presented approximately a 2.7-fold lower binding affinity to the mGLP-1R than GLP-1 (X18 $IC_{50} = 6.37 \pm 3.15$ nM, X19 $IC_{50} = 6.53 \pm 2.55$ nM). Analogues X20 and X21 presented approximately a 2.9- and 2.8-fold lower binding affinity to the mGLP-1R than GLP-1 respectively (X20 $IC_{50} = 6.88 \pm 3.20$ nM, X21 $IC_{50} = 6.65 \pm 2.10$ nM).

When OXM analogues X17 to X21 were compared to OXM, they presented approximately a higher affinity to the mGLP-1R that ranged from 5.8-fold for X20, 6-fold for X21, 6.2-fold for X19, 6.3-fold for X18 and 7.3-fold for X17.

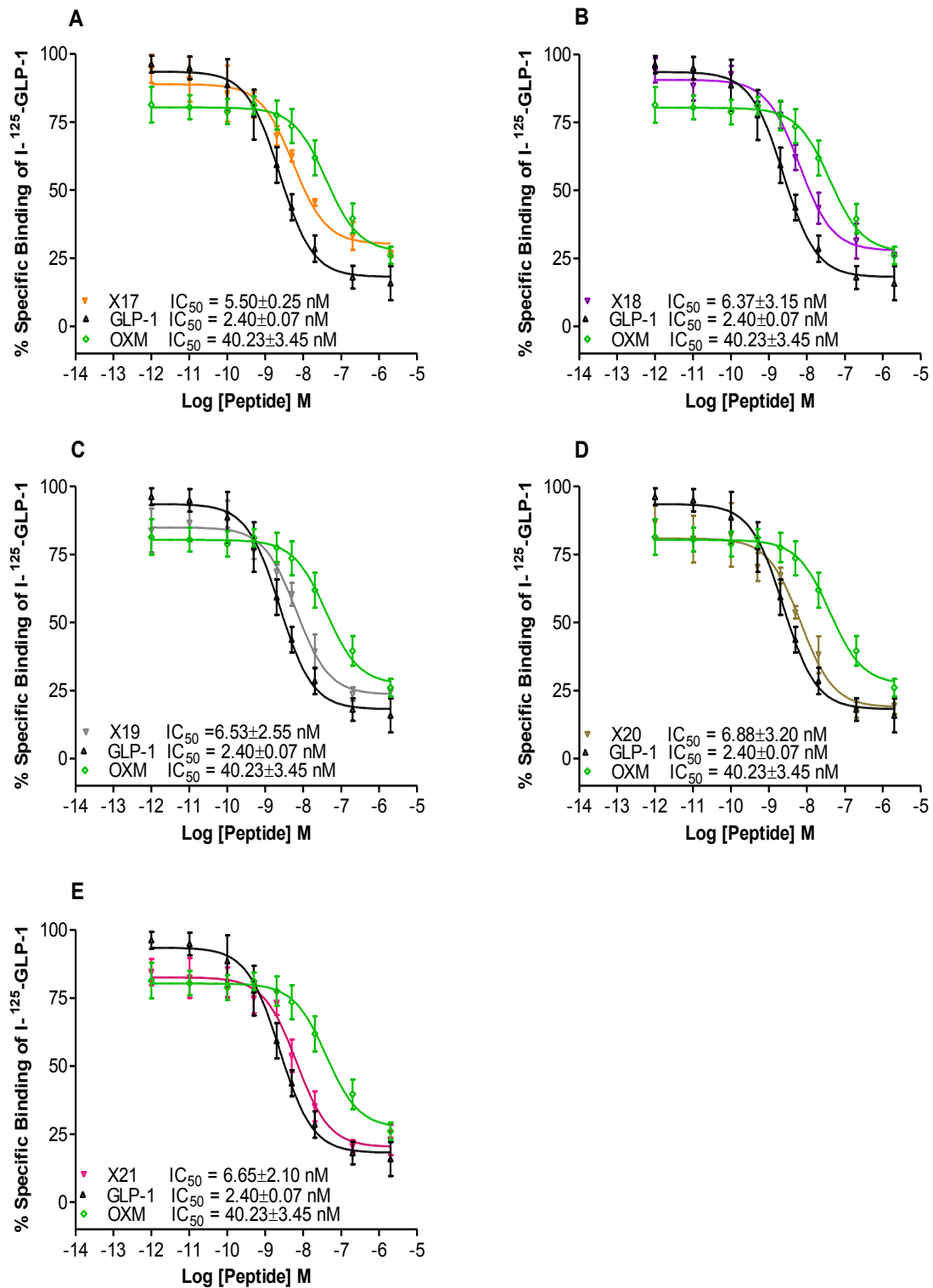


Figure 48: Competitive receptor binding affinity of GLP-1, OXM and 5 OXM analogues A: X17, B: X18, C: X19, D: X20, E: X21 to the mGLP-1R. Cell membrane preparations from mouse lung overexpressing the mGLP-1R were the source of the receptors. I¹²⁵-GLP-1 was used as the competing peptide. IC₅₀ values calculated from a minimum of three separate experiments. Values shown are mean ± SEM.

	GCGR		GLP-1R	
	hGCGR binding affinity IC ₅₀ (nM) ± SEM	mGCGR binding affinity IC ₅₀ (nM) ± SEM	hGLP-1R binding affinity IC ₅₀ (nM) ± SEM	mGLP-1R binding affinity IC ₅₀ (nM) ± SEM
GLP-1	>10000	>10000	0.31±0.07	2.40±0.07
GCG	0.52±0.05	1.35±0.17	>10000	220.0±10.0
OXM	8.84±0.47	5.87±0.31	121.0±3.40	40.23±3.45
X6	0.82±0.02	6.66±0.80	1.83±0.03	3.95±1.63
X7	0.63±0.16	7.11±0.34	1.66±0.15	3.41±0.07
X8	0.60±0.04	5.54±0.55	2.54±0.01	4.32±0.49
X9	0.62±0.04	6.65±1.04	2.69±0.50	5.49±0.50
X10	0.85±0.32	4.79±0.66	2.57±0.35	4.20±0.25
X11	0.55±0.03	5.46±0.79	2.93±0.36	5.42±2.90
X12	0.73±0.01	4.74±0.11	3.28±0.50	4.88±1.95
X13	0.62±0.06	5.32±1.05	3.40±1.20	4.48±0.24
X14	0.77±0.06	5.97±0.45	3.66±0.93	10.21±2.30
X15	0.75±0.06	6.16±1.05	3.84±1.27	2.45±0.05
X16	0.76±0.15	6.74±1.20	3.65±1.45	7.99±0.30
X17	0.94±0.10	6.99±1.20	2.54±0.18	5.50±0.25
X18	0.85±0.03	6.78±0.35	2.93±0.76	6.37±3.15
X19	0.77±0.12	7.27±1.07	3.29±0.50	6.53±2.55
X20	0.83±0.15	7.84±1.40	3.26±0.90	6.88 ±3.20
X21	0.75±0.01	6.92±0.40	3.48±0.50	6.65±2.10

Table 7: A summary of IC₅₀ values for GLP-1, GCG, OXM and OXM analogues X6-X21 at both the GCGR and GLP-1R (human and mouse receptors). Half-maximal concentrations values are shown as mean ± SEM.

5.3.2. Effect of oxyntomodulin, glucagon and oxyntomodulin analogues on the human glucagon receptor mediated cAMP accumulation

The ability of OXM, GCG and GLP-1 to stimulate cAMP accumulation in CHO cells overexpressing the hGCGR was investigated. The potencies of GCG and OXM are shown graphically in Figure 49 and summarised in Table 8. The bioactivity of OXM at the hGCGR was 9-fold lower than GCG (OXM EC_{50} = 0.512 ± 0.080 nM, GCG EC_{50} = 0.056 ± 0.007 nM). Glucagon-like peptide-1 presented a >2000-fold lower bioactivity at the hGCGR when compared with GCG (GLP-1 EC_{50} = 114.50 ± 5.30 nM).

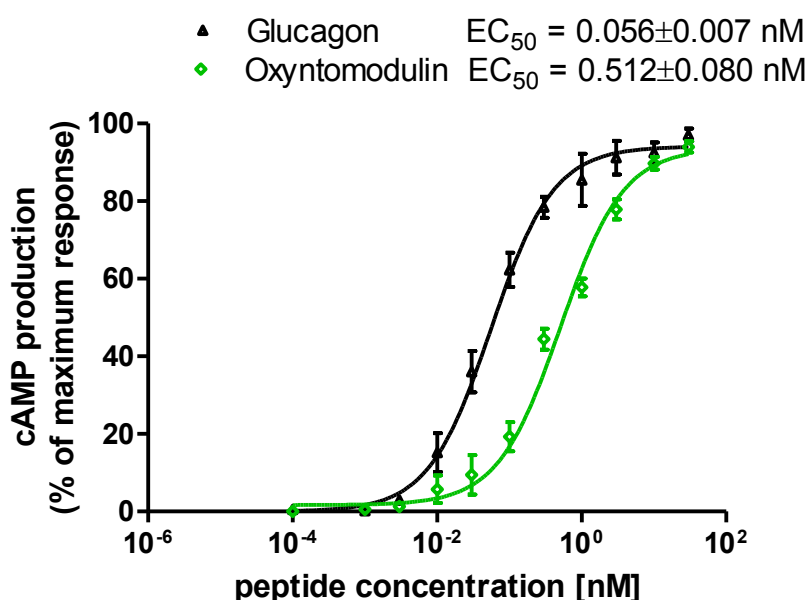


Figure 49: hGCGR-mediated cAMP by GCG and OXM. hGCGR overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

The *in vitro* potencies of 16 OXM analogues (X6 to X21) at stimulating cAMP accumulation at the GCGR were compared with GCG and OXM (Figure 50, Figure 51, Figure 52, Figure 53, Table 8).

Analogue X6-X9

The bioactivity of analogue X6 at the hGCGR was 3.1-fold higher than GCG (X6 $EC_{50} = 0.018 \pm 0.007$ nM) while analogue X7 demonstrated an approximately 9.3-fold higher bioactivity at the hGCGR than GCG (X7 $EC_{50} = 0.006 \pm 0.001$ nM) (Figure 50). Analogues X8 and X9 stimulated a cAMP response with a potency approximately 3.7- and 2.8-fold higher than GCG respectively (X8 $EC_{50} = 0.015 \pm 0.005$ nM, X9 $EC_{50} = 0.020 \pm 0.001$ nM).

Analogues X6, X7, X8 and X9 had a higher bioactivity at the hGCGR when compared to OXM that ranged from 28.4- and 25.6-fold for analogues X6 and X9 respectively, to 34.1-fold for analogue X8 and 85.3-fold for analogue X7.

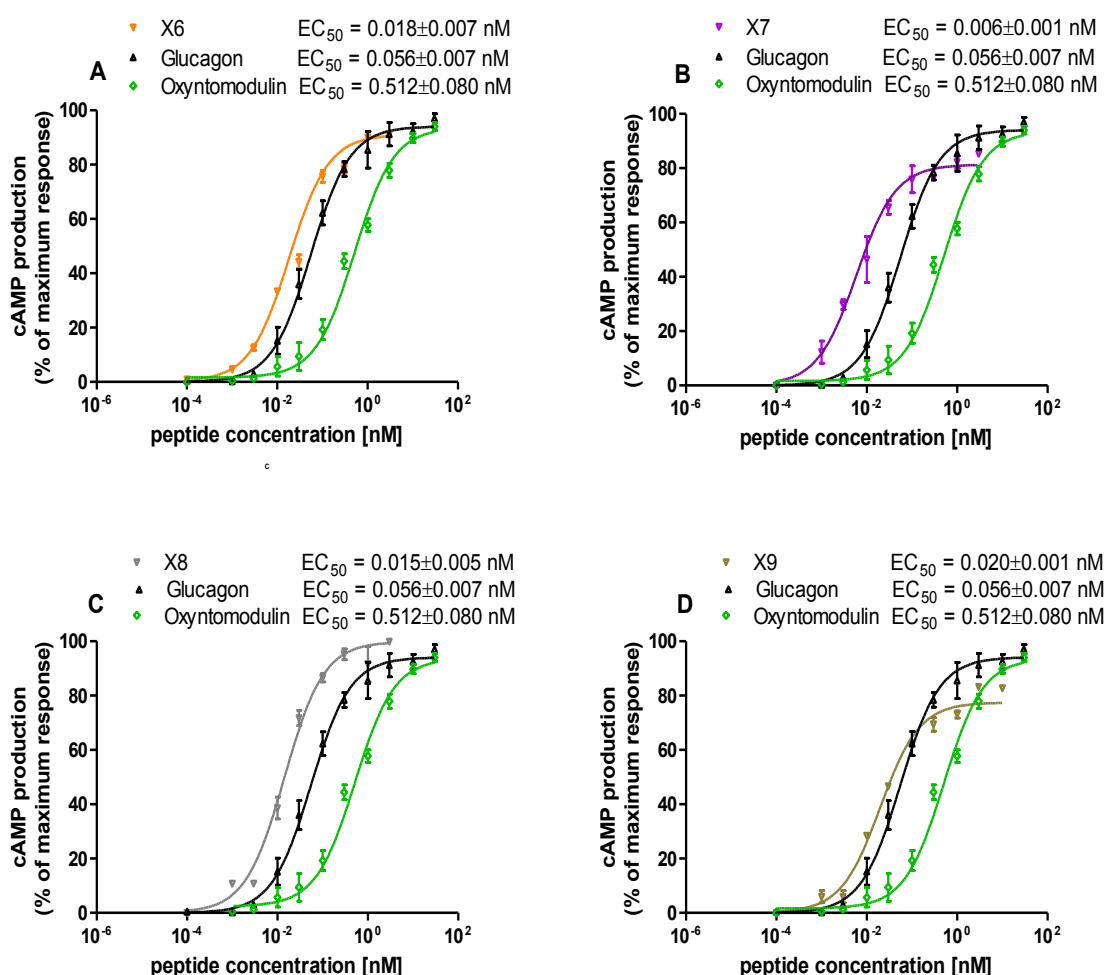


Figure 50: hGCGR-mediated cAMP accumulation by GCG, OXM and 4 OXM analogues A: X6, B: X7, C: X8, D: X9. hGCGR overexpressed in CHO cells. Each peptide concentrations tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X10-X13

Analogue X10 and X11 were 4.7- and 3.7-fold more bioactive than GCG at stimulating the hGCGR (X10 $EC_{50} = 0.012 \pm 0.009$ nM, X11 $EC_{50} = 0.015 \pm 0.008$ nM) (Figure 51). In a similar pattern analogues X12 and X13 had a 6.2-fold higher bioactivity at the hGCGR when compared to GCG (X12 $EC_{50} = 0.009 \pm 0.001$ nM, X13 $EC_{50} = 0.009 \pm 0.009$ nM).

Analogue X10, X11, X12 and X13 had a higher bioactivity at the hGCGR when compared to OXM that ranged from 42.7- and 34.1-fold for analogues X10 and X11 respectively, to 56.9-fold for analogues X12 and X13.

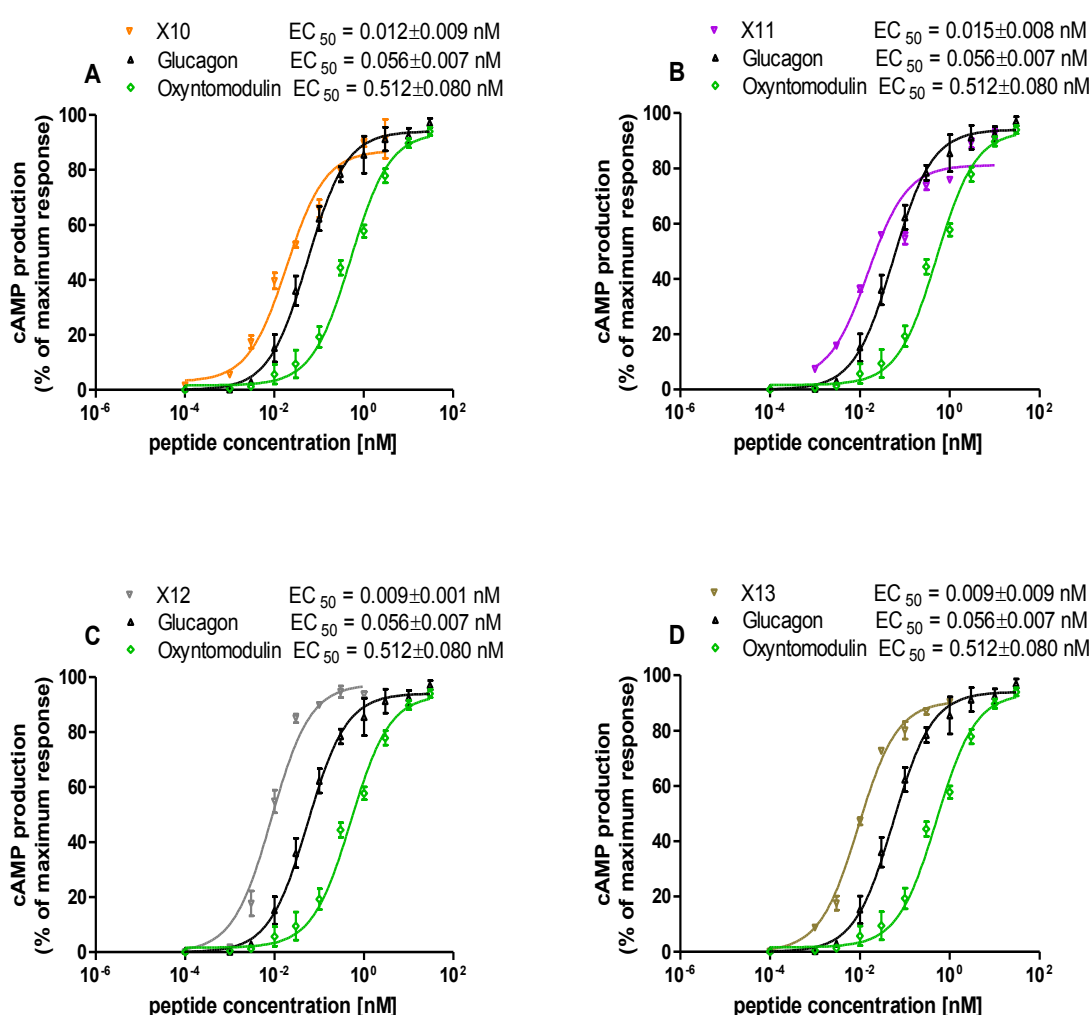


Figure 51: hGCGR-mediated cAMP accumulation by GCG, OXM and 4 OXM analogues A: X10, B: X11, C: X12, D: X13. hGCGR overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X14-X16

Analogue X14 was 3.7-fold more bioactive than GCG at stimulating the hGCGR (X14 $EC_{50} = 0.015 \pm 0.006$ nM) (Figure 52). Analogues X15 and X16 were 4.7-fold more bioactive than GCG at stimulating the hGCGR (X15 $EC_{50} = 0.012 \pm 0.005$ nM, X16 $EC_{50} = 0.012 \pm 0.004$ nM).

Analogues X14, X15 and X16 had a higher bioactivity at the hGCGR when compared to OXM that ranged from 34.1-fold for X14 to 42.7-fold for analogues X15 and X16.

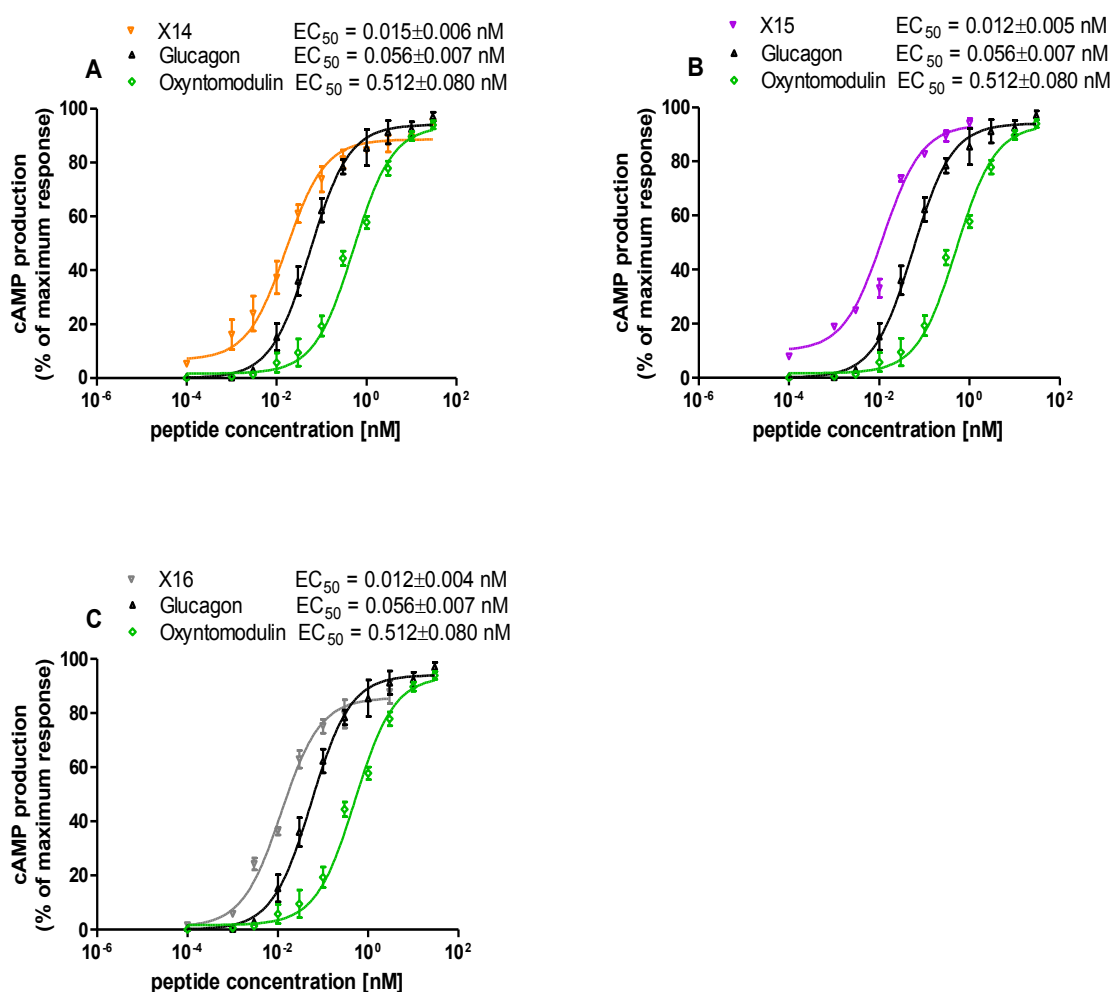


Figure 52: hGCGR-mediated cAMP by GCG, OXM and 3 OXM analogues A: X14, B: X15, C: X16. hGCGR overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogues X17-X21

The bioactivity of analogue X17 at the hGCGR was 4.3-fold higher than GCG (X17 $EC_{50} = 0.013 \pm 0.004$ nM) while analogue X18 demonstrated an approximately 6.2-fold higher bioactivity at the hGCGR than GCG (X18 $EC_{50} = 0.009 \pm 0.001$ nM) (Figure 53). Analogues X19 and X21 stimulated a cAMP response with a 4.7-fold higher potency than GCG (X19 $EC_{50} = 0.012 \pm 0.001$ nM, X21 $EC_{50} = 0.012 \pm 0.004$ nM). Analogue X20 was 4.3 fold more potent than GCG (X20 $EC_{50} = 0.013 \pm 0.005$ nM).

Analogues X17, X18, X19, X20 and X21 had a higher bioactivity at the hGCGR when compared to OXM that ranged from 39.4 fold for analogues X17 and X20, to 42.7-fold for analogues X19 and X21 and 56.9-fold for analogue X18.

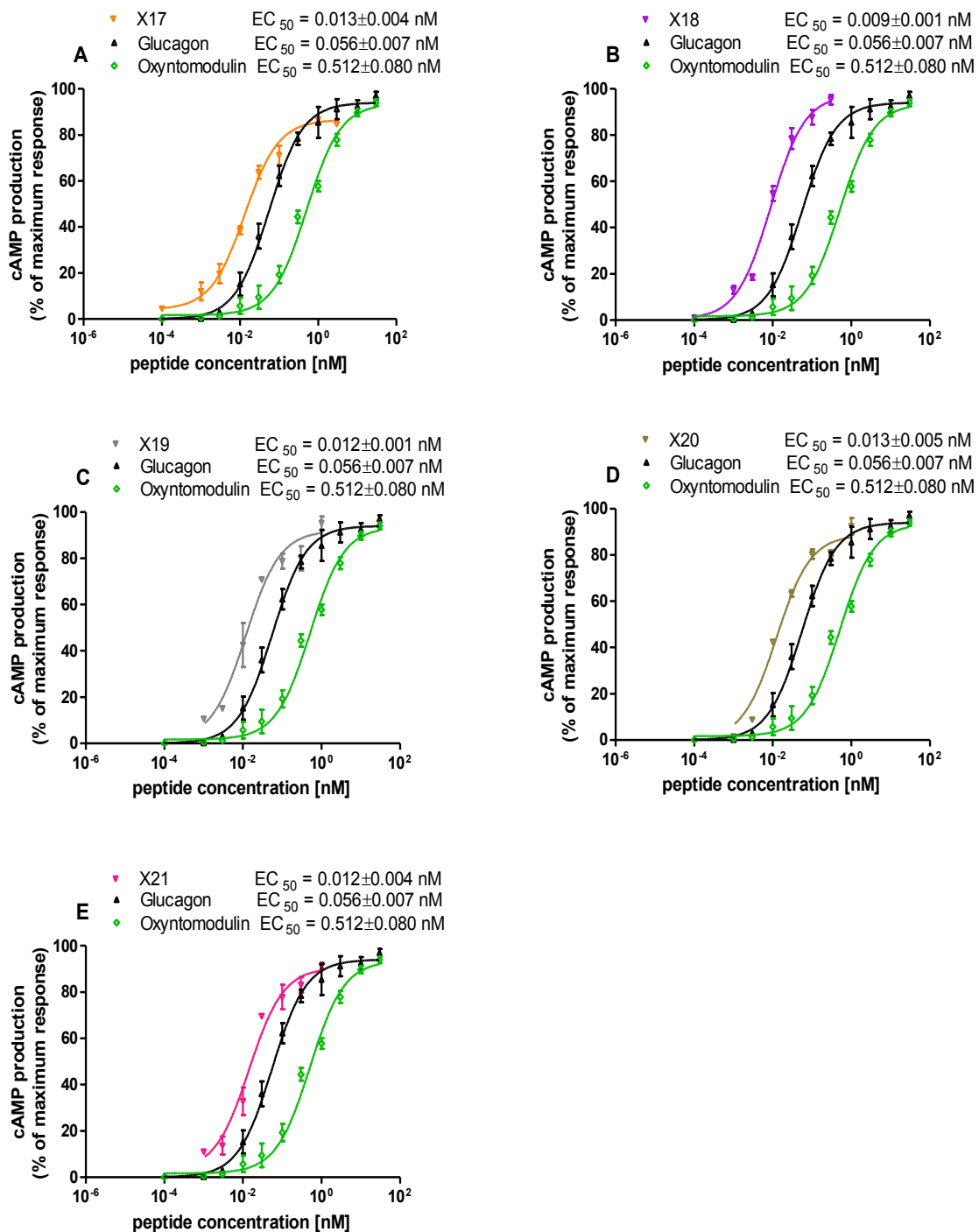


Figure 53: hGCGR-mediated cAMP accumulation by GCG, OXM and 5 OXM analogues A: X17, B: X18, C: X19, D: X20, E: X21. hGCGR overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

5.3.3. Effect of oxyntomodulin, GLP-1 and oxyntomodulin analogues on the human GLP-1 receptor mediated cAMP accumulation

The ability of OXM, GCG and GLP-1 to stimulate cAMP accumulation in CHO cells overexpressing the hGLP-1R was investigated. The potencies of GLP-1 and OXM are shown graphically in Figure 54 and summarised in Table 8. The bioactivity of OXM was approximately 13-fold lower than GLP-1 (OXM EC_{50} = 6.531 ± 1.940 nM, GLP-1 EC_{50} = 0.504 ± 0.020 nM). Glucagon presented a 359-fold lower bioactivity approximately at the hGLP-1R (GCG EC_{50} = 180.70 ± 3.511 nM) when compared to GLP-1.

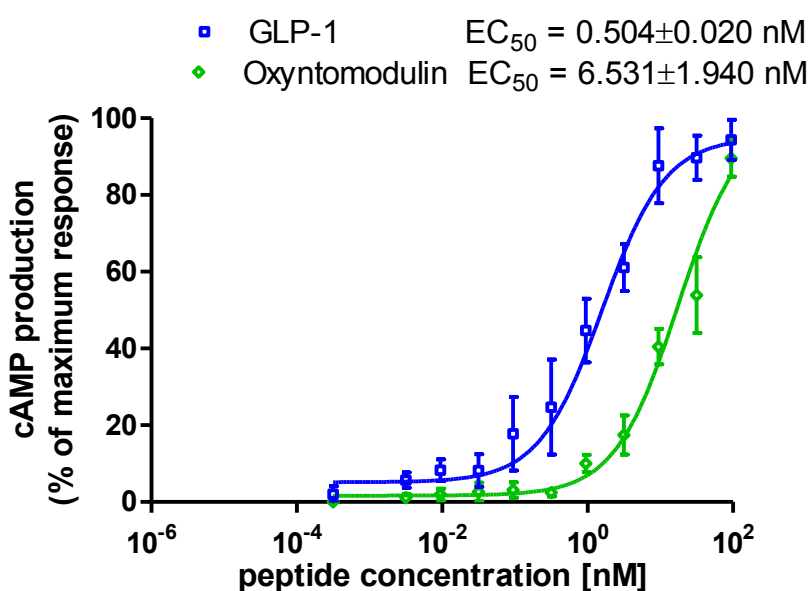


Figure 54: hGLP-1R-mediated cAMP accumulation by GLP-1 and OXM. hGLP-1R overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

The *in vitro* potencies of 16 OXM analogues (X6 to X21) at stimulating cAMP accumulation at the hGLP-1R were compared with GLP-1 and OXM (Figure 55, Figure 56, Figure 57, Figure 58, Table 8).

Analogue X6-X9

The bioactivity of analogues X6 and X7 at the hGLP-1R was approximately 2.4-fold lower than GLP-1 (X6 EC_{50} = 1.192 ± 0.261 nM, X7 EC_{50} = 1.20 ± 0.315 nM) (Figure 55). Analogues X8 and X9 stimulated a cAMP response at the hGLP-1R with a potency approximately 2.1 and 2-fold lower than GLP-1 respectively (X8 EC_{50} = 1.046 ± 0.260 nM, X9 EC_{50} = 1.015 ± 0.070 nM).

Analogues X6, X7, X8 and X9 had a higher bioactivity at the hGLP-1R when compared to OXM that ranged from 5.5- and 5.4-fold for analogues X6 and X7, to 6.2-fold for analogue X8 and 6.4-fold for analogues X9 and X10.

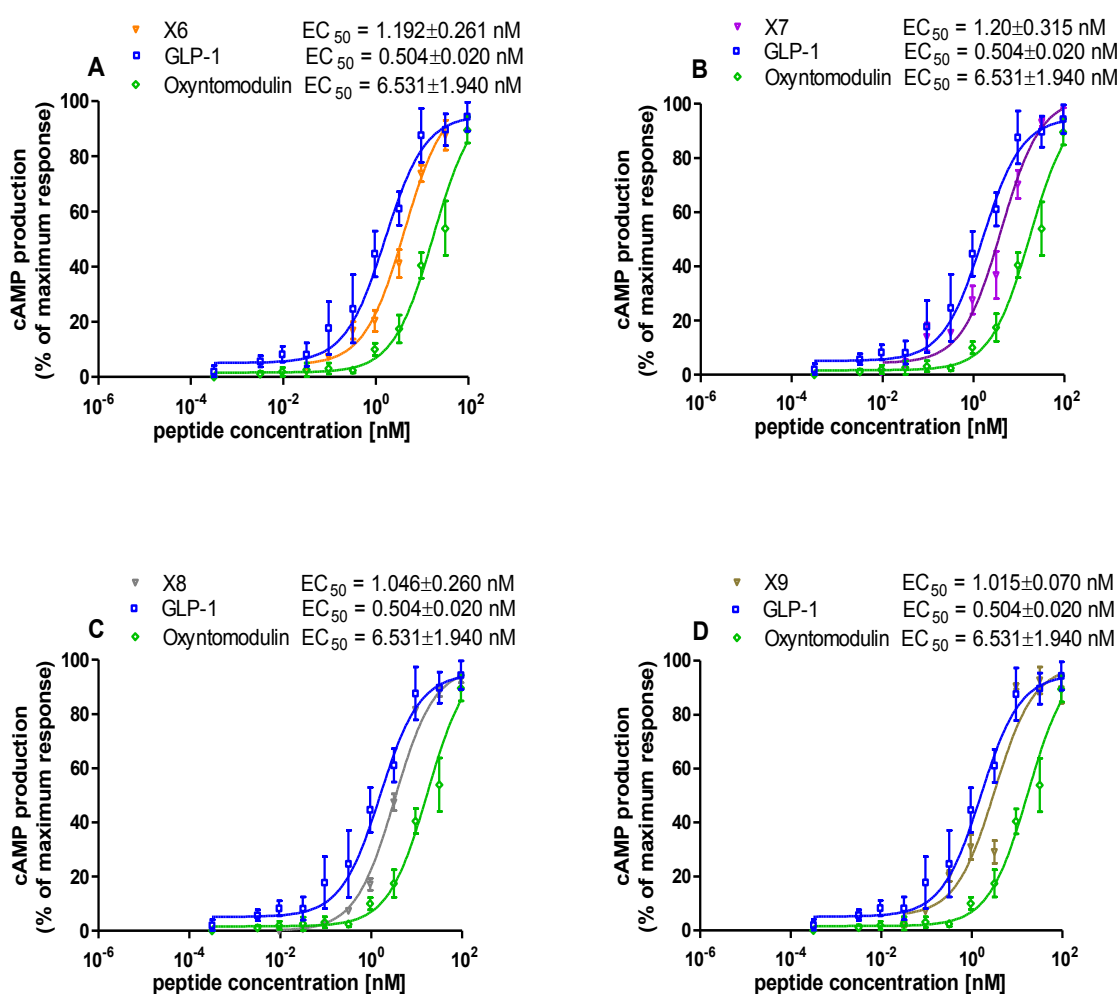


Figure 55: hGLP-1R-mediated cAMP accumulation by GLP-1, OXM and 4 OXM analogues A: X6, B: X7, C: X8, D: X9. hGLP-1R overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X10-X13

Analogue X10 and X11 were 2- and 1.6-fold respectively less bioactive than GLP-1 at stimulating the hGLP-1R (X10 EC_{50} = 1.020 ± 0.119 nM, X11 EC_{50} = 0.783 ± 0.130 nM) (Figure 56). Analogue X12 and X13 were 1.2-fold less bioactive than GLP-1 at stimulating the hGLP-1R (X12 EC_{50} = 0.60 ± 0.081 nM, X13 EC_{50} = 0.596 ± 0.180 nM).

Analogue X10, X11, X12 and X13 had a higher bioactivity at the hGLP-1R when compared to OXM that ranged from 6.4- and 8.3-fold for analogues X10 and X11 respectively, to 10.9- and 11-fold for analogues X12 and X13 respectively.

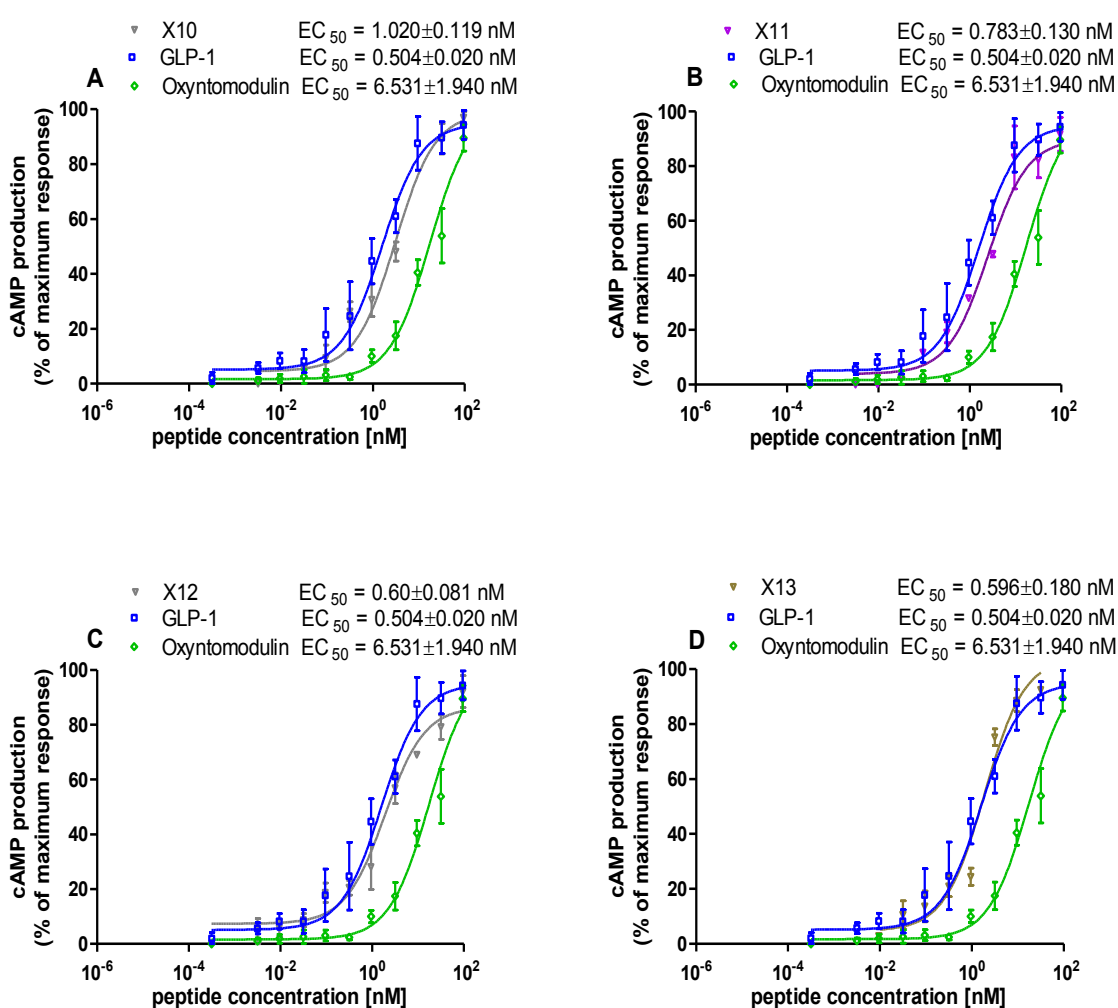


Figure 56: hGLP-1R-mediated cAMP accumulation by GLP-1, OXM and 4 OXM analogues A: X10, B: X11, C: X12, D: X13. hGLP-1R overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogue X14-X16

Analogue X14 was 1.8-fold less bioactive than GLP-1 at stimulating the hGLP-1R (X14 $EC_{50} = 0.925 \pm 0.170$ nM) while analogue X16 had a 1.5-fold lower bioactivity at the hGLP-1R than GLP-1 (X16 $EC_{50} = 0.749 \pm 0.050$ nM) (Figure 57). Analogue X15 presented an *in vitro* potency at the hGLP-1R almost equal to the one of native GLP-1 (X15 $EC_{50} = 0.512 \pm 0.090$ nM).

Analogue X14, X15 and X16 had a higher bioactivity at the hGLP-1R when compared to OXM that was 7.1-fold, 12.8-fold and 8.7-fold for analogues X14, X15 and X16 respectively.

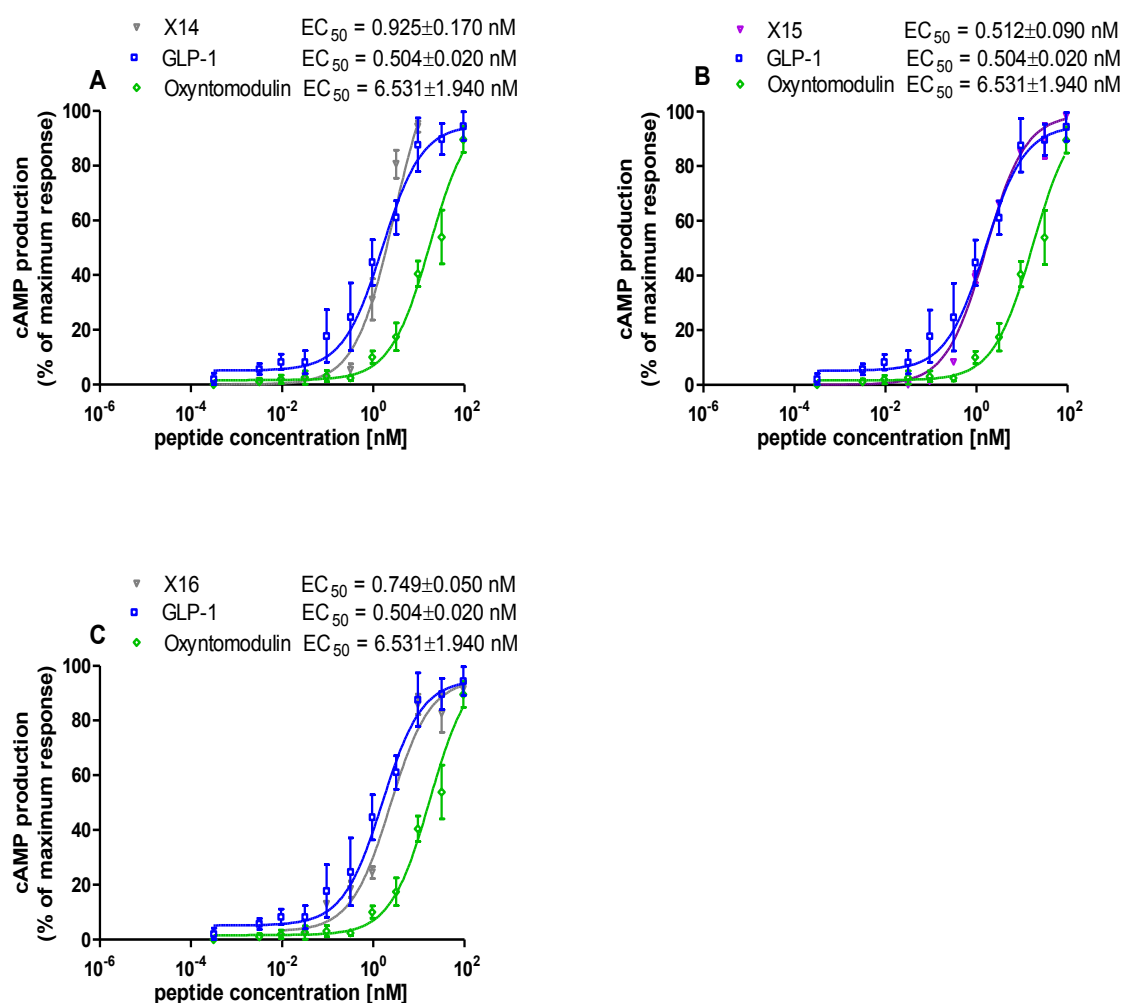


Figure 57: hGLP-1R-mediated cAMP accumulation by GLP-1, OXM and 3 OXM analogues A: X14, B: X15, C: X16. hGLP-1R overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Analogues X17-X21

The bioactivity of analogue X17 at the hGLP-1R was 2.6-fold lower than GLP-1 (X17 $EC_{50} = 1.311 \pm 0.415$ nM) (Figure 58). Analogues X18, X19 and X21 were 2.3-fold less bioactive than GLP-1 at the hGLP-1R (X18 $EC_{50} = 1.136 \pm 0.380$ nM, X19 $EC_{50} = 1.176 \pm 0.360$ nM, X21 $EC_{50} = 1.144 \pm 0.560$ nM). Analogue X20 presented a 2-fold lower potency at the hGLP-1R when compared to GLP-1 (X20 $EC_{50} = 1.018 \pm 0.280$ nM).

Analogues X17 to X21 had a higher bioactivity at the hGLP-1R when compared to OXM that ranged from 5-fold for analogue X17, 5.6-fold for X19, 5.7-fold for X18 and X21 and 6.4-fold for analogue X20.

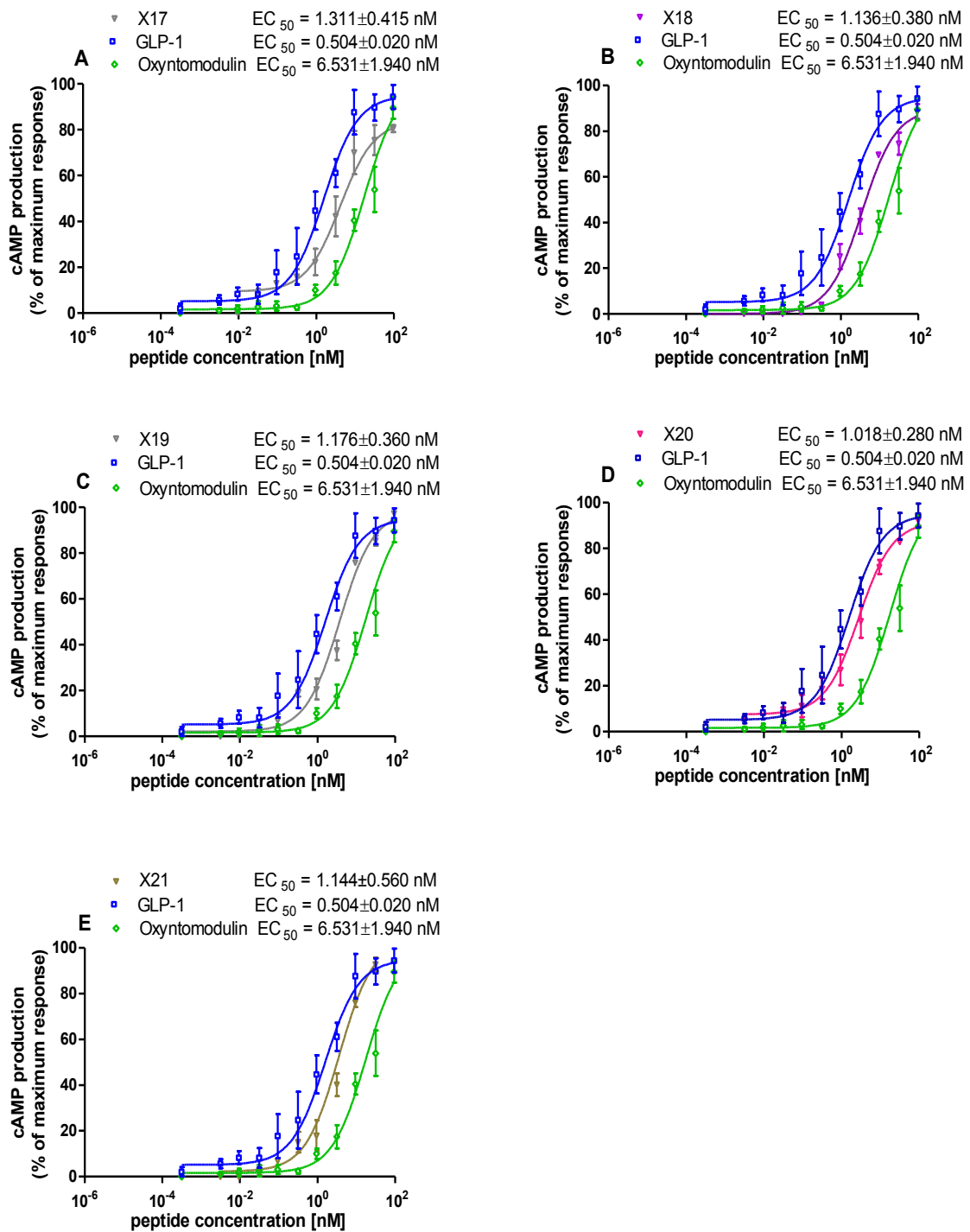


Figure 58: hGLP-1R-mediated cAMP accumulation by GLP-1, OXM and 5 OXM analogues A: X17, B: X18, C: X19, D: X20, E: X21. hGLP-1R overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

	hGCGR	hGLP-1R
	cAMP bioactivity EC ₅₀ (nM) ± SEM	cAMP bioactivity EC ₅₀ (nM) ± SEM
GLP-1	114.50±5.30	0.504±0.020
GCG	0.056±0.007	180.70±3.511
OXM	0.512±0.080	6.531±1.940
X6	0.018±0.007	1.192±0.261
X7	0.006±0.001	1.20±0.315
X8	0.015±0.005	1.046±0.260
X9	0.020±0.001	1.015±0.070
X10	0.012±0.009	1.020±0.119
X11	0.015±0.008	0.783±0.130
X12	0.009±0.001	0.60±0.081
X13	0.009±0.009	0.596±0.180
X14	0.015±0.006	0.925±0.170
X15	0.012±0.005	0.512±0.090
X16	0.012±0.004	0.749±0.050
X17	0.013±0.004	1.311±0.415
X18	0.009±0.001	1.136±0.380
X19	0.012±0.001	1.176±0.360
X20	0.013±0.005	1.018±0.280
X21	0.012±0.004	1.144±0.560

Table 8: Summary of EC₅₀ values for GLP-1, GCG, OXM and OXM analogues X6-X21 at both the hGCGR and hGLP-1R. Half-maximal concentrations values are shown as mean ± SEM.

5.3.4. Effect of exendin-4 and oxyntomodulin analogues X6 to X21 on food intake in fasted mice

Analogues X6-X9

Subcutaneous administration of EX-4 (10 nmol/kg) and 4 OXM analogues (X6 to X9) at 50 nmol/kg showed that all tested agents significantly reduced food intake to fasted C57BL/6J mice over the first hour post-injection compared with the vehicle control group (Figure 59). At the period of 1-2 hours, EX-4 and analogue X9 significantly reduced food intake in comparison to the vehicle control group. Exendin-4 was the only one that significantly inhibited food intake in the 2-4 hours post-injection. At the 4-8 hours period, EX-4 and analogues X7 and X9 significantly inhibited food intake in comparison to the vehicle control group. There were no significant differences in food intake between vehicle control group and the other treatments in the 8-24 hour and 24 - 48 hour interval.

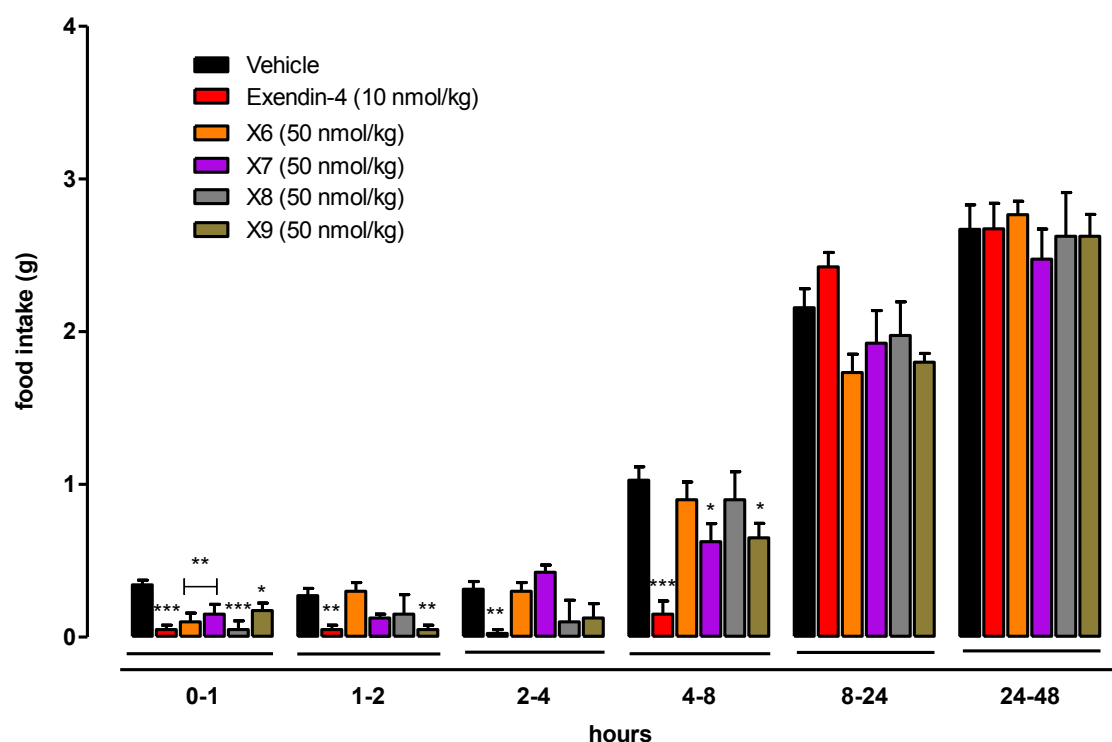


Figure 59: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X6-X9) (50 nmol/kg), on acute food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour and 8-24 and 24-48 hour intervals. n=7-8 per group. Statistics: One way ANOVA with Dunnett's post-hoc test *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ vs. vehicle.

Analysis of cumulative food intake data showed that EX-4 (10 nmol/kg) and OXM analogues X7, X8 and X9 (50 nmol/kg), significantly reduced food intake 0-2 hours post-injection compared with the

vehicle control group (Figure 60). The groups did not present statistically significant differences in the inhibition of food intake when they were compared between them.

Cumulative food intake at 0-4 hours post-injection was significantly reduced in EX-4 and OXM groups X8 and X9 when compared to the vehicle control group (Figure 60). Test groups X6 and X7 ate significantly more than the EX-4 group ($p<0.01$ for both analogues).

At the 0-24 hour's period cumulative food intake of groups EX-4 and X9 demonstrated that they significantly reduced the food intake when compared to vehicle control group ($p<0.001$ and $p<0.01$ respectively). Test groups X6, X7 and X8 did not significantly reduced food intake during this time period when compared to the vehicle control group and in fact consumed significantly more food than EX-4 group ($p<0.05$ for analogues X6 and X7 and $p<0.01$ for analogue X8 respectively).

Cumulative food intake data over 48 hours demonstrated that none of the OXM analogues significantly reduced the food intake when compared to the vehicle control group. Test group EX-4 significantly reduced their food intake in the 0-48 hours period when compared to the vehicle control group ($p<0.01$).

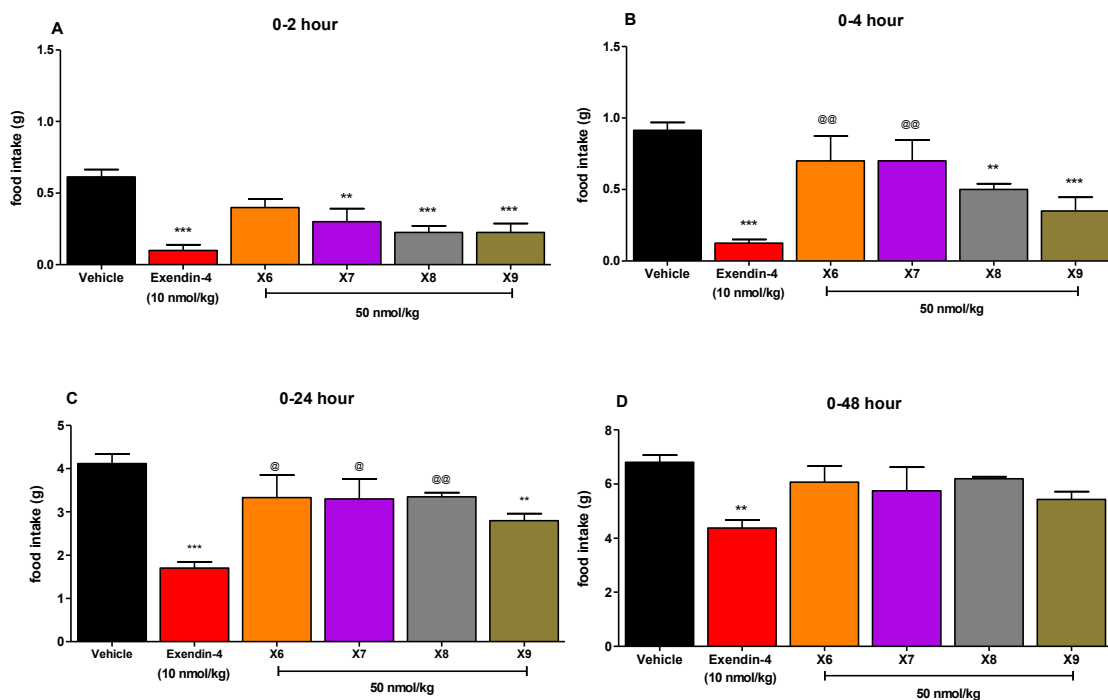


Figure 60: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X6-X9) (50 nmol/kg) on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for A: 0-2 hours, B: 0-4 hours, C: 0-24 hours and D: 0-48 hours. $n=7-8$ mice per group. Statistics: Comparisons versus vehicle were performed by one-way ANOVA with Dunnett's correction test while comparisons between groups were performed by one-way ANOVA with Tukey's correction test. **= $p<0.01$, *= $p<0.001$ vs. vehicle; @= $p<0.05$, @@= $p<0.01$ vs. EX-4.**

Investigation of the body weight changes that occurred between the periods of -16 to 48 hours demonstrated that test groups EX-4, X7 and X9 had a significant reduction in body weight when compared to the vehicle control group ($p<0.001$, $p<0.001$ and $p<0.01$ respectively) (Figure 61). The average body weight reduction of the EX-4 group was 1.80 ± 0.20 g compared to vehicle-treated controls; X7 group lost 1.20 ± 0.10 g and X9 group lost 0.90 ± 0.20 g.

When the body weight change of the OXM analogues treated groups of mice was compared to the respective change of the EX-4 group, it was shown that groups X6 and X8 had a significantly less body weight reduction ($p<0.001$ and $p<0.01$ respectively) while the body weight reduction of analogues X7 and X9 did differ significantly from EX-4 group.

When the OXM analogues were compared between them it was shown that only analogue X7 group had a significantly greater body weight reduction when compared to the analogue X6 group ($p<0.05$) over the 2 day period.

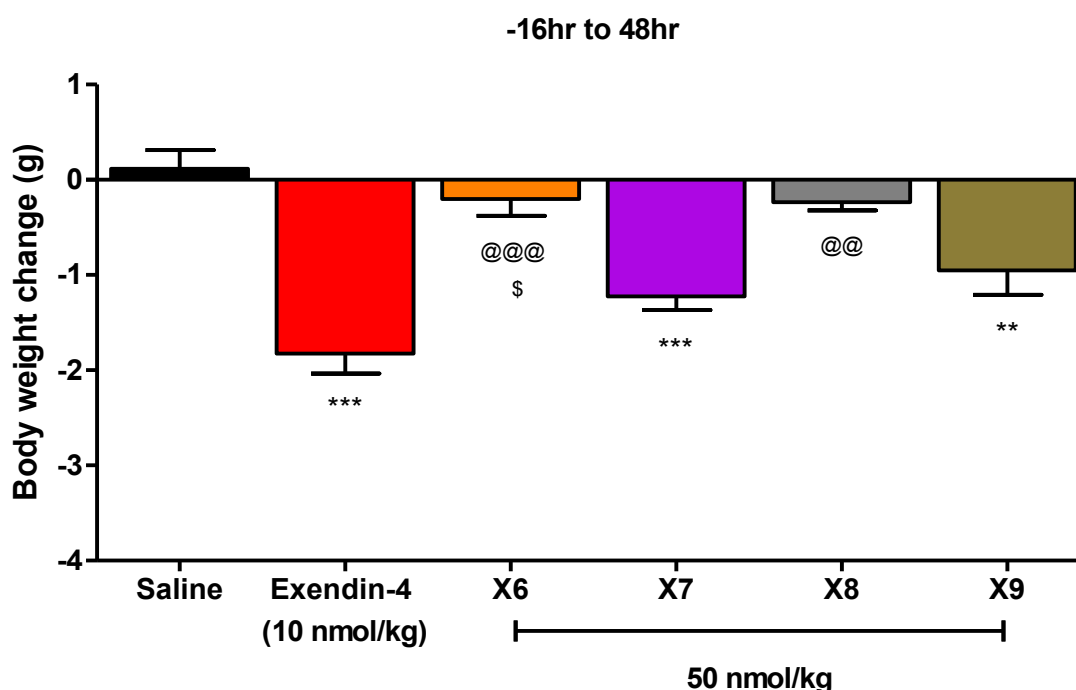


Figure 61: Body weight change over a 2 day period following a single SC dose of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X6-X9) (50 nmol/kg). Body weight change measured to accuracy of 0.1g compared to body weight at day 0. Error bars are $\pm$ SEM. Statistics: Comparisons versus vehicle were performed by one-way ANOVA with Dunnett's correction test while comparisons between groups were performed by one-way ANOVA with Tukey's correction test. **= $p<0.01$, ***= $p<0.001$ vs. vehicle; @@= $p<0.01$, @= $p<0.001$ vs. EX-4; \$= $p<0.05$ vs. X7.

Analogues X10-X16

Subcutaneous administration of EX-4 (10 nmol/kg) and 7 OXM analogues (X10 to X16) at 50 nmol/kg to fasted C57BL/6J mice, demonstrated that over the first hour post-injection only animals treated with EX-4, analogue X11, X12, X13 and X15 significantly reduced their food intake injection compared with the vehicle control group ($p<0.001$, $p<0.05$, $p<0.05$, $p<0.01$ and $p<0.01$ respectively) (Figure 62). None of the analogues tested caused a significant reduction of food intake in any of the later intervals, while EX-4 continued to inhibit food intake until the 8-24 hour interval.

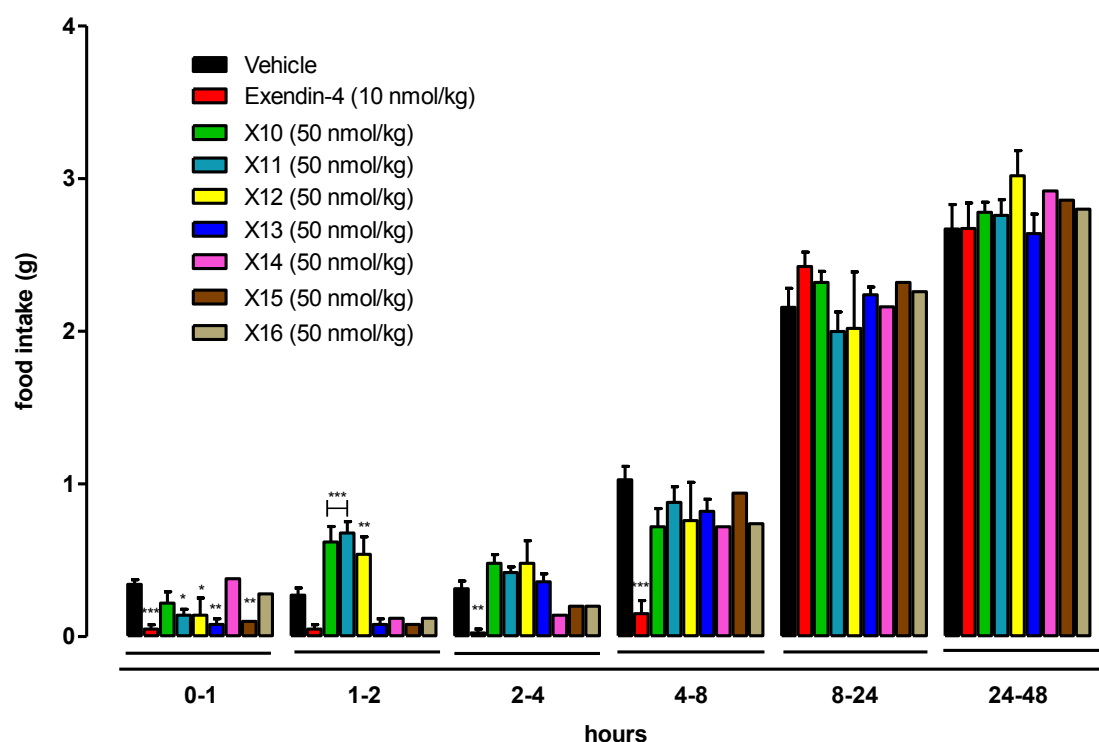


Figure 62: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X10-X16) (50 nmol/kg), on acute food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour and 8-24 and 24-48 hour intervals. $n=7-8$ per group. Statistics: One way ANOVA with Dunnett's post-hoc test $*=p<0.05$, $**=p<0.01$, $***=p<0.001$ vs. vehicle.

When the anorectic effect of EX-4 and the OXM analogues was compared between the groups, during the first hour post-injection, it was shown that only animals treated with analogue X14 ate significantly more than the EX-4 group ($p<0.01$) (Figure 63). Furthermore X14 group consumed significantly more than X11, X12, X13 and X15 groups ($p<0.05$, $p<0.01$, $p<0.01$ and $p<0.01$ respectively).

During the 1-2 hours period, the comparison of food intake between the EX-4 and the OXM analogues groups demonstrated that animals treated with X13, X14, X15 and X16 did not have a significantly different food intake in comparison to EX-4. Furthermore, animals treated with analogues X13, X14, X15 and X16 had a significantly lower food intake when compared to groups X10, X11 and X12.

In the 2-4 hours period post-injection, the food intake of animals treated with analogues X14, X15 and X16 did not differ in a statistical significant manner when compared to EX-4. On the contrary, animals treated with X10, X11, X12 and X13 consumed significantly more food than EX-4 group. Groups of animals treated with X14, X15 and X16 presented a lower statistically significant food intake when compared with analogues X10 and X12. Furthermore, analogue X14 had a statistically significant lower food intake when compared to analogue X11.

During the 4-8 hours period, test groups X10 to X16 consumed significantly more food than EX-4 group.

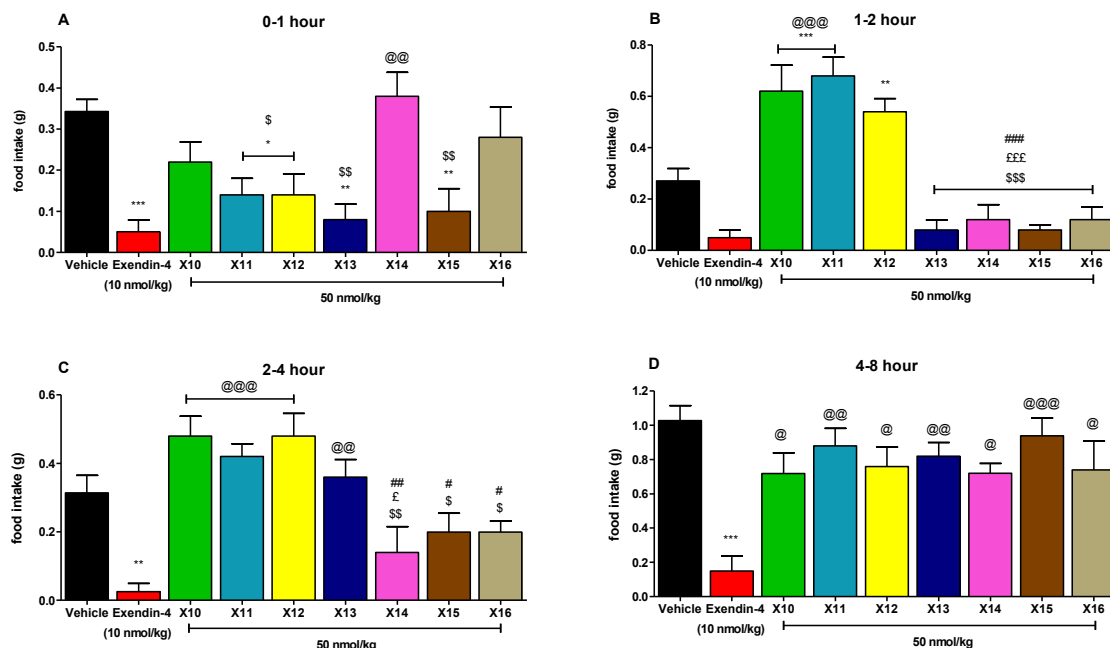


Figure 63: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X10-X16) (50 nmol/kg), on acute food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour and 8-24 and 24-48 hour intervals. $n=7-8$ per group. Statistics: One way ANOVA with Tukey's post-hoc test $*=p<0.05$, $**=p<0.01$, $***=p<0.001$ vs. vehicle; $@=p<0.05$, $@@=p<0.01$, $@@@=p<0.001$ vs. EX-4. A: $\$=p<0.05$, $\$\$=p<0.01$ vs. x14; B: $\$ \$ \$=p<0.001$ vs. X10, $\text{£££}=p<0.001$ vs. X11, $\text{###}=p<0.001$ vs. X12; C: $\$=p<0.05$, $\$ \$=p<0.01$ vs. X10, $\text{£}=p<0.05$ vs. X11, $\#=p<0.05$, $\text{###}=p<0.01$ vs. X12.

Analysis of cumulative food intake data showed that EX-4 (10 nmol/kg) and OXM analogues X12, X13 and X15 (50 nmol/kg), significantly reduced food intake 0-2 hours post-injection compared with the vehicle control group (Figure 64). The EX-4 and OXM groups X10-X16 did not present statistically significant differences in the inhibition of food intake when they were compared between them.

Cumulative food intake at 0-4 hours post-injection was significantly reduced in EX-4 and OXM group X15 when compared to the vehicle control group ($p<0.001$ and $p<0.01$ respectively). Test groups X10,

X11, X12 and X14 ate significantly more than the EX-4 group while groups X13, X15 and X16 did not differ from EX-4. X11 group consumed significantly more food than X15 group ($p<0.01$).

At the 0-24 hour's period, cumulative food intake of test group EX-4 was significantly lower than the food intake when compared to vehicle control group ($p<0.001$) while none of the analogues tested caused a significant inhibition of food intake. Test groups X10 to X16 consumed significantly more food than EX-4 group ($p<0.001$ for all analogues).

Cumulative food intake data over 48 hours demonstrated that none of the OXM analogues reduced significantly the food intake when compared to the vehicle control group. Only test group EX-4 significantly reduced their food intake in the 0-48 hours period when compared to vehicle control group ($p<0.01$). Furthermore, groups X10 to X16 consumed significantly more food than EX-4 group ($p<0.001$ for all groups).

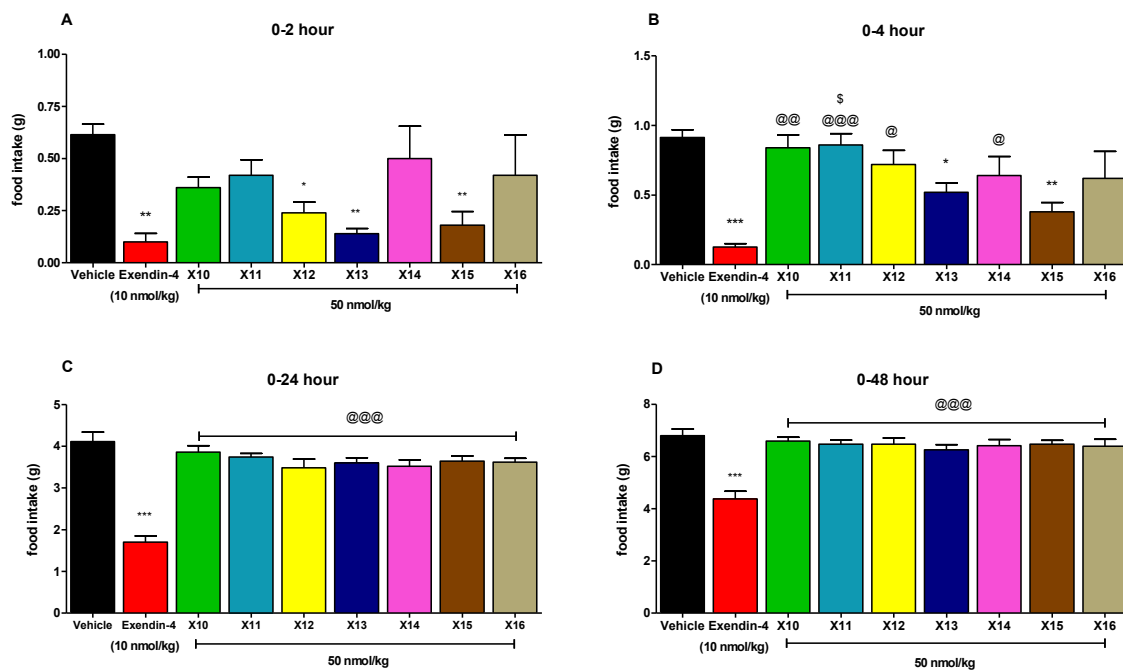


Figure 64: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X10-X16) (50 nmol/kg) on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for A: 0-2 hours, B: 0-4 hours, C: 0-24 hours and D: 0-48 hours. $n=7-8$ mice per group. Statistics: One way ANOVA with Tukey's post-hoc test $*=p<0.05$, $**=p<0.01$, $***=p<0.001$ vs. vehicle; $@=p<0.05$, $@@=p<0.01$, $@@@=p<0.001$ vs. EX-4. B: $\$=p<0.05$ vs. X15.

Investigation of the body weight changes that occurred between the periods of -16 to 48 hours showed that EX-4 and analogue X10 groups had a significant reduction in body weight when compared to the vehicle control group ($p<0.001$ and $p<0.05$ respectively) (Figure 65). The average body weight reduction of the EX-4 group was 1.8 ± 0.2 g compared to vehicle-treated controls while X10 group lost 0.70 ± 0.20 g.

When the body weight change of the OXM analogues treated groups of mice was compared to the respective change of the EX-4 group, it was shown that all OXM analogues injected groups had a significantly less body weight reduction.

When the OXM analogues were compared between them it was shown that there was no statistical significant difference in the body weight reduction recorded over the 2 day period.

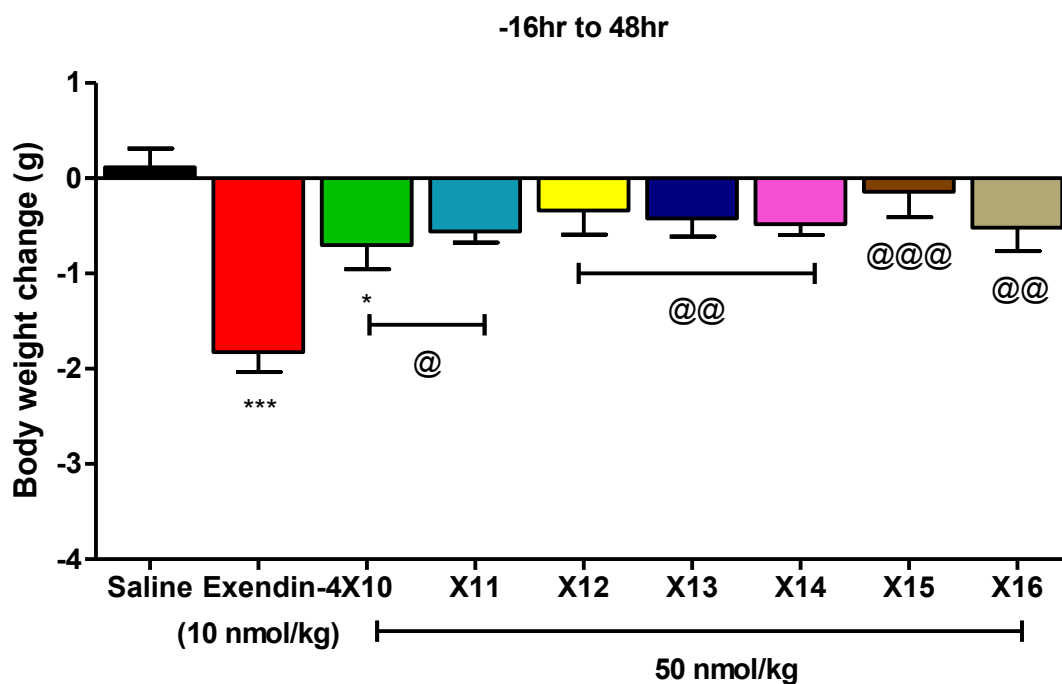


Figure 65: Body weight change over a 2 day period following a single SC dose of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X10-X16) (50 nmol/kg). Body weight change measured to accuracy of 0.1g compared to body weight at day 0. Error bars are $\pm$ SEM. Statistics: Comparisons versus vehicle were performed by one-way ANOVA with Dunnett's correction test while comparisons between groups were performed by one-way ANOVA with Tukey's correction test. *= $p < 0.05$, ***= $p < 0.001$ vs. vehicle; @= $p < 0.05$, @@= $p < 0.01$, @@@= $p < 0.001$ vs. EX-4.

Analogues X17-X21

Subcutaneous administration of EX-4 (10 nmol/kg) and 5 OXM analogues (X17 to X21) at 50 nmol/kg to fasted DIO C57BL/6J mice, demonstrated that over the first hour post-injection only animals treated with EX-4, analogues X18, X20 and X21 had a significantly reduced food intake compared to the vehicle control group ($p<0.001$, $p<0.001$, $p<0.01$ and $p<0.001$ respectively) (Figure 66). At the period of 1-2 hours, animals treated with analogues EX-4, X18, X19, X20 and X21 had a significantly lower food intake in comparison to the vehicle control group. Exendin-4 and analogue X21 were the only test items that resulted in a significant inhibition of food intake in the 2-4 hours post-injection ($p<0.001$ for both peptides). At the 4-8 hours period, again, only EX-4 and analogue X21 significantly inhibited food intake in comparison to the vehicle control group ($p<0.001$ for both peptides). There were no significant differences in food intake between the vehicle control group and the other treatments in the 8-24 hour and 24 - 48 hour interval.

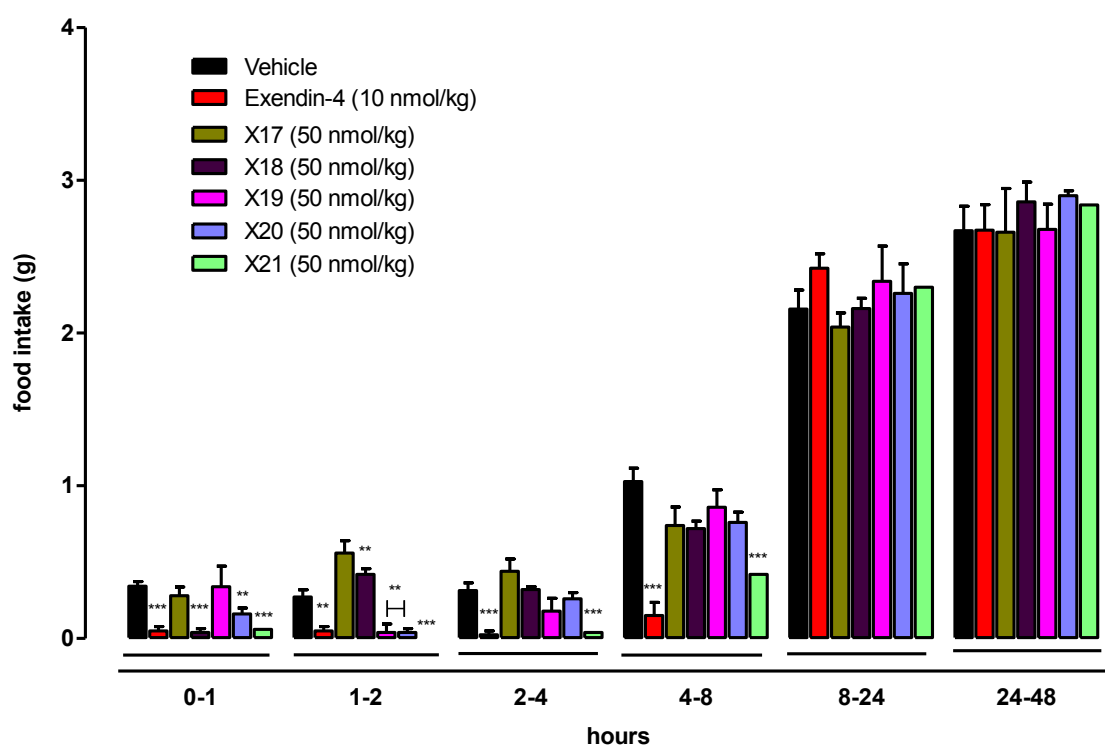


Figure 66: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X17-X21) (50 nmol/kg), on acute food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour and 8-24 and 24-48 hour intervals. $n=7-8$ per group. Statistics: One way ANOVA with Dunnett's post-hoc test *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ vs. vehicle.

When the anorectic effect of EX-4 and the OXM analogues was compared between the groups, during the first hour post-injection, it was shown that only animals treated with analogues X17 and X19 ate significantly more than the EX-4 group ($p<0.05$ and $p<0.001$ respectively) (Figure 67). Furthermore

animals treated with analogues X18 and X21 had a significantly lower food intake when compared to analogues X17 and X19.

During the 1-2 hours period, the comparison of food intake between the EX-4 and the OXM groups X17-X21 demonstrated that animals treated with X19, X20 and X21 did not have a significantly different food intake in comparison to EX-4. Furthermore, animals treated with analogues X18, X19, X20 and X21 had a significantly lower food intake when compared to group X17 ($p < 0.001$ for all peptides). Animals treated with agents X19, X20 and X21 had also a significantly lower food intake when compared to group X18 ($p < 0.001$ for all peptides).

In the 2-4 hours period post-injection, the food intake of animals treated with analogues X19 and X21 did not differ in a statistical significant manner when compared to EX-4. On the contrary, animals treated with X17, X18 and X20 consumed significantly more food than EX-4 group. Groups of animals treated with X19 and X21 presented a lower statistically significant food intake when compared with analogues X17. Furthermore, analogue X21 had a statistically significant lower food intake when compared to analogue X18 and X20.

During the 4-8 hours period, test groups X17, X18, X19 and X20 consumed significantly more food than EX-4 group. Analogue X21 had a statistically significant lower food intake when compared to analogue X19 ($p < 0.05$).

In the 8-24 hours and 24-48 hours period the food intake of mice treated with EX-4 and OXM analogues did not differ in a statistical significant manner when compared to the vehicle control group.

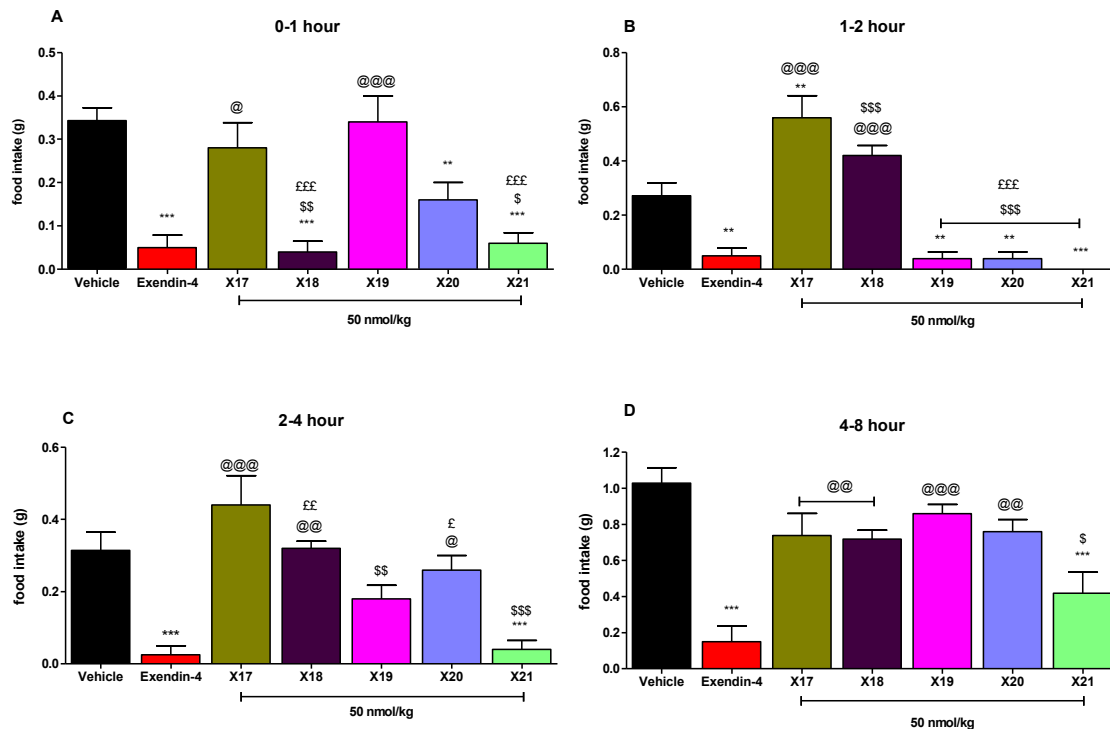


Figure 67: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X17-X21) (50 nmol/kg), on acute food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour and 8-24 and 24-48 hour intervals. $n=7-8$ per group. Statistics: One way ANOVA with Tukey's post-hoc test $*=p<0.05$, $**=p<0.01$, $***=p<0.001$ vs. vehicle; $@=p<0.05$, $@@=p<0.01$, $@@@=p<0.001$ vs. EX-4. A: $$$$=p<0.01$ vs. X17, $£££=p<0.001$ vs. X19; B: $$$$=p<0.01$ vs. X17, $£££=p<0.001$ vs. X18; C: $$$=p<0.01$, $$$$=p<0.001$ vs. X17, $£=p<0.05$, $£££=p<0.001$ vs. X21; D: $\$=p<0.05$ vs. X19.

Analysis of cumulative food intake data showed that both EX-4 (10 nmol/kg) and OXM analogues X18, X19, X20 and X21 (50 nmol/kg), all significantly reduced food intake 0-2 hours post-injection compared with the vehicle control group (Figure 68). Analogue X17 consumed significantly more food than X21 group ($p<0.05$). No statistically significant differences in food intake were observed between EX-4 and analogues X17-X21 in the 0-2 hour interval.

Cumulative food intake at 0-4 hours post-injection was significantly reduced in EX-4 and all OXM analogues groups, except for analogue X17, when compared to the vehicle control group. All OXM analogues groups, except for X21, ate significantly more than the EX-4 group. Mice treated with analogues X18, X20 and X21 had a significantly greater food intake inhibition when compared to analogue X17. Furthermore, analogue X21 had a significantly greater food intake inhibition than analogues X18, X19 and X20.

Over the 0-24 hour's period, cumulative food intake of test groups EX-4, X18 and X21 were significantly reduced when compared to vehicle control group ($p<0.001$, $p<0.05$ and $p<0.001$ respectively). Test

groups X17 to X21 consumed significantly more food during this time period than EX-4 group. The food intake inhibition during the same period was statistically significantly greater for analogue X21 when compared to analogue X19.

Cumulative food intake data over 48 hours demonstrated that only EX-4 and X21 groups had a significantly lower food intake than the vehicle control group ($p<0.001$ and $p<0.01$ respectively). Groups X17 to X21 consumed significantly more food than EX-4 group over 48 hours ($p<0.001$ for X17, X18, X19 and X20; $p<0.05$ for X21).

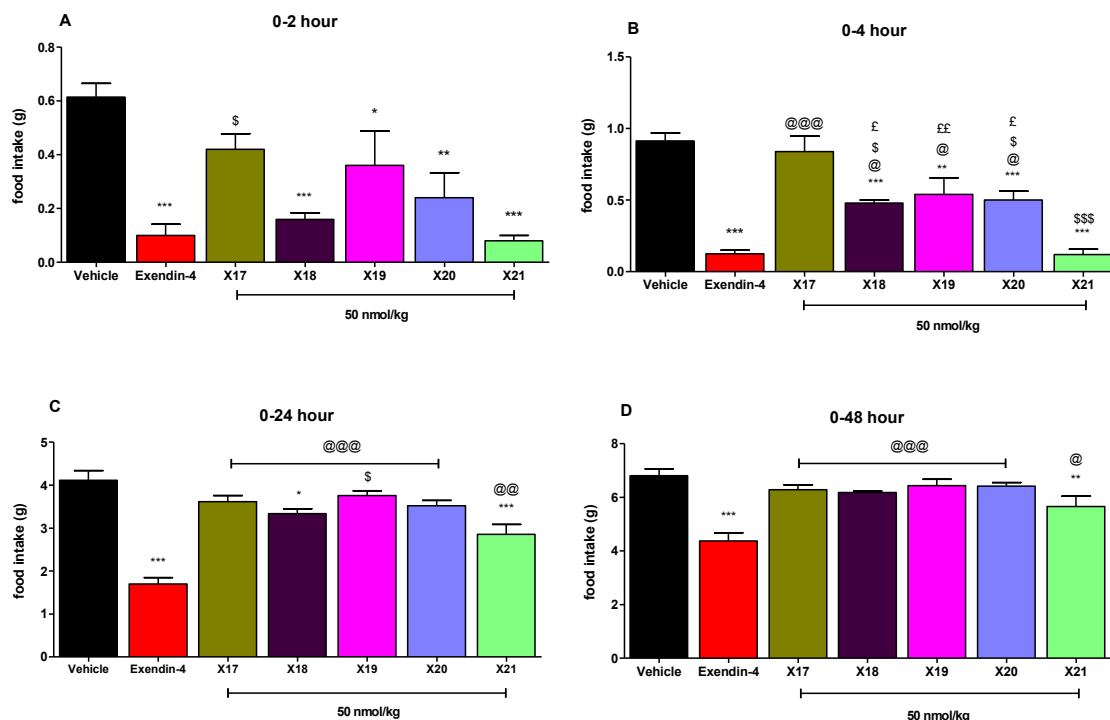


Figure 68: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X17-X21) (50 nmol/kg) on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for A: 0-2 hours, B: 0-4 hours, C: 0-24 hours and D: 0-48 hours. $n=7-8$ mice per group. Statistics: One way ANOVA with Tukey's post-hoc test $*=p<0.05$, $**=p<0.01$, $***=p<0.001$ vs. vehicle; $@=p<0.05$, $@@=p<0.01$, $@@@=p<0.001$ vs. EX-4. A: $\$=p<0.05$ vs. X21; B: $\$=p<0.05$, $$$$=p<0.001$ vs. X17, $\pounds=p<0.05$, $\pounds\pounds=p<0.01$ vs. X21; C: $\$=p<0.01$ vs. X21.

Investigation of the body weight changes that occurred between the periods of -16 to 48 hours demonstrated that test groups EX-4 and X21 had a significant reduction in body weight than the vehicle control group ($p<0.001$ and $p<0.01$ respectively) (Figure 69). The average body weight reduction of the EX-4 group was 1.80 ± 0.20 g compared to vehicle-treated controls while X21 group lost 1.20 ± 0.30 g.

No significant change in body weight over the 2 day period was observed between the OXM analogues treated groups and EX-4 group, or between the OXM analogue treatment groups.

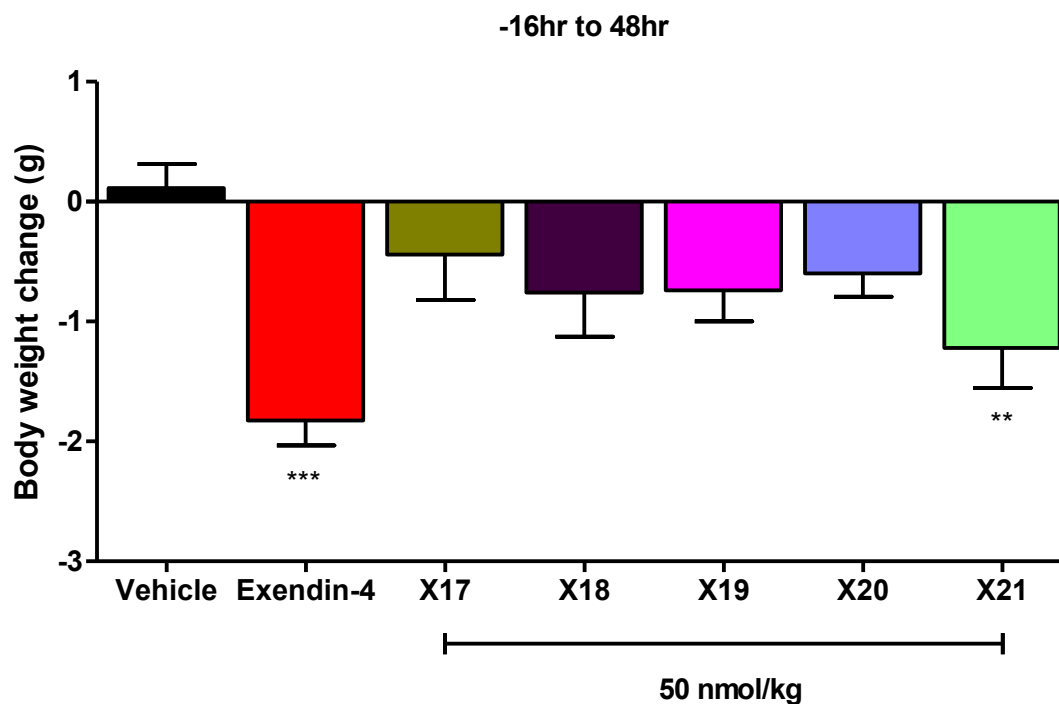


Figure 69: Body weight change over a 2 day period following a single SC dose of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X17-X21) (50 nmol/kg). Body weight change measured to accuracy of 0.1g compared to body weight at day 0. Error bars are $\pm$ SEM. Statistics: Comparisons versus vehicle were performed by one-way ANOVA with Dunnett's correction test while comparisons between groups were performed by one-way ANOVA with Tukey's correction test. **= $p < 0.01$, ***= $p < 0.001$ vs. vehicle.

5.3.5. Investigation of the pharmacokinetic properties of oxyntomodulin analogues X6 to X21 in an *in vivo* constant infusion model

The time-courses of the mean plasma concentrations of analogues X6 to X21 during the IV primed-continuous peptide infusion were measured by RIA. Plateau values were observed between 30 and 60 minutes after the start of the infusion of 9 nmol/hr of the peptide. The PK parameters and the steady-state concentration of analogues X6 to X21 are shown graphically in Figure 73, Figure 77, Figure 81 and Figure 85 and presented in Table 9. The steady-state concentrations of analogues X6 to X21 ranged between 7.2 ± 0.1 pmol/ml (analogue X11) to 40.0 ± 11.7 pmol/ml (analogue X7).

Analogue X14 presented the lowest and analogue X18 the highest MCR (4.7 ± 0.7 ml/min/kg and 13.2 ± 0.5 ml/min/kg respectively). Analogues X14 and X15 had a significantly lower MCR when compared to the MCR of the OXM ($p < 0.05$ for both analogues) (Figure 78).

The half-life of OXM analogues X6-X21 ranged between 5.4 ± 0.2 minutes (analogue X6) to 15.4 ± 0.4 minutes (analogue X18). Analogues X6, X7 and X8 had a significantly lower half-life than OXM ($p < 0.001$ for X6 and $p < 0.01$ for X7 and X8). Analogue X18 had a significantly higher half-life when compared with OXM ($p < 0.05$).

The V_D of OXM analogues X6-X21 ranged between 63.0 ± 7.0 ml/kg (analogue X7) to 237.0 ± 6.5 ml/kg (analogue X18). Analogues X6, X7, X8, X9 X13 and X14 had a significantly lower V_D when compared to OXM ($p < 0.05$, $p < 0.01$, $p < 0.01$, $p < 0.05$, $p < 0.01$ and $p < 0.01$ respectively).

	Metabolic clearance rate (ml/min/kg)	Half-life (min)	Volume of distribution (ml/kg)	Steady state concentration (pmol/ml)
OXM	12.8±3.2	12.0±2.1	231.6±94.0	19.7±5.8
X6	10.4±0.3	5.4±0.2	80.1±51.1	31.8±4.6
X7	6.3±0.8	7.1±0.6	63.0±7.0	40.0±11.7
X8	8.0±3.6	6.7±0.7	73.8±27.3	17.7±0.7
X9	6.8±1.2	8.4±0.9	80.4±6.2	10.2±1.9
X10	11.1±3.5	8.4±0.2	135.2±45.0	11.2±0.3
X11	11.8±3.7	9.9±0.5	173.3±63.3	7.2±0.1
X12	8.3±1.9	10.9±0.3	131.6±32.3	16.8±0.5
X13	6.0±0.4	11.7±0.1	101.3±5.6	19.0±1.5
X14	4.7±0.7	14.8±0.6	100.3±18.9	29.0±2.5
X15	5.4±1.5	15.4±0.4	117.6±29.9	18.3±0.9
X16	10.8±0.2	15.1±0.4	234.6±7.8	18.8±1.4
X17	8.4±3.2	11.9±0.3	142.9±52.6	32.7±0.3
X18	13.2±0.5	12.5±0.8	237.0±6.5	10.8±0.2
X19	8.5±1.1	12.9±1.0	158.0±7.9	15.7±2.3
X20	10.4±1.9	12.5±0.30	178.60±15.	12.4±1.4
X21	6.2±3.4	14.4±3.40	144.4±20.30	33.3±6.7

Table 9: Pharmacokinetic parameters (MCR, Half-life and Volume of distribution) and steady-state concentration of OXM and OXM analogues X6-X21 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean ± S.E.M.

Analogues X6-X9

Analogue X7 presented the lowest and analogue X6 the highest MCR (6.3 ± 0.8 ml/min/kg and 10.4 ± 0.3 ml/min/kg respectively) (Figure 70). The MCR of analogues X8 and X9 was 8.0 ± 3.6 ml/min/kg and 6.8 ± 1.2 ml/min/kg respectively. Analogues X7 and X9 had a significantly lower MCR when compared to the MCR of the OXM ($p < 0.05$ for both analogues). The MCR of analogues X6-X9 did not differ statistically when they were compared between them.

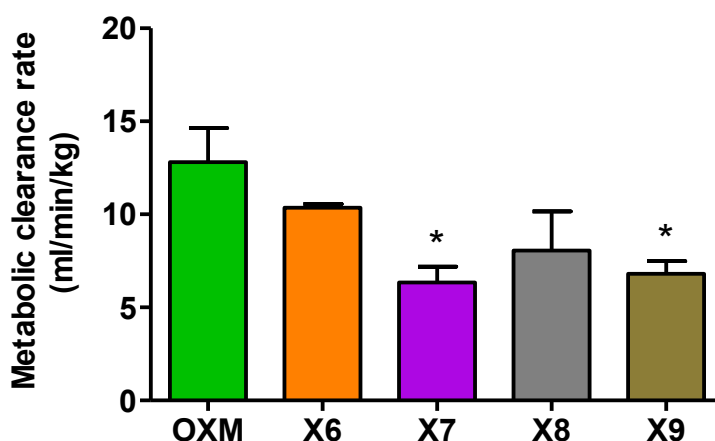


Figure 70: Metabolic clearance rate in ml/min/kg of OXM and OXM analogues X6-X9 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes ($n=4$). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test. *= $p < 0.05$ vs. OXM.

Analogue X6 presented the lowest half-life (5.4 ± 0.2 min) between analogues X6 to X9 and analogue X9 demonstrated the highest half-life of this group of peptides (8.4 ± 0.9 min) (Figure 71). All analogues (X6 to X9) had a significantly lower half-life than OXM ($p < 0.001$ for analogues X6, X7, X8 and $p < 0.01$ for X9).

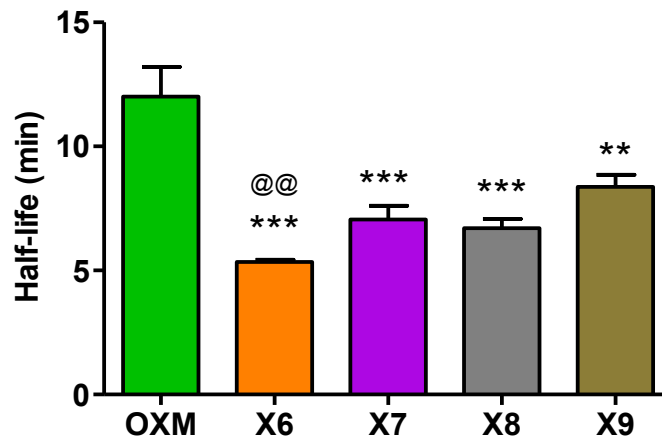


Figure 71: Half-life in minutes of OXM and OXM analogues X6-X9 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test. **= $p<0.01$, ***= $p<0.001$ vs. OXM; @@= $p<0.01$ between OXM analogues.

Analogue X7 presented the lowest V_D (63.0 ± 7.0 ml/kg) while analogues X6 and X9 had a similar V_D of 80.0 ml/kg approximately (Figure 72). All analogues (X6 to X9) had a significantly lower V_D when compared to OXM ($p<0.01$ for all analogues). The V_D of analogues X6-X9 did not differ statistically when they were compared between them.

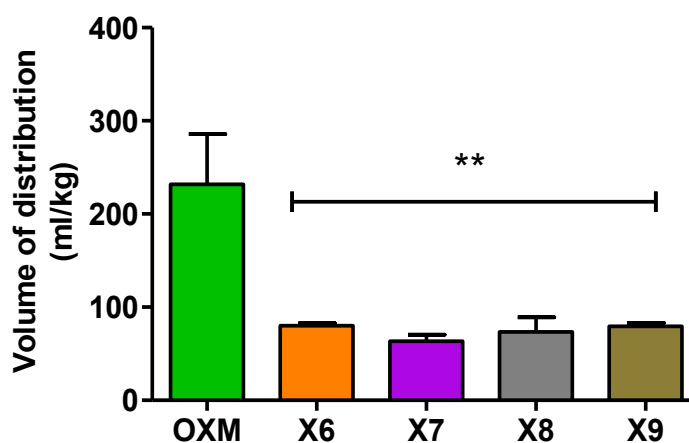


Figure 72: Volume of distribution in ml/kg of OXM and OXM analogues X6-X9 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test. **= $p<0.01$ vs. OXM.

Plasma concentrations of analogues X6 to X9 are shown in Figure 73. The steady-state concentrations of OXM analogues X6 and X7 were 31.8 ± 4.6 and 40.0 ± 11.7 pmol/ml respectively (Figure 73). Analogues X8 and X9 reached a steady-state concentration of 17.7 ± 0.7 and 10.2 ± 1.9 pmol/ml respectively.

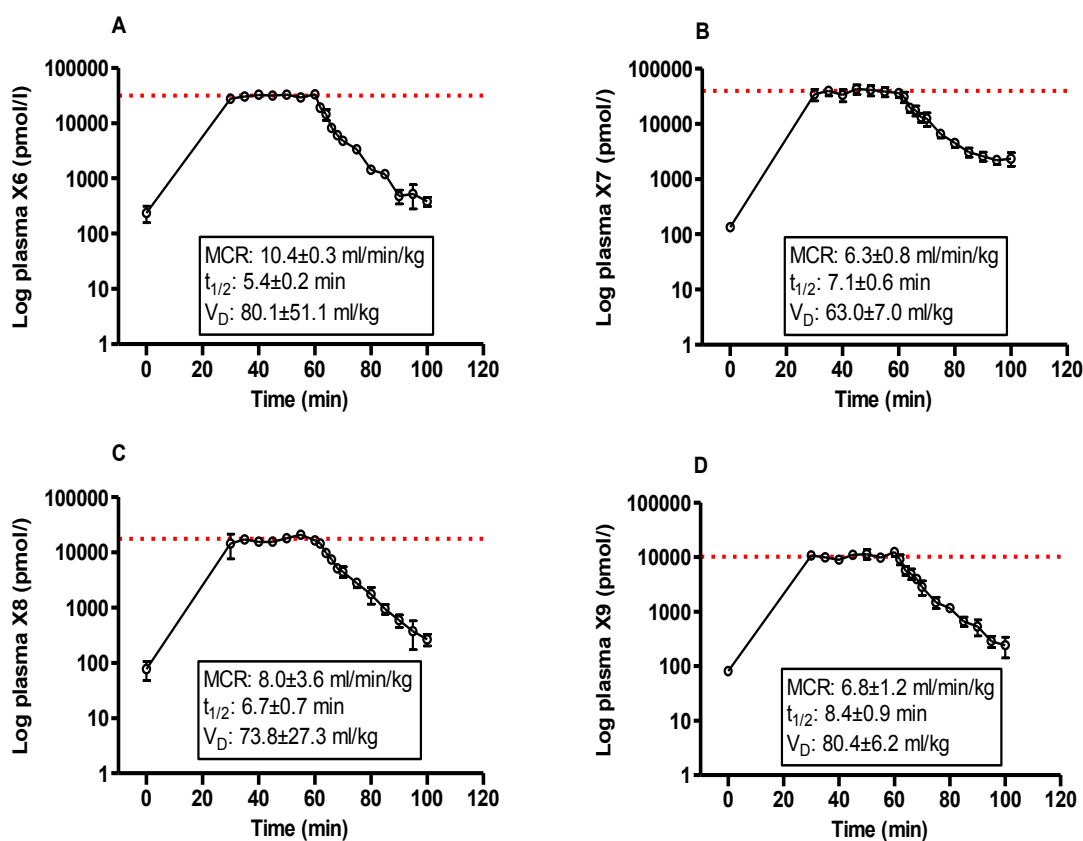


Figure 73: Plasma concentration of analogues X6-X9 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). A: X6, B: X7, C: X8 and D: X9. Infusion begins at “0” time-point and lasts until “60” minute at which point the infusion is stopped. Values are shown as mean. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

Analogues X10-X13

Analogue X13 presented the lowest and analogue X11 the highest MCR (6.0 ± 0.4 ml/min/kg and 11.8 ± 3.7 ml/min/kg respectively) between analogues X10 to X13 (Figure 74). The MCR of analogues X10-X13 did not differ statistically when they were compared to the MCR of OXM or when they were compared to each other.

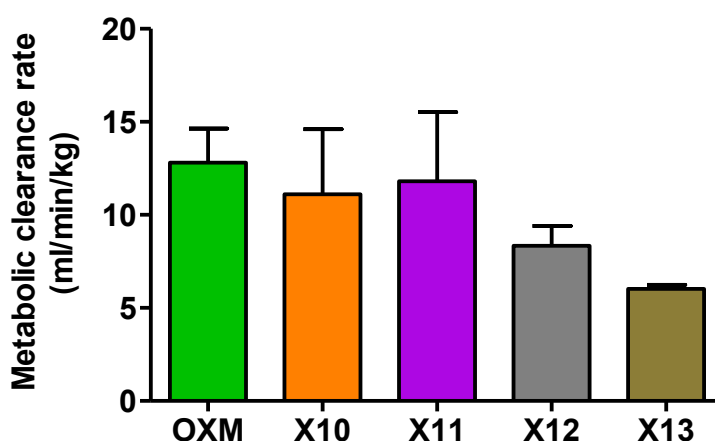


Figure 74: Metabolic clearance rate in ml/min/kg of OXM and OXM analogues X10-X13 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test.

Analogue X10 presented the lowest half-life (8.4 ± 0.2 min) between analogues X10 to X13 and analogue X13 demonstrated the highest half-life of this group of peptides (11.7 ± 0.1 min) (Figure 75). Only analogue X10 had a statistically significant lower half-life when the analogues were compared to OXM ($p < 0.01$ for X10). When the OXM analogues were compared between them, analogue X10 had a statistically significant lower half-life when compared to X11 ($p < 0.05$), to X12 ($p < 0.001$) and X13 ($p < 0.001$). Furthermore, analogue X13 had a statistically significant longer half-life than analogue X11 ($p < 0.05$).

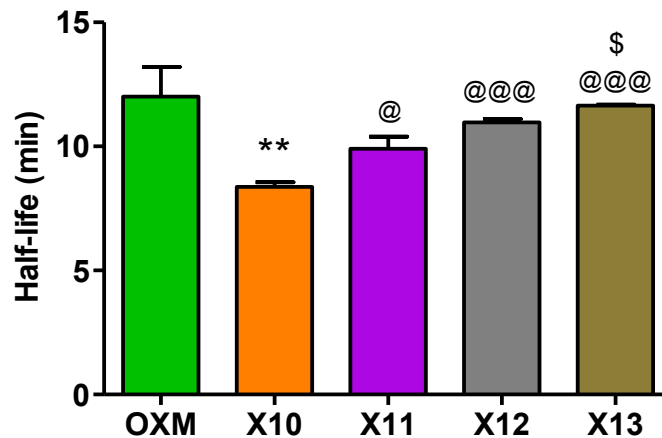


Figure 75: Half-life in minutes of OXM and OXM analogues X10-X13 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test. **= $p < 0.01$ vs. OXM; @= $p < 0.05$, @@@= $p < 0.001$ vs. X10; \$= $p < 0.05$ vs. X11.

Analogue X13 presented the lowest V_D (101.3 ± 5.6 ml/kg) while analogue X11 presented the highest V_D (173.3 ± 63.3 ml/kg) (Figure 76). The V_D of analogues X10-X13 did not differ statistically when they were compared to the V_D of OXM or when they were compared between them.

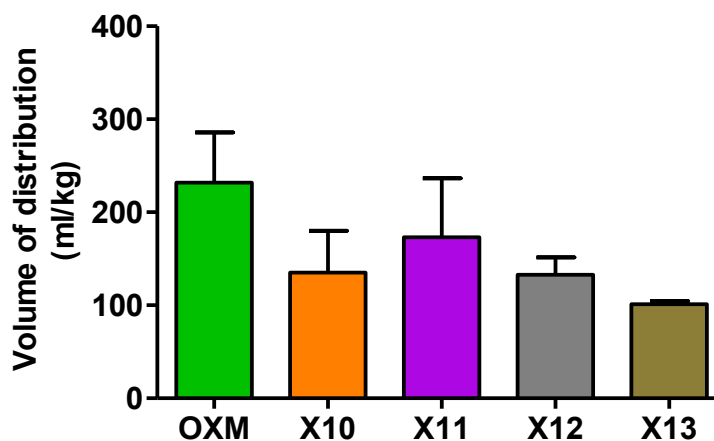


Figure 76: Volume of distribution in ml/kg of OXM and OXM analogues X10-X13 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test.

The steady-state concentrations of analogues X11 and X13 ranged between 7.2 pmol/ml (analogue X11) to 19.0 pmol/ml (analogue X13) (Figure 77). Analogues X10 and X12 reached a steady-state concentration of 11.2 and 16.8 pmol/ml respectively. Plasma concentrations of analogues X10 to X13 are shown in Figure 82.

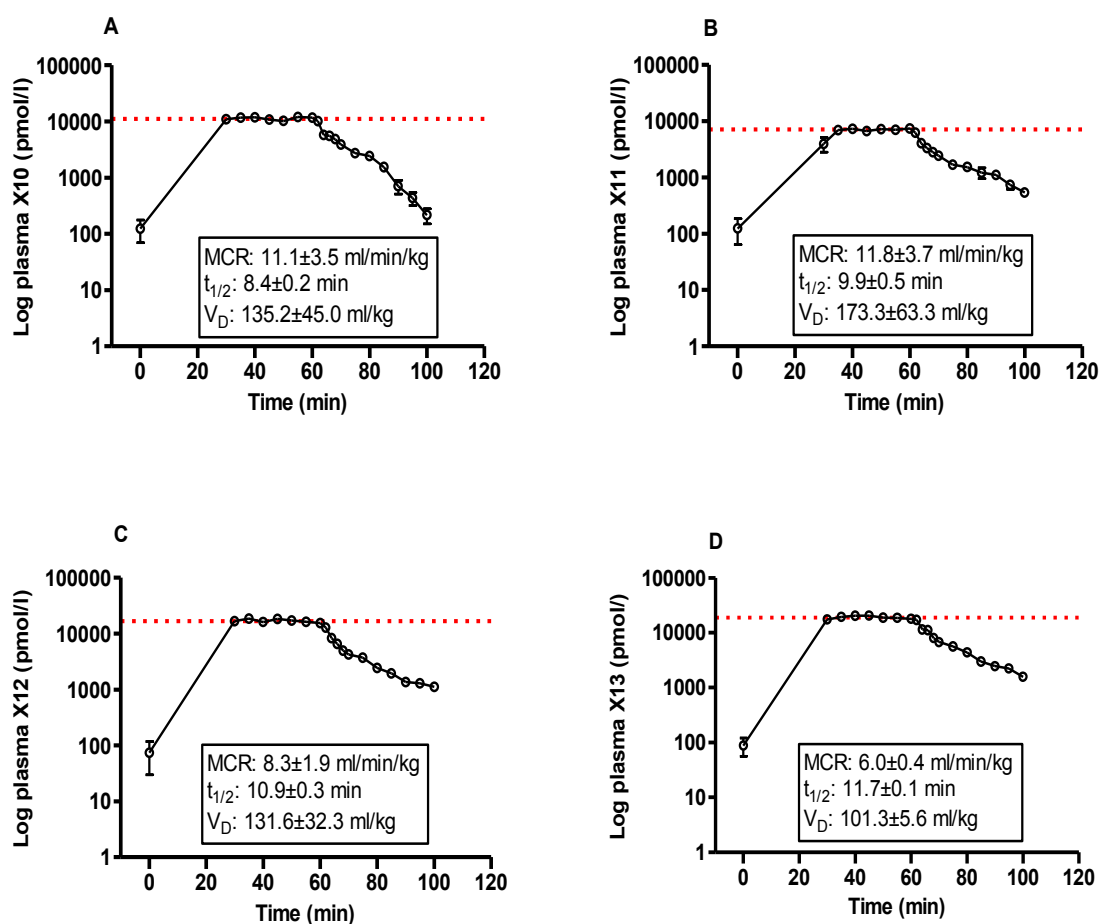


Figure 77: Plasma concentration of analogues X10-X13 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). A: X10, B: X11, C: X12 and D: X13. Infusion begins at “0” time-point and lasts until “60” minute at which point the infusion is stopped. Values are shown as mean. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

Analogues X14-X16

Analogue X14 presented the lowest and analogue X16 the highest MCR (4.7 ± 0.7 ml/min/kg and 10.8 ± 0.2 ml/min/kg respectively) (Figure 78). The MCR of analogues X14 and X15 (MCR for X15: 5.4 ± 1.5 ml/min/kg) were statistically significant lower than the MCR of OXM ($p < 0.01$ for both analogues) and the MCR of analogue X16 ($p < 0.05$ for both analogues).

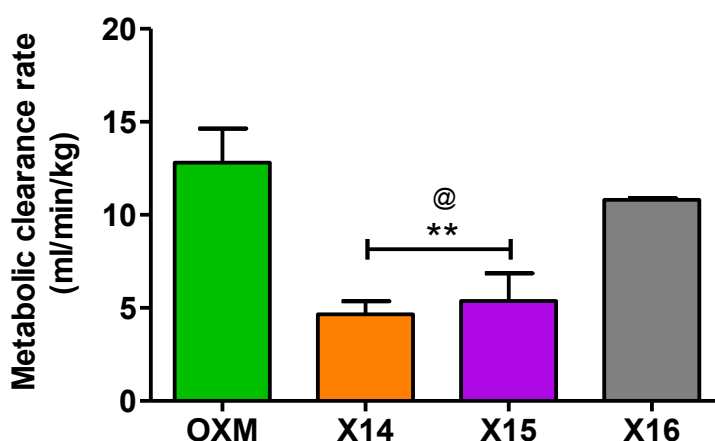


Figure 78: Metabolic clearance rate in ml/min/kg of OXM and OXM analogues X14-X16 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes ($n=4$). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test. . **= $p < 0.01$ vs. OXM; @= $p < 0.05$ vs. X16.

All 3 analogues, X14-X16, presented a half-life of approximately 15.0 minutes which did not differ significantly between them (Figure 79). Analogue X15 presented the highest half-life (15.4 ± 0.4 min) between analogues X14-X16. Analogues X14 to X16 had a significantly higher half-life when compared to OXM ($p < 0.05$ for all analogues).

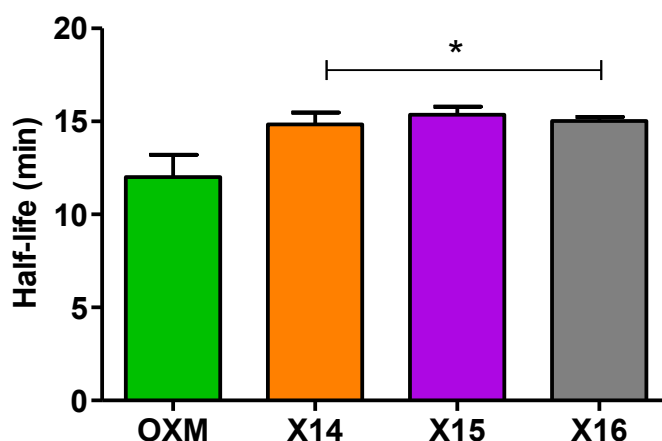


Figure 79: Half-life in minutes of OXM and OXM analogues X14-X16 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test. *=p<0.05 vs. OXM.

Analogue X16 presented the highest V_D (234.6 $\pm$ 7.8 ml/kg) while analogues X14 and X15 presented a V_D of 100.3 $\pm$ 18.9 and 117.6 $\pm$ 29.9 ml/kg respectively (Figure 80). The V_D of analogues X14-X16 did not differ statistically when they were compared to the V_D of OXM. The V_D of analogues X14 and X15 was statistically significant lower than the V_D of analogue X16 (p<0.01 for both analogues).

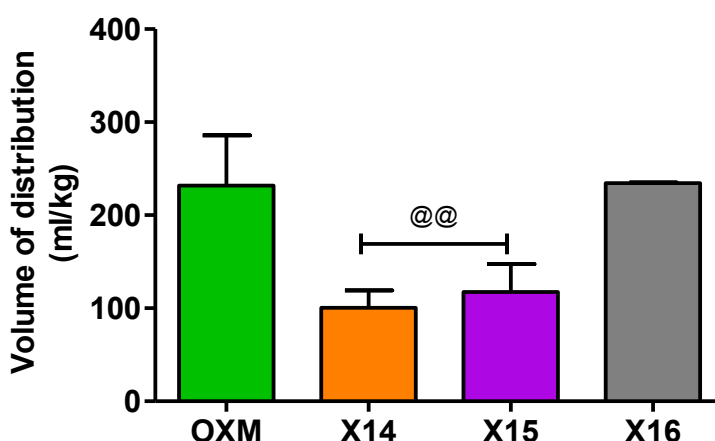


Figure 80: Volume of distribution in ml/kg of OXM and OXM analogues X14-X16 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test. @@=p<0.01 vs. X16.

The steady-state concentrations of OXM analogues X15 and X16 ranged between 18.3-18.8 pmol/ml approximately while analogue X14 reached a steady-state concentration of 29.0 ± 2.5 pmol/ml (Figure 81). Plasma concentrations of analogues X14 to X16 are shown in Figure 87.

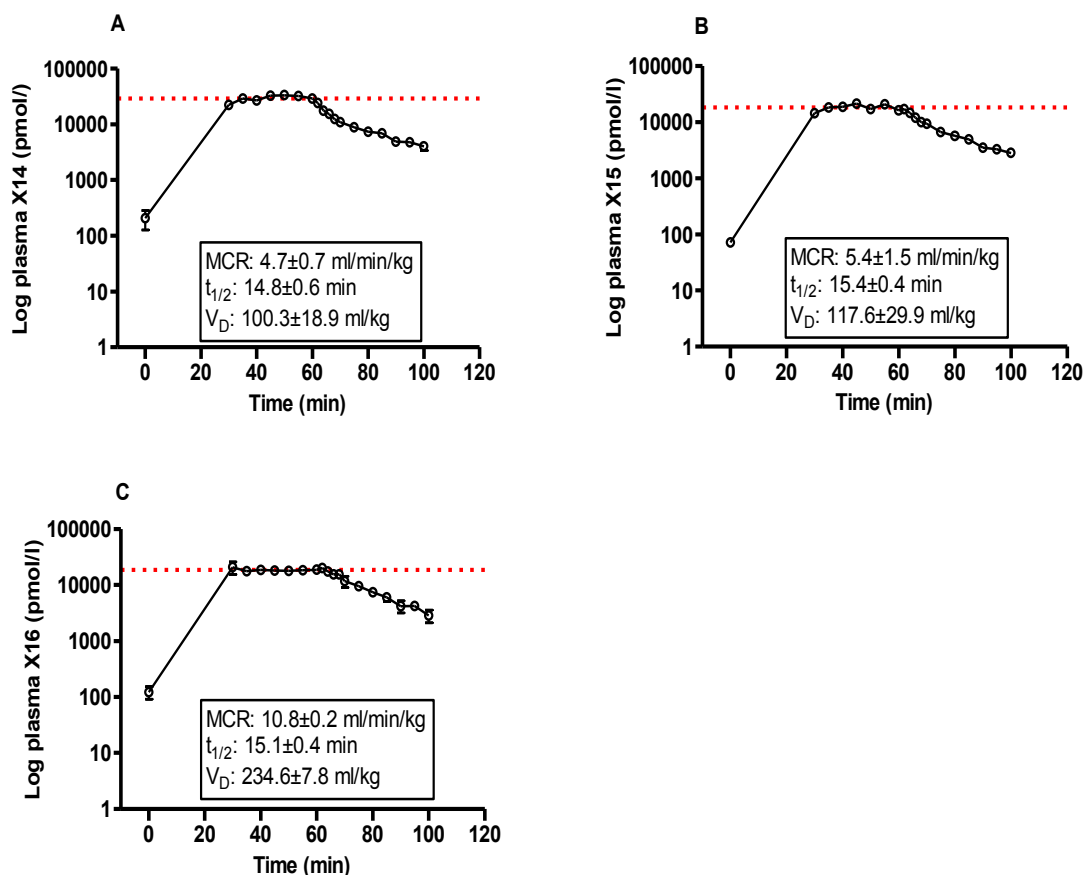


Figure 81: Plasma concentration of analogues X14-X16 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). A: X14, B: X15 and C: X16. Infusion begins at “0” time-point and lasts until “60” minute at which point the infusion is stopped. Values are shown as mean. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

Analogues X17-X21

Analogue X21 presented the lowest and analogue X18 the highest MCR (6.2 ± 3.4 ml/min/kg and 13.2 ± 0.5 ml/min/kg respectively) (Figure 82). The MCR of analogues X17, X19 were similar (8.4 ± 3.2 and 8.5 ± 1.1 ml/min/kg respectively). Analogue X20 presented an MCR of 10.4 ± 1.9 ml/min/kg. When the analogues X17 to X21 were compared to OXM only analogue X21 presented a statistically significant lower MCR ($p < 0.05$). When the OXM analogues were compared between them, analogue X18 presented a significantly higher MCR when compared to the MCR of X21 ($p < 0.05$).

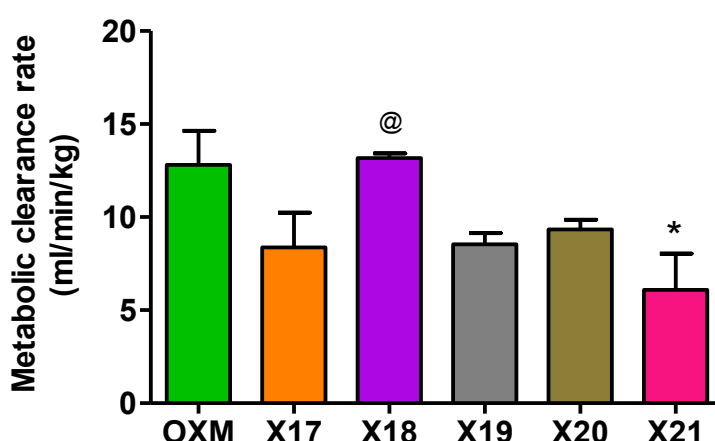


Figure 82: Metabolic clearance rate in ml/min/kg of OXM and OXM analogues X17-X21 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes ($n=4$). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test. . $*$ = $p < 0.05$ vs. OXM; $@$ = $p < 0.05$ vs. X21.

Analogues X17 to X20 presented a half-life of approximately 12-13 minutes and analogue X21 had a half-life of 14.4 ± 3.4 minutes (Figure 83). The half-life of analogues X17 to X21 did not differ significantly between them or when they were compared to the half-life of OXM or when they were compared between them.

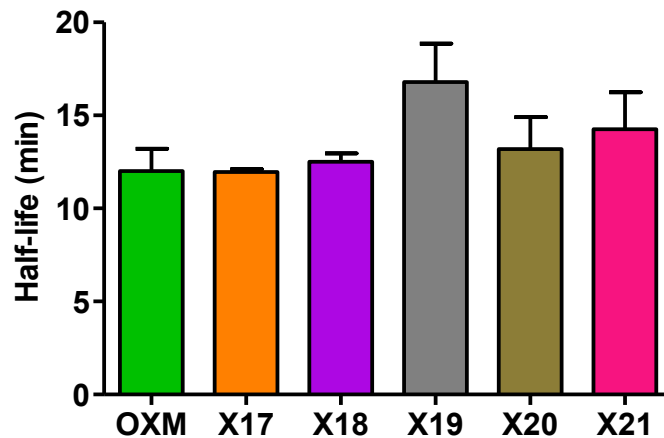


Figure 83: Half-life in minutes of OXM and OXM analogues X17-X21 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test.

Analogues X17 and X21 presented a V_D of approximately 143-144.0 ml/kg respectively (Figure 84). Analogue X18 presented the highest V_D of 237.0 ± 6.5 ml/kg. The V_D of analogues X17-X21 did not differ statistically when they were compared to the V_D of OXM or when they were compared between them.

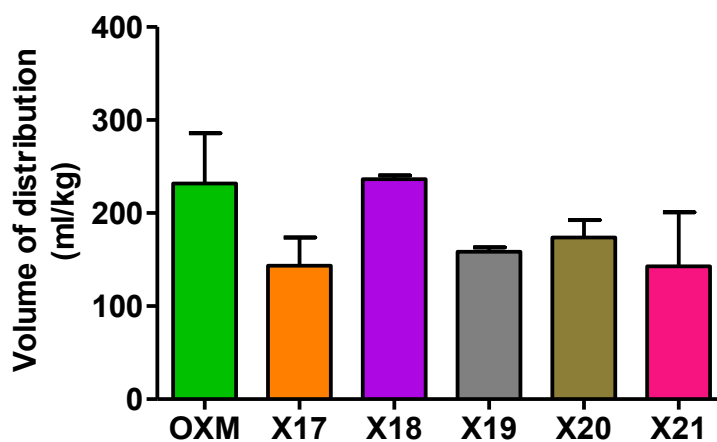


Figure 84: Volume of distribution in ml/kg of OXM and OXM analogues X17-X21 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test.

The steady-state concentrations of OXM analogues X18 to X20 ranged between 10.8 to 15.7 pmol/ml while analogues X17 and X21 reached a steady-state concentration of 33.0 pmol/ml approximately (Figure 85). Plasma concentrations of analogues X17 to X21 are shown in Figure 92.

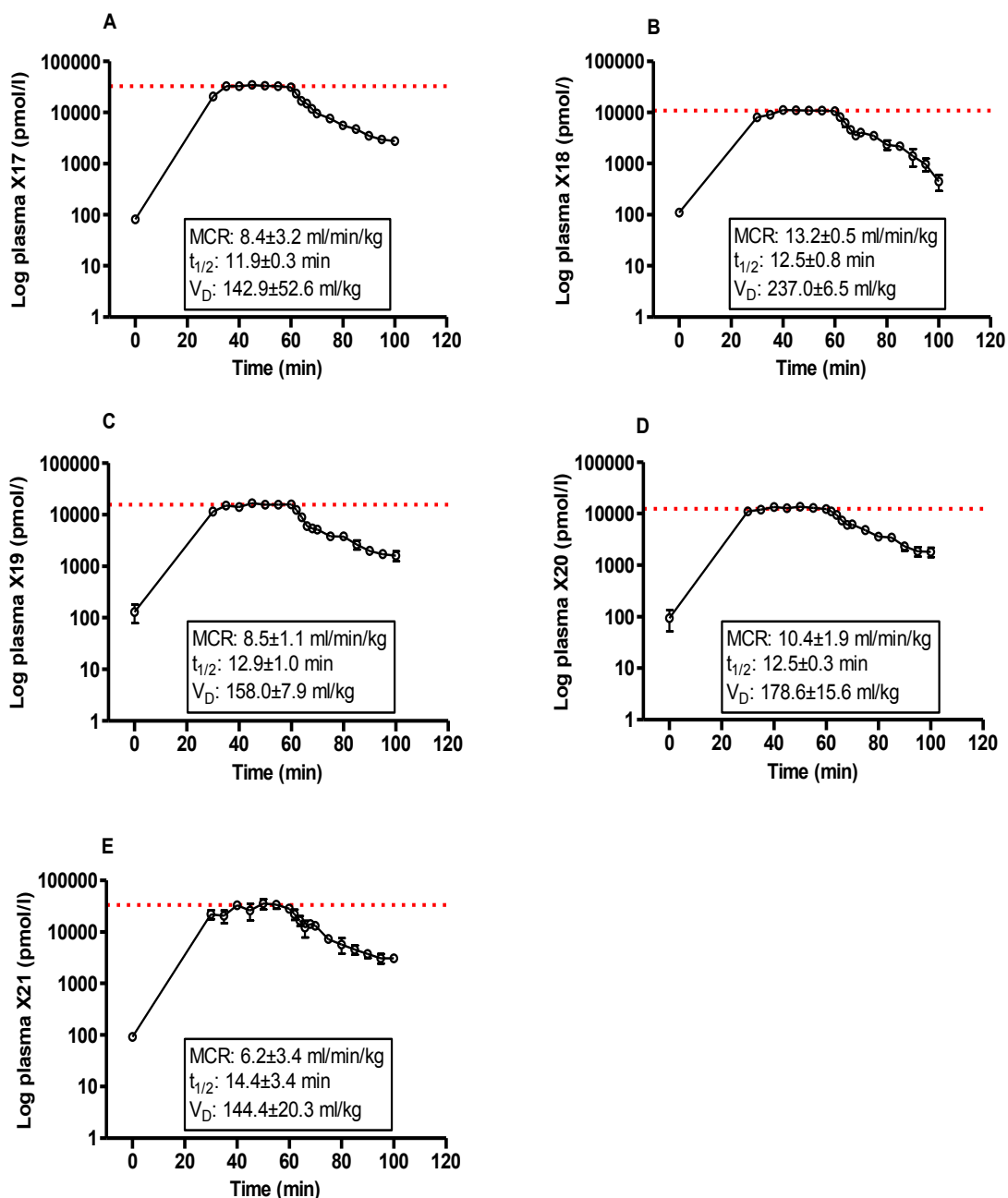


Figure 85: Plasma concentration of analogues X17-X21 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). A: X17, B: X18, C: X19, D: X20 and E: X21. Infusion begins at “0” time-point and lasts until “60” minute at which point the infusion is stopped. Values are shown as mean. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

5.3.6. Investigation of the pharmacokinetic properties of oxyntomodulin analogues X6 to X21 following single subcutaneous bolus injection in male Wistar rats

The PK profiles of OXM analogues X6 to X21, when administered as a 1 mg 1:1 molar ratio Zn:NaCl (saline) SC injection to male Wistar rats, were investigated (Figure 86, Figure 87, Figure 88, Figure 89).

OXM analogues X6 to X9

The T_{max} was 30 minutes post-injection for analogues X6 and X7 and 3 hours for analogues X8 and X9 (Figure 86). Plasma levels of analogues X6 and X7 were 72% and 58% of C_{max} respectively at 3 hours post-injection and had dropped further to 49% and 51% of C_{max} by 24 hours post-injection. Plasma levels of analogues X8 and X9 were 64% and 70% of C_{max} respectively at 24 hours post-injection and had dropped further to 6% and 14% of C_{max} by 48 hours post-injection. Analogues X6 to X9 became undetectable in plasma after 48 hours (LLOQ was 148±31, 278±14, 153±27, 390±19 pmol/L respectively).

OXM analogues X10 to X13

The T_{max} was 30 minutes post-injection for analogues X10 and X11 and 3 hours for analogues X12 and X13 (Figure 87). Plasma levels of analogues X10 and X11 were 60% and 82% of C_{max} respectively at 3 hours post-injection and had dropped further to 51% and 70% of C_{max} by 24 hours post-injection. Analogues X10 and X11 were detectable in plasma samples up to 48 hours post-injection with 40% and 32% of C_{max} remaining respectively.

Plasma levels of analogues X12 and X13 were 67% and 70% of C_{max} at 30 minutes and continued to rise until 3 hours post-injection. Plasma levels of analogues X12 and X13 were 67% and 70% of C_{max} respectively at 24 hours post-injection and had dropped further to 56% and 50% of C_{max} by 48 hours post-injection. Analogues X10, X11 and X12 were undetectable at 96-hour and at 7 days while analogue X13 retained 19% of its C_{max} at 96 hours and became undetectable at 7 days (LLOQ was 243±16, 458±25, 140±21, 342±26 pmol/L respectively).

OXM analogues X14 to X16

The T_{max} was 24 hours post-injection for all analogues (Figure 88). Plasma levels of analogues X14, X15 and X16 at 30 minutes post-injection were 50%, 37% and 36% of C_{max} respectively, at 3 hours post-injection were 85%, 92%, 91% of C_{max} respectively and continued to rise until 24 hours post-injection. Plasma levels of analogues X14 to X16 at 48 hours post-injection dropped to 98%, 81% and

41% of C_{max} respectively. At 96 hours post-injection analogues X14 and X15 were still detectable in the plasma retaining 25% and 14% of C_{max} respectively. Analogue X16 became undetectable in plasma at 96 hours. Analogues X14, X15 and X16 were undetectable in plasma at 7 days (LLOQ was 179±34, 215±12 and 167±28 pmol/L respectively).

OXM analogues X17 to X21

The T_{max} was 3 hours post-injection for analogues X17 and X19 and 24 hours post-injection for analogues X18, X20 and X21 (Figure 89). Plasma levels of analogues X17 and X19 were 77% and 75% of C_{max} at 30 minutes and continued to rise until 3 hours post-injection. Plasma levels of analogues X17 and X19 were 90% and 93% of C_{max} respectively at 24 hours post-injection and had dropped further to 61% and 47% of C_{max} by 48 hours post-injection. Analogues X17 and X19 became undetectable in plasma after 96 hours post-injection (LLOQ was 239±21, 167±38 pmol/L respectively).

Plasma levels of analogues X18, X20 and X21 at 30 minutes post-injection were 91%, 72% and 47% of C_{max} respectively, at 3 hours post-injection were 94%, 96%, 86% of C_{max} respectively and continued to rise until 24 hours post-injection. Plasma levels of analogues X18, X20 and X21 were 55%, 53% and 86% of C_{max} respectively at 48 hours post-injection. Analogue X18 became undetectable in plasma after 96 hours post-injection (LLOQ was 367±38 pmol/L). Analogues X20 and X21 were detectable in plasma up to 96 hours with 20% and 16% of C_{max} remaining respectively and became undetectable at 7 days (LLOQ was 289±25 and 195±44 pmol/L respectively).

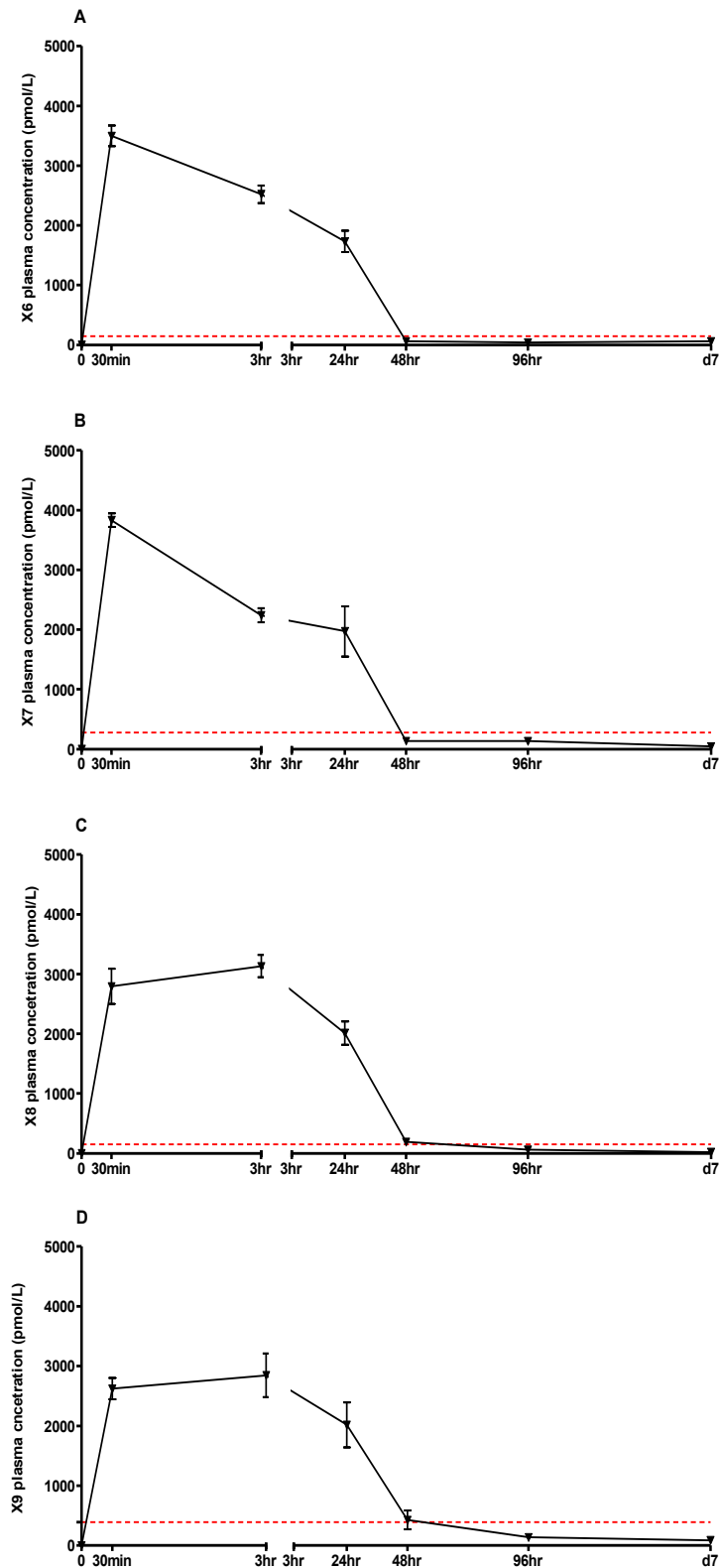


Figure 86: Line graphs illustrating the pharmacokinetic profile of A: X6 B: X7, C: X8 and D: X9 after a single 1 mg SC injection of peptide (formulated in 1:1 Zn:NaCl slow release diluent). Peptide concentration measured in rat plasma using RIA and expressed as mean concentration $\pm$ SEM pmol/L (n=4). The lower limits of quantification (LLOQ) for each RIA are shown as horizontal dashed lines. Interpolated values which fall below this level are still plotted.

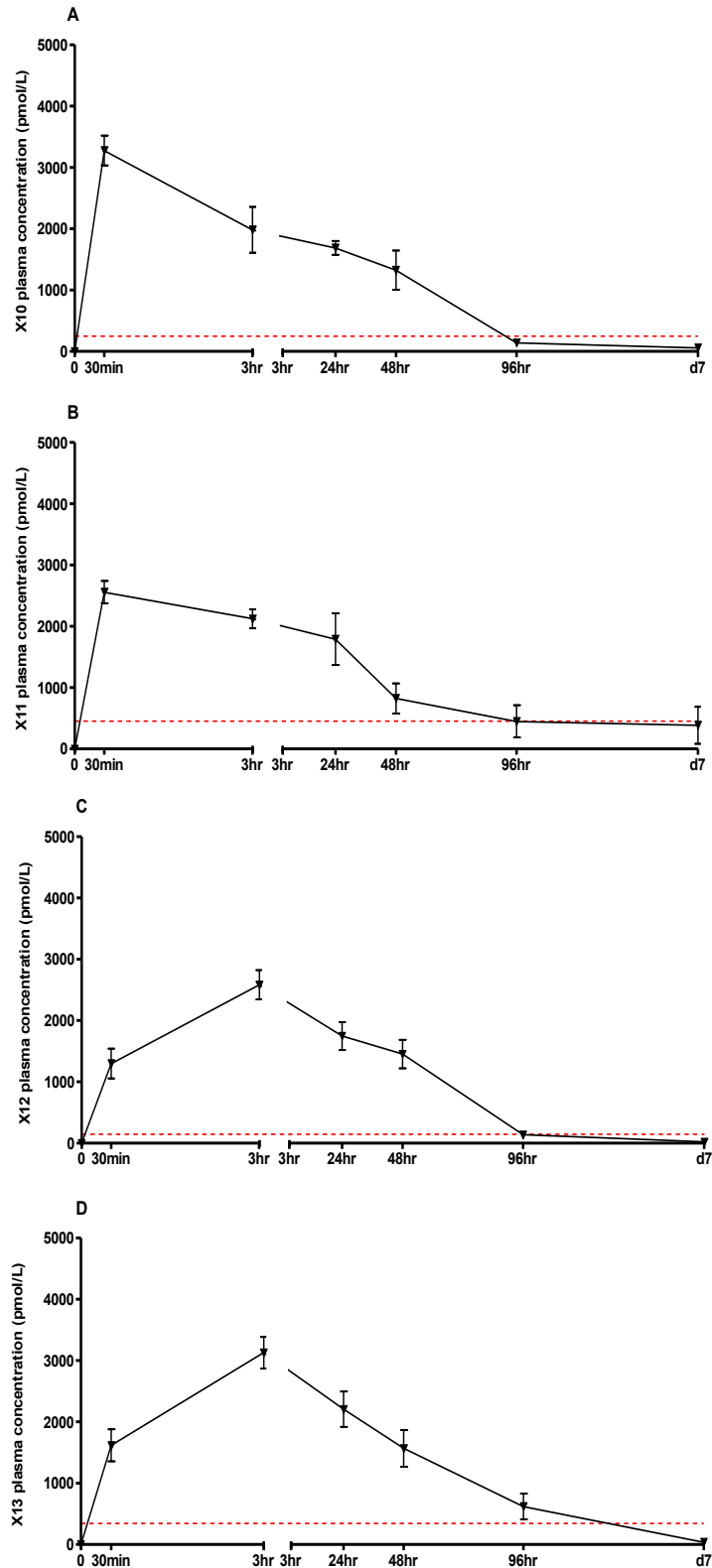


Figure 87: Line graphs illustrating the pharmacokinetic profile of A: X10, B: X11, C: X12 and D: X13 after a single 1 mg SC injection of peptide (formulated in 1:1 Zn:NaCl slow release diluent). Peptide concentration measured in rat plasma using RIA and expressed as mean concentration $\pm$ SEM pmol/L (n=4). The lower limits of quantification (LLOQ) for each RIA are shown as horizontal dashed lines. Interpolated values which fall below this level are still plotted.

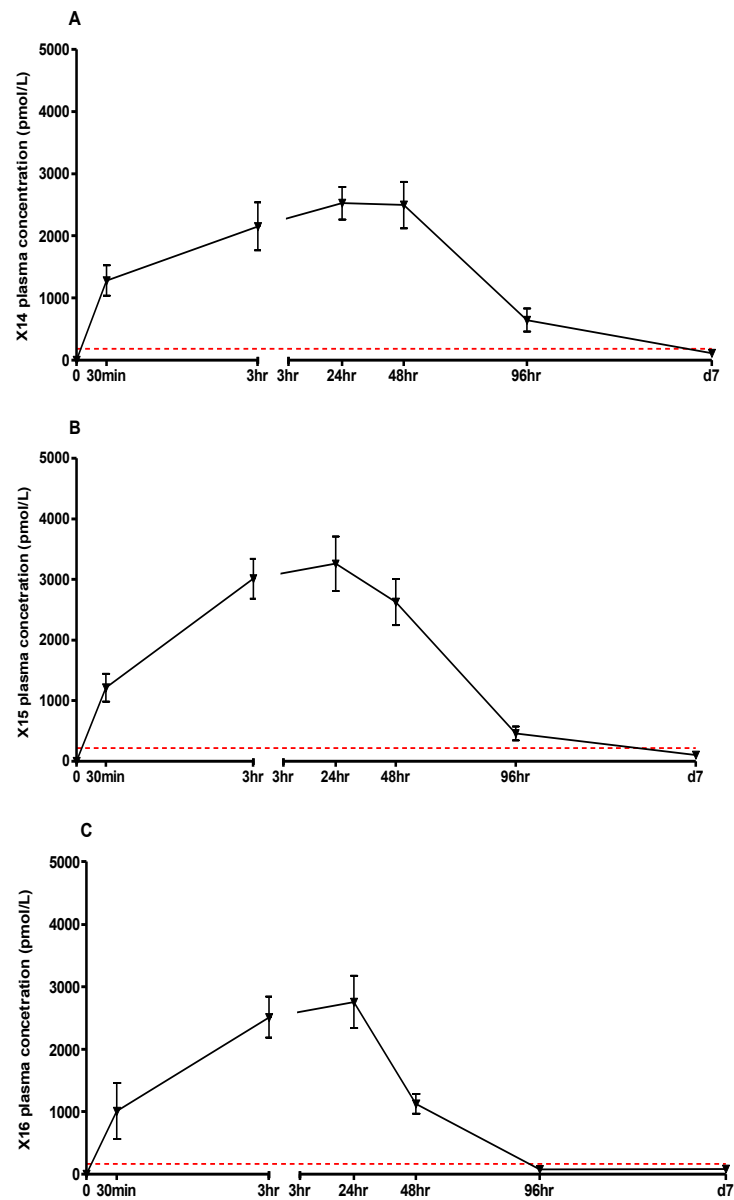


Figure 88: Line graphs illustrating the pharmacokinetic profile of A: X14, B: X15 and C: X16 after a single 1 mg SC injection of peptide (formulated in 1:1 Zn:NaCl slow release diluent). Peptide concentration measured in rat plasma using RIA and expressed as mean concentration $\pm$ SEM pmol/L (n=4). The lower limits of quantification (LLOQ) for each peptide in the RIA are shown as horizontal dashed lines. Interpolated values which fall below this level are still plotted.

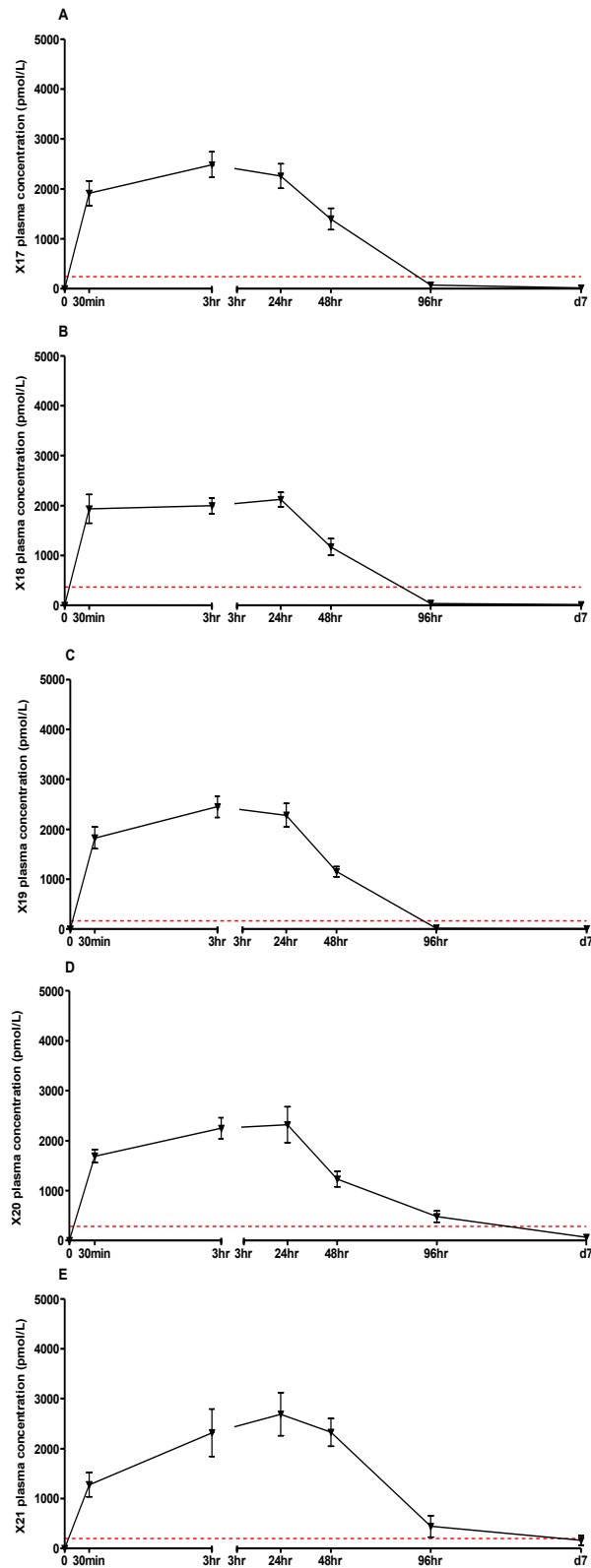


Figure 89: Line graphs illustrating the pharmacokinetic profile of A: X17 B: X18, C: X19, D: X20 and E: X21 after a single 1 mg SC injection of peptide (formulated in 1:1 Zn:NaCl slow release diluent). Peptide concentration measured in rat plasma using RIA and expressed as mean concentration $\pm$ SEM pmol/L (n=4). The lower limits of quantification (LLOQ) for each peptide in the RIA are shown as horizontal dashed lines. Interpolated values which fall below this level are still plotted.

5.3.7. The chronic effect of exendin-4 and oxyntomodulin analogues X7, X9, X10, X16, X18 and X21 on food intake and body weight in C57BL/6J mice

The effect of OXM analogues on food intake was investigated in a 6 day chronic study in mice. Analogues were dissolved in ZnCl_2 solution prepared in saline at zinc to peptide ratio of 1:1. A single SC administration of EX-4 (10 nmol/kg) and analogue X21 (50 nmol/kg) caused an inhibition of food intake at 6 days when compared to the vehicle control group ($p<0.001$ and $p<0.01$ respectively) (Figure 90). No statistical difference in mean food intake over 6 days was observed between the EX-4 (50 nmol/kg) and X21 groups (10 nmol/kg) (13 ± 0.9 g and 15.1 ± 1.7 g respectively, $p=0.2857$). Other analogues tested (X7, X9, X10, X16 and X18) caused no statistically significant inhibition of food intake compared to vehicle and the groups ate significantly more food compared to the EX-4 group.

Administration of EX-4 (50 nmol/kg) and all OXM analogues (X7, X9, X10, X16, X18 and X21) (10 nmol/kg) resulted in a significantly reduced body weight at 6 days post-injection when compared to the vehicle control group (Figure 91). Body weight loss over 6 days was greatest in animals treated with EX-4 (-0.7 ± 0.4 g) and X21 (1.6 ± 0.2 g). A multiple comparison statistical test showed analogues X9, X10 and X18 lost a significantly smaller amount of body weight than EX-4 and all OXM analogues lost significantly less weight than analogue X21.

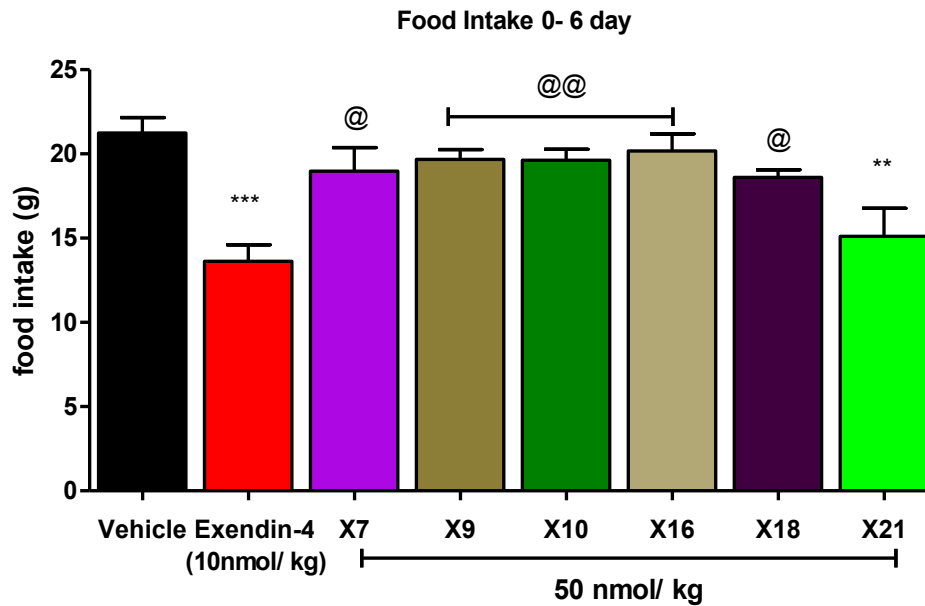


Figure 90: Chronic 6 day effect of a single SC injection of EX-4 (10 nmol/kg) and OXM analogues X7, X9, X10, X16, X18 and X21 (50 nmol/kg), on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-6 days. n=5-6 mice per group Statistics: One way ANOVA with Tukey's post-hoc test. **= $p < 0.01$, ***= $p < 0.001$ vs. vehicle; @= $p < 0.05$, @@= $p < 0.01$ vs. EX-4.

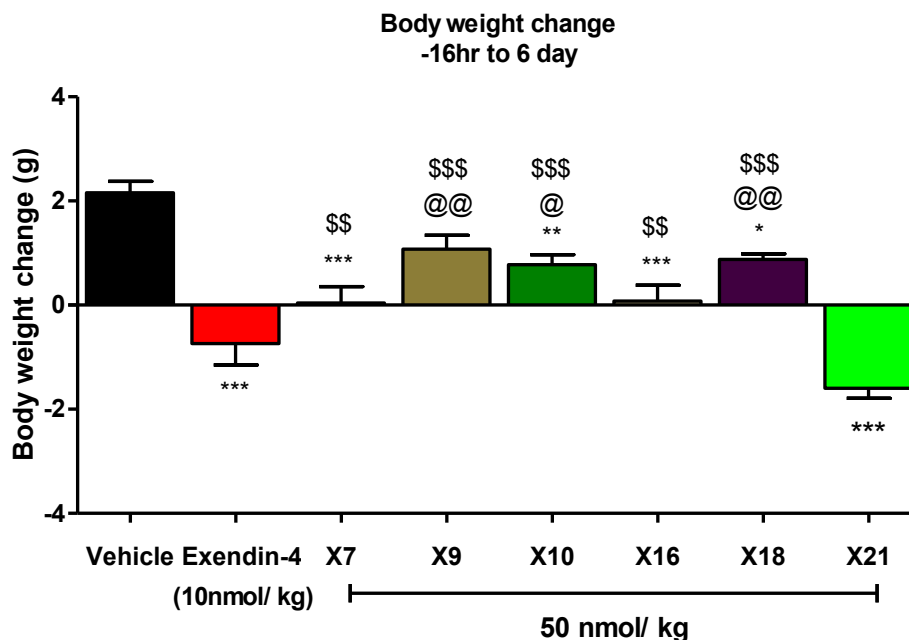


Figure 91: Chronic 6 day effect of a single SC injection of EX-4 (10 nmol/kg) and OXM analogues X7, X9, X10, X16, X18 and X21 (50 nmol/kg), on body weight loss in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 6 days. n=5-6 mice per group Statistics: One way ANOVA with Tukey's post-hoc test. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ vs. vehicle; @= $p < 0.05$, @@= $p < 0.01$ vs. EX-4; \$= $p < 0.01$, \$\$\$= $p < 0.001$ vs. X21.

5.4. Discussion

The goal of this chapter was to produce an OXM analogue that would cause a higher body weight loss in rodents compared to analogues X1-X5. The strategy to achieve this goal was 2-fold in this chapter; increasing GLP-1R binding and potency and improving the PK profile of the analogues. Valuable lessons learned from analogues X1-X5 included the fact that introduction of a poly-His tail can improve the PK profile of the analogues and that the flexibility around positions 29-30 is important for zinc binding.

In order to achieve the goal regarding the GLP-1R, substitutions were made in positions 16 and 23. The substitution of Ser¹⁶ of native OXM for Glu¹⁶ in this series of analogues was made in an effort to increase the binding affinity of the OXM analogues to the GLP-1R. Glutamic acid at this position is a feature found in EX-4. In the GLP-1 molecule, Gly¹⁶ causes a kink at the α -helical region which may not be optimal for GLP-1 binding (247). The second substitution done in this chapter was the replacement of Val²³ from Ile²³. Isoleucine in position 23 is a common feature of both GLP-1 and EX-4 and it has been shown to be located on the hydrophobic face of the α -helix and to be important for the binding of the molecule to the GLP-1R (219).

The effort to improve the PK profile of the analogues by increasing their half-life and their zinc binding was centred on changes made in the C-terminal; mainly through investigating the effect of the poly-His tail and point substitutions of position 29 and 30. Threonine at position 29 is normally found in native GCG and OXM and was preserved in analogues X17 to X21. In analogues X6 to X16 position 29 is occupied by Gly in an effort to increase the flexibility of the poly-His tail (and increase GLP-1R binding). For the same reason, analogues X10 to X16 had a second Gly residue introduced at position 30, while analogues X6 to X9 and X17 to X21 retained a His amino acid. Analogues X14 to X16 had further small additions in their C-terminal tail by adding a terminal Ala, Ala-NH₂ or Gln-NH₂ respectively.

In order to assess whether Glu¹⁶ and Ile²³ were indeed beneficial, I compared the results of the binding affinity and potency to the GLP-1R of analogues X6 and X7 to the ones of analogues X1 and X2 (differing only at positions 16 and 23). The binding affinity to both the hGLP-1R and mGLP-1R of analogues X6 and X7 was higher than the one exhibited by analogues X1 and X2 (2-3-fold for the mGLP-1R and 3-4-fold for hGLP-1R). In the cAMP accumulation assay at the hGLP-1R, X6 was 3-fold more potent than X1 and X7 was 1.2-fold more potent than X2. These results confirmed my initial hypothesis and these positions will be preserved in the next generation of analogues in chapter 6.

The higher affinity and potency at the GLP-1R as exhibited by X6 and X7 did not however translate in a higher biologic effect. The *in vivo* anorectic effect of analogues X6 and X7 in an acute feeding study

in mice did not differ from the one exhibited by analogues X1 and X2 with similarities in the body weight loss at 48 hours between the peptides. This is most probably the result of the fact that analogues X1, X2, X6 and X7 exhibited similar PK properties. Thus, in this case, the beneficial effects of enhancing GLP-1R binding and potency are hindered by an inadequate PK profile. Hence, it becomes evident that the PK profile of analogues is a critical factor in drug development.

The strategy to improve the PK profile of the analogues was in itself 3-fold; increasing the number of His residues in the poly-His tail, modifying positions 29-30 and introducing the helix capping. The effect of an increasing number of His molecules at the C-terminal of analogues X6 and X7 was investigated by designing the peptides X8 and X9 which differ only at positions 32 and 33. The effect of the introduction of His³² and His³²-His³³ was evident in the studies evaluating the PK profile of the analogues. In the continuous infusion study, where no zinc is present in the solution, analogues X8 and X9 presented an improved half-life and lower MCR in comparison to X6 and X7 while the effect on the V_D was variable. In the SC PK study where analogues are administered in a zinc formulation, X8 and X9 presented a T_{max} at 3 hours, instead of the T_{max} of 30 minutes noted in analogues X6 and X7. This is an indirect sign that analogues X8 and X9 have a higher binding to zinc with a more controlled and prolonged release from the SC depot.

The conclusion is that increasing the number of His residues, I have been able to improve the PK profile of the analogues and this poly-His tail will be preserved in the next generation of analogues. This improvement in the PK profile however was transient and analogues X6-X9 were undetectable at 48 hours. Furthermore, the slight improvement in the PK profile of X8 and X9 did not translate in a higher anorectic effect overall when tested in an acute feeding study in mice.

In order to further improve the PK profile, analogues X11-X16 were designed with a progressively even longer poly-His tail. At the same time, analogues X10-X15 had also helix-capping by Ala and a Gly³⁰-Gly³¹. As stated previously, helix capping may improve the stability of the α -helix and the introduction of Gly³⁰ and Gly³¹ in analogues X10 to X16 aimed at investigating whether a more flexible poly-His tail can bind more readily to zinc molecules.

Analogues X10, X11 and X12 differed in their C-terminal from X3, X4 and X5 respectively only at the terminal Ala. The introduction of the Ala after the terminal His residue of the C-terminal resulted in an improvement of the PK parameters (half-life, MCR and V_D) of X10-X12 in comparison to X3-X5. More specifically the increase in half-life and V_D was approximately 2-fold and 3-fold respectively. The improvement in the MCR was marginal.

An important improvement was seen in the SC PK study of analogues X12 and X13 in comparison to X10 and X11; the T_{max} of X12 and X13 was at 3 hours instead of 30 minutes noted in analogues X10 and X11. The delayed plasma T_{max} levels indicate higher zinc binding of analogues. The improved PK profile and GLP-1R potency of analogues X10–X13 when compared to X3-X5 failed to demonstrate an improvement in the bioactivity of the former group.

Analogues X14-X16 were designed in order to fine-tune helix capping by comparing Ala, Glu-NH₂ and Gln-NH₂. The T_{max} for X14-X16 was 24 hours and X14 was shown at 96 hours post-administration to retain 25% of its C_{max} plasma levels. In regards to the PK properties of analogues X10-X16, the continuous infusion study in rats has demonstrated that there was a steady increase in the half-life of the analogues as an increasing number of His amino acids were added at the C-terminal (half-lives of X10, X12 and X14: 8.4±0.2, 10.9±0.3 and 14.8±0.6 min respectively). In a similar fashion, the MCR of the analogues of analogues X10-X16 decreased progressively (MCR of X10, X12 and X14: 11.1±3.5, 8.3±1.9 and 4.7±0.7 ml/min/kg respectively). The V_D of the peptides varied with a slight reduction exhibited as the poly-His tail increased in number.

The main function of the poly-His tail is to bond to zinc and cause a delayed release from the SC depot. The gradual improvement in the PK profile of analogues X10 –X16, as measured in the continuous infusion study where peptides are administered in a non-zinc preparation, remains elusive. The hypothesis is that the poly-His tail protects the peptide from degradation from peptidases targeting the C-terminal of the molecule and that helix-capping residues further stabilise the helix thus increasing its half-life. Further research into the degradation pattern of these peptides can provide more information about this phenomenon.

The *in vitro* studies performed of X14-X16 have shown that small changes at the C-terminal can alter both the binding and the efficacy at the GLP-1R. Analogue X15 had a higher binding affinity and potency at the GLP-1R when compared to X14 and X16. Analogues X14-X16 differ only in positions 36 and 37; analogue X14 has Ala³⁶, X15 has Glu³⁶-NH₂³⁷ and X16 has Gln³⁶NH₂³⁷. This is an indication that the increased flexibility of the poly-His tail, possibly due to Gly²⁹.Gly³⁰, may interfere with the binding of the helix to the receptor and its activation or that there is another unknown mechanism in play.

The anorectic effect of analogues X10-X16, as demonstrated in an acute feeding study in mice, was higher in groups X10, X11 and X16; however it did not reach significance levels when peptide groups were compared between them. It is interesting to note that the binding affinity of analogue X10 for the GLP-1R is almost 2-fold higher and its half-life is 2-fold lower when compared to X16; the body weight loss of both peptides at 48 hours is similar which may suggest that the PK is equally important as potency at the GLP-1R in terms of the bioactivity of the molecule.

Overall, the introduction of helix-capping in analogues X14-X16 has resulted in an improvement of analogues' half-life and their PK profile when administered in zinc-containing formulation. Analogue X15 has Glu³⁶NH₂³⁷ residues and exhibited the longer half-life, with an unexpected improvement in binding and potency at the GLP-1R. For these reasons the Glu³⁶NH₂³⁷ helix capping will be introduced in the next generation of analogues in chapter 6.

In chapter 4, Thr²⁹ was replaced by Gly²⁹ in an effort to increase GLP-1R binding and potency. Having achieved an improvement in the binding and potency to the GLP-1R in analogues X6-X16 when compared to the previous generation (X1-X5), through Glu¹⁶ and Ile²³, the role of Thr²⁹ was revisited. Analogues X17 to X21 were designed in order to investigate if Thr²⁹ can improve the PK profile of the analogues. Threonine and Gly are significantly different in terms of their size; Gly is small with a simple side-chain making it less likely to contribute to a direct interaction with the GLP-1 or GCGR while Thr is a larger molecule with a polar side chain. In theory, the poly-His tail connected to the helix of the molecule through Thr²⁹ would be less flexible to move in all directions.

The PK profile of X17-X21 as examined in the continuous infusion study demonstrated that X21, possessing a 5-His tail, demonstrated the highest half-life of 14.4 minutes and the lowest MCR (6.2 ml/min/kg). The half-life of analogues X17-X21 progressively increased as the His amino acids of the C-terminal tail increased in number, but overall were still lower than the ones of X14-X16 that have C-terminal helix capping. The PK profile of the analogues X17-X21, when administered SC in a formula that contained zinc, displayed a gradual rise of the plasma concentration of the peptide over the first 24 hours with the 48 hours value being 50% or more of the 24-hour T_{max} level. The plasma levels of analogue X21 at 48 hours were 86% of the 24-hour C_{max} level and furthermore it was still detectable in plasma at 96 hours post-administration. These features of analogue X21 indicate that Thr²⁹ may have a beneficial effect on the PK profile of the analogues, independently from helix capping.

Overall, the only analogues still present at 96 hours so far, were X13, X14, X15, X20 and X21. However, the PK properties are not the only critical factor when considering the bioactivity of a peptide. Analogue X13 presented a 3-hour burst that is considered to be a possible cause of nausea and vomiting and thus this analogue is not considered a valid drug candidate. Analogues X14 and X15 presented a low V_D and when tested in an acute feeding study in mice their anorectic effect was not significant after the first hour post-administration. Although the V_D does not correspond to an actual body fluid compartment, it does provide a measure of the extent of distribution of a drug. A low V_D that approximates plasma volume or extracellular fluid volume usually indicates that the drug's distribution is restricted to a particular compartment (the plasma or extracellular fluid). Analogue

X20's anorectic effect was only significant for 2 hours post-administration and the body weight loss at 48 hours was not different from the vehicle control group.

Analogues X7, X9, X10, X16, X18 and X21 were selected, among the analogues developed in this chapter, to be tested in a chronic feeding study in mice. The reason for selecting these particular analogues was that they have previously demonstrated the higher body weight loss when tested in an acute feeding study in mice. However, as the goal of this thesis was to develop an anti-obesity medication that ideally would be administered once weekly, the full anorectic effect of the analogues over one week needed to be determined. In this chronic study, analogues were given in a zinc-containing formulation and their PK properties and their ability to bind readily to zinc would determine whether they would be present in the circulation long enough to cause a sustainable weight loss effect.

In this chronic feeding study, only EX-4 and analogue X21 showed a significant anorectic effect over 6 days when compared to the vehicle control group ($p < 0.001$ and $p < 0.01$ respectively). Furthermore, analogue X21 caused a significant body weight loss of 1.6 ± 0.2 g at 6 days when compared to the vehicle control group ($p < 0.001$). In this series of analogues tested, analogue X21 was not the one with the highest GLP-1R or GCGR binding; however it exhibited the highest bioactivity thanks to its PK profile as shown by its long half-life and by its zinc binding ability that allowed the peptide to be still present in the circulation at 96 hours. This highlights once again how PK can influence decision-making processes in drug discovery.

The OXM analogues developed in this chapter provide the basis for further development of a drug candidate that would be used as an anti-obesity medication. The point substitutions made in the analogues of this chapter aimed at improving the affinity and the potency at the GLP-1R and the PK profile of the peptide. This second generation of analogues resulted in an increase binding and potency to the GLP-1R while at the same time the peptides were able to demonstrate a longer presence in the plasma *in vivo*. Further structural changes can possibly increase the bioactivity of the analogues in order to make them suitable for an *in vivo* therapeutic medication.

5.5. Conclusions

Point substitutions at positions 16 and 23 (Glu¹⁶-Ile²³) have proven to be beneficial for the binding and potency to the GLP-1R and future analogues will retain these substitutions. In regards to the C-terminal poly-His tail, it was shown from analogues X10-X16 that a longer poly-His tail and helix-capping.

The OXM analogue X21 which is a GCG/GLP-1 dual agonist presents a higher binding affinity to the GLP-1R and GCGR when compared to native OXM. It's bioefficacy at the GLP-1R is 4-fold lower than EX-4 while at the GCGR it is approximately 4-fold more potent than native GCG. The PK studies performed have demonstrated that X21 has a half-life of 14.4 minutes with a MCR of 6.2 ml/min/kg and a V_D of 144 ml/kg. When administered in a preparation that contains zinc, analogue X21 demonstrated that it presents a T_{max} level at 24 hours and is still present in the circulation at 96 hours post-administration. Furthermore it was demonstrated that it is able to cause a significant inhibition of food intake both in an acute and in a chronic feeding study in mice.

Chapter 6

Investigation of amino acid substitutions at positions 24, 29, 30 and at the C-terminal

6.1. Modification of positions 24, 29, 30 and of the C-terminal poly-Histidine tail

A number of novel peptide analogues, X22-X26, were designed and synthesised in this chapter taking into account the observed effects of Glu¹⁶, Ile²³ and of the poly-His tail as documented in chapter 5 with the aim of identifying an optimal anti-obesity therapeutic agent. The goal of this chapter was to further improve the GLP-1R binding affinity and potency of the OXM analogues (through changes in position 24) and also improve their PK profile with a longer duration of action (through point substitutions in positions 29, 30 and the C-terminal).

Each of these analogues was tested at several functional parameters including *in vitro* and *in vivo* experiments. The *in vitro* tests included testing the analogues' binding affinity to both the GLP-1R and GCGR and their ability to induce a cAMP response at the human GLP-1 and GCG overexpressing cell lines. The *in vivo* experiments aimed at testing the analogues' ability to inhibit rodent food intake in an acute and chronic setting. An assessment of the molecules PK properties in the *in vivo* constant infusion model and of the plasma concentration (when administered in a solution with zinc) was also completed. The OXM analogue that has demonstrated the highest bioactivity in inhibiting food intake and causing weight loss in both mice and rats was tested in an oral glucose tolerance test in order to investigate its effect on plasma glucose.

6.1.1. Position 24

Previous unpublished work in the Department has revealed that Glu-Gln substitutions in the GLP-1 molecule have yielded analogues with a higher bioactivity in inhibiting food intake. Investigation of the mid-section of the EX-4 molecule which includes position 15-24 demonstrated that this section is a linking region which is critical for the bioactivity of EX-4 (353). In specific, Glu²⁴ lies in the hydrophilic surface of the α -helix of EX-4 and in it was used in the OXM analogues of this chapter instead of the native Gln²⁴ in an effort to further improve the binding and potency to the GLP-1R (261).

6.1.2. Position 29

A further change in position 29 was made in order to investigate whether the combination of Gly²⁹-Gly³⁰ in this series of analogues would be able to enhance the PK profile of the analogues. In analogues X23-X26 the native Thr²⁹ was preserved as it has been shown from analogues X17-X21 that it can also result in prolongation of the PK profile independently of helix-capping.

6.1.3. Position 30

In analogue X22, position 30 was occupied by Gly in an effort to investigate the effect of the combination of Gly²⁹-Gly³⁰. In analogues X23-X25, position 30 was occupied by a His residue and in analogue X26 it was occupied by Gly trying to investigate what is the optimal flexibility of the poly-His tail for zinc binding.

6.1.4. Position 35-37 (Helix capping)

Helix-capping was introduced in analogues X10-X16 of chapter 5 and was shown to be beneficial in improving the PK profile of the analogues. In this chapter, analogues X22 and X24-X26 had helix-capping at the C-terminal with the use of Glu-NH₂.

6.1.5. C-terminal poly-Histidine tail

As demonstrated from the studies in the previous chapter, a poly His-tail in the C-terminal of the molecule can improve the PK profile of the analogues; the hypothesis being that His binds to the zinc molecules in the preparation resulting in a slow release from the SC depot. All analogues designed in this chapter (Table 10) have a variable His C-terminal tail to investigate if this further improves the PK profile.

6.1.6. Aims

Hypothesis:

Rational modifications to the amino acid sequence of OXM will result in an OXM analogue more efficacious at inhibiting food intake and promoting weight loss than previously developed OXM analogues.

Aims:

To determine:

- Investigate effects of rational point substitutions to the sequence of OXM analogues on the agonist properties at the GLP-1R and GCGR.
- The effect of specific changes to the structure of OXM on its *in vivo* PK parameters administered in solutions with and without zinc.

- Investigate the effect of SC administration of OXM analogues to mice on food intake and body in an acute feeding study.
- Investigate the effect of SC administration of OXM analogues on food intake and body weight in a chronic feeding study in both mice and rats.
- Investigate the effect of peripheral administration of analogue X26 on plasma glucose by an Intraperitoneal Glucose Tolerance Test (IPGTT) in mice.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
GCG	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T								
GLP1	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R	NH2						
EX4	H	G	E	G	T	F	T	S	D	L	S	K	Q	M	E	E	E	A	V	R	L	F	I	E	W	L	K	N	G	G	P	S	S	G	A	P	P
OXM	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	K	R	N	R	N	N	I	A
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
X22	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	E	W	L	L	N	G	G	H	H	H	H	H	E	NH2
X23	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	E	W	L	L	N	T	H	H	H	H	H			
X24	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	E	W	L	L	N	T	H	H	H	H	H	E	NH2	
X25	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	E	W	L	L	N	T	H	H	H	H	H	H	E	NH2
X26	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	E	R	R	A	H	E	F	I	E	W	L	L	N	T	G	H	H	H	H	H	E	NH2

Table 10: Amino acid sequences of peptides used in chapter 6. Blue filled residues are conserved residues across the GLP-1 and GCG peptides; yellow letters are amino acids derived from the GLP-1 sequence; red letters are amino acids derived from the EX-4 sequence; grey filled residues represent point substitutions.

6.2. Materials and Methods

6.2.1. Custom design of peptide analogues

Five analogues of OXM were designed and synthesised as described in section 2.2. The amino acid sequences of these analogues as well as EX-4 and the naturally occurring OXM, GLP-1 and GCG are shown in Table 10.

6.2.2. Competitive receptor binding assays

6.2.2.1. Glucagon receptor binding assays

6.2.2.1.1. Receptor binding of ligands to the human glucagon receptor

Binding studies were completed as described in section 2.6.5, using CHO cell membrane (cells overexpressing the hGCGR as the source of GCGR (section 2.6.2). ¹²⁵I-GCG was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean ± SEM.

6.2.2.1.2. Receptor binding of ligands to the mouse glucagon receptor

Binding studies were completed as described in section 2.6.5, using mouse liver cell membranes as the source of GCGR (section 2.6.1). ¹²⁵I-GCG was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean ± SEM.

6.2.2.2. GLP-1 receptor binding assays

6.2.2.2.1. Receptor binding of ligands to the human GLP-1 receptor

Binding studies were completed as described in section 2.6.4, using CHO cell membrane (cells overexpressing the hGLP-1R as the source of GLP-1R (section 2.6.2). ¹²⁵I-GLP-1 was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean ± SEM.

6.2.2.2.2. Receptor binding of ligands to the mouse GLP-1 receptor

Binding studies were completed as described in section 2.6.4, using mouse lung cell membrane as the source of GLP-1R (section 2.6.1). ^{125}I -GLP-1 was used as the competing peptide for all studies. Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

6.2.3. cAMP accumulation studies

6.2.3.1. *In vitro* bioactivity studies at the human glucagon receptor

The ability of ligands to stimulate cAMP production was investigated as described in section 2.7 using CHO cells overexpressing the hGCGR (section 2.6.2) as the source of GCGR. EC₅₀ values were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

6.2.3.2. *In vitro* bioactivity studies at the human GLP-1 receptor

The ability of ligands to stimulate cAMP production was investigated as described in section 2.7 using CHO cells overexpressing the hGLP-1R (section 2.2) as the source of GLP-1R. EC₅₀ values were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate. Values are shown as mean $\pm$ SEM.

6.2.4. Acute feeding studies in mice

The effect of OXM analogues on food intake were investigated in fasted mice, as described in section 2.4. Peptides were administered via SC injection and food intake measured at 1, 2, 4, 8 and 24 and 48 hours post-injection, unless otherwise stated. Statistical analysis of results was completed as described in section 2.9, results are shown as mean $\pm$ SEM (n=7-8 per group). Doses administered were chosen based on the observations from previous feeding studies (not shown). Exendin-4 (10 nmol/kg) was used as a positive control and OXM analogues were administered at 50 nmol/kg.

6.2.5. Chronic feeding studies in mice

The effect of OXM analogues on food intake was investigated in a 6 day chronic study in mice. Analogues were dissolved in ZnCl₂ solution prepared in saline at a zinc to peptide ratio of 1:1. Peptides were administered via SC injection at a dosage of 50 nmol/kg at 0900 to overnight fasted DIO C57BL/6J mice (av. body weight 40.2 g). Exendin-4 was administered at a dosage of 10 nmol/kg without zinc. Food intake and body weight were measured at times -16h, 48h and 6 days.

6.2.6. Chronic feeding studies in rats

The effect of OXM analogues on food intake was investigated in a 6 day chronic study in rats. Analogues were dissolved in ZnCl₂ prepared in a saline at zinc to peptide ratio of 1:1. Peptides were administered via SC injection at a dosage of 50 nmol/ kg at 0900 to male Wistar rats fasted for 24 hours (av. body weight 560 g). Exendin-4 was administered at a dosage of 10 nmol/kg without zinc. Doses were chosen accordingly to the acute feeding studies described above. Food intake and body weight were measured at times -16h, 48h, 72h and 6 days. Each group contained 5-6 rats and data shown is mean ± SEM (g).

6.2.7. Pharmacokinetics

6.2.7.1. Investigation of the pharmacokinetic properties in an *in vivo* constant infusion model

Studies were carried out to determine the *in vivo* PK properties (MCR, half-life, Volume of distribution) of OXM analogues with the use of the IV constant infusion model in male Wistar rats (section 3.2.2). Plasma peptide concentrations were determined by RIA (section 2.8).

6.2.7.2. Investigation of the pharmacokinetic properties following single subcutaneous bolus injection in male Wistar rats

Studies were carried out to investigate the formation of a slow release depot and measure plasma clearance following a SC administration (section 2.5.1). Male Wistar rats were stratified by body weight and placed into groups of 5 rats of equal mean body weight. Each rat was administered 1 mg of peptide (50 mg/ml) reconstituted in 1:1 molar ratio Zn:NaCl (saline) at time 0. Plasma peptide concentrations were determined by RIA (section 2.8).

6.2.8. Glucose tolerance test in mice

An IPGTT study was performed. Naïve mice (fed ad libitum) received a single SC injection of saline, OXM analogue X26 (100 nmol/kg) or EX-4 (20 nmol/kg) at 0700 on the day of study and then fasted until 1300 (T₀). Tail tip samples of blood were obtained 30 min before glucose administration. At 1300 (T₀), each mouse was injected IP with 2 g/kg glucose solution. Tail tip samples of blood (removing clot of previous tip) were then obtained at time points: 0 min, 30 min and 90 min in order to measure glucose using handheld CONTOUR glucometer (Bayer, Canada). A strip was inserted into the

glucometer, then a drop of blood from the tail tip was placed on the measuring strip and the blood glucose concentration was shown and recorded.

6.3. Results

6.3.1. Third generation of oxyntomodulin analogues

6.3.1.1. Binding affinities of oxyntomodulin, glucagon and oxyntomodulin analogues to the glucagon receptor

6.3.1.1.1. Binding to membrane preparation of CHO cells overexpressing human glucagon receptor

Binding assays were completed as described in section 6.2.2.1.1. Binding studies using ^{125}I -GCG routinely resulted in greater than 90% specific binding to a cell membrane preparation of CHO cells overexpressing the hGCGR. The binding affinities of OXM and GCG to the hGCGR are shown graphically in Figure 92 and summarised in Table 11. Oxyntomodulin was shown to bind to the hGCGR with approximately 17-fold lower affinity than GCG (OXM $\text{IC}_{50} = 9.19 \pm 0.78 \text{ nM}$, GCG $\text{IC}_{50} = 0.55 \pm 0.07 \text{ nM}$).

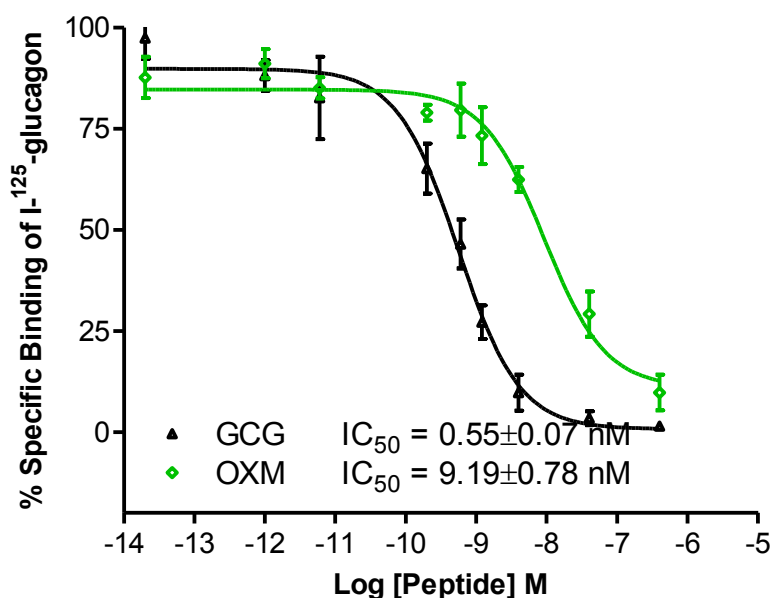


Figure 92: Competitive receptor binding affinity of GCG and OXM to the hGCGR. Purified cell membranes of CHO cells overexpressing the hGCGR were the source of the receptors. $\text{I}^{125}\text{-GCG}$ was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 5 OXM analogues (X22-X26) to the hGCGR were tested and compared with endogenous ligands (Figure 93, Table 11).

Analogues X22, X24 and X25 had a 1.4-fold lower affinity to the hGCGR than GCG (X22 $IC_{50} = 0.79 \pm 0.15$ nM, X24 $IC_{50} = 0.78 \pm 0.09$ nM, X25 $IC_{50} = 0.77 \pm 0.17$ nM) (Figure 93). Analogues X23 and X26 had a 1.3-fold lower affinity to the hGCGR than GCG (X23 $IC_{50} = 0.74 \pm 0.08$ nM, X26 $IC_{50} = 0.72 \pm 0.05$ nM).

Analogues X22 to X26 demonstrated a higher binding affinity to the hGCGR than OXM that ranged from 11.6-fold for X22, 11.8-fold for X24, 11.9-fold for X25, 12.4-fold for X23 and 12.8-fold for X26.

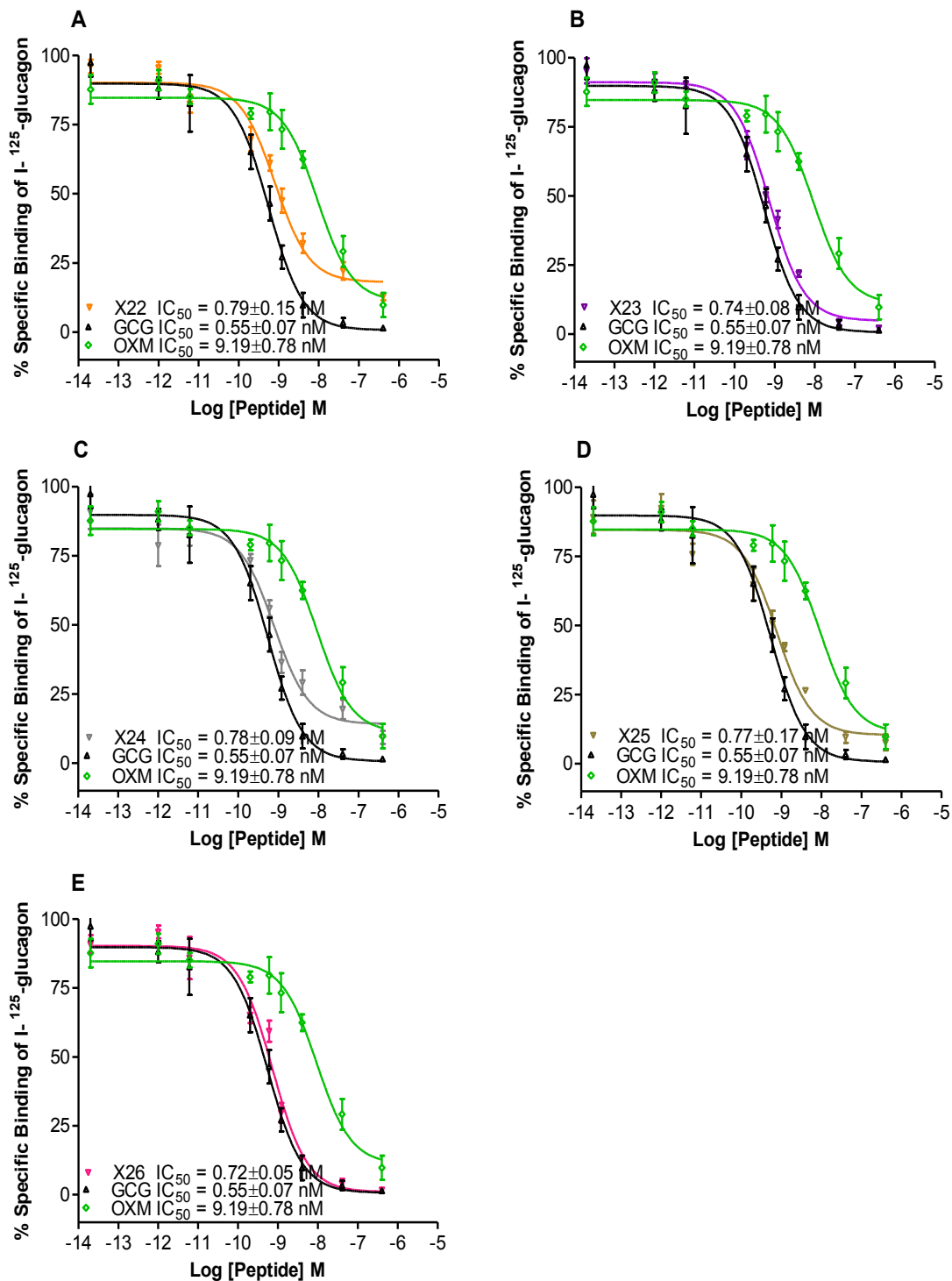


Figure 93: Competitive receptor binding affinity of GCG, OXM and 5 OXM analogues A: X22, B: X23, C: X24, D: X25, E: X26 to the hGCGR. Purified cell membranes of CHO cells overexpressing the hGCGR were the source of the receptors. I¹²⁵-GCG was used as the competing peptide. IC₅₀ values calculated from a minimum of three separate experiments. Values shown are mean ± SEM.

6.3.1.1.2. Binding to membrane preparation of mouse liver cells overexpressing mouse glucagon receptor

Binding assays were completed as described in section 6.2.2.1.2. Binding studies with I^{125} -GCG routinely shown greater than 90% specific binding to membranes prepared from mouse liver tissue. The binding affinities of GCG and OXM to the mGCGR are shown graphically in Figure 94 and summarised in Table 11. The affinity of OXM was 4.8-fold lower than GCG (GCG IC_{50} = 1.27 ± 0.23 nM, OXM IC_{50} = 6.04 ± 0.02 nM).

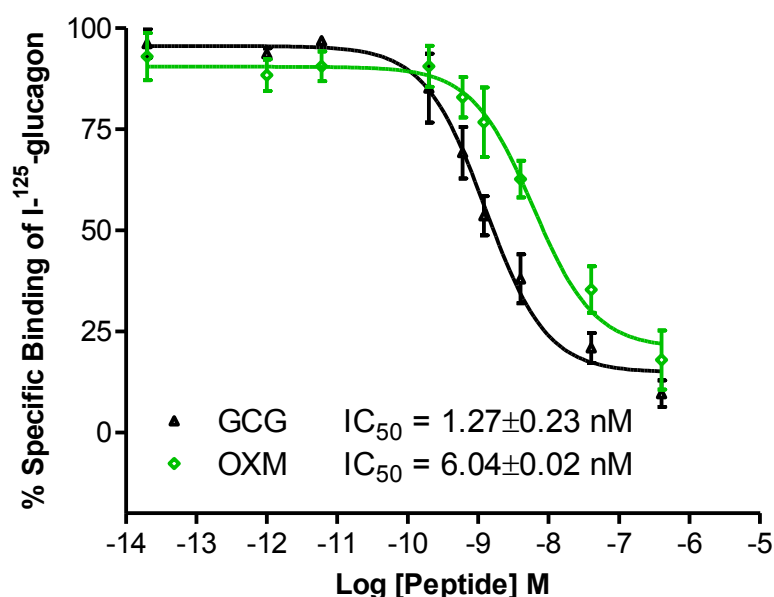


Figure 94: Competitive receptor binding affinity of GCG and OXM to the mGCGR. Cell membrane preparations from mouse liver overexpressing the mGCGR were the source of the receptors. I^{125} -GCG was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 5 OXM analogues (X22-X26) to the mGCGR were tested and compared with endogenous ligands (Figure 95, Table 11).

Analogues X22 and X23 presented approximately a 5.5-fold lower affinity to the mGCGR than GCG (X22 IC_{50} = 7.01 ± 1.42 nM, X23 IC_{50} = 6.93 ± 0.43 nM) while analogues X24 to X26 had a 5.4 fold lower affinity than GCG (X24 IC_{50} = 6.84 ± 0.16 nM, X25 IC_{50} = 6.92 ± 0.10 nM, X26 IC_{50} = 6.82 ± 0.27 nM) (Figure 95).

When compared to OXM, analogues X23-X26 presented approximately a 1.1-fold lower affinity to the mGCGR while X22 had a 1.2-fold lower affinity than OXM.

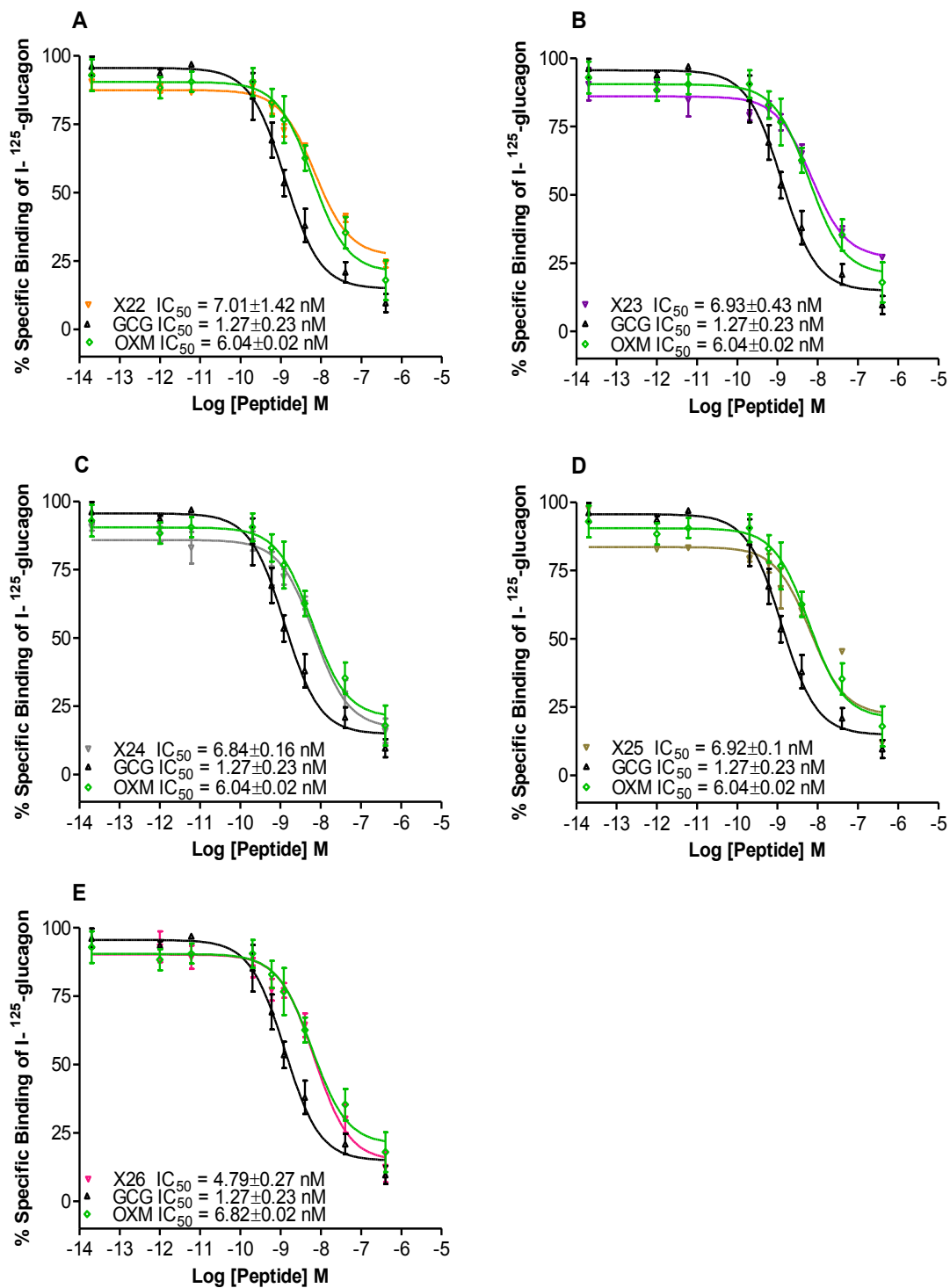


Figure 95: Competitive receptor binding affinity of GCG, OXM and 5 OXM analogues A: X22, B: X23, C: X24, D: X25, E: X26 to the mGCGR. Cell membrane preparations from mouse liver overexpressing the mGCGR were the source of the receptors. I¹²⁵-GCG was used as the competing peptide. IC₅₀ values calculated from a minimum of three separate experiments. Values shown are mean ± SEM.

6.3.1.2. Binding affinities of oxyntomodulin, GLP-1 and oxyntomodulin analogues to the GLP-1 receptor

6.3.1.2.1. Binding to membrane preparation of CHO cells overexpressing human GLP-1 receptor

Binding assays to the hGLP-1R were completed as described in section 6.2.2.2.1. Binding studies using ^{125}I -GLP-1 routinely resulted in greater than 90% specific binding to a cell membrane preparation of CHO cells overexpressing the hGLP-1R. The binding affinities of GLP-1 and OXM to the hGLP-1R are shown graphically in Figure 96 and summarised in Table 11. Oxyntomodulin was shown to bind to the hGLP-1R with approximately 418-fold lower affinity than GLP-1 (OXM $\text{IC}_{50}=125.3\pm2.20$ nM, GLP-1 $\text{IC}_{50}=0.30\pm0.02$ nM).

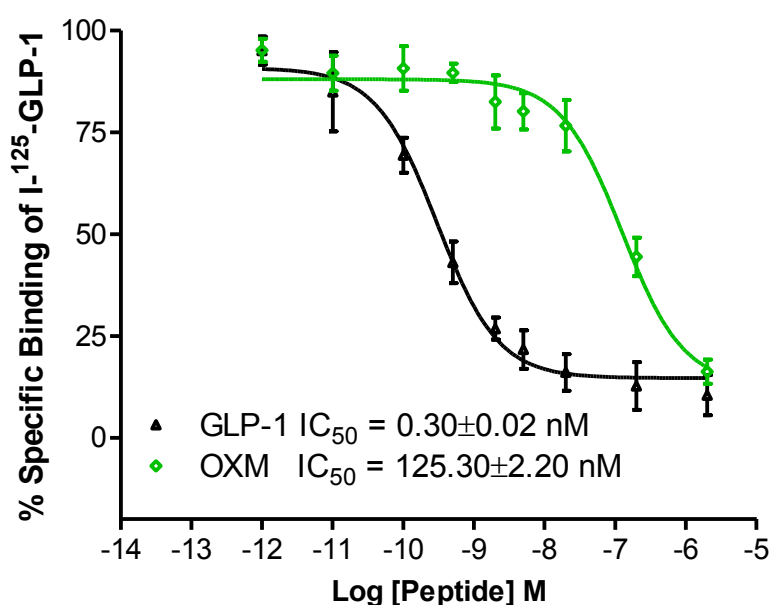


Figure 96: Competitive receptor binding affinity of GLP-1 and OXM to the hGLP-1R. Purified cell membranes of CHO cells overexpressing the hGLP-1R were the source of the receptors. ^{125}I -GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 5 OXM analogues (X22-X26) to the hGLP-1R were tested and compared with endogenous ligands (Figure 97, Table 11).

Analogues X22 and X25 had a 3.3-fold and 3.2-fold lower affinity than GLP-1 to the hGLP-1R (X22 $\text{IC}_{50}=0.98\pm0.15$ nM, X25 $\text{IC}_{50}=0.96\pm0.29$ nM) (Figure 97). Analogues X23, X24 and X26 had a 2.9-, 2.8- and 3-fold lower affinity than GLP-1 to the hGLP-1R (X23 $\text{IC}_{50}=0.88\pm0.19$ nM, X24 $\text{IC}_{50}=0.85\pm0.09$ nM, X26 $\text{IC}_{50}=0.89\pm0.26$ nM). When OXM analogues X22 to X26 were compared to

OXM, they presented a higher affinity to the hGLP-1R that ranged from 127.9-fold for X22, 130.5-fold for X25, 140.8-fold for X26, 142.4-fold for X23 and 147.4-fold for X24.

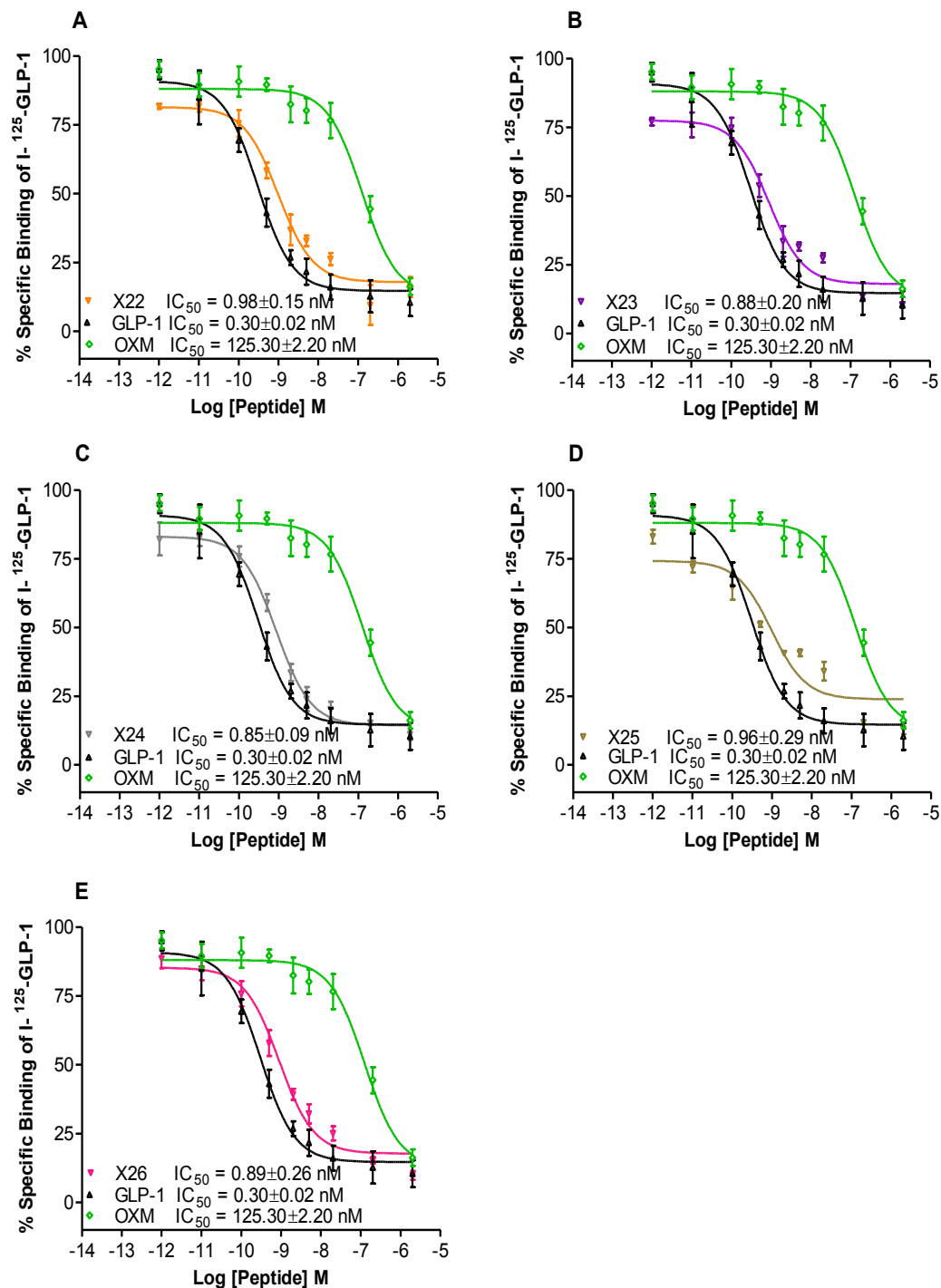


Figure 97: Competitive receptor binding affinity of GLP-1, OXM and 5 OXM analogues A: X22, B: X23, C: X24, D: X25, E: X26 to the hGLP-1R. Purified cell membranes of CHO cells overexpressing the hGLP-1R were the source of the receptors. ^{125} I-GLP-1 was used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

6.3.1.2.2. Binding to membrane preparation of mouse lung cells overexpressing mouse GLP-1 receptor

Binding assays were completed as described in section 6.2.2.2.2. Binding studies with I^{125} -GLP-1 routinely shown greater than 90% specific binding to membranes prepared from mouse lung tissue. Oxyntomodulin bound to the mGLP-1R with a 20-fold lower affinity approximately in comparison to GLP-1 (OXM IC_{50} = 43.58 ± 6.22 nM, GLP-1 IC_{50} = 2.23 ± 0.14 nM). The binding affinities of GLP-1 and OXM to the mGP-1R are shown graphically in Figure 98 and summarised in Table 11.

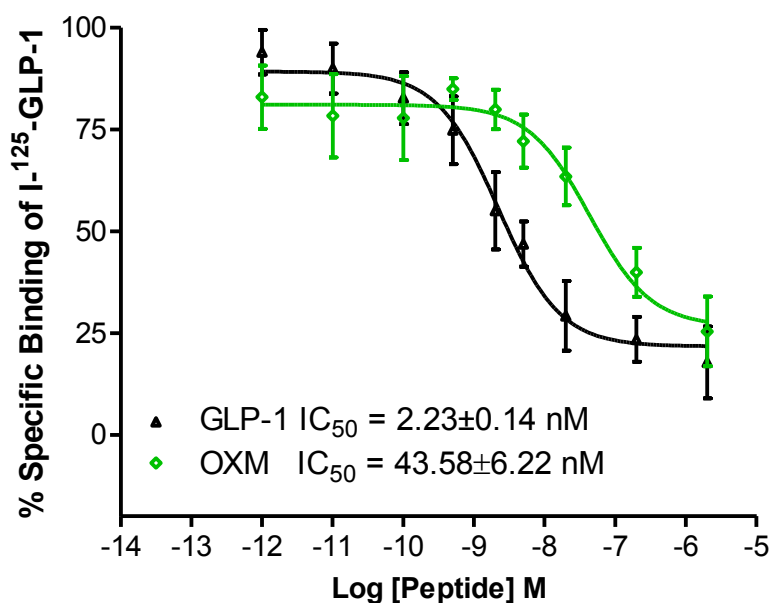


Figure 98: Competitive receptor binding affinity of GLP-1 and OXM to the mGLP-1R. Cell membrane preparations from mouse lung overexpressing the mGLP-1R were the source of the receptors. I^{125} -GLP-1 used as the competing peptide. IC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

Binding affinities of 5 OXM analogues (X22-X26) to the mGLP-1R were tested and compared with endogenous ligands (Figure 99, Table 11).

Analogue X22 had a 1.7-fold lower affinity to the mGLP-1R than GLP-1 (X22 IC_{50} = 3.89 ± 0.08 nM) (Figure 99). Analogues X23 and X25 had a 1.6-fold lower affinity to the mGLP-1R than GLP-1 (X23 IC_{50} = 3.57 ± 0.64 nM, X25 IC_{50} = 3.64 ± 0.66 nM). Analogues X24 and X26 had a 1.5-fold lower affinity than GLP-1 to the mGLP-1R (X24 IC_{50} = 3.27 ± 0.10 nM, X26 IC_{50} = 3.45 ± 0.09 nM).

When OXM analogues X22 to X26 were compared to OXM, they presented a higher affinity to the mGLP-1R that ranged from 11.2-fold for X22, 12-fold for X25, 12.2-fold for X23, 12.6-fold for X26 and 13.3-fold for X24.

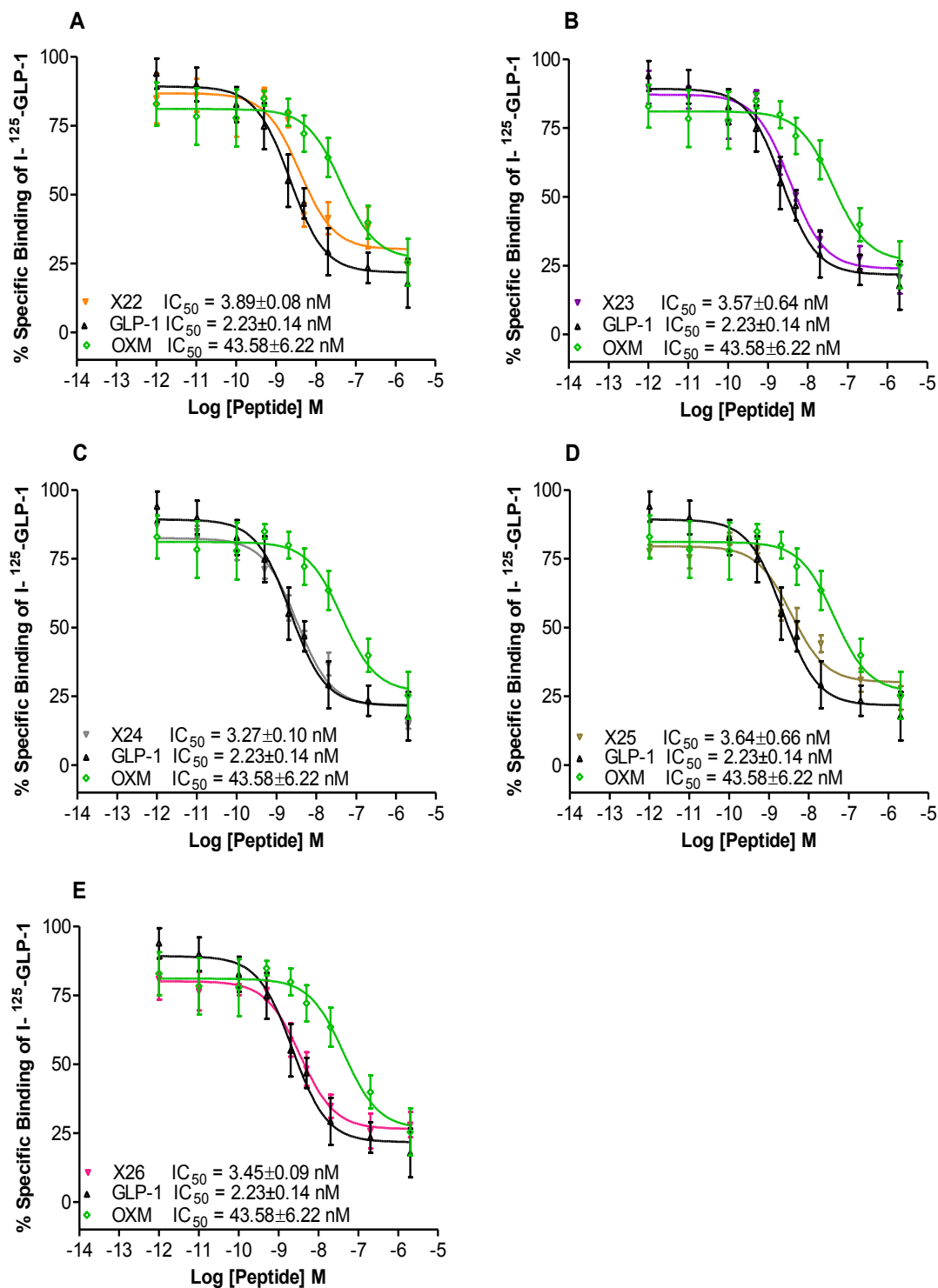


Figure 99: Competitive receptor binding affinity of GLP-1, OXM and 5 OXM analogues A: X22, B: X23, C: X24, D: X25, E: X26 to the mGLP-1R. Cell membrane preparations from mouse lung overexpressing the mGLP-1R were the source of the receptors. I¹²⁵-GLP-1 was used as the competing peptide. IC₅₀ values calculated from a minimum of three separate experiments. Values shown are mean ± SEM.

	GCGR		GLP-1R	
	hGCGR binding affinity IC ₅₀ (nM) ± SEM	mGCGR binding affinity IC ₅₀ (nM) ± SEM	hGLP-1R binding affinity IC ₅₀ (nM) ± SEM	mGLP-1R binding affinity IC ₅₀ (nM) ± SEM
GLP-1	>10000	>10000	0.30±0.02	2.23±0.14
GCG	0.55±0.07	1.27±0.23	>10000	220.0±10.0
OXM	9.19±0.78	6.04±0.02	125.30±2.20	43.58±6.22
X22	0.79±0.15	7.01±1.42	0.98±0.15	3.89±0.08
X23	0.74±0.08	6.93±0.43	0.88±0.19	3.57±0.64
X24	0.78±0.09	6.84±0.16	0.85±0.09	3.27±0.10
X25	0.77±0.17	6.92±0.10	0.96±0.29	3.64±0.66
X26	0.72±0.05	6.82±0.27	0.89±0.26	3.45±0.09

Table 11: A summary of IC₅₀ values for GLP-1, GCG, OXM and OXM analogues X22-X26 at both the GCGR and GLP-1R (human and mouse receptors). Half-maximal concentrations values are shown as mean ± SEM.

6.3.2. Effect of oxyntomodulin, glucagon and oxyntomodulin analogues on the human glucagon receptor mediated cAMP accumulation

The ability of OXM and GCG to stimulate cAMP accumulation in CHO cells overexpressing the hGCGR was investigated. The potencies of OXM and GCG are shown graphically in Figure 100 and summarised in Table 12. The bioactivity of OXM at the hGCGR was 9-fold lower than GCG (OXM EC_{50} = 0.495 ± 0.091 nM, GCG EC_{50} = 0.053 ± 0.009 nM). Glucagon-like peptide-1 presented a >2000-fold lower bioactivity at the hGCGR when compared with GCG (GLP-1 EC_{50} = 114.50 ± 5.30 nM).

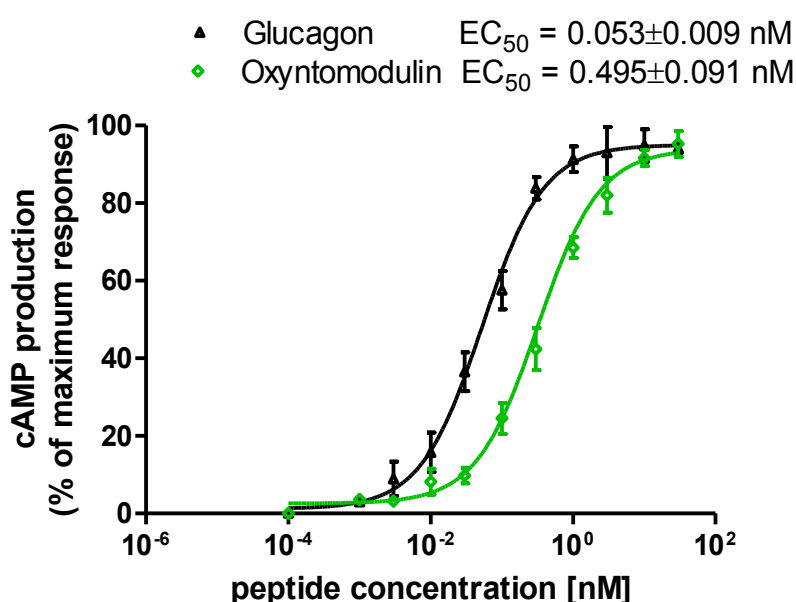


Figure 100: hGCGR-mediated cAMP accumulation by GCG and OXM. hGCGR overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

The *in vitro* potencies of 5 OXM analogues (X22 to X26) at stimulating cAMP accumulation at the hGCGR were compared with GCG and OXM (Figure 101, Table 12).

Analogues X22 and X24 presented a potency at the hCGR that was 4.4-fold higher than GCG (X22 EC_{50} = 0.012 ± 0.004 nM, X24 EC_{50} = 0.012 ± 0.004 nM) (Figure 101). Analogue X23 had a 4.1-fold higher bioactivity at the hCGR when compared to GCG (X23 EC_{50} = 0.013 ± 0.003 nM). The bioactivity of the OXM analogues X25 and X26 at the hGCGR was 4.8-fold approximately higher than native GCG (X25 EC_{50} = 0.011 ± 0.002 nM, X26 EC_{50} = 0.011 ± 0.002 nM).

Analogue X22-X26 had a higher bioactivity at the hGCGR when compared to OXM that ranged from 38.1-fold for X23, 41.3-fold for X22 and X24 and 45-fold for X25 and X26.

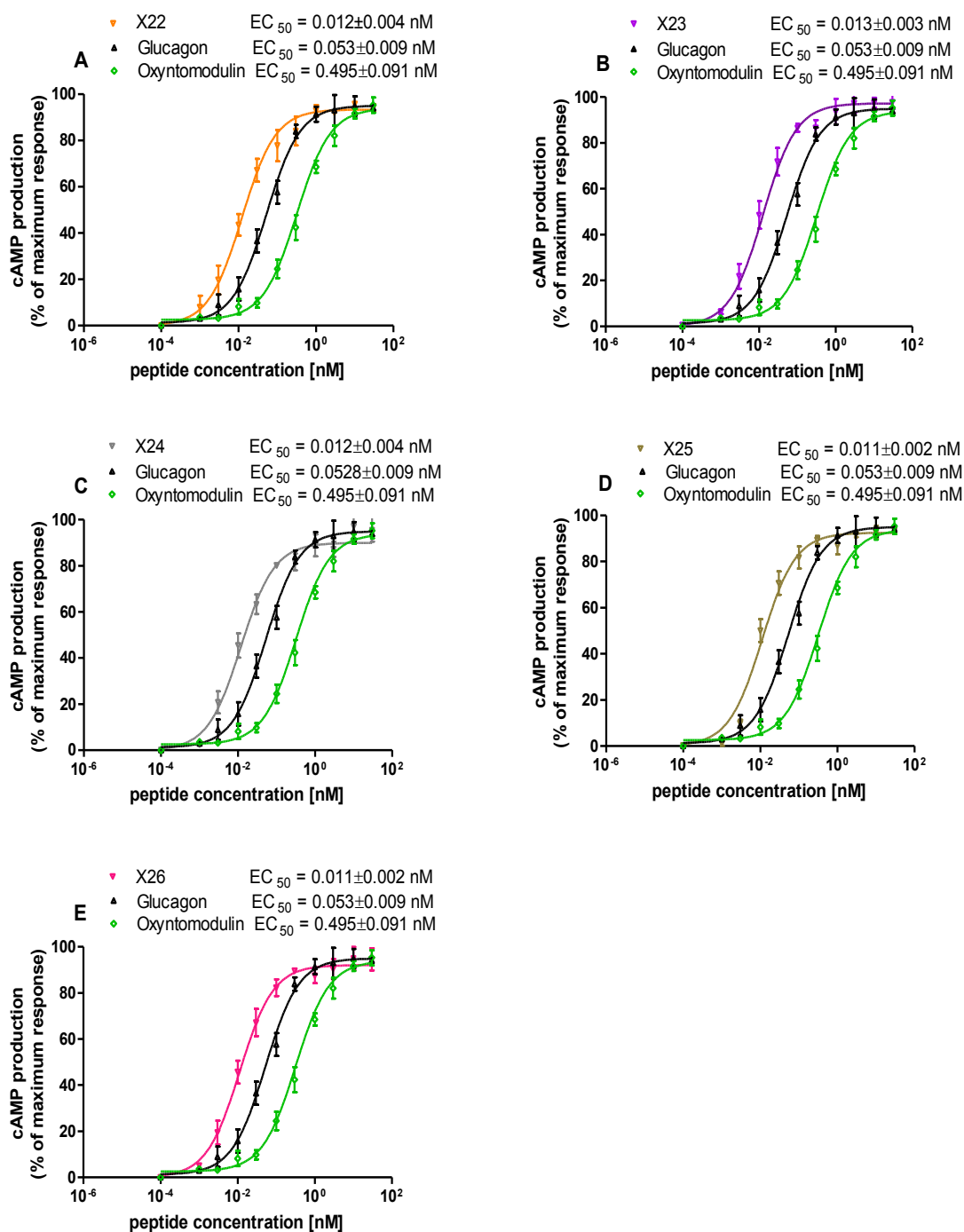


Figure 101: hGCGR-mediated cAMP accumulation by GCG and OXM and 5 OXM analogues. A: X22, B: X23, C: X24, D: X25, E: X26. hGCGR overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC₅₀ values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

6.3.3. Effect of oxyntomodulin, GLP-1 and oxyntomodulin analogues on the human GLP-1 receptor mediated cAMP accumulation

The ability of OXM, GCG and GLP-1 to stimulate cAMP accumulation in CHO cells overexpressing the hGLP-1R was investigated. The potencies of OXM and GLP-1 are shown graphically in Figure 102 and summarised in Table 12. The bioactivity of OXM at the hGLP-1R was 12.4-fold lower than GLP-1 (OXM EC_{50} = 6.886 ± 1.285 nM, GLP-1 EC_{50} = 0.554 ± 0.054 nM). Glucagon presented a 326-fold lower bioactivity at the hGLP-1R (GCG EC_{50} = 180.70 ± 3.511 nM) when compared to GLP-1.

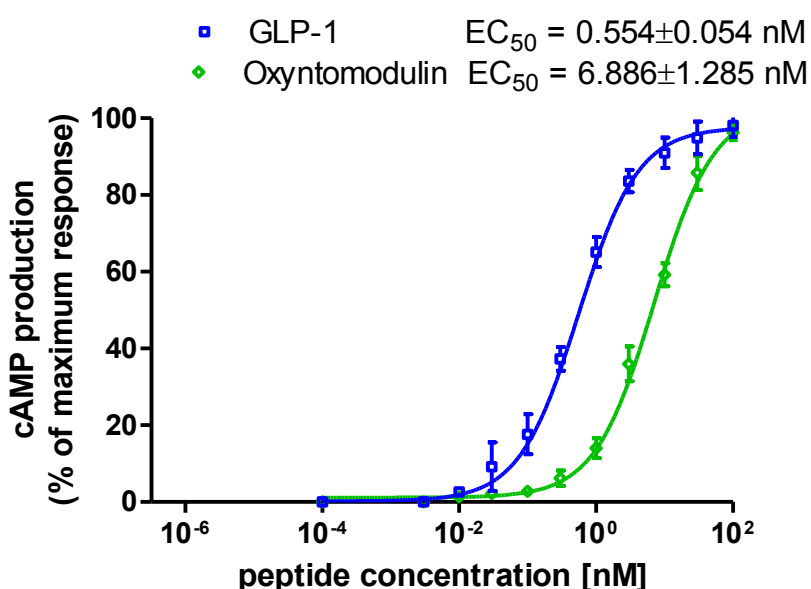


Figure 102: hGLP-1R-mediated cAMP accumulation by GLP-1 and OXM. hGLP-1R overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated as a mean from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

The *in vitro* potencies of 5 OXM analogues (X22 to X26) at stimulating cAMP accumulation at the hGLP-1R were compared with GLP-1 and OXM (Figure 103, Table 12).

Analogue X22 had approximately a 0.9-fold lower bioactivity at the hGLP-1R when compared to GLP-1 (X22 EC_{50} = 0.645 ± 0.617 nM) (Figure 103). Analogues X23, X24 and X26 presented a potency at the hGLP-1R that was 0.7-fold lower approximately when compared to the one of GLP-1 (X23 EC_{50} = 0.813 ± 0.159 nM, X24 EC_{50} = 0.834 ± 0.050 nM, X26 EC_{50} = 0.829 ± 0.141 nM). The bioactivity of the OXM analogue X25 at the hGLP-1R was 0.6-fold lower than native GLP-1 (X25 EC_{50} = 0.902 ± 0.130 nM).

Analogue X22 to X26 had a higher bioactivity at the hGLP-1R when compared to OXM that ranged from 7.6 fold for X25, 8.3-fold for X24 and X26, 8.5-fold for X23 and 10.7-fold for X22.

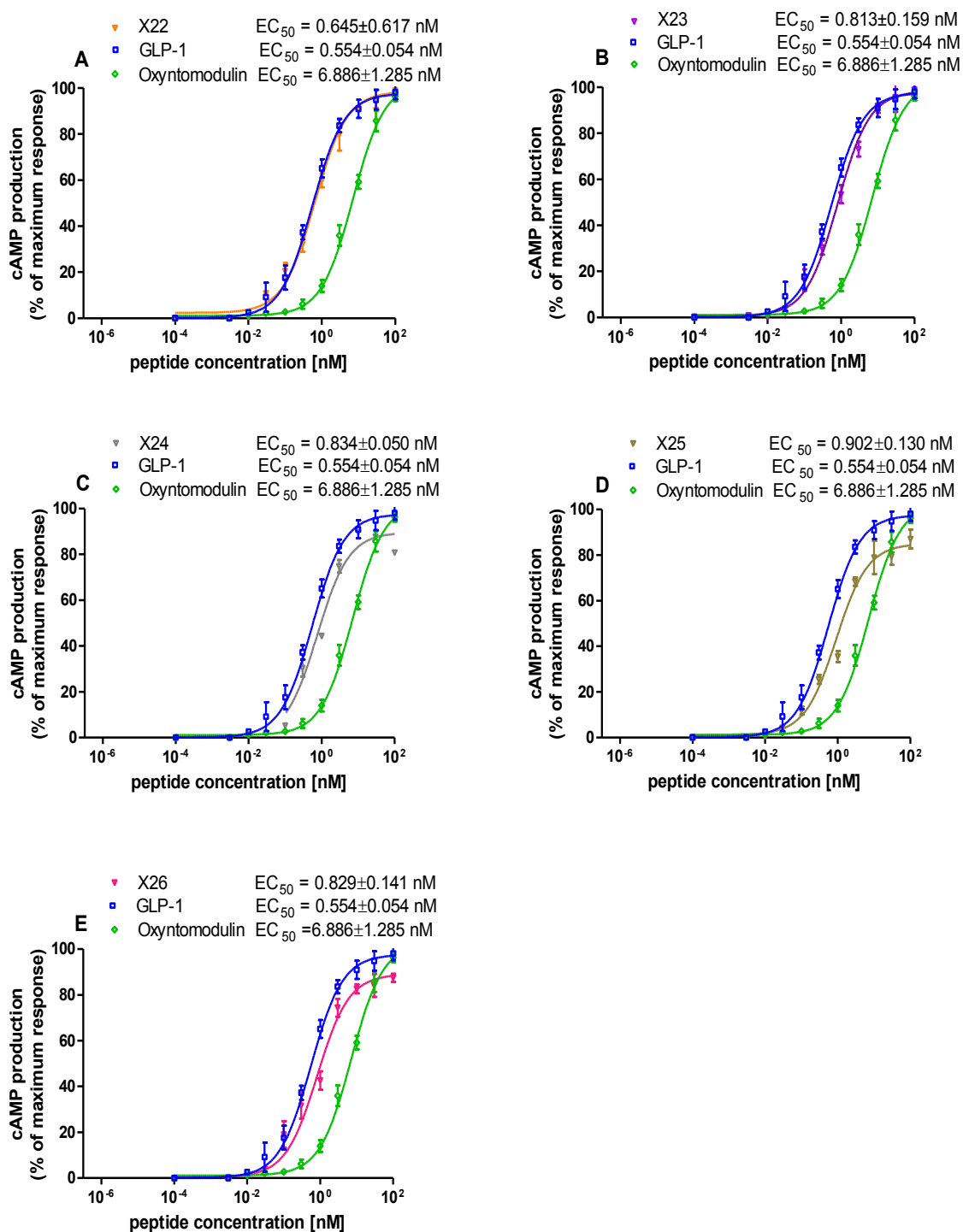


Figure 103: hGLP-1R-mediated cAMP accumulation by GLP-1, OXM and 5 OXM analogues A: X22, B: X23, C: X24, D: X25, E: X26. hGLP-1R overexpressed in CHO cells. Each peptide concentration tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments. Values shown are mean $\pm$ SEM.

	hGCGR	hGLP-1R
	cAMP bioactivity EC ₅₀ (nM) ± SEM	cAMP bioactivity EC ₅₀ (nM) ± SEM
GLP-1	114.50±5.30	0.554±0.054
GCG	0.053±0.009	180.70±3.511
OXM	0.495±0.091	6.886±1.285
X22	0.012±0.004	0.645±0.617
X23	0.013±0.003	0.813±0.159
X24	0.012±0.004	0.834±0.050
X25	0.011±0.002	0.902±0.130
X26	0.011±0.002	0.829±0.141

Table 12: Summary of EC₅₀ values for GLP-1, GCG, OXM and OXM analogues X22-X26 at both the hGCGR and hGLP-1R. Half-maximal concentrations values are shown as mean ± SEM.

6.3.4. Effect of exendin-4 and oxyntomodulin analogues X22 to X26 on food intake in fasted mice

Subcutaneous administration of EX-4 (10 nmol/kg) and 5 OXM analogues (X22 to X26) at 50 nmol/kg showed that all tested agents significantly reduced food intake to fasted DIO C57BL/6J mice over the 0-1 and 1-2 hour period post-injection compared with the vehicle control group (Figure 104). At the period of 2-4 hours, EX-4 and all OXM analogues significantly reduced food intake in comparison to the vehicle control group; analogues X23 and X25 being less potent than the rest of OXM analogues.

Exendin-4, X23 and X26 significantly inhibited food intake in the 4-8 hours post-injection at a level of significance of $p < 0.001$. Analogues X22, X24 and X25 significantly inhibited food intake in comparison to the vehicle control group, in the 4-8 hours post-injection, at a level of significance of $p < 0.01$.

There were no significant differences in food intake between the vehicle control group and the other treatment groups in the 8-24 hour and 24-48 hour interval.

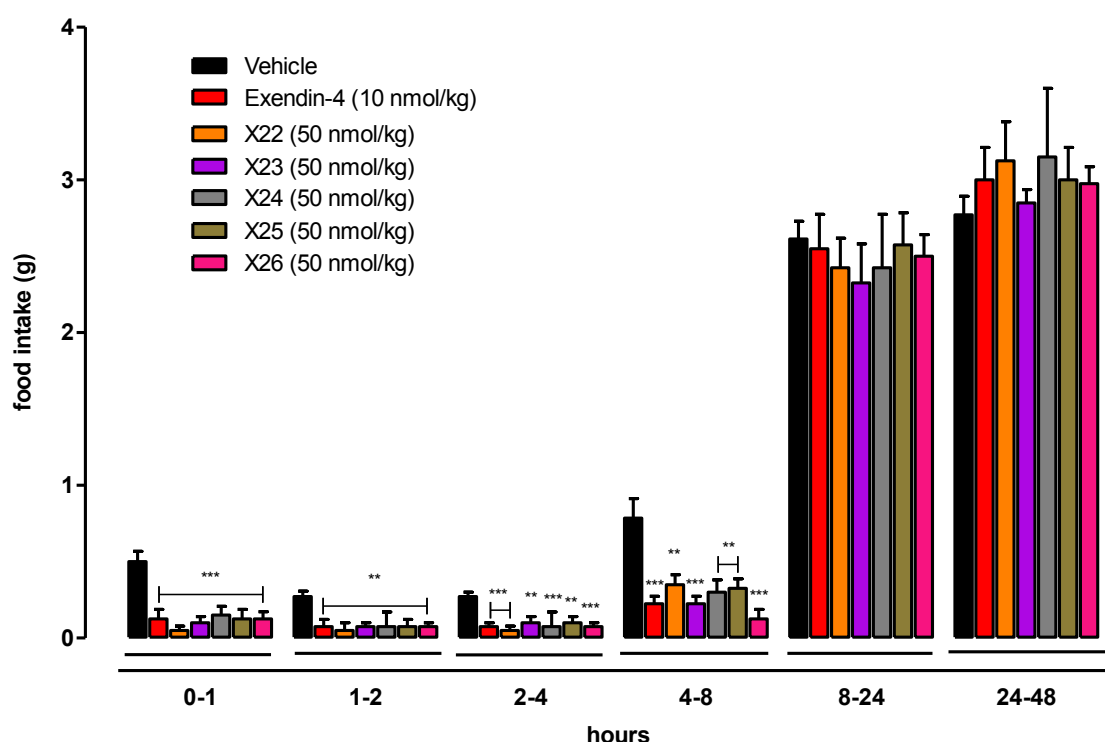


Figure 104: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on acute food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour and 8-24 and 24-48 hour intervals. $n=7-8$ per group. Statistics: One way ANOVA with Dunnett's post-hoc test $*=p < 0.05$, $**=p < 0.01$, $***=p < 0.001$ vs. vehicle.

When the anorectic effect of OXM analogues was compared to the one of EX-4, it was shown that there was no significant difference in the time periods 0-1, 1-2, 2-4 and 4-8 hours (Figure 105). Furthermore when the anorectic effect of the OXM analogues was compared between them, there was also no significant difference in the above-mentioned time periods.

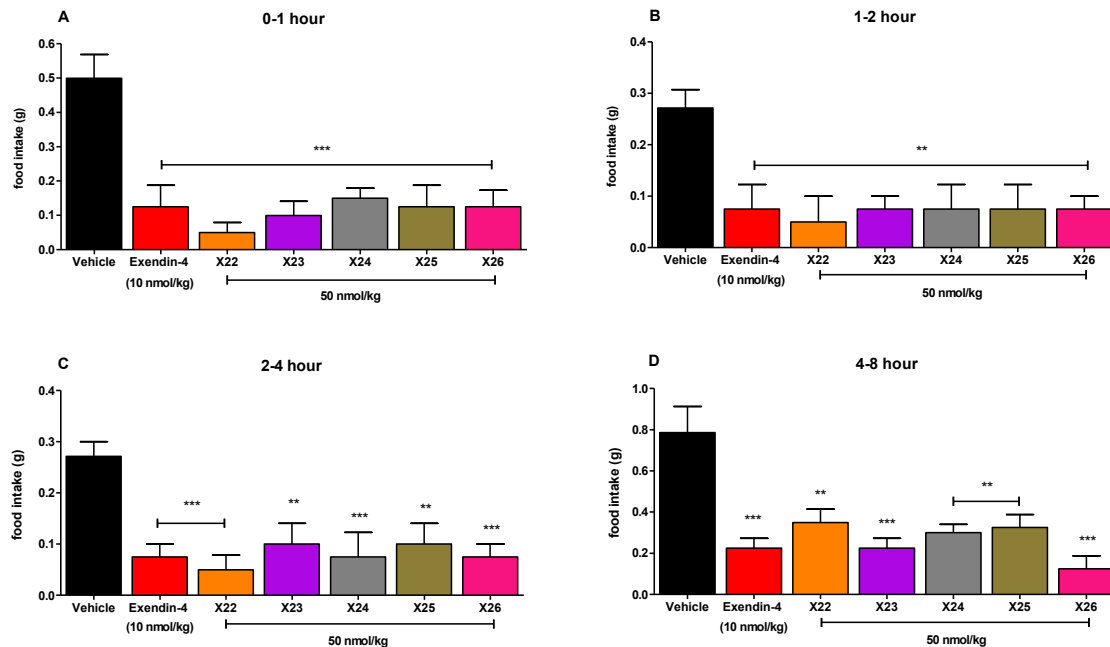


Figure 105: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on acute food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour and 8-24 and 24-48 hour intervals. $n=7-8$ per group. Statistics: One way ANOVA with Tukey's post-hoc test $**=p<0.01$, $***=p<0.001$ vs. vehicle.

Analysis of cumulative food intake data showed that both EX-4 (10 nmol/kg) and OXM analogues X22-X26 (50 nmol/kg), all significantly reduced food intake during the time periods of 0-2, 0-4 and 0-24 hours post-injection compared with the vehicle control group (Figure 106). The EX-4 and OXM analogues groups did not present statistically significant differences in the inhibition of food intake when they were compared between them.

Cumulative food intake at 0-48 hours post-injection was significantly reduced in EX-4 and OXM groups X22-X26 when compared to the vehicle control group ($p<0.05$ for EX-4 and analogues X22-X5, $p<0.01$ for X26).

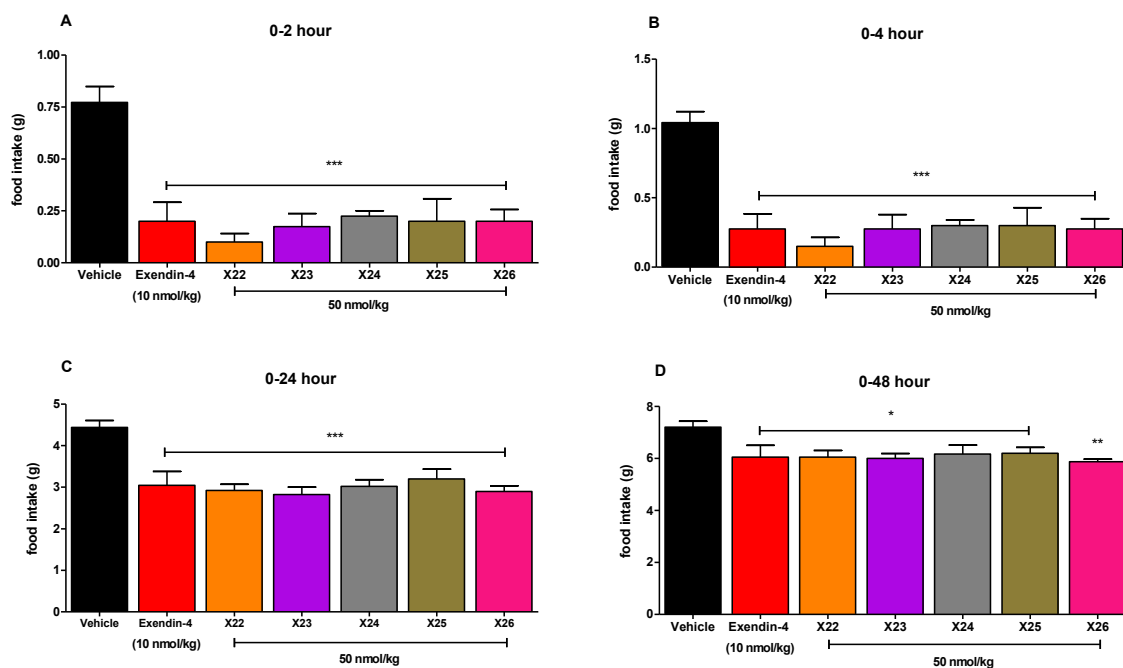


Figure 106: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg) on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for A: 0-2 hours, B: 0-4 hours, C: 0-24 hours and D: 0-48 hours. $n=7-8$ mice per group. Statistics: One way ANOVA with Tukey's post-hoc test $*=p<0.05$, $**=p<0.01$, $***=p<0.001$ vs. vehicle.

Investigation of the body weight changes that occurred between the periods of -16 to 48 hours showed that test groups EX-4, X22, X23 and X24 had a significant reduction in body weight when compared to the vehicle control group ($p<0.05$ for all peptides) (Figure 107). The average body weight reduction of the EX-4 group was 1.30 ± 0.30 g compared to vehicle-treated controls; X22 group lost 1.22 ± 0.30 g, X23 group lost 1.32 ± 0.10 g and X24 group lost 1.50 ± 0.50 g.

The body weight reduction demonstrated by X25 and X26 was significantly greater when compared to the vehicle control group ($p<0.01$ and $p<0.001$ respectively). The average body weight reduction of mice treated with X25 and X26, when compared to vehicle-treated controls, was 1.75 ± 0.30 g and 2.12 ± 0.40 g respectively.

There was no significant difference in the body weight change over the 2 day period between the OXM analogues-treated groups and the EX-4 group. No significant difference in the body weight change was detected when the OXM analogues were compared between them over the 2 day period.

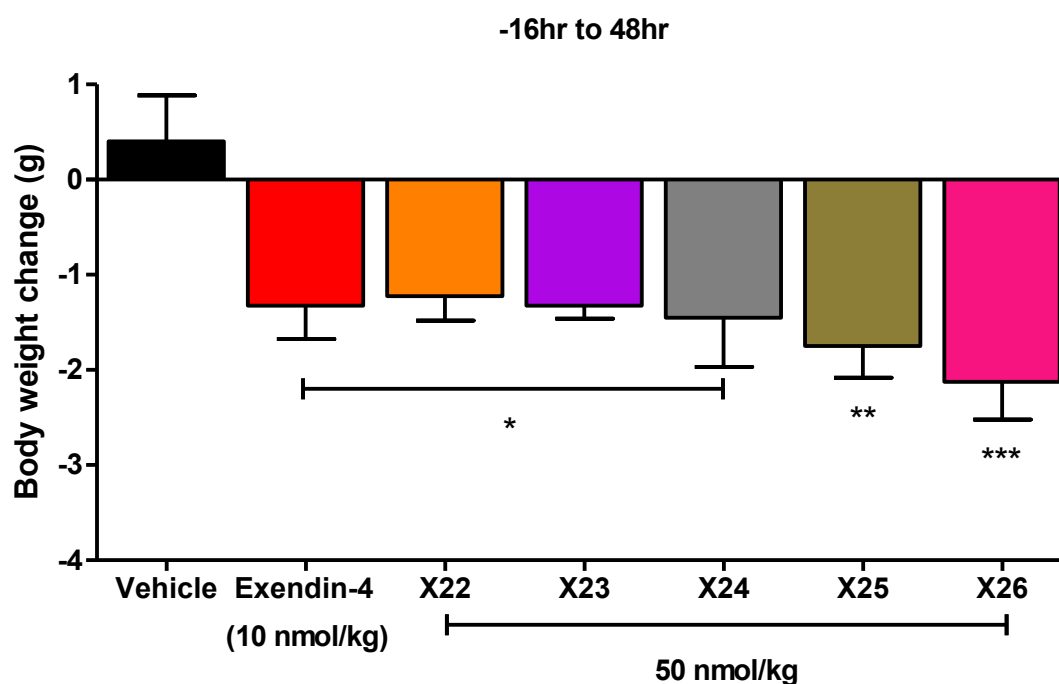


Figure 107: Body weight change over a 2 day period following a single SC dose of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg). Body weight change measured to accuracy of 0.1g compared to body weight at day 0. Error bars are $\pm$ SEM. Statistics: Comparisons versus vehicle were performed by one-way ANOVA with Dunnett's correction test while comparisons between groups were performed by one-way ANOVA with Tukey's correction test. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$.

6.3.5. Investigation of the pharmacokinetic properties in an *in vivo* constant infusion model

The time-courses of the mean plasma concentrations of analogues X22 to X26 during the IV primed-continuous peptide infusion were measured by RIA. Steady-state concentration levels were observed between 30 and 60 minutes after the start of the infusion of 9 nmol/hr of the peptide. The PK parameters and the steady-state concentration of analogues X22 to X26 are shown graphically in Figure 111 and presented in Table 13.

	Metabolic clearance rate (ml/min/kg)	Half-life (min)	Volume of distribution (ml/kg)	Steady state concentration (pmol/ml)
OXM	12.8±3.2	12.0±2.1	231.6±94.0	19.7±5.8
X22	6.2±2.0	16.1±2.8	142.0±43.5	14.0±2.4
X23	8.5±1.1	13.0±1.0	158.0±7.9	15.6±2.3
X24	5.1±0.8	16.5±1.6	123.0±26.1	31.9±3.3
X25	5.8±0.9	15.9±1.5	133.0±30.6	24.5±0.2
X26	5.4±0.2	21.3±3.5	164.0±31.3	24.9±0.1

Table 13: Pharmacokinetic parameters (MCR, Half-life and Volume of distribution) and steady-state concentration of OXM and OXM analogues X22-X26 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean ± S.E.M.

Analogues X24 and X26 presented an MCR of 5.1±0.8 and 5.4±0.2 ml/min/kg respectively (Figure 108). The MCR of analogue X24 was the lowest MCR recorded amongst analogues X22-X26. The MCR of X22 and X23 were 6.2±2.0 and 8.5±1.1 ml/min/kg respectively. Analogues X25 (5.8±0.9 ml/min/kg) and X26 had a 2-fold lower MCR when compared to the MCR of the OXM approximately but was not statistically significant. The MCR of analogues X22-X26 did not differ statistically when they were compared between them.

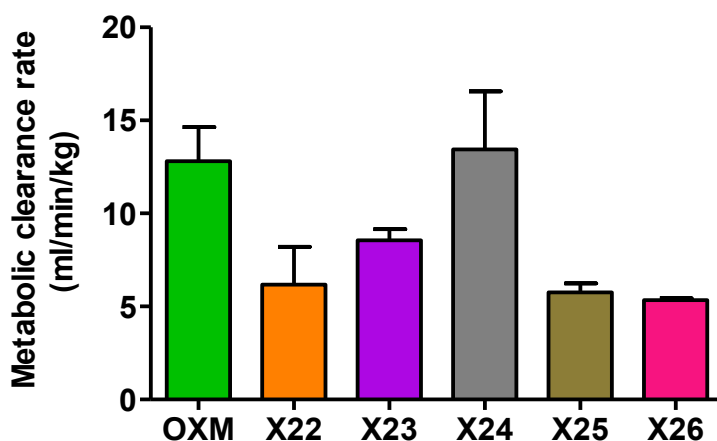


Figure 108: Metabolic clearance rate in ml/min/kg of OXM and OXM analogues X22-X26 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test.

The half-life of OXM analogues X22-X26 ranged between 13.0 $\pm$ 1.0 minutes (analogue X23) and 21.3 $\pm$ 3.5 minutes (analogue X26) (Figure 109). Analogue X22 had a half-life of 16.1 $\pm$ 2.8 minutes, analogue X24 had a half-life of 16.5 $\pm$ 1.6 minutes and analogue X25 presented a half-life of 15.9 $\pm$ 1.5 minutes. All analogues tested in this chapter had a higher half-life than OXM, however only X26 demonstrated a statistically significant difference ($p < 0.05$ for analogue X26).

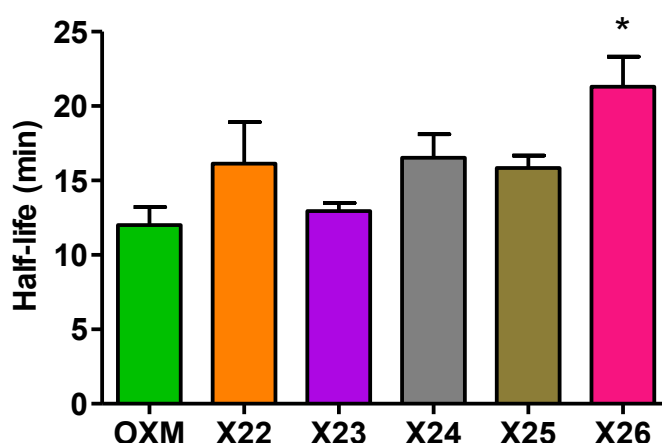


Figure 109: Half-life in minutes of OXM and OXM analogues X22-X26 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test. *= $p < 0.05$ vs. OXM.

The V_D of OXM analogues X22-X26 ranged between 123.0 ± 2.1 ml/kg (analogue X24) and 164.0 ± 31.3 ml/kg (analogue X26) (Figure 110). The V_D of X22, X23 and X25 was 142.0 ± 43.5 , 158.0 ± 7.9 and 133.0 ± 30.6 ml/kg respectively. The V_D of OXM analogues in this chapter did not differ significantly from OXM or when they were compared between them.

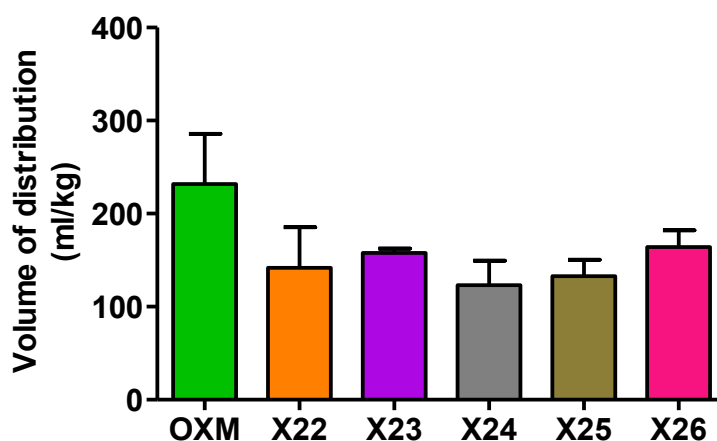


Figure 110: Volume of distribution in ml/kg of OXM and OXM analogues X22-X26 during continuous IV infusion in anaesthetised male Wistar rats for 60 minutes (n=4). Values are shown as mean $\pm$ S.E.M. Comparisons of OXM analogues with OXM were performed by one-way ANOVA with Dunnett's correction test while comparisons between the OXM analogues were performed by one-way ANOVA with Tukey's correction test.

The steady-state concentrations of analogues X22 to X26 ranged between 14.0 ± 2.4 pmol/ml (analogue X22) and 31.9 ± 3.3 pmol/ml (analogue X24) (Figure 111). Analogues X25 and X26 presented a steady-state concentration of 25.0 pmol/ml approximately. Plasma concentrations of analogues X21 to X26 are shown in Figure 119.

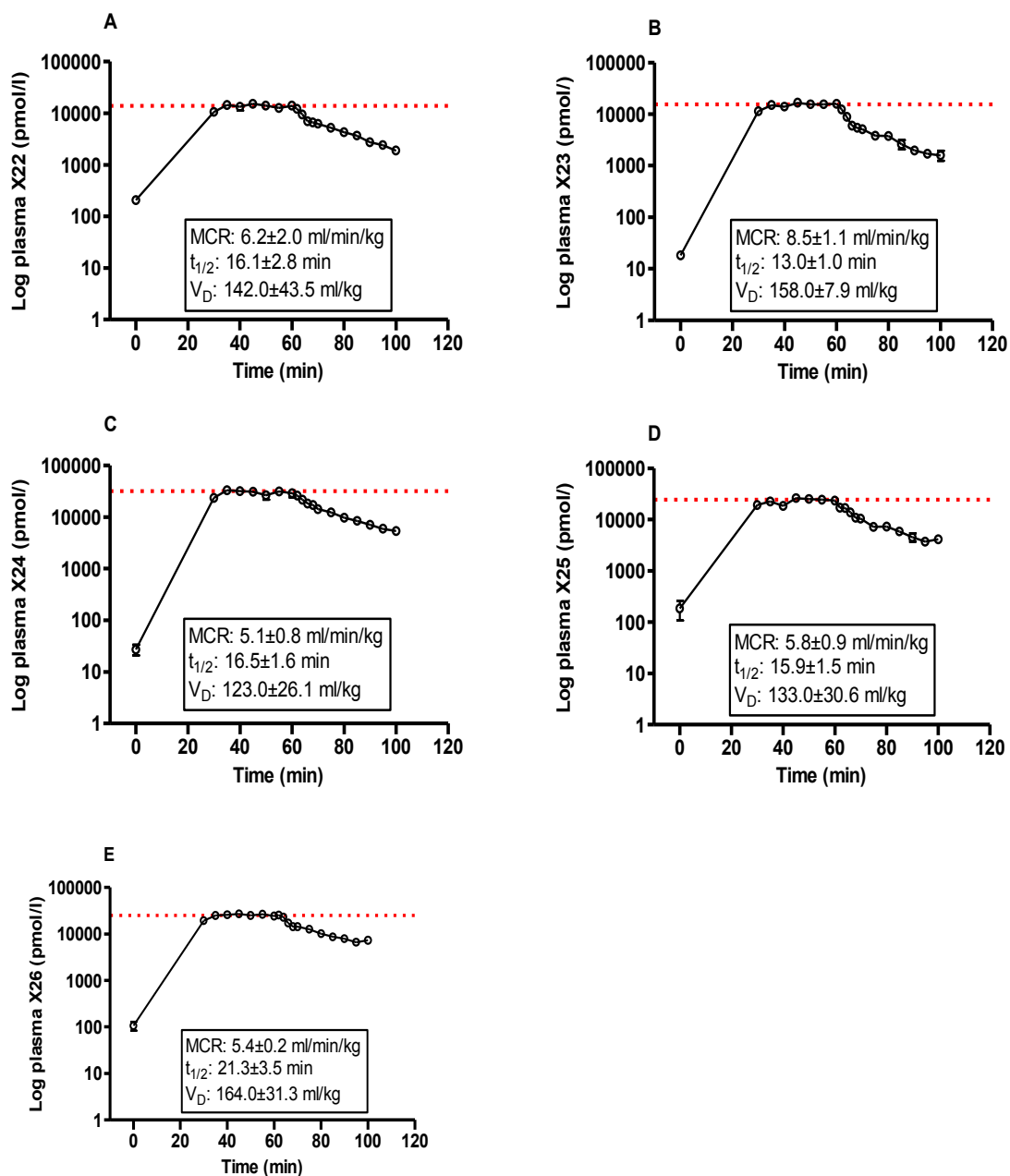


Figure 111: Plasma concentration of analogues X22-X26 during continuous IV infusion in anesthetised male Wistar rats for 60 minutes (n=4). A: X22, B: X23, C: X24, D: X25 and E: X26. Infusion begins at “0” time-point and lasts until “60” minute at which point the infusion is stopped. Values are shown as mean. MCR: Metabolic clearance rate, $t_{1/2}$: Half-life, V_D : Volume of distribution. The red dotted line indicates the steady-state level achieved during the infusion phase.

6.3.6. Investigation of the pharmacokinetic properties of oxyntomodulin analogues X22 to X26 following single subcutaneous bolus injection in male Wistar rats

The PK profiles of OXM analogues X22 to X26, when administered as a 1 mg 1:1 molar ratio Zn:NaCl (saline) SC injection to male Wistar rats, were investigated (Figure 112). The T_{max} was 24 hours post-injection for all analogues. Plasma levels of analogues X22 to X26 at 30 minutes post-injection were 36%, 54%, 58%, 66% and 62% of C_{max} respectively, at 3 hours post-injection were 79%, 99%, 89%, 95% and 88% of C_{max} respectively and continued to rise until 24 hours post-injection. The 48 hour plasma levels of analogue X22 were 37% of C_{max} and had dropped further to 7% of C_{max} by 96 hours post-injection. Plasma levels of analogues X23 to X26 were between 87%-95% of C_{max} at 48 hours post-injection and had dropped further to 23%, 42%, 72%, and 60% of C_{max} respectively by 96 hours post-injection. Analogues X22 and X23 were undetectable in plasma at 7 days (LLOQ was 156 ± 28 , 197.0 ± 35.0 pmol/L respectively). Analogues X24, X25 and X26 were still detectable in plasma up to 7 days with 14%, 43% and 63% of the C_{max} remaining respectively (LLOQ was 187 ± 55 , 243 ± 71 and 161 ± 62 pmol/L respectively).

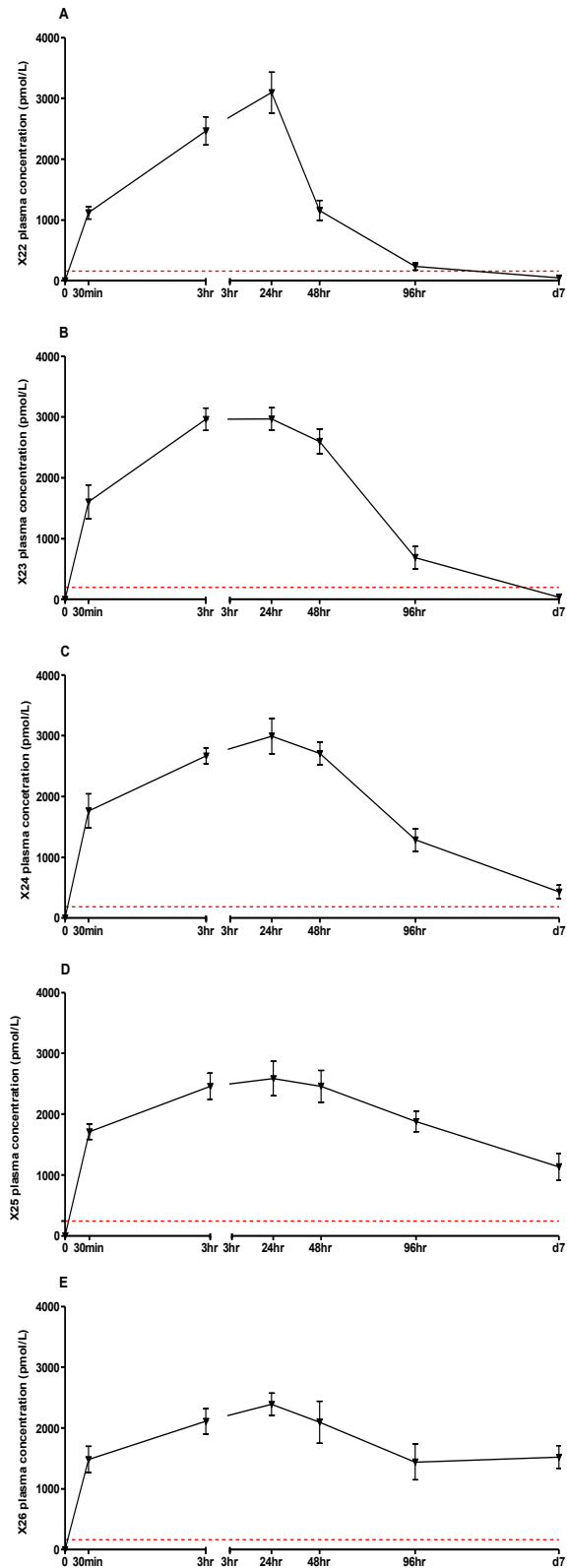


Figure 112: Line graphs illustrating the pharmacokinetic profile of A: X22 B: X23, C: X24, D: X25 and E: X26 after a single 1 mg SC injection of peptide (formulated in 1:1 Zn:NaCl slow release diluent). Peptide concentration measured in rat plasma using RIA and expressed as mean concentration $\pm$ SEM pmol/L (n=4). The lower limits of quantification (LLOQ) for each peptide in the RIA are shown as horizontal dashed lines. Interpolated values which fall below this level are still plotted.

6.3.7. The chronic effect of exendin-4 and oxyntomodulin analogues X22 to X26 on food intake and body weight in C57BL/6J mice

The effect of OXM analogues on food intake was investigated in a 6 day chronic study in mice. Analogues were dissolved in ZnCl₂ solution prepared in saline at a zinc to peptide ratio of 1:1. Subcutaneous administration of EX-4 (10 nmol/kg) and 5 OXM analogues (X22 to X26) at 50 nmol/kg to fasted DIO C57BL/6J mice, demonstrated that all treatment groups significantly reduced their food intake over a period of 6 days compared with the vehicle control group ($p<0.001$ for EX-4, X24 and X26; $p<0.01$ for X22, X23 and X25) (Figure 113).

When the anorectic effect of OXM analogues was compared to EX-4, it was demonstrated that X24 and X26 did not have a statistically significant different effect. Analogues X22, X23 and X25 presented a greater food intake than EX-4 over 6 days. Comparisons between groups demonstrated that analogue X26 had a significantly greater anorectic effect than analogues X22, X23, X24 and X25. The mean food intake of EX-4 group over 6 days was 13.60 ± 0.90 g and of X26 group was 12.60 ± 0.70 g.

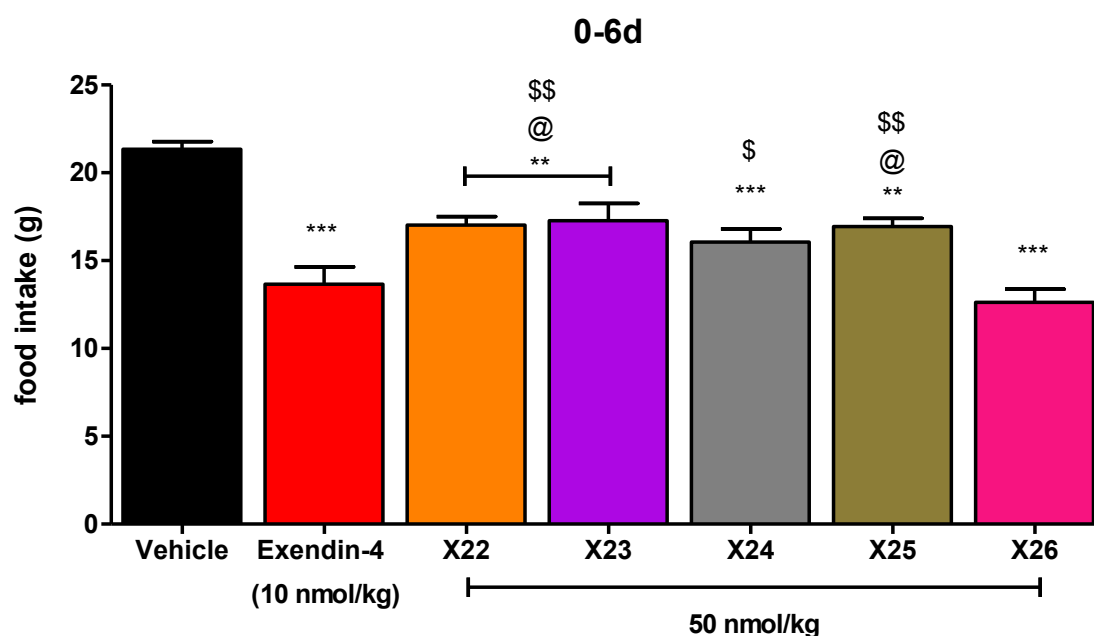


Figure 113: Chronic 6 day effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-6 days. $n=5-6$ mice per group. Statistics: One way ANOVA with Tukey's post-hoc test. **= $p<0.01$, ***= $p<0.001$ vs. vehicle; @= $p<0.05$ vs. EX-4; \$= $p<0.05$, \$\$= $p<0.01$ vs. X26.

Administration of EX-4 and analogues X22-X26 reduced body weight at 6 days when compared to the vehicle control group, however only mice treated with X26 demonstrated a statistically significant effect ($p<0.01$) (Figure 114). The mean body weight loss of mice treated with X26 over 6 days was 2.48 ± 0.80 g.

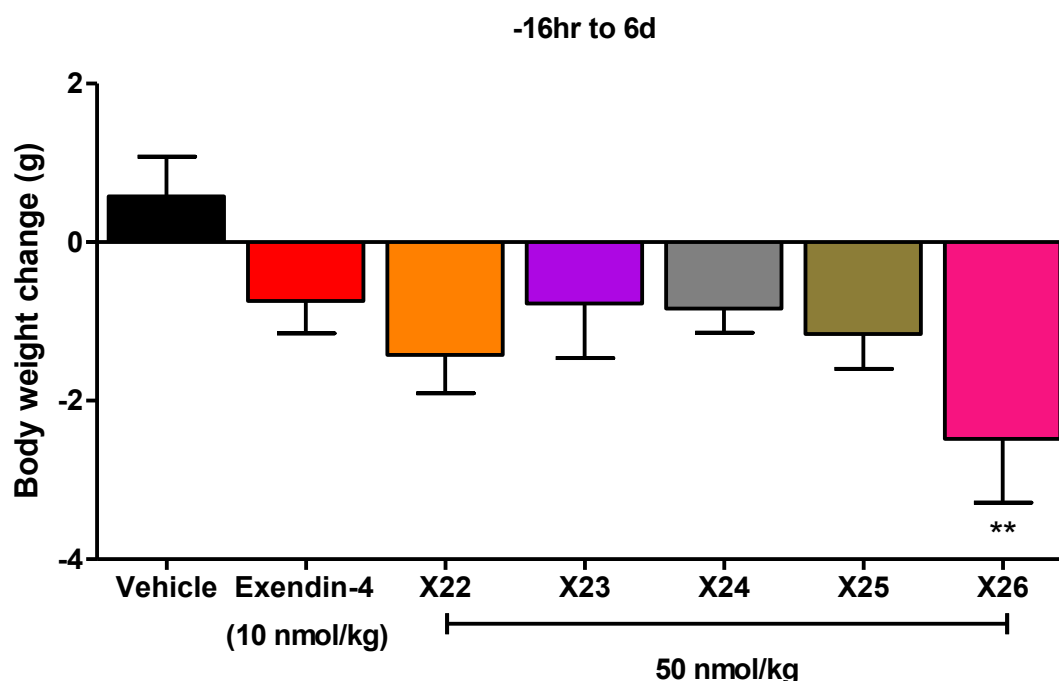


Figure 114: Chronic 6 day effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on body weight loss in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 6 days. $n=5-6$ mice per group Statistics: One way ANOVA with Tukey's post-hoc test. **= $p<0.01$ vs. vehicle.

Analysis of cumulative food intake data showed that both EX-4 (10 nmol/kg) and all OXM analogues (50 nmol/kg), all significantly reduced food intake 0-2 hours post-injection compared with the vehicle control group ($p<0.001$) (Figure 115).

Cumulative food intake at 0-8 and 0-24 hours post-injection was significantly reduced in EX-4 and OXM groups X22, X24 and X26 at a level of significance of $p<0.001$. Analogues X23 and X25 caused a significant food intake inhibition compared to vehicle control group ($p<0.05$ and $p<0.01$ respectively) in the 0-8 hours period and in the 0-24 hours period ($p<0.01$ for both analogues).

In the 24-48 hour time period, only EX-4 and analogues X22 and X26 significantly reduced food intake compared to vehicle control group ($p<0.05$, $p<0.05$ and $p<0.01$ respectively).

Exendin-4 and analogues X22 and X26 caused the higher significant food intake inhibition in the 0-48 hours period ($p<0.001$). Analogue X24 also reached a significant food intake inhibition albeit lower than X26 ($p<0.01$). X23 group consumed significantly more food than X26 group.

Mice treated with analogues EX-4, X25 and X26 had a significantly greater food intake inhibition when compared to vehicle control group in the 48 hours-6 days' time period ($p<0.001$, $p<0.05$ and $p<0.001$ respectively).

The EX-4 and the OXM groups X22-X26 did not present statistically significant differences in the inhibition of food intake when they were compared between them in the time periods 0-2 hours, 0-8 hours, 0-24 hours, 24-48 hours, 0-48 hours and 48 hours-6 days.

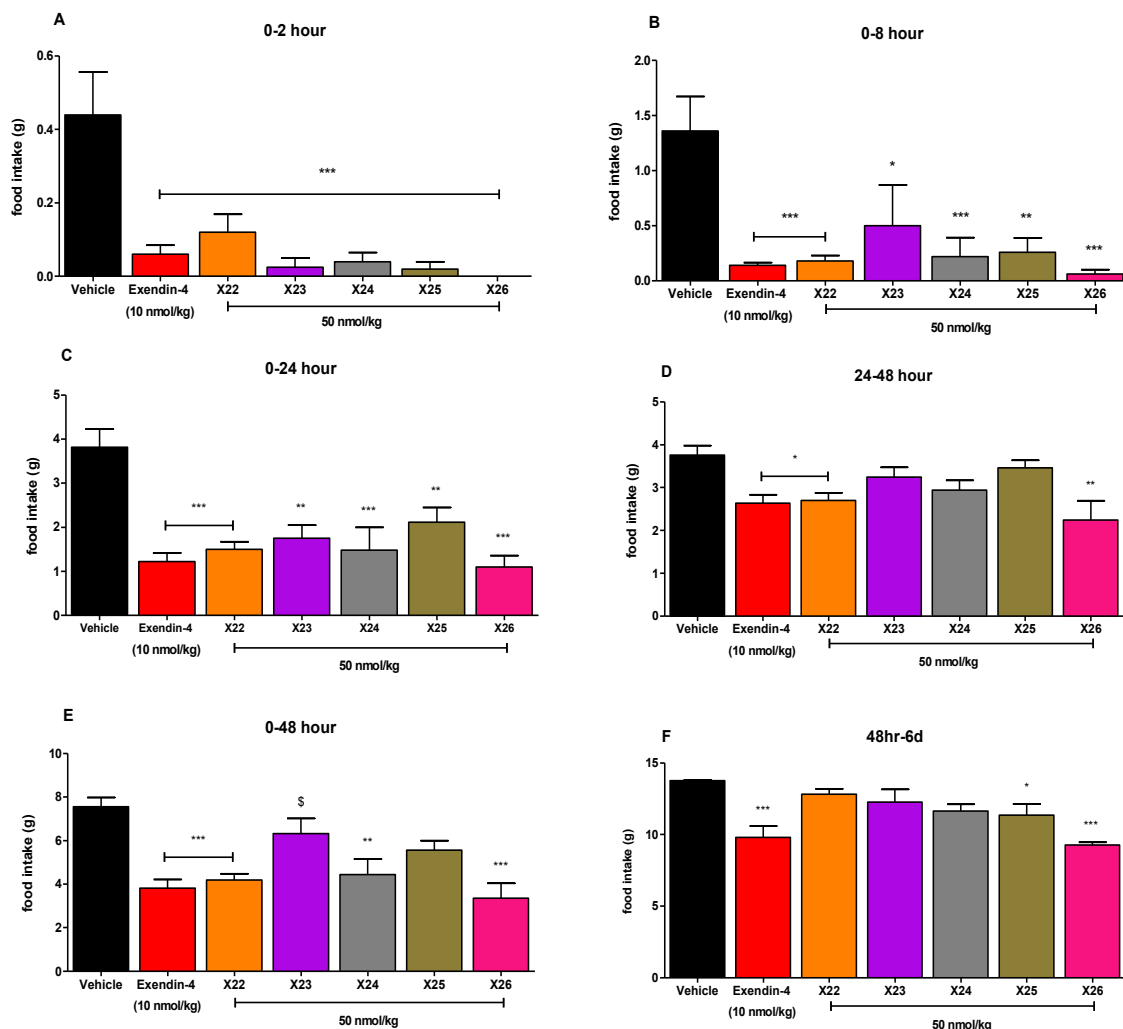


Figure 115: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on cumulative food intake in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for A: 0-2 hours, B: 0-8 hours, C: 0-24 hours, D: 24-48 hours, E: 0-48 hours and F: 48 hours-6 days. $n=7-8$ mice per group. Statistics: One way ANOVA with Tukey's post-hoc test $*=p<0.05$, $**=p<0.01$, $***=p<0.001$ vs. vehicle; E: $\$=p<0.05$ vs. X26.

Administration of X22 and X26 significantly reduced body weight at 48 hours when compared to the vehicle control group ($p<0.05$ and $p<0.001$ respectively) (Figure 116). The mean body weight loss of X22 group and X26 group over 48 hours was 2.40 ± 0.40 g and 3.40 ± 0.70 g respectively.

In the 48 hour to 6 days period, there was no statistical significant difference in the body weight change between the treatment groups and the vehicle control group.

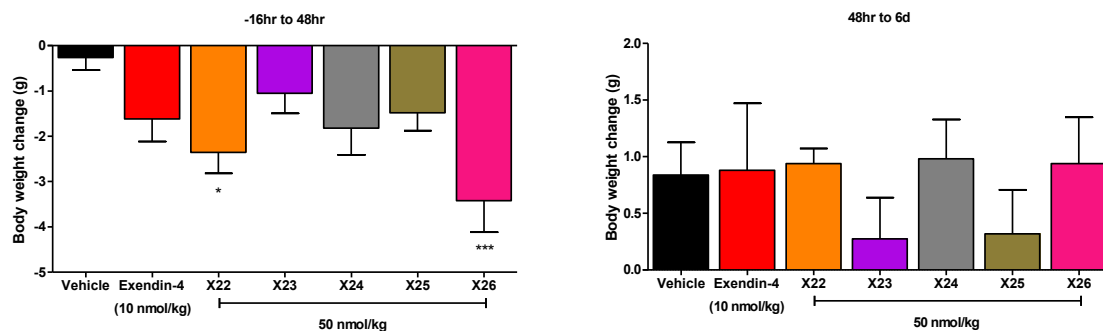


Figure 116: Chronic 6 day effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on body weight loss in overnight fasted DIO C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 6 days. $n=5-6$ mice per group. Statistics: One way ANOVA with Tukey's post-hoc test. $*=p<0.05$, $***=p<0.001$ vs. vehicle.

6.3.8. The chronic effect of exendin-4 and oxyntomodulin analogues X22 to X26 on food intake and body weight in Wistar rats

The effect of OXM analogues on food intake was investigated in a 6 day chronic study in rats. Analogues were dissolved in ZnCl_2 prepared in a saline at zinc to peptide ratio of 1:1. Subcutaneous administration of EX-4 (10 nmol/kg) and 5 OXM analogues (X22 to X26) at 50 nmol/kg to fasted Wistar rats showed that none of the treatment groups reduced food intake significantly over a period of 6 days compared with the vehicle control group (Figure 117). When the anorectic effect of OXM analogues was compared to EX-4, it was demonstrated that the OXM analogues did not have a statistically significant different effect.

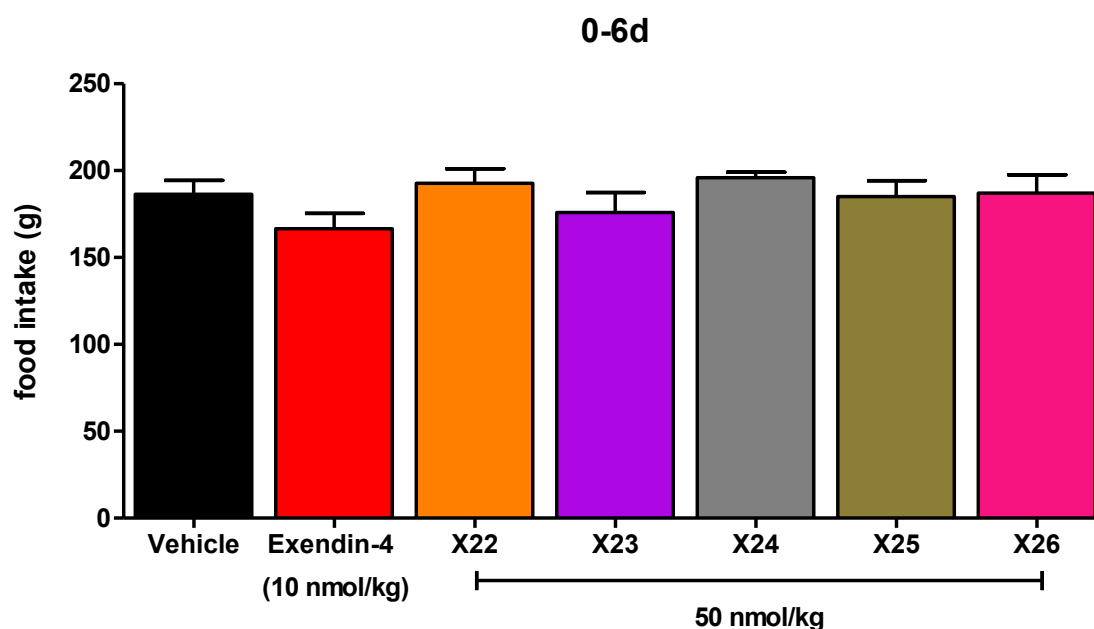


Figure 117: Chronic 6 day effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on cumulative food intake in overnight fasted Wistar rats. Data shown as mean $\pm$ SEM food intake in rats for 0-6 days. n=5-6 rats per group Statistics: One way ANOVA with Tukey's post-hoc test.

Analysis of body weight change over 6 days demonstrated that administration of analogues X22 and X26 significantly reduced body weight at 6 days when compared to the vehicle control group ($p < 0.01$ for both analogues) (Figure 118). Animals treated with analogue X23 also presented a significant reduction of their body weight in comparison to the vehicle control group ($p < 0.05$).

The mean body weight loss of rats treated with X22 and X26 over 6 days was 2.75 ± 1.25 g and 4.0 ± 1.50 g respectively. Animals treated with analogue X23 increased their body weight only by 1.50 ± 4.70 g while the vehicle control group gained 13.20 ± 4.60 g over the 6 days period.

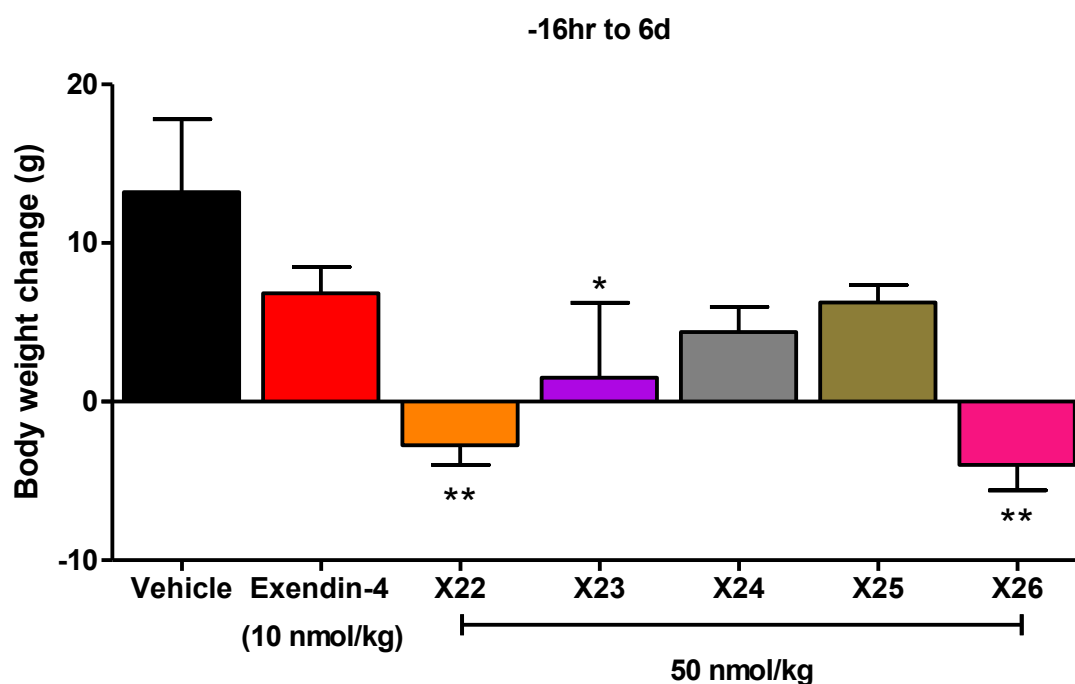


Figure 118: Chronic 6 day effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on body weight loss in overnight fasted Wistar rats. Data shown as mean $\pm$ SEM food intake in rats for 6 days. $n=5-6$ rats per group. Statistics: One way ANOVA with Tukey's post-hoc test. $*=p<0.05$, $**=p<0.01$ vs. vehicle.

Analysis of cumulative food intake data showed that only EX-4 (10 nmol/kg) significantly reduced food intake 0-2 hours post-injection compared with the vehicle control group ($p<0.001$) (Figure 119). Groups X23, X24, X25 and X26 consumed significantly more food than EX-4.

Cumulative food intake at 0-8 hours post-injection was significantly reduced in EX-4 and X26 treated groups ($p<0.001$ and $p<0.05$ respectively). Groups X24 and X25 consumed significantly more food than EX-4 ($p<0.01$ and $p<0.05$ respectively).

In the 0-24 hour time period, only EX-4 and analogues X22, X24 and X25 significantly reduced food intake compared to vehicle control group ($p<0.01$, $p<0.05$, $p<0.05$ and $p<0.01$ respectively). There were no significant differences in the food intake between groups and EX-4.

Exendin-4 was the only agent that caused a significant food intake inhibition in the 0-48 hours period ($p<0.05$). The EX-4 and the OXM X22-X26 groups did not present statistically significant differences when compared to the vehicle control group in the inhibition of food intake in the 24-48 hours' time period. In the 48 hours-6 days' time period group X22 consumed significantly more food than EX-4 group.

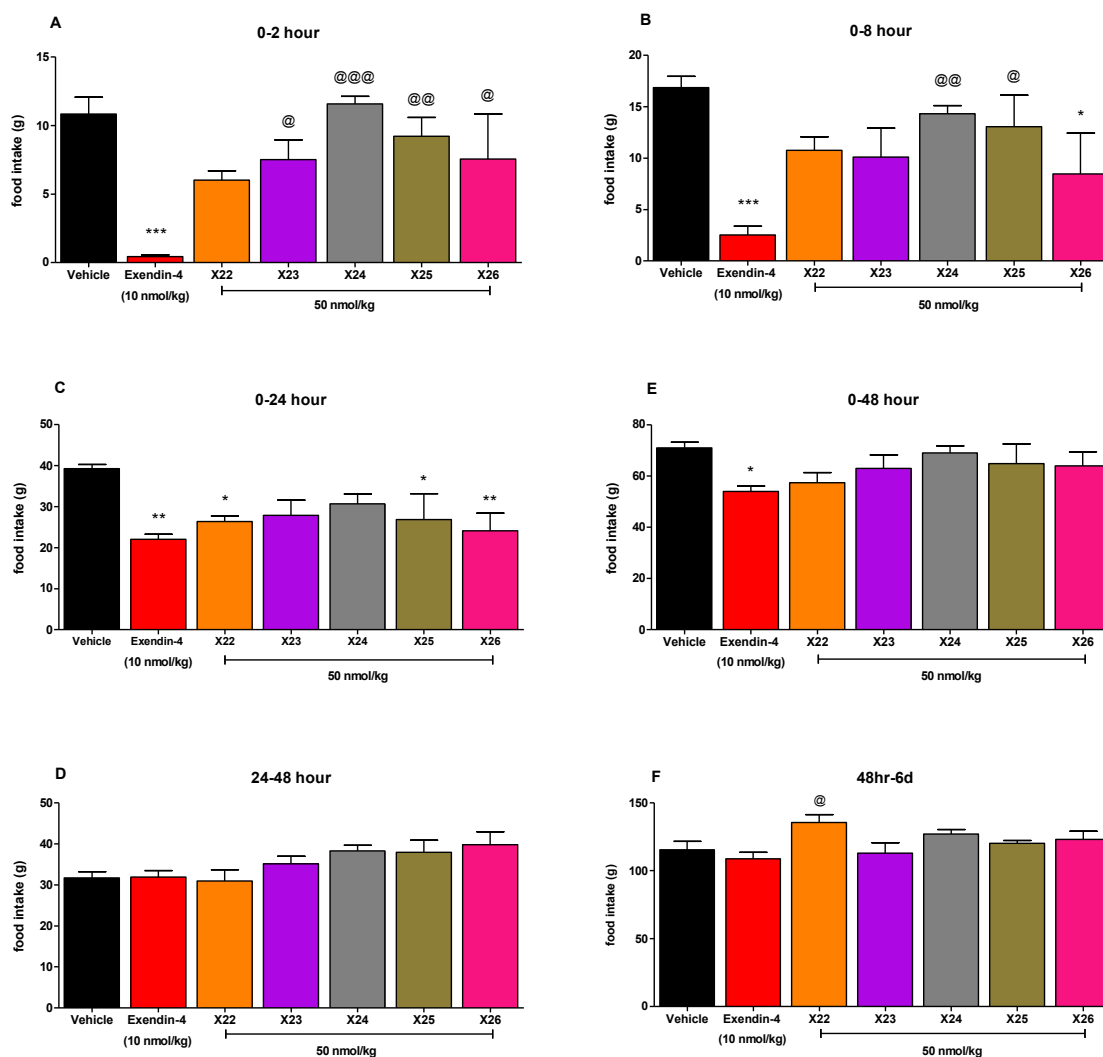


Figure 119: Effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on cumulative food intake in overnight fasted Wistar rats. Data shown as mean $\pm$ SEM food intake in rats for A: 0-2 hours, B: 0-8 hours, C: 0-24 hours, D: 24-48 hours, E: 0-48 hours and F: 48 hours-6 days. n=7-8 rats per group. Statistics: One way ANOVA with Tukey's post-hoc test *= $p<0.05$, **= $p<0.01$, *= $p<0.001$ vs. vehicle; @= $p<0.05$, @@= $p<0.01$, @@@= $p<0.001$ vs. EX-4.**

Administration of X22 and X26 significantly reduced body weight at 48 hours when compared to the vehicle control group ($p<0.01$ for both analogues) (Figure 120). The mean body weight loss of X22 and X26 treated groups over 48 hours was 8.25 ± 0.80 g and 7.25 ± 1.60 g respectively.

In the 48 hour to 6 days period, there was no statistical significant difference in the body weight change between the treatment groups and the vehicle control group.

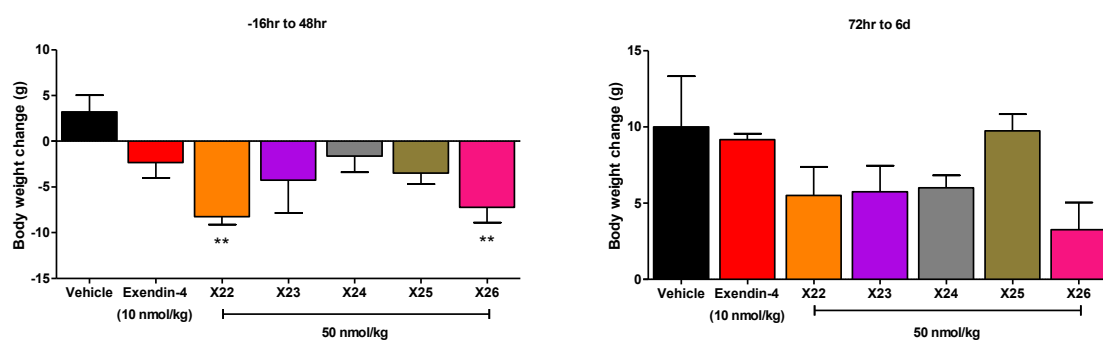


Figure 120: Chronic 6 day effect of a single SC injection of vehicle (saline), EX-4 (10 nmol/kg) and OXM analogues (X22-X26) (50 nmol/kg), on body weight loss in overnight fasted Wistar rats. Data shown as mean $\pm$ SEM food intake in rats for 6 days. n=5-6 rats per group. Statistics: One way ANOVA with Tukey's post-hoc test. **= $p < 0.01$ vs. vehicle.

6.3.9. Glucose tolerance test study in mice acutely treated with oxyntomodulin analogue X26

In order to assess the effect of analogue X26 on glucose levels, a glucose tolerance test was performed in DIO C57BL/6J mice that have been treated with a SC injection of saline, EX-4 (20 nmol/kg) or OXM analogue X26 (100 nmol/kg) as described in section 6.2.8 (Figure 121). Analogue X26 reduced the plasma glucose spike following administration of IP glucose (2 g/kg), at both 30 and 90 minutes post glucose IP administration mice had significantly lower blood glucose levels than vehicle controls ($p<0.001$ for both time-points). Exendin-4 has also demonstrated a significantly lower glucose levels in blood at 30 and 90 min post glucose IP injection, compared to the vehicle control group ($p<0.001$ and $p<0.01$ for both time-points).

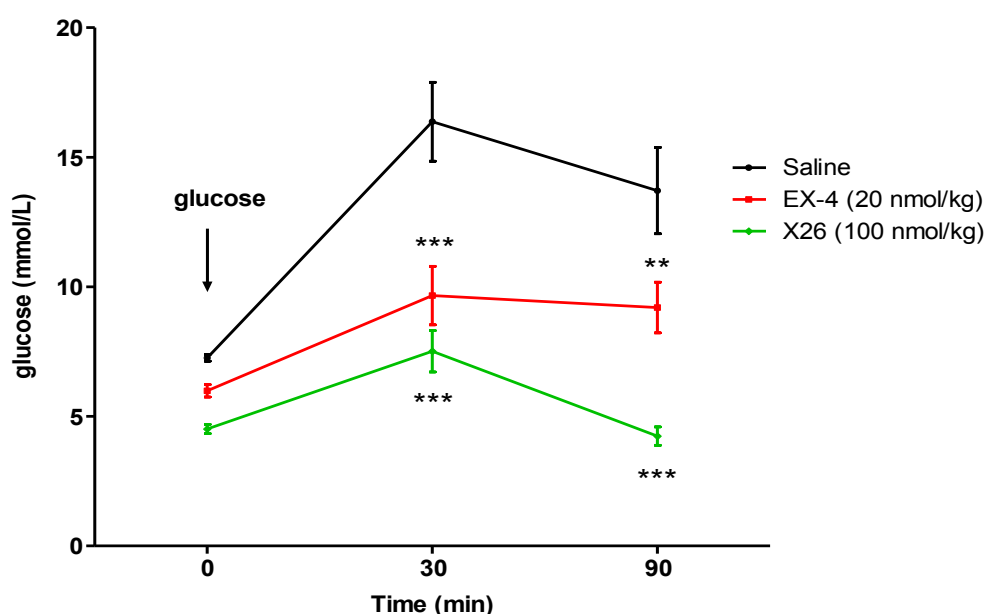


Figure 121: Glucose measurement (mmol/L) of tail tip samples of blood at times 0, 30 and 90 after 2g/kg glucose IP injection at time 0 in DIO C57BL/6J mice that have been treated with a subcutaneous injection of saline, EX-4 (20 nmol/kg) or OXM analogue X26 (100 nmol/kg). Statistics: 2-way ANOVA of 30 and 90 minute time points with Bonferroni correction, all groups compared with vehicle. **= $p<0.01$, ***= $p<0.001$.

6.4. Discussion

The studies described in this chapter tested 5 OXM analogues, X22-X26, that were designed based on the obtained results from the previous chapters of this thesis. Analogues, X22-X26, have point substitutions that were aimed at improving GLP-1R affinity and potency while at the same time improving the PK properties of the molecules. The aim was to identify the analogue that presented the higher bioactivity in a feeding study in rodents; that caused the higher body weight loss in comparison to the other treatment groups.

In this chapter there were two new studies that were introduced in comparison to the previous chapters. Firstly, a chronic feeding study in rats was performed in order to investigate the bioefficacy of the OXM analogues in more than one species and correlate the subcutaneous PK findings (performed in rats and given in a zinc-formulation) with the *in vivo* effects in rats and when given also in a zinc-formulation. Secondly, an IP glucose tolerance test was performed in mice with analogue X26 in order to investigate the effect of the analogue on the glucose plasma levels.

The OXM analogues used in this chapter were based on 2 OXM analogues developed previously in chapter 5, analogues X15 and X21. The reason for selecting these 2 analogues as a starting point was based on the results obtained in the PK studies. These analogues have proven to have a long half-life when compared with the other analogues (half-life of 15.35 and 14.4 min respectively for X15 and X21 respectively). Furthermore, when these peptides were administered SC in rodents in a solution containing zinc, they were able to show significant plasma levels at 24 and 48 hours. Analogue X21 also demonstrated significant weight loss in comparison to the vehicle control group when injected SC in a feeding study in mice in a solution containing zinc.

The point substitutions that were used in this chapter included position 24 and the C-terminal. As it was previously discussed, Gln²⁴ was substituted by Glu²⁴ in an effort to increase GLP-1R affinity and potency. Position 29 of the native OXM molecule is occupied by Thr while in the EX-4 molecule this position is occupied by Gly. Receptor binding studies in the previous chapter demonstrated that Gly²⁹ may offer a higher affinity and potency at the GLP-1R but can compromise the PK profile of the peptide. Thus in this chapter analogues X23-X26 retained the native Thr²⁹. Analogue X22 is the only analogue in this chapter with a Gly²⁹ and was designed in order to investigate the effect of Gly²⁹-Gly³⁰ and to allow direct comparisons with X15 (they differ only in position 24).

The C-terminal changes that were implemented aimed to increase the half-life of the peptide and prolong its presence in the plasma. Analogues X22-X26 retained a poly His-tail that has previously been shown to offer improved PK profile. Furthermore, all OXM analogues tested in this chapter used

a Glu-NH₂ extension after the terminal His molecule as it has been demonstrated in the previous chapter that it further improves the PK profile of the molecule by increasing its half-life. With the introduction of Thr²⁹, the flexibility of the C-terminal chain was decreased and in order to account for this loss of flexibility, the position 30 of analogue X26 has been switched from His to Gly.

In order to investigate the effect of introducing Glu²⁴ in the primary structure of my analogues, I developed analogues X22 and X23 which are based on X15 and X21 respectively with the sole difference being the introduction of Glu²⁴ in the 3rd generation analogues. The receptor binding assays performed in this chapter demonstrated that in comparison to X15, analogue X22 is able to bind to the hGLP-1R with a 4-fold higher affinity approximately while binding to the mGLP-1R was not improved. This improvement in the binding of the GLP-1R by X22, did not however translate to an increased potency to the GLP-1R at the cAMP accumulation assays compared to X15. Hence the introduction of Glu²⁴ seems to play a negative role in GLP-1R activation in the presence of Gly²⁹-Gly³⁰.

The beneficial effect of Glu²⁴ in GLP-1R affinity and potency was more obvious when it was introduced in analogue X21. Analogue X23 has the same primary structure as X21 with the exception of Glu²⁴. The binding affinity of X23 for the GLP-1R was approximately 2-fold higher for the mouse receptor and 3-fold higher for the human receptor, when compared to X21. The potency at the GLP-1R cAMP assay of X23 was 1.8 times higher than X21. The *in vivo* effect of analogue X23 was investigated in an acute feeding study in mice. Food intake inhibition and body weight loss exhibited by X23 was similar to the results exhibited by analogue X21 with an average body weight loss of 1.3 g at 48 hours for X23.

The design of the primary structure of X24 was aimed at investigating the influence of the Glu-NH₂ tail when it is introduced after the terminal His of the C-terminal of analogue X23. Analogue X24 did not exhibit significant differences when compared to X23, in its ability to bind and activate the GLP-1R or the GCGR. This observation fits well with the hypothesis made when designing the analogue; the addition of Glu-NH₂ should not influence these characteristics as it lies far away from the region that interacts with the receptor.

The PK properties of analogue X24 were improved in comparison to X23 albeit in a non-significant fashion. The half-life of analogues X23 and X24 was 13.0±1.0 and 16.5±1.6 minutes respectively. The MCR of analogue X24 was lower than X23 (5.1±0.8 vs. 8.5±1.1 ml/min/kg respectively). When administered in zinc solution analogues X23 and X24 demonstrated a similar behaviour until the 96-hour time point where the plasma levels were at 23% and 42% of their respective C_{max} levels respectively.

The slight improvement in the PK properties of analogue X24 in comparison to X23 did not translate in a significant improvement of its bioactivity both in an acute and in a chronic feeding study in mice. Both analogues had a significant reduction in food intake and body weight when compared to the vehicle control group but no significant difference was observed between them. The same similarity in the observed bioactivity was true when these analogues were tested in a chronic feeding study in rats.

Analogues' X25 design is based on the primary structure of X24 with the introduction of an extra His at the poly His-tail in order to reach the same number of His amino acids as X22. The PK properties of analogue X25 and X24 did not differ significantly when they were tested in the constant infusion rat model where they were given IV without zinc. However, when they were administered in a zinc containing formula, analogue X25 demonstrated an improvement of its plasma concentration profile. More specifically, X25 exhibited a slower release from the SC depot with a slower rate of fall in its plasma concentration after the 24-hour C_{max} level. Plasma concentration of analogue X25 at 96 hours post administration was 72% of its C_{max} level with a significant level of the peptide being present even at 7 days post-administration.

In agreement with the PK characteristics, analogues X24 and X25 demonstrated a similar food intake inhibition and body weight reduction when compared to the vehicle control group in an acute feeding study in mice where they were injected without zinc. In a chronic feeding study in mice, injected in a zinc containing formula, analogue X25 demonstrated a higher reduction of body weight in the period of 48h-6d. This reduction however was admittedly non-significant and the overall body weight reduction and food intake inhibition of X24 and X25 at 6 days did not differ.

The substitution of His³⁰ of analogue X25 for Gly³⁰ resulted in the development of analogue X26. The hypothesis for this His³⁰ to Gly³⁰ substitution was that as Gly is smaller and more flexible than His, it would offer an increased range of motion of the C-terminal poly His tail, thus enhancing its ability to bind to the zinc molecules found in the zinc-formulation. Analogue X26 presented an improved PK profile in comparison to X25 both in the constant infusion rat model and the SC PK. The half-life of X26 was 21.3±3.5 min in comparison to the 15.9±1.5 min of X25. The MCR of analogues X25 and X26 was similar (5.8±0.9 vs. 5.4±0.2 ml/min/kg respectively) while X26 exhibited a non-significant increase in its V_D (133±30.6 vs. 164±31.3 ml/kg respectively). A more significant difference in the PK characteristics of X25 and X26 was observed in the SC PK study. Analogue X26 demonstrated a plasma concentration of approximately 2000 pmol/L at day 7 post-injection corresponding at 63% of its C_{max} level while analogue X25 had a plasma level of 43% of its C_{max} level.

Analogue X26 demonstrated a significant difference in its bioactivity in comparison to the rest of the analogues tested in this chapter. When tested in an acute feeding study in mice, X26 was shown to significantly inhibit food intake for 48 hours when compared to the vehicle control group. Furthermore X26 was shown to cause a significant reduction of body weight when compared to the vehicle control group (average body weight reduction of X26: 2.12 ± 0.4 g). Although in this study the food intake inhibition and body weight reduction effects of X26 were not significantly different from the rest of the analogues, it did demonstrate a consistently higher bioactivity.

The difference in the bioactivity of X26 and the rest of the analogues was proven in the chronic feeding study performed in mice. Analogue X26 demonstrated a significantly greater anorectic effect both against the control group and analogues X22-X26. Furthermore, the body weight reduction at 6 days exhibited by X26 was 2.48 ± 0.8 g; higher than any other analogue tested in a similar study in this thesis. Analogues X25 and X26 were the only ones that were shown to be able to significantly inhibit food intake in the 48hr-6d period of this study.

In order to confirm that the bioactivity of the OXM analogues tested in this thesis is not species specific, a chronic feeding study of the analogues X22-X26 was performed in rats. In this study there was no difference in the 6-day food intake between OXM analogues and the control group while, of note, is that both X22 and X26 were able to significantly reduce body weight at 6 days (mean body weight loss for X22 and X26: 2.75 ± 1.25 g and 4 ± 1.5 g respectively). With this study analogue X26 has confirmed its anorectic effect and the ability to cause a body weight reduction in multiple species (rats and mice). Taking into account the *in vitro* results on the cAMP accumulation assays in human cell lines, analogue X26 is predicted to be able to exert its anorectic effect also in humans.

The effects of GLP-1R agonists such as the OXM analogues developed in this thesis on food intake inhibition and weight loss add an extra benefit for these drug candidates as they could offer a small improvement on the control of T2DM. There are several existing therapies targeting the GLP-1R currently on the market. These include GLP-1R agonists based on GLP-1 and EX-4 (Liraglutide, Exenatide and Bydureon) as well as inhibitors of DPP4 (Sitagliptin, Vildagliptin, Saxagliptin and Linagliptin) which work to increase levels of active endogenous GLP-1 post-prandially.

However, GCGR agonists have an opposing effect to GLP-1 with regard to glucose homeostasis. With the correct ratio between GLP-1R and GCGR actions, glucose homeostasis could remain under control. The endogenous GLP-1/GCG dual agonist OXM has been shown to have beneficial effects on body weight through actions at the GCGR while simultaneously activating GLP-1R to stimulate insulin production and treat diabetes (271).

For the above-mentioned reasons, when designing a dual GLP-1/GCG dual agonist, it is necessary to evaluate the effect of the drug in glucose homeostasis. Analogue X26 designed in this chapter has consistently shown to be able to inhibit food intake and reduce body weight in a statistical significant level both in mice and rats. For that reason it was selected to be tested in a study aimed to investigate its effect on glucose homeostasis. The glucose tolerance test was performed in DIO mice that received a SC injection of analogue X26 at a dose of 100 nmol/kg. The mice treated with X26 sustained significantly lower glucose levels in blood at 30 min post glucose IP injection, compared to the vehicle control group, and maintained the levels stable until 90 min, thus presenting an acceptable blood glucose profile. These IPGTT results show that X26 could be a safe anti-obesity therapeutic agent, from glucose homeostasis point of view.

All the studies performed in this chapter aimed at selecting the drug candidate with the highest bioactivity which is the result of the PK/PD properties of the compound. Although analogue X26 may not present the highest receptor binding affinity or potency at the GLP-1R/GCGR in comparison to the other analogues, it has demonstrated a better PK profile with a longer half-life and low MCR. As a result it is able to stay for longer in the circulation and exert its biological effect.

6.5. Conclusions

Investigations in this thesis provide interesting observations on how the PK/PD relationship can influence the bioactivity of peptides. Data presented in this chapter have identified OXM analogue X26 as a novel and valuable pharmaceutical approach for the treatment of obesity. Analogue X26 presents an 8.3– and 45-fold higher potency approximately at the GLP-1R and GCGR respectively when compared to native OXM. When administered in a zinc containing formula, X26 is present in the circulation 7 days post-injection. Furthermore, it has demonstrated a significantly longer half-life than OXM (21.3 vs. 12 min). Its ability to inhibit food intake and reduce body weight has been shown in two rodent species; mice and rats.

Chapter 7

General discussion

Obesity is one of the most important health problems of the 21st century. The rapid increase of its prevalence is mainly due to a combination of factors; genetic predisposition, sedentary way of life and high availability of high calorie food. Unfortunately, available treatments such as lifestyle interventions are either unsuccessful or difficult to maintain and surgery is associated with morbidity and mortality and is not an option for all patients. This fact highlights the need for a pharmacological therapy for obesity.

The signals to the brain regulating body weight and energy balance originate in multiple sources including the gut. The importance of the gut hormones in the regulation of the body weight is especially evident in the post-bariatric surgery changes that have been documented. The higher plasma concentrations of specific gut hormones such as GLP-1 and OXM have been associated with the significant and sustained weight loss observed after bariatric procedures such as RYGB. Studies in humans have demonstrated that it is possible to replicate the weight loss effects by peripherally administering gut hormones such as OXM and GLP-1.

Gut released peptides play an important role in energy homeostasis and thus represent a target based on which an anti-obesity medication can be developed. The sequences of native gut hormones have been discovered many decades ago but unfortunately native gut hormones cannot be used as drug treatments due to their inadequate PK profile. In specific, GLP-1, GCG and OXM have a half-life of only 4, 2 and 12 minutes approximately. Administering native gut hormones would require a dosing regimen that would make the use of the drug impractical.

By improving the PK profile of native gut hormones new therapeutic agents have been developed. Exendin-4 is an artificially synthesised version of GLP-1 offering a 20-fold increase in the half-life of GLP-1 and is currently in the market as a treatment of diabetes mellitus. Another example of GLP-1 agonist in the market is Liraglutide that is a long acting GLP-1 agonist. Unfortunately, Liraglutide usage is limited by the occurrence of nausea that is dose-dependent, thus hindering the use of the drug to its full potentials. Glucagon, by itself, is not a suitable anti-obesity therapeutic agent as it would cause significant hyperglycemia.

On the other hand, OXM is a native gut hormone that is active in both the GLP-1R and GCGR, albeit with less potency than native ligands. These dual agonism effects of OXM have been shown to cause a body weight loss that is caused by 2 different sources; food intake inhibition and increased EE. Such a mechanism of action increases the potential for body weight loss without relying exclusively on food intake inhibition. Studies of GLP-1/GCG drug candidates have confirmed that dual agonism can produce a higher body weight loss (354).

The goal of this thesis was to develop a potent anti-obesity agent based on native OXM that would be able to be administered with an acceptable dosage regimen such as once weekly. Various substitutions (Glu¹⁶, Ile²³ and Glu²⁴) were made mainly at the mid-molecule and C-terminal of OXM in order to be able to develop an OXM agonist that would have a higher potency at the GLP-1R. Position 21 (Glu²¹) offered a higher potency at the GCGR. In order to investigate the effect of point substitutions of the primary structure of the analogues on receptor affinity and binding, receptor binding assays and cAMP accumulation assays to both the GCGR and GLP-1R were performed. Through these novel modifications my analogue X26, developed in chapter 6, was shown to be 8.3- and 45-fold more potent approximately at the GLP-1R and GCGR respectively when compared to native OXM. To further investigate what was the *in vivo* effect of the developed analogues, acute feeding studies in mice were performed. In these studies analogues were given SC in a non-zinc formulation and X26 was shown over 48 hours to cause a significant weight loss of 2.12 g and food intake inhibition.

In order to be able to investigate the PK characteristics of my analogues I developed a novel *in vivo* rat model. This model uses a constant IV infusion of the peptide and the PK characteristics (half-life, MCR and the V_D) of the analogues can be measured. These data allow drawing accurate conclusions in regards to the effect of the point substitutions on its plasma concentration over time and the dosage regimen that would be required to maintain therapeutic plasma levels. The constant infusion rat model was validated by measuring the PK properties of 4 peptides, GCG, GLP-1, OXM and EX-4. The results obtained in the 2nd chapter were consistent with previous published literature. All 26 OXM analogues developed in chapters 4, 5 and 6 were tested for their *in vivo* PK properties and the PK results guided the design of new analogues from one generation to the other. The importance of the PK data is highlighted by the frequent discovery of potent analogues at the GLP-1R that failed to demonstrate a significant body weight loss in rodents due to their inadequate PK profile. Analogue X26 demonstrated a significantly longer half-life than OXM (21.3 vs. 12 min respectively) and a lower MCR (5.4 vs. 12.8 ml/min/kg respectively).

In order to achieve the goal for an anti-obesity drug that would be administered SC once weekly; there was a need to significantly improve the PK characteristics of the analogues. This goal was achieved by making changes in the C-terminal of the analogues (X26: poly-His tail and Glu³⁶-NH₂³⁷) and by using a zinc formulation that creates a SC depot and allows for a gradual release of the peptide into the bloodstream. The imidazole ring of histidine is able to form complexes with positively charged zinc and form complexes that stabilise the molecule and prolong its half-life. The benefit of such a strategy is two-fold; firstly there is a gradual rise in the plasma concentration of the peptide thus avoiding the burst effect which is associated with nausea and secondly there is a prolonged duration of action as the peptide is released constantly over many days. Such analogues require less frequent

administration, may have a prolonged bioavailability and have a therapeutically advantageous PK profile.

The PK/PD relationship of the peptides when given in a zinc formulation was investigated by measuring the PK profile of the analogues when given SC in a zinc formulation *in vivo* in rats and with a chronic 6 day feeding study both in rats and mice (given also SC in a zinc formulation). These studies were complementary; analogues with good PK profiles and showing high potency in the *in vitro* tests demonstrated a high bioactivity in the feeding studies. Analogue X26 was still detectable at plasma 7 days post-administration in rats when given SC in a zinc formulation. Its PK profile showed a gradual rise over 24 hours with a T_{max} at 24 hours and a subsequent slow decline with 63% of the drugs' C_{max} remaining in the circulation at 7 days.

To ascertain that the *in vivo* results of X2 were not species specific, I performed a 6-day feeding study both in rats and mice (given in a zinc formulation). Analogue X26 significantly inhibited food intake and reduced body weight over 6 days in mice (-2.48 ± 0.80 g). In the 6 day feeding study in rats analogue X26 significantly inhibited food intake only for 24 hours but was still able to produce a weight loss of 4.0 ± 1.50 g over 6 days.

These results together with the ones of the cAMP accumulation assays in human cell lines provide evidence that analogue X26 should be effective in demonstrating similar weight loss effects when given in humans. It is important to note that cell lines overexpressing a particular receptor are not always able to predict the *in vivo* biological effects due to inherent inadequacies of the cell lines. However, *in vitro* assays provide a valuable, fast and ethical tool that can be used to screen drug candidates. When lead analogues are finally selected they can be tested in human trials for definitive confirmation of their biological effects.

In order to transfer the discoveries of this thesis in an anti-obesity drug development program, a number of factors will need to be further explored. Drug formulation aiming to enter the market needs to fulfil certain characteristics in order to make the drug a safe and successful alternative to existing drugs. Such characteristics include an easy route of administration ex: oral, SC injection, a safe profile and a prolonged duration of action that maximises the dosing intervals. Analogue X26 in a zinc formulation could be given SC in humans once weekly if human trials confirmed similar plasma levels over 7 days. In human trials the safety of the drug formulation would be tested and the occurrence of side-effects recorded. An excessive amount of zinc can cause damage to the lysosomes and subsequent necrosis in the surrounding tissues. I expect that the small amount of zinc given in my formulation should not cause such issues in humans and it has been shown that His chains are not very immunogenic; posing a small risk of unwanted allergic reactions (355).

Dual GLP-1R/GCGR agonists present more safety concerns that will need to be elucidated. The action solely on the GCGR can aggravate hyperglycaemia; however analogue X26 was not shown to cause hyperglycaemia in an IPGTT in mice thanks to its concomitant action on the GLP-1R. Furthermore, the improvement of the potency at the hGLP-1R of X26 was much more significant than the one at the hGCGR compared to native OXM. More information, if needed, about the effect of chronic peptide administration on glucose homeostasis could be collected by a chronic feeding study in mice with repeated SC peptide administration and IPGTTs performed on regular time periods. Such chronic studies, with repeated SC peptide administration, could also provide more information about the body weight loss effect of analogue X26 when given over a prolonged period of time.

Drugs designed for use in humans based on GLP-1R agonists have two other potentially limiting factors; causing nausea and vomiting and their effect on pancreas (356). Liraglutide, a GLP-1R agonist, is well known to be associated with causing nausea and vomiting when given in humans. Such side-effects might be minimal and appear in a small subset of the population receiving the drug or they can be severe and result in drug withdrawal from the market. Unfortunately, nausea and vomiting cannot be assessed in rodents as they lack the anatomy and physiology necessary for vomiting (357). Indirect signs of discomfort during a study in rodents are signs such as piloerection and pica. As an adjunct to such indirect series before going into phase I human trials, drug discovery programs also use testing in other species, such as dogs, where nausea and vomiting can be evaluated.

Over the past few years, a new safety concern has emerged for GLP-1R agonists; the risk of causing proliferative changes on pancreatic duct cells potentially leading to pancreatitis and/or pancreatic cancer (358). Obesity and weight loss are known to be associated with cholelithiasis which may lead to the development of pancreatitis (359). The SCALE study has shown that the risk of pancreatitis was 0.4% in the liraglutide group vs. <0.1% in the placebo group (360). The presence of conflicting evidence and the lack of well-powered randomized trials has led regulators to conclude that although they can't exclude such a relationship, for the moment there is not enough evidence to confirm causal relationship between GLP-1 agonist therapies and pancreatitis/pancreatic cancer (361). Long-term chronic feeding studies in rodents with close monitoring of pancreatitis markers such as lipase and amylase levels can help assessing the effect of X26 on the pancreas. At the end of such studies the pancreata can be harvested for further histopathological examination.

GLP-1R agonists have also been associated with decreased fluid intake which in combination to nausea and vomiting can lead to a risk of dehydration in the clinical setting (362). A study from Gutzwiller et al. reported hypodipsia in humans after iv injection of GLP-1 while McKay et al. showed that ICV injected GLP-1R agonists affected unstimulated, overnight intake in the absence of food, in rats (362,

363). The exact mechanisms underlying peripheral or central GLP-1R-mediated hypodipsia remain to be elucidated although vagal afferents that synapse on NTS neurons and structures such as amygdala, AP, nucleus accumbens, PVN and supraoptic nucleus have possibly been implicated in regulation of water intake (362).

The PK data collected in this thesis have helped select the most promising analogue, X26, as a candidate anti-obesity drug for humans. However, the usefulness of the PK data and the rat model developed in this thesis does not stop here. Once all the safety parameters have been addressed, as much as possible in animals, candidate drugs go into human trials for further testing. One of the important decisions at that time would be the selection of the appropriate dosage to be given in humans, which depends on the PK characteristics of the drug. The accurate prediction of the ideal human dose is very difficult but the *in vivo* PK data collected by the rat model developed in this thesis can help make informed decisions (364). Allometric scaling and regression analysis have been shown to help predict human *in vivo* PK parameters and in order to increase the accuracy of predictions, PK data from more than one animal species are used (365). However, even single-species, rat *in vivo* PK data, can help make preliminary decisions about human PK parameters (366). Using published methodology the predicted human *in vivo* half-life of X26, if it is not given in a zinc formulation, would be approximately 95 minutes (in a 100-kg individual) (366). Further testing of X26 in my rat model when given with zinc could provide the necessary rat PK data to extrapolate the human *in vivo* PK characteristics of X26 when given with the zinc formulation.

In conclusion work presented in this thesis demonstrates that OXM analogue X26 could be a novel anti-obesity therapeutic agent; it has a longer duration of action (improved PK profile) and higher bioactivity than native OXM. The PK characteristics of analogues are important in a drug discovery screening program and the *in vivo* rat model developed in this thesis can be used to assess them.

Appendices

Appendix A: Amino Acids

Ala (A) Alanine

Asp (D) Aspartic acid

Glu (E) Glutamic acid

Phe (F) Phenylalanine

Gly (G) Glycine

His (H) Histidine

Ile (I) Isoleucine

Lys (K) Lysine

Leu (L) Leucine

Met (M) Methionine

Asn (N) Asparagine

Pro (P) Proline

Gln (Q) Glutamine

Arg (R) Arginine

Ser (S) Serine

Thr (T) Threonine

Val (V) Valine

Trp (W) Tryptophan

Tyr (Y) Tyrosine

Appendix B: General Principle of a Radioimmunoassay

All RIA's used were derived and maintained by Professor MA Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College) unless otherwise stated. All reagents and materials other than peptides were supplied by Sigma-Aldrich (Dorset, UK). The principle of RIA is the competition between a radioactive and nonradioactive antigen for a fixed number of antibody binding sites. When unlabelled antigen from standards of samples and a fixed amount of labelled antigen are allowed to react with a constant and limiting amount of antibody, decreasing amounts of labelled antigen are bound to the antibody as the amount of unlabelled antigen is increased. The RIA is incubated and allowed to reach equilibrium, according to the equation:



Ag = unlabelled antigen

Ab = antibody

Separation of the bound from the free antigen is achieved by addition of either dextran coated charcoal (free label is contained in the charcoal pellet following centrifugation) or using a primary-secondary antibody complex (free label is contained in the supernatant following centrifugation). The secondary antibody is derived from an animal species different from that used to generate the primary antibody. After incubation and separation, the bound and free label is counted in a γ -counter. The data are used to construct a standard curve from which the values of the unknowns can be obtained by interpolation.

Inter-assay variation can be calculated by assaying aliquots of the same sample in each assay performed and comparing the concentrations obtained in each. To measure and correct for baseline drift, tubes with no sample ('zero' tubes) are placed at regular intervals throughout the assay and standard curves are performed at the beginning and end of each assay. The general structure of the RIA is outlined in Table 14 which shows the content of the tubes according to their designation.

The following tubes are important for the assessment and performance of the assay:

Non-specific binding: low binding indicates adequate label integrity.

½ X: assesses if greater sensitivity could be achieved by adding half the volume of label.

2X: assesses if greater sensitivity could be achieved by adding double the volume of label.

Zero tubes: allows assessment of assay drift.

Excess antibody: assesses the immunological integrity of the labelled peptide.

Tube number	Designation
1-2	NSB
3-4	1/2X
5-6	2X
7-8	Zero
9-10	Zero
11-12	Standard 1
13-14	Standard 2
15-16	Standard 3
17-18	Standard 4
19-20	Standard 5
21-22	Standard 6
23-24	Standard 7
25-26	Standard 8
27-28	Standard 9
29-30	Standard 10
31-32	Zero
33-...	Samples
Standard curve	
Last 2 tubes	Excess Ab

Table 14: The general structure of the radioimmunoassay

References

1. Keys A, Fidanza F, Karvonen MJ, Kimura N, Taylor HL. Indices of relative weight and obesity. *Journal of chronic diseases*. 1972;25(6):329-43. Epub 1972/07/01.
2. Excellence NIHC. Obesity: identification, assessment and management of overweight and obesity in children, young people and adults. United Kingdom 2014.
3. McPherson K, Marsh T, Brown M. Foresight report on obesity. *Lancet*. 2007;370(9601):1755; author reply Epub 2007/11/27.
4. McPherson R. Genetic contributors to obesity. *The Canadian journal of cardiology*. 2007;23 Suppl A:23A-7A. Epub 2007/12/06.
5. Kopelman P. Health risks associated with overweight and obesity. *Obesity reviews : an official journal of the International Association for the Study of Obesity*. 2007;8 Suppl 1:13-7. Epub 2007/02/24.
6. Dietary reference values for food energy and nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy. *Reports on health and social subjects*. 1991;41:1-210. Epub 1991/01/01.
7. Nutritional aspects of the development of cancer. Report of the Working Group on Diet and Cancer of the Committee on Medical Aspects of Food and Nutrition Policy. *Reports on health and social subjects*. 1998;48:i-xiv, 1-274. Epub 1998/05/26.
8. Nutritional aspects of cardiovascular disease. Report of the Cardiovascular Review Group Committee on Medical Aspects of Food Policy. *Reports on health and social subjects*. 1994;46:1-186. Epub 1994/01/01.
9. Makris A, Foster GD. Dietary approaches to the treatment of obesity. *The Psychiatric clinics of North America*. 2011;34(4):813-27. Epub 2011/11/22.
10. Rucker D, Padwal R, Li SK, Curioni C, Lau DC. Long term pharmacotherapy for obesity and overweight: updated meta-analysis. *BMJ*. 2007;335(7631):1194-9.
11. Administration USFaD. FDA approves weight-management drug Saxenda. 2014; Available from: <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm427913.htm>.
12. Camilleri M, Toouli J, Herrera MF, Kulseng B, Kow L, Pantoja JP, et al. Intra-abdominal vagal blocking (VBLOC therapy): clinical results with a new implantable medical device. *Surgery*. 2008;143(6):723-31.
13. Sarr MG, Billington CJ, Brancatisano R, Brancatisano A, Toouli J, Kow L, et al. The EMPOWER study: randomized, prospective, double-blind, multicenter trial of vagal blockade to induce weight loss in morbid obesity. *Obesity surgery*. 2012;22(11):1771-82.

14. Guyenet SJ, Schwartz MW. Clinical review: Regulation of food intake, energy balance, and body fat mass: implications for the pathogenesis and treatment of obesity. *The Journal of clinical endocrinology and metabolism*. 2012;97(3):745-55. Epub 2012/01/13.
15. Rosenbaum M, Murphy EM, Heymsfield SB, Matthews DE, Leibel RL. Low dose leptin administration reverses effects of sustained weight-reduction on energy expenditure and circulating concentrations of thyroid hormones. *The Journal of clinical endocrinology and metabolism*. 2002;87(5):2391-4. Epub 2002/05/08.
16. Leibel RL, Rosenbaum M, Hirsch J. Changes in energy expenditure resulting from altered body weight. *The New England journal of medicine*. 1995;332(10):621-8. Epub 1995/03/09.
17. Arone LJ, Mackintosh R, Rosenbaum M, Leibel RL, Hirsch J. Autonomic nervous system activity in weight gain and weight loss. *The American journal of physiology*. 1995;269(1 Pt 2):R222-5. Epub 1995/07/01.
18. Rosenbaum M, Hirsch J, Murphy E, Leibel RL. Effects of changes in body weight on carbohydrate metabolism, catecholamine excretion, and thyroid function. *The American journal of clinical nutrition*. 2000;71(6):1421-32. Epub 2000/06/06.
19. Rissanen P, Franssila-Kallunki A, Rissanen A. Cardiac parasympathetic activity is increased by weight loss in healthy obese women. *Obesity research*. 2001;9(10):637-43. Epub 2001/10/12.
20. Rosenbaum M, Goldsmith R, Bloomfield D, Magnano A, Weimer L, Heymsfield S, et al. Low-dose leptin reverses skeletal muscle, autonomic, and neuroendocrine adaptations to maintenance of reduced weight. *The Journal of clinical investigation*. 2005;115(12):3579-86. Epub 2005/12/03.
21. Rosenbaum M, Vandenborne K, Goldsmith R, Simoneau JA, Heymsfield S, Joannisse DR, et al. Effects of experimental weight perturbation on skeletal muscle work efficiency in human subjects. *American journal of physiology Regulatory, integrative and comparative physiology*. 2003;285(1):R183-92. Epub 2003/03/01.
22. Berthoud HR. Mind versus metabolism in the control of food intake and energy balance. *Physiology & behavior*. 2004;81(5):781-93. Epub 2004/07/06.
23. Kenny PJ. Reward mechanisms in obesity: new insights and future directions. *Neuron*. 2011;69(4):664-79. Epub 2011/02/23.
24. Stratford TR, Kelley AE. Evidence of a functional relationship between the nucleus accumbens shell and lateral hypothalamus subserving the control of feeding behavior. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1999;19(24):11040-8. Epub 1999/12/14.

25. Barone FC, Wayner MJ, Tsai WH, Zarco de Coronado I. Effects of ventral tegmental area stimulation and microiontophoretic application of dopamine and norepinephrine on hypothalamic neurons. *Brain research bulletin*. 1981;7(2):181-93. Epub 1981/08/01.
26. Buchwald H, Avidor Y, Braunwald E, Jensen MD, Pories W, Fahrbach K, et al. Bariatric surgery: a systematic review and meta-analysis. *JAMA*. 2004;292(14):1724-37. Epub 2004/10/14.
27. Chikunguwo S, Brethauer S, Nirujogi V, Pitt T, Udomsawaengsup S, Chand B, et al. Influence of obesity and surgical weight loss on thyroid hormone levels. *Surgery for obesity and related diseases : official journal of the American Society for Bariatric Surgery*. 2007;3(6):631-5; discussion 5-6. Epub 2007/11/21.
28. Shin AC, Berthoud HR. Food reward functions as affected by obesity and bariatric surgery. *Int J Obes (Lond)*. 2011;35 Suppl 3:S40-4. Epub 2011/09/23.
29. Schouten R, Rijs CS, Bouvy ND, Hameeteman W, Koek GH, Janssen IM, et al. A multicenter, randomized efficacy study of the EndoBarrier Gastrointestinal Liner for presurgical weight loss prior to bariatric surgery. *Annals of surgery*. 2010;251(2):236-43. Epub 2009/10/28.
30. Ochner CN, Gibson C, Shanik M, Goel V, Geliebter A. Changes in neurohormonal gut peptides following bariatric surgery. *Int J Obes (Lond)*. 2011;35(2):153-66. Epub 2010/07/14.
31. Ackroff K, Yiin YM, Sclafani A. Post-oral infusion sites that support glucose-conditioned flavor preferences in rats. *Physiology & behavior*. 2010;99(3):402-11. Epub 2009/12/23.
32. Mojsov S, Weir GC, Habener JF. Insulinotropin: glucagon-like peptide I (7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas. *The Journal of clinical investigation*. 1987;79(2):616-9.
33. Clapham JC. Central control of thermogenesis. *Neuropharmacology*. 2012;63(1):111-23.
34. Silva JE. The multiple contributions of thyroid hormone to heat production. *The Journal of clinical investigation*. 2001;108(1):35-7.
35. Lowell BB, Spiegelman BM. Towards a molecular understanding of adaptive thermogenesis. *Nature*. 2000;404(6778):652-60.
36. Westerterp KR. Diet induced thermogenesis. *Nutrition & metabolism*. 2004;1(1):5.
37. Ravussin E, Lillioja S, Anderson TE, Christin L, Bogardus C. Determinants of 24-hour energy expenditure in man. Methods and results using a respiratory chamber. *The Journal of clinical investigation*. 1986;78(6):1568-78.
38. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiological reviews*. 2004;84(1):277-359.
39. Lean ME. Brown adipose tissue in humans. *The Proceedings of the Nutrition Society*. 1989;48(2):243-56.

40. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes*. 2009;58(7):1526-31.
41. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *The New England journal of medicine*. 2009;360(15):1509-17.
42. Lee P, Swarbrick MM, Zhao JT, Ho KK. Inducible brown adipogenesis of supraclavicular fat in adult humans. *Endocrinology*. 2011;152(10):3597-602.
43. Rothwell NJ, Stock MJ. Effects of age on diet-induced thermogenesis and brown adipose tissue metabolism in the rat. *Int J Obes*. 1983;7(6):583-9.
44. Bartness TJ, Bamshad M. Innervation of mammalian white adipose tissue: implications for the regulation of total body fat. *The American journal of physiology*. 1998;275(5 Pt 2):R1399-411.
45. Bottcher H, Furst P. Decreased white fat cell thermogenesis in obese individuals. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity*. 1997;21(6):439-44.
46. Nnodim JO, Lever JD. Neural and vascular provisions of rat interscapular brown adipose tissue. *The American journal of anatomy*. 1988;182(3):283-93.
47. Bianco AC, Silva JE. Optimal response of key enzymes and uncoupling protein to cold in BAT depends on local T3 generation. *The American journal of physiology*. 1987;253(3 Pt 1):E255-63.
48. Nicholls DG, Locke RM. Thermogenic mechanisms in brown fat. *Physiological reviews*. 1984;64(1):1-64.
49. Jones BJ, Tan T, Bloom SR. Minireview: Glucagon in stress and energy homeostasis. *Endocrinology*. 2012;153(3):1049-54. Epub 2012/02/02.
50. Morales A, Lachuer J, Duchamp C, Vera N, Georges B, Cohen-Adad F, et al. Tissue-specific modulation of rat glucagon receptor mRNA by thyroid status. *Molecular and cellular endocrinology*. 1998;144(1-2):71-81. Epub 1998/12/24.
51. Wynne K, Stanley S, McGowan B, Bloom S. Appetite control. *The Journal of endocrinology*. 2005;184(2):291-318.
52. Suzuki K, Simpson KA, Minnion JS, Shillito JC, Bloom SR. The role of gut hormones and the hypothalamus in appetite regulation. *Endocrine journal*. 2010;57(5):359-72.
53. Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature*. 2000;404(6778):661-71.
54. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell*. 1997;88(1):131-41.

55. Tao YX. Molecular mechanisms of the neural melanocortin receptor dysfunction in severe early onset obesity. *Molecular and cellular endocrinology*. 2005;239(1-2):1-14.
56. Clark JT, Kalra PS, Crowley WR, Kalra SP. Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. *Endocrinology*. 1984;115(1):427-9.
57. Leibowitz SF, Hammer NJ, Chang K. Hypothalamic paraventricular nucleus lesions produce overeating and obesity in the rat. *Physiology & behavior*. 1981;27(6):1031-40.
58. Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, Jones KR, et al. Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. *Nature neuroscience*. 2003;6(7):736-42.
59. Unger TJ, Calderon GA, Bradley LC, Sena-Esteves M, Rios M. Selective deletion of Bdnf in the ventromedial and dorsomedial hypothalamus of adult mice results in hyperphagic behavior and obesity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2007;27(52):14265-74.
60. Schwartz GJ, Salorio CF, Skoglund C, Moran TH. Gut vagal afferent lesions increase meal size but do not block gastric preload-induced feeding suppression. *The American journal of physiology*. 1999;276(6 Pt 2):R1623-9.
61. Schwartz GJ. The role of gastrointestinal vagal afferents in the control of food intake: current prospects. *Nutrition*. 2000;16(10):866-73.
62. Koda S, Date Y, Murakami N, Shimbara T, Hanada T, Toshinai K, et al. The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats. *Endocrinology*. 2005;146(5):2369-75.
63. Nakagawa A, Satake H, Nakabayashi H, Nishizawa M, Furuya K, Nakano S, et al. Receptor gene expression of glucagon-like peptide-1, but not glucose-dependent insulinotropic polypeptide, in rat nodose ganglion cells. *Autonomic neuroscience : basic & clinical*. 2004;110(1):36-43.
64. Nelson DW, Sharp JW, Brownfield MS, Raybould HE, Ney DM. Localization and activation of glucagon-like peptide-2 receptors on vagal afferents in the rat. *Endocrinology*. 2007;148(5):1954-62.
65. Grill HJ, Kaplan JM. The neuroanatomical axis for control of energy balance. *Frontiers in neuroendocrinology*. 2002;23(1):2-40.
66. Ellacott KL, Halatchev IG, Cone RD. Characterization of leptin-responsive neurons in the caudal brainstem. *Endocrinology*. 2006;147(7):3190-5.
67. Saad MF, Riad-Gabriel MG, Khan A, Sharma A, Michael R, Jinagouda SD, et al. Diurnal and ultradian rhythmicity of plasma leptin: effects of gender and adiposity. *The Journal of clinical endocrinology and metabolism*. 1998;83(2):453-9.
68. Leal-Cerro A, Considine RV, Peino R, Venegas E, Astorga R, Casanueva FF, et al. Serum immunoreactive-leptin levels are increased in patients with Cushing's syndrome. *Hormone and*

metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme. 1996;28(12):711-3.

69. Baskin DG, Breininger JF, Schwartz MW. Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. *Diabetes*. 1999;48(4):828-33.
70. Sahu A. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. *Frontiers in neuroendocrinology*. 2003;24(4):225-53.
71. Pellemounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, et al. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science*. 1995;269(5223):540-3.
72. Faggioni R, Fuller J, Moser A, Feingold KR, Grunfeld C. LPS-induced anorexia in leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice. *The American journal of physiology*. 1997;273(1 Pt 2):R181-6.
73. Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, et al. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell*. 1996;84(3):491-5.
74. Coleman DL. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia*. 1978;14(3):141-8.
75. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, et al. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science*. 1995;269(5223):543-6.
76. Munzberg H. Leptin-signaling pathways and leptin resistance. *Forum of nutrition*. 2010;63:123-32.
77. Banks WA. Anorectic effects of circulating cytokines: role of the vascular blood-brain barrier. *Nutrition*. 2001;17(5):434-7.
78. Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochemical and biophysical research communications*. 1996;221(2):286-9.
79. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, et al. Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. *The Journal of biological chemistry*. 2003;278(41):40352-63.
80. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature*. 2003;423(6941):762-9.
81. Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood*. 2000;96(5):1723-32.

82. Spranger J, Kroke A, Mohlig M, Bergmann MM, Ristow M, Boeing H, et al. Adiponectin and protection against type 2 diabetes mellitus. *Lancet*. 2003;361(9353):226-8.
83. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochemical and biophysical research communications*. 1999;257(1):79-83.
84. Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, et al. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. *Diabetologia*. 2003;46(4):459-69.
85. Polonsky KS, Given BD, Van Cauter E. Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. *The Journal of clinical investigation*. 1988;81(2):442-8.
86. Air EL, Benoit SC, Blake Smith KA, Clegg DJ, Woods SC. Acute third ventricular administration of insulin decreases food intake in two paradigms. *Pharmacology, biochemistry, and behavior*. 2002;72(1-2):423-9.
87. Corp ES, Woods SC, Porte D, Jr., Dorsa DM, Figlewicz DP, Baskin DG. Localization of 125I-insulin binding sites in the rat hypothalamus by quantitative autoradiography. *Neuroscience letters*. 1986;70(1):17-22.
88. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*. 1999;402(6762):656-60.
89. Hosoda H, Kojima M, Kangawa K. Biological, physiological, and pharmacological aspects of ghrelin. *Journal of pharmacological sciences*. 2006;100(5):398-410.
90. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, et al. Ghrelin enhances appetite and increases food intake in humans. *The Journal of clinical endocrinology and metabolism*. 2001;86(12):5992.
91. Otto B, Cuntz U, Fruehauf E, Wawarta R, Folwaczny C, Riepl RL, et al. Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. *European journal of endocrinology / European Federation of Endocrine Societies*. 2001;145(5):669-73.
92. Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, et al. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *The New England journal of medicine*. 2002;346(21):1623-30.
93. Tschöp M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature*. 2000;407(6806):908-13.
94. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. *Nature*. 2001;409(6817):194-8.

95. Wang L, Saint-Pierre DH, Tache Y. Peripheral ghrelin selectively increases Fos expression in neuropeptide Y - synthesizing neurons in mouse hypothalamic arcuate nucleus. *Neuroscience letters*. 2002;325(1):47-51.
96. Chen HY, Trumbauer ME, Chen AS, Weingarth DT, Adams JR, Frazier EG, et al. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology*. 2004;145(6):2607-12.
97. Lawrence CB, Snape AC, Baudoin FM, Luckman SM. Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. *Endocrinology*. 2002;143(1):155-62.
98. Gibbs J, Young RC, Smith GP. Cholecystokinin decreases food intake in rats. *Journal of comparative and physiological psychology*. 1973;84(3):488-95.
99. Liddle RA, Goldfine ID, Rosen MS, Taplitz RA, Williams JA. Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. *The Journal of clinical investigation*. 1985;75(4):1144-52.
100. Cummings DE, Overduin J. Gastrointestinal regulation of food intake. *The Journal of clinical investigation*. 2007;117(1):13-23. Epub 2007/01/04.
101. Blevins JE, Baskin DG. Hypothalamic-brainstem circuits controlling eating. *Forum of nutrition*. 2010;63:133-40.
102. Moran TH, Baldessarini AR, Salorio CF, Lowery T, Schwartz GJ. Vagal afferent and efferent contributions to the inhibition of food intake by cholecystokinin. *The American journal of physiology*. 1997;272(4 Pt 2):R1245-51.
103. Moran TH, Katz LF, Plata-Salaman CR, Schwartz GJ. Disordered food intake and obesity in rats lacking cholecystokinin A receptors. *The American journal of physiology*. 1998;274(3 Pt 2):R618-25.
104. Wank SA. Cholecystokinin receptors. *The American journal of physiology*. 1995;269(5 Pt 1):G628-46.
105. Owyang C, Heldsinger A. Vagal control of satiety and hormonal regulation of appetite. *Journal of neurogastroenterology and motility*. 2011;17(4):338-48.
106. Tatemoto K, Mutt V. Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. *Nature*. 1980;285(5764):417-8.
107. Lin S, Boey D, Herzog H. NPY and Y receptors: lessons from transgenic and knockout models. *Neuropeptides*. 2004;38(4):189-200.
108. Ekblad E, Sundler F. Distribution of pancreatic polypeptide and peptide YY. *Peptides*. 2002;23(2):251-61.

109. Eberlein GA, Eysselein VE, Schaeffer M, Layer P, Grandt D, Goebell H, et al. A new molecular form of PYY: structural characterization of human PYY(3-36) and PYY(1-36). *Peptides*. 1989;10(4):797-803.
110. Parker RM, Herzog H. Regional distribution of Y-receptor subtype mRNAs in rat brain. *The European journal of neuroscience*. 1999;11(4):1431-48.
111. Asakawa A, Inui A, Yuzuriha H, Ueno N, Katsuura G, Fujimiya M, et al. Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. *Gastroenterology*. 2003;124(5):1325-36.
112. Kojima S, Ueno N, Asakawa A, Sagiya K, Naruo T, Mizuno S, et al. A role for pancreatic polypeptide in feeding and body weight regulation. *Peptides*. 2007;28(2):459-63.
113. Adrian TE, Bloom SR, Bryant MG, Polak JM, Heitz PH, Barnes AJ. Distribution and release of human pancreatic polypeptide. *Gut*. 1976;17(12):940-44.
114. Uhe AM, Szmukler GI, Collier GR, Hansky J, O'Dea K, Young GP. Potential regulators of feeding behavior in anorexia nervosa. *The American journal of clinical nutrition*. 1992;55(1):28-32.
115. Jorde R, Burhol PG. Fasting and postprandial plasma pancreatic polypeptide (PP) levels in obesity. *Int J Obes*. 1984;8(5):393-7.
116. Wisen O, Bjorvell H, Cantor P, Johansson C, Theodorsson E. Plasma concentrations of regulatory peptides in obesity following modified sham feeding (MSF) and a liquid test meal. *Regulatory peptides*. 1992;39(1):43-54.
117. Glaser B, Zoghlin G, Pienta K, Vinik AI. Pancreatic polypeptide response to secretin in obesity: effects of glucose intolerance. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme*. 1988;20(5):288-92.
118. Lassmann V, Vague P, Vialettes B, Simon MC. Low plasma levels of pancreatic polypeptide in obesity. *Diabetes*. 1980;29(6):428-30.
119. Holst JJ. Glucagon-like Peptide 1 (GLP-1): An Intestinal Hormone, Signalling Nutritional Abundance, with an Unusual Therapeutic Potential. *Trends in endocrinology and metabolism: TEM*. 1999;10(6):229-35.
120. Holst JJ, Bersani M, Johnsen AH, Kofod H, Hartmann B, Orskov C. Proglucagon processing in porcine and human pancreas. *The Journal of biological chemistry*. 1994;269(29):18827-33. Epub 1994/07/22.
121. Orskov C, Bersani M, Johnsen AH, Hojrup P, Holst JJ. Complete sequences of glucagon-like peptide-1 from human and pig small intestine. *J Biol Chem*. 1989;264(22):12826-9. Epub 1989/08/05.

122. Buhl T, Thim L, Kofod H, Orskov C, Harling H, Holst JJ. Naturally-Occurring Products of Proglucagon-111-160 in the Porcine and Human Small-Intestine. *Journal of Biological Chemistry*. 1988;263(18):8621-4.
123. Orskov C, Buhl T, Rabenhøj L, Kofod H, Holst JJ. Carboxypeptidase-B-like processing of the C-terminus of glucagon-like peptide-2 in pig and human small intestine. *FEBS Lett*. 1989;247(2):193-6. Epub 1989/04/24.
124. Kervran A, Blache P, Bataille D. Distribution of oxyntomodulin and glucagon in the gastrointestinal tract and the plasma of the rat. *Endocrinology*. 1987;121(2):704-13.
125. Elrick H, Stimmler L, Hlad CJ, Jr., Arai Y. Plasma Insulin Response to Oral and Intravenous Glucose Administration. *J Clin Endocrinol Metab*. 1964;24:1076-82. Epub 1964/10/01.
126. Creutzfeldt M. Candidate hormones of the gut. XV. Insulin-releasing factors of the gastrointestinal mucosa (Incretin). *Gastroenterology*. 1974;67(4):748-50. Epub 1974/10/01.
127. Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. *Gastroenterology*. 2007;132(6):2131-57. Epub 2007/05/15.
128. Green BD, Flatt PR. Incretin hormone mimetics and analogues in diabetes therapeutics. *Best Pract Res Clin Endocrinol Metab*. 2007;21(4):497-516. Epub 2007/12/07.
129. McIntyre N HC, Turner DS. Intestinal factors in the control of insulin secretion. *J Clin Endocrinol Metab*. 1965;25(10):1317-24.
130. McIntyre N HC, Turner DS. New interpretation of oral glucose tolerance. *Lancet* 1964;4(2):20-1.
131. Elrick H SL, Hlad CJ, Arai Y. . Plasma insulin responses to oral and intravenous glucose administration. *J Clin Endocrinol Metab*. 1964;24:1076-82.
132. Orskov C, Rabenhøj L, Wettergren A, Kofod H, Holst JJ. Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans. *Diabetes*. 1994;43(4):535-9.
133. Gallwitz B, Schmidt WE, Conlon JM, Creutzfeldt W. Glucagon-like peptide-1(7-36)amide: characterization of the domain responsible for binding to its receptor on rat insulinoma RINm5F cells. *Journal of molecular endocrinology*. 1990;5(1):33-9.
134. Takahashi H, Manaka H, Suda K, Fukase N, Sekikawa A, Eguchi H, et al. Hyperglycaemia but not hyperinsulinaemia prevents the secretion of glucagon-like peptide-1 (7-36 amide) stimulated by fat ingestion. *Scandinavian journal of clinical and laboratory investigation*. 1991;51(6):499-507.
135. Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V. Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. *The Journal of endocrinology*. 1993;138(1):159-66.

136. Brubaker PL. The glucagon-like peptides: pleiotropic regulators of nutrient homeostasis. *Annals of the New York Academy of Sciences*. 2006;1070:10-26.
137. Buteau J, El-Assaad W, Rhodes CJ, Rosenberg L, Joly E, Prentki M. Glucagon-like peptide-1 prevents beta cell glucolipotoxicity. *Diabetologia*. 2004;47(5):806-15.
138. Farilla L, Bulotta A, Hirshberg B, Li Calzi S, Khoury N, Noushmehr H, et al. Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. *Endocrinology*. 2003;144(12):5149-58.
139. Yusta B, Baggio LL, Estall JL, Koehler JA, Holland DP, Li H, et al. GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress. *Cell metabolism*. 2006;4(5):391-406.
140. Holz GG, Kuhlreiter WM, Habener JF. Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7-37). *Nature*. 1993;361(6410):362-5.
141. Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF. Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proceedings of the National Academy of Sciences of the United States of America*. 1987;84(10):3434-8.
142. Kreyman B, Williams G, Ghatei MA, Bloom SR. Glucagon-like peptide-1 7-36: a physiological incretin in man. *Lancet*. 1987;2(8571):1300-4.
143. Heller RS, Kieffer TJ, Habener JF. Insulinotropic glucagon-like peptide I receptor expression in glucagon-producing alpha-cells of the rat endocrine pancreas. *Diabetes*. 1997;46(5):785-91.
144. Fehmann HC, Habener JF. Functional receptors for the insulinotropic hormone glucagon-like peptide-I(7-37) on a somatostatin secreting cell line. *FEBS letters*. 1991;279(2):335-40.
145. Scrocchi LA, Brown TJ, McCluskey N, Brubaker PL, Auerbach AB, Joyner AL, et al. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nature medicine*. 1996;2(11):1254-8.
146. Wang Z, Wang RM, Owji AA, Smith DM, Ghatei MA, Bloom SR. Glucagon-like peptide-1 is a physiological incretin in rat. *The Journal of clinical investigation*. 1995;95(1):417-21. Epub 1995/01/01.
147. Hui H, Nourparvar A, Zhao X, Perfetti R. Glucagon-like peptide-1 inhibits apoptosis of insulin-secreting cells via a cyclic 5'-adenosine monophosphate-dependent protein kinase A- and a phosphatidylinositol 3-kinase-dependent pathway. *Endocrinology*. 2003;144(4):1444-55.
148. Xu G, Stoffers DA, Habener JF, Bonner-Weir S. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes*. 1999;48(12):2270-6.
149. Holst JJ. Glucagonlike peptide 1: a newly discovered gastrointestinal hormone. *Gastroenterology*. 1994;107(6):1848-55.

150. Tan TM, Field BC, McCullough KA, Troke RC, Chambers ES, Salem V, et al. Coadministration of glucagon-like peptide-1 during glucagon infusion in humans results in increased energy expenditure and amelioration of hyperglycemia. *Diabetes*. 2013;62(4):1131-8. Epub 2012/12/19.
151. Wettergren A, Schjoldager B, Mortensen PE, Myhre J, Christiansen J, Holst JJ. Truncated GLP-1 (proglucagon 78-107-amide) inhibits gastric and pancreatic functions in man. *Digestive diseases and sciences*. 1993;38(4):665-73.
152. Wettergren A, Wojdemann M, Meisner S, Stadil F, Holst JJ. The inhibitory effect of glucagon-like peptide-1 (GLP-1) 7-36 amide on gastric acid secretion in humans depends on an intact vagal innervation. *Gut*. 1997;40(5):597-601.
153. Larsen PJ, Tang-Christensen M, Holst JJ, Orskov C. Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. *Neuroscience*. 1997;77(1):257-70.
154. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, et al. A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature*. 1996;379(6560):69-72.
155. Tang-Christensen M, Larsen PJ, Goke R, Fink-Jensen A, Jessop DS, Moller M, et al. Central administration of GLP-1-(7-36) amide inhibits food and water intake in rats. *The American journal of physiology*. 1996;271(4 Pt 2):R848-56.
156. Yamamoto H, Kishi T, Lee CE, Choi BJ, Fang H, Hollenberg AN, et al. Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2003;23(7):2939-46.
157. Abbott CR, Monteiro M, Small CJ, Sajedi A, Smith KL, Parkinson JR, et al. The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. *Brain research*. 2005;1044(1):127-31.
158. Osaka T, Endo M, Yamakawa M, Inoue S. Energy expenditure by intravenous administration of glucagon-like peptide-1 mediated by the lower brainstem and sympathoadrenal system. *Peptides*. 2005;26(9):1623-31.
159. Tachibana T, Oikawa D, Adachi N, Boswell T, Furuse M. Intracerebroventricular injection of glucagon-like peptide-1 changes lipid metabolism in chicks. *Comparative biochemistry and physiology Part A, Molecular & integrative physiology*. 2007;147(4):1104-8.
160. Ayala JE, Bracy DP, James FD, Julien BM, Wasserman DH, Drucker DJ. The glucagon-like peptide-1 receptor regulates endogenous glucose production and muscle glucose uptake independent of its incretin action. *Endocrinology*. 2009;150(3):1155-64.

161. Szayna M, Doyle ME, Betkey JA, Holloway HW, Spencer RG, Greig NH, et al. Exendin-4 decelerates food intake, weight gain, and fat deposition in Zucker rats. *Endocrinology*. 2000;141(6):1936-41.
162. Kolterman OG, Buse JB, Fineman MS, Gaines E, Heintz S, Bicsak TA, et al. Synthetic exendin-4 (exenatide) significantly reduces postprandial and fasting plasma glucose in subjects with type 2 diabetes. *The Journal of clinical endocrinology and metabolism*. 2003;88(7):3082-9.
163. Tourrel C, Bailbe D, Lacorne M, Meile MJ, Kergoat M, Portha B. Persistent improvement of type 2 diabetes in the Goto-Kakizaki rat model by expansion of the beta-cell mass during the prediabetic period with glucagon-like peptide-1 or exendin-4. *Diabetes*. 2002;51(5):1443-52.
164. Parkes DG, Pittner R, Jodka C, Smith P, Young A. Insulinotropic actions of exendin-4 and glucagon-like peptide-1 in vivo and in vitro. *Metabolism: clinical and experimental*. 2001;50(5):583-9.
165. Egan JM, Clocquet AR, Elahi D. The insulinotropic effect of acute exendin-4 administered to humans: comparison of nondiabetic state to type 2 diabetes. *The Journal of clinical endocrinology and metabolism*. 2002;87(3):1282-90.
166. Thorens B, Porret A, Buhler L, Deng SP, Morel P, Widmann C. Cloning and functional expression of the human islet GLP-1 receptor. Demonstration that exendin-4 is an agonist and exendin-(9-39) an antagonist of the receptor. *Diabetes*. 1993;42(11):1678-82.
167. Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, et al. Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. *The Journal of biological chemistry*. 1993;268(26):19650-5.
168. Furuta M, Carroll R, Martin S, Swift HH, Ravazzola M, Orci L, et al. Incomplete processing of proinsulin to insulin accompanied by elevation of Des-31,32 proinsulin intermediates in islets of mice lacking active PC2. *J Biol Chem*. 1998;273(6):3431-7. Epub 1998/03/07.
169. Furuta M, Zhou A, Webb G, Carroll R, Ravazzola M, Orci L, et al. Severe defect in proglucagon processing in islet A-cells of prohormone convertase 2 null mice. *J Biol Chem*. 2001;276(29):27197-202. Epub 2001/05/18.
170. Quesada I, Todorova MG, Soria B. Different metabolic responses in alpha-, beta-, and delta-cells of the islet of Langerhans monitored by redox confocal microscopy. *Biophys J*. 2006;90(7):2641-50. Epub 2006/01/10.
171. Gromada J, Bokvist K, Ding WG, Barg S, Buschard K, Renstrom E, et al. Adrenaline stimulates glucagon secretion in pancreatic A-cells by increasing the Ca²⁺ current and the number of granules close to the L-type Ca²⁺ channels. *J Gen Physiol*. 1997;110(3):217-28. Epub 1997/09/01.

172. MacDonald PE, De Marinis YZ, Ramracheya R, Salehi A, Ma X, Johnson PR, et al. A K ATP channel-dependent pathway within alpha cells regulates glucagon release from both rodent and human islets of Langerhans. *PLoS Biol.* 2007;5(6):e143. Epub 2007/05/17.
173. Franklin I, Gromada J, Gjinovci A, Theander S, Wollheim CB. Beta-cell secretory products activate alpha-cell ATP-dependent potassium channels to inhibit glucagon release. *Diabetes.* 2005;54(6):1808-15. Epub 2005/05/28.
174. Olsen HL, Theander S, Bokvist K, Buschard K, Wollheim CB, Gromada J. Glucose stimulates glucagon release in single rat alpha-cells by mechanisms that mirror the stimulus-secretion coupling in beta-cells. *Endocrinology.* 2005;146(11):4861-70. Epub 2005/08/06.
175. Ravier MA, Rutter GA. Glucose or insulin, but not zinc ions, inhibit glucagon secretion from mouse pancreatic alpha-cells. *Diabetes.* 2005;54(6):1789-97. Epub 2005/05/28.
176. Dunning BE, Foley JE, Ahren B. Alpha cell function in health and disease: influence of glucagon-like peptide-1. *Diabetologia.* 2005;48(9):1700-13. Epub 2005/09/01.
177. Meier JJ, Kjems LL, Veldhuis JD, Lefebvre P, Butler PC. Postprandial suppression of glucagon secretion depends on intact pulsatile insulin secretion: further evidence for the inraislet insulin hypothesis. *Diabetes.* 2006;55(4):1051-6. Epub 2006/03/29.
178. Bollheimer LC, Landauer HC, Troll S, Schweimer J, Wrede CE, Scholmerich J, et al. Stimulatory short-term effects of free fatty acids on glucagon secretion at low to normal glucose concentrations. *Metabolism.* 2004;53(11):1443-8. Epub 2004/11/13.
179. Ahren B. Autonomic regulation of islet hormone secretion--implications for health and disease. *Diabetologia.* 2000;43(4):393-410. Epub 2000/05/20.
180. Dumonteil E, Magnan C, Ritz-Laser B, Ktorza A, Meda P, Philippe J. Glucose regulates proinsulin and prosomatostatin but not proglucagon messenger ribonucleic acid levels in rat pancreatic islets. *Endocrinology.* 2000;141(1):174-80. Epub 1999/12/30.
181. Parmley WW, Glick G, Sonnenblick EH. Cardiovascular effects of glucagon in man. *The New England journal of medicine.* 1968;279(1):12-7.
182. Campos RV, Lee YC, Drucker DJ. Divergent tissue-specific and developmental expression of receptors for glucagon and glucagon-like peptide-1 in the mouse. *Endocrinology.* 1994;134(5):2156-64.
183. Dunphy JL, Taylor RG, Fuller PJ. Tissue distribution of rat glucagon receptor and GLP-1 receptor gene expression. *Molecular and cellular endocrinology.* 1998;141(1-2):179-86.
184. Hansen LH, Abrahamsen N, Nishimura E. Glucagon receptor mRNA distribution in rat tissues. *Peptides.* 1995;16(6):1163-6.

185. Svoboda M, Tastenoy M, Vertongen P, Robberecht P. Relative quantitative analysis of glucagon receptor mRNA in rat tissues. *Molecular and cellular endocrinology*. 1994;105(2):131-7.
186. Pohl SL, Birnbaumer L, Rodbell M. Glucagon-sensitive adenyl cyclase in plasma membrane of hepatic parenchymal cells. *Science*. 1969;164(3879):566-7.
187. Wakelam MJ, Murphy GJ, Hruby VJ, Houslay MD. Activation of two signal-transduction systems in hepatocytes by glucagon. *Nature*. 1986;323(6083):68-71.
188. Bataille D, Gespach C, Tatemoto K, Marie JC, Coudray AM, Rosselin G, et al. Bioactive enteroglucagon (oxyntomodulin): present knowledge on its chemical structure and its biological activities. *Peptides*. 1981;2 Suppl 2:41-4.
189. Bataille D, Tatemoto K, Coudray AM, Rosselin G, Mutt V. [Bioactive "enteroglucagon" (oxyntomodulin): evidence for a C-terminal extension of the glucagon molecule]. *Comptes rendus des seances de l'Academie des sciences Serie III, Sciences de la vie*. 1981;293(6):323-8. "Enteroglucagon" bioactif (oxyntomoduline) : evidence pour une extension C-terminale de la molecule de glucagon.
190. Bell GI, Sanchez-Pescador R, Laybourn PJ, Najarian RC. Exon duplication and divergence in the human preproglucagon gene. *Nature*. 1983;304(5924):368-71. Epub 1983/07/03.
191. Audousset-Puech MP, Dufour M, Kervran A, Jarrousse C, Castro B, Bataille D, et al. Solid-phase peptide synthesis of human(Nle-27)-oxyntomodulin. Preliminary evaluation of its biological activities. *FEBS letters*. 1986;200(1):181-5. Epub 1986/05/05.
192. Holst JJ. Enteroglucagon. *Annual review of physiology*. 1997;59:257-71.
193. Bataille D, Dalle S, Blache P, Bergeron F. [Post-translational maturation of proglucagon: variations in tissues and regulation pathways]. *Journees annuelles de diabetologie de l'Hotel-Dieu*. 1998;127-40. Maturation post-traductionnelle du proglucagon: variations tissulaires et voies de regulation.
194. Strader AD, Woods SC. Gastrointestinal hormones and food intake. *Gastroenterology*. 2005;128(1):175-91.
195. Baldissera FG, Holst JJ, Knuhtsen S, Hilsted L, Nielsen OV. Oxyntomodulin (glicentin-(33-69)): pharmacokinetics, binding to liver cell membranes, effects on isolated perfused pig pancreas, and secretion from isolated perfused lower small intestine of pigs. *Regulatory peptides*. 1988;21(1-2):151-66.
196. Ghatei MA, Uttenthal LO, Christofides ND, Bryant MG, Bloom SR. Molecular forms of human enteroglucagon in tissue and plasma: plasma responses to nutrient stimuli in health and in disorders of the upper gastrointestinal tract. *The Journal of clinical endocrinology and metabolism*. 1983;57(3):488-95. Epub 1983/09/01.

197. Anini Y, Fu-Cheng X, Cuber JC, Kervran A, Chariot J, Roz C. Comparison of the postprandial release of peptide YY and proglucagon-derived peptides in the rat. *Pflugers Archiv : European journal of physiology*. 1999;438(3):299-306.
198. Le Quellec A, Kervran A, Blache P, Ciurana AJ, Bataille D. Oxyntomodulin-like immunoreactivity: diurnal profile of a new potential enterogastrone. *The Journal of clinical endocrinology and metabolism*. 1992;74(6):1405-9.
199. Dakin CL, Small CJ, Batterham RL, Neary NM, Cohen MA, Patterson M, et al. Peripheral oxyntomodulin reduces food intake and body weight gain in rats. *Endocrinology*. 2004;145(6):2687-95.
200. Horvath TL. The hardship of obesity: a soft-wired hypothalamus. *Nature neuroscience*. 2005;8(5):561-5.
201. Baggio LL, Huang Q, Brown TJ, Drucker DJ. Oxyntomodulin and glucagon-like peptide-1 differentially regulate murine food intake and energy expenditure. *Gastroenterology*. 2004;127(2):546-58.
202. Couce ME, Green D, Brunetto A, Achim C, Lloyd RV, Burguera B. Limited brain access for leptin in obesity. *Pituitary*. 2001;4(1-2):101-10. Epub 2002/02/05.
203. Wynne K, Bloom SR. The role of oxyntomodulin and peptide tyrosine-tyrosine (PYY) in appetite control. *Nature clinical practice Endocrinology & metabolism*. 2006;2(11):612-20.
204. Dakin CL, Gunn I, Small CJ, Edwards CM, Hay DL, Smith DM, et al. Oxyntomodulin inhibits food intake in the rat. *Endocrinology*. 2001;142(10):4244-50.
205. Fehmann HC, Jiang J, Schweinfurth J, Wheeler MB, Boyd AE, 3rd, Goke B. Stable expression of the rat GLP-I receptor in CHO cells: activation and binding characteristics utilizing GLP-I(7-36)-amide, oxyntomodulin, exendin-4, and exendin(9-39). *Peptides*. 1994;15(3):453-6.
206. Bataille D, Gespach C, Coudray AM, Rosselin G. "Enteroglucagon": a specific effect on gastric glands isolated from the rat fundus. Evidence for an "oxyntomodulin" action. *Bioscience reports*. 1981;1(2):151-5. Epub 1981/02/01.
207. Maida A, Lovshin JA, Baggio LL, Drucker DJ. The glucagon-like peptide-1 receptor agonist oxyntomodulin enhances beta-cell function but does not inhibit gastric emptying in mice. *Endocrinology*. 2008;149(11):5670-8. Epub 2008/08/02.
208. Schjoldager B, Mortensen PE, Myhre J, Christiansen J, Holst JJ. Oxyntomodulin from distal gut. Role in regulation of gastric and pancreatic functions. *Digestive diseases and sciences*. 1989;34(9):1411-9. Epub 1989/09/01.
209. Jarrousse C, Bataille D, Jeanrenaud B. A pure enteroglucagon, oxyntomodulin (glucagon 37), stimulates insulin release in perfused rat pancreas. *Endocrinology*. 1984;115(1):102-5.

210. Dakin CL, Small CJ, Park AJ, Seth A, Ghatei MA, Bloom SR. Repeated ICV administration of oxyntomodulin causes a greater reduction in body weight gain than in pair-fed rats. *American journal of physiology Endocrinology and metabolism*. 2002;283(6):E1173-7.
211. Wynne K, Park AJ, Small CJ, Meeran K, Ghatei MA, Frost GS, et al. Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial. *International journal of obesity*. 2006;30(12):1729-36.
212. Cohen MA, Ellis SM, Le Roux CW, Batterham RL, Park A, Patterson M, et al. Oxyntomodulin suppresses appetite and reduces food intake in humans. *The Journal of clinical endocrinology and metabolism*. 2003;88(10):4696-701.
213. Wynne K, Park AJ, Small CJ, Patterson M, Ellis SM, Murphy KG, et al. Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. *Diabetes*. 2005;54(8):2390-5.
214. Elangbam CS. Review paper: Current strategies in the development of anti-obesity drugs and their safety concerns. *Veterinary pathology*. 2009;46(1):10-24.
215. Di Marzo V, Matias I. Endocannabinoid control of food intake and energy balance. *Nature neuroscience*. 2005;8(5):585-9.
216. Traynor K. Panel advises against rimonabant approval. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists*. 2007;64(14):1460-1.
217. Cooke D, Bloom S. The obesity pipeline: current strategies in the development of anti-obesity drugs. *Nature reviews Drug discovery*. 2006;5(11):919-31.
218. Lopez de Maturana R, Donnelly D. The glucagon-like peptide-1 receptor binding site for the N-terminus of GLP-1 requires polarity at Asp198 rather than negative charge. *FEBS letters*. 2002;530(1-3):244-8.
219. Adelhorst K, Hedegaard BB, Knudsen LB, Kirk O. Structure-activity studies of glucagon-like peptide-1. *The Journal of biological chemistry*. 1994;269(9):6275-8.
220. Morrison KL, Weiss GA. Combinatorial alanine-scanning. *Curr Opin Chem Biol*. 2001;5(3):302-7. Epub 2001/08/02.
221. Sturm NS, Lin Y, Burley SK, Krstenansky JL, Ahn JM, Azizeh BY, et al. Structure-function studies on positions 17, 18, and 21 replacement analogues of glucagon: the importance of charged residues and salt bridges in glucagon biological activity. *Journal of medicinal chemistry*. 1998;41(15):2693-700. Epub 1998/07/21.

222. Parker JC, Andrews KM, Rescek DM, Massefski W, Jr., Andrews GC, Contillo LG, et al. Structure-function analysis of a series of glucagon-like peptide-1 analogs. *The journal of peptide research : official journal of the American Peptide Society*. 1998;52(5):398-409.
223. Gallwitz B, Witt M, Paetzold G, Morys-Wortmann C, Zimmermann B, Eckart K, et al. Structure/activity characterization of glucagon-like peptide-1. *European journal of biochemistry / FEBS*. 1994;225(3):1151-6.
224. Carles-Bonnet C, Martinez J, Jarrousse C, Aumelas A, Niel H, Bataille D. H-Lys-Arg-Asn-Lys-Asn-Asn-OH is the minimal active structure of oxyntomodulin. *Peptides*. 1996;17(3):557-61. Epub 1996/01/01.
225. Wright SM, Aronne LJ. Obesity in 2010: the future of obesity medicine: where do we go from here? *Nature reviews Endocrinology*. 2011;7(2):69-70.
226. Powell AG, Apovian CM, Aronne LJ. New drug targets for the treatment of obesity. *Clinical pharmacology and therapeutics*. 2011;90(1):40-51.
227. Birnbaumer L, Yatani A, VanDongen AM, Graf R, Codina J, Okabe K, et al. G protein coupling of receptors to ionic channels and other effector systems. *British journal of clinical pharmacology*. 1990;30 Suppl 1:13S-22S.
228. Drews J. Drug discovery: a historical perspective. *Science*. 2000;287(5460):1960-4.
229. Ballesteros JA, Weinstein H. Analysis and refinement of criteria for predicting the structure and relative orientations of transmembranal helical domains. *Biophysical journal*. 1992;62(1):107-9.
230. Egan JM, Montrose-Rafizadeh C, Wang Y, Bernier M, Roth J. Glucagon-like peptide-1(7-36) amide (GLP-1) enhances insulin-stimulated glucose metabolism in 3T3-L1 adipocytes: one of several potential extrapancreatic sites of GLP-1 action. *Endocrinology*. 1994;135(5):2070-5.
231. Ishihara T, Nakamura S, Kaziro Y, Takahashi T, Takahashi K, Nagata S. Molecular cloning and expression of a cDNA encoding the secretin receptor. *The EMBO journal*. 1991;10(7):1635-41.
232. Harmar AJ. Family-B G-protein-coupled receptors. *Genome biology*. 2001;2(12):REVIEWS3013.
233. Josefsson LG. Evidence for kinship between diverse G-protein coupled receptors. *Gene*. 1999;239(2):333-40. Epub 1999/11/05.
234. Kolakowski LF, Jr. GCRDb: a G-protein-coupled receptor database. *Receptors & channels*. 1994;2(1):1-7.
235. Hoare SR. Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. *Drug discovery today*. 2005;10(6):417-27.
236. Segre GV, Goldring SR. Receptors for secretin, calcitonin, parathyroid hormone (PTH)/PTH-related peptide, vasoactive intestinal peptide, glucagonlike peptide 1, growth hormone-releasing

hormone, and glucagon belong to a newly discovered G-protein-linked receptor family. Trends in endocrinology and metabolism: TEM. 1993;4(10):309-14.

237. Bazarsuren A, Grauschopf U, Wozny M, Reusch D, Hoffmann E, Schaefer W, et al. In vitro folding, functional characterization, and disulfide pattern of the extracellular domain of human GLP-1 receptor. Biophysical chemistry. 2002;96(2-3):305-18.

238. Dong M, Pinon DI, Asmann YW, Miller LJ. Possible endogenous agonist mechanism for the activation of secretin family G protein-coupled receptors. Molecular pharmacology. 2006;70(1):206-13. Epub 2006/03/15.

239. Dong M, Gao F, Pinon DI, Miller LJ. Insights into the structural basis of endogenous agonist activation of family B G protein-coupled receptors. Molecular endocrinology. 2008;22(6):1489-99. Epub 2008/03/29.

240. Goke R, Fehmann HC, Goke B. Glucagon-like peptide-1(7-36) amide is a new incretin/enterogastrone candidate. European journal of clinical investigation. 1991;21(2):135-44.

241. Gros L, Demirpence E, Jarrousse C, Kervran A, Bataille D. Characterization of binding sites for oxyntomodulin on a somatostatin-secreting cell line (RIN T3). Endocrinology. 1992;130(3):1263-70.

242. Thorens B. Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(18):8641-5.

243. Tibaduiza EC, Chen C, Beinborn M. A small molecule ligand of the glucagon-like peptide 1 receptor targets its amino-terminal hormone binding domain. The Journal of biological chemistry. 2001;276(41):37787-93. Epub 2001/08/11.

244. Glucagon receptor family: GLP-1 receptor [database on the Internet]. 2014 [cited 26/10/2014]. Available from: <http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?objectId=249>.

245. Runge S, Thogersen H, Madsen K, Lau J, Rudolph R. Crystal structure of the ligand-bound glucagon-like peptide-1 receptor extracellular domain. The Journal of biological chemistry. 2008;283(17):11340-7.

246. Underwood CR, Garibay P, Knudsen LB, Hastrup S, Peters GH, Rudolph R, et al. Crystal structure of glucagon-like peptide-1 in complex with the extracellular domain of the glucagon-like peptide-1 receptor. The Journal of biological chemistry. 2010;285(1):723-30.

247. Neidigh JW, Fesinmeyer RM, Prickett KS, Andersen NH. Exendin-4 and glucagon-like-peptide-1: NMR structural comparisons in the solution and micelle-associated states. Biochemistry. 2001;40(44):13188-200.

248. Fehmann HC, Jiang J, Schweinfurth J, Dorsch K, Wheeler MB, Boyd AE, 3rd, et al. Ligand-specificity of the rat GLP-I receptor recombinantly expressed in Chinese hamster ovary (CHO-) cells. *Zeitschrift fur Gastroenterologie*. 1994;32(4):203-7.
249. Kieffer TJ, Heller RS, Unson CG, Weir GC, Habener JF. Distribution of glucagon receptors on hormone-specific endocrine cells of rat pancreatic islets. *Endocrinology*. 1996;137(11):5119-25.
250. Runge S, Wulff BS, Madsen K, Brauner-Osborne H, Knudsen LB. Different domains of the glucagon and glucagon-like peptide-1 receptors provide the critical determinants of ligand selectivity. *British journal of pharmacology*. 2003;138(5):787-94. Epub 2003/03/19.
251. Hjorth SA, Adelhorst K, Pedersen BB, Kirk O, Schwartz TW. Glucagon and glucagon-like peptide 1: selective receptor recognition via distinct peptide epitopes. *The Journal of biological chemistry*. 1994;269(48):30121-4.
252. Thornton K, Gorenstein DG. Structure of glucagon-like peptide (7-36) amide in a dodecylphosphocholine micelle as determined by 2D NMR. *Biochemistry*. 1994;33(12):3532-9.
253. Murage EN, Schroeder JC, Beinborn M, Ahn JM. Search for alpha-helical propensity in the receptor-bound conformation of glucagon-like peptide-1. *Bioorganic & medicinal chemistry*. 2008;16(23):10106-12.
254. Suzuki S, Kawai K, Ohashi S, Mukai H, Yamashita K. Comparison of the effects of various C-terminal and N-terminal fragment peptides of glucagon-like peptide-1 on insulin and glucagon release from the isolated perfused rat pancreas. *Endocrinology*. 1989;125(6):3109-14.
255. Gefel D, Hendrick GK, Mojsov S, Habener J, Weir GC. Glucagon-like peptide-I analogs: effects on insulin secretion and adenosine 3',5'-monophosphate formation. *Endocrinology*. 1990;126(4):2164-8.
256. Knudsen LB, Pridal L. Glucagon-like peptide-1-(9-36) amide is a major metabolite of glucagon-like peptide-1-(7-36) amide after in vivo administration to dogs, and it acts as an antagonist on the pancreatic receptor. *European journal of pharmacology*. 1996;318(2-3):429-35.
257. Mojsov S. Structural requirements for biological activity of glucagon-like peptide-I. *International journal of peptide and protein research*. 1992;40(3-4):333-43.
258. Kieffer TJ, Habener JF. The glucagon-like peptides. *Endocrine reviews*. 1999;20(6):876-913.
259. Thum A, Hupe-Sodmann K, Goke R, Voigt K, Goke B, McGregor GP. Endoproteolysis by isolated membrane peptidases reveal metabolic stability of glucagon-like peptide-1 analogs, exendins-3 and -4. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association*. 2002;110(3):113-8. Epub 2002/05/16.

260. Al-Sabah S, Donnelly D. A model for receptor-peptide binding at the glucagon-like peptide-1 (GLP-1) receptor through the analysis of truncated ligands and receptors. *British journal of pharmacology*. 2003;140(2):339-46. Epub 2003/09/13.
261. Runge S, Schimmer S, Oschmann J, Schiodt CB, Knudsen SM, Jeppesen CB, et al. Differential structural properties of GLP-1 and exendin-4 determine their relative affinity for the GLP-1 receptor N-terminal extracellular domain. *Biochemistry*. 2007;46(19):5830-40.
262. Unson CG, Wu CR, Fitzpatrick KJ, Merrifield RB. Multiple-site replacement analogs of glucagon. A molecular basis for antagonist design. *The Journal of biological chemistry*. 1994;269(17):12548-51. Epub 1994/04/29.
263. Drucker DJ. Minireview: the glucagon-like peptides. *Endocrinology*. 2001;142(2):521-7. Epub 2001/02/13.
264. Druce MR, Minnion JS, Field BC, Patel SR, Shillito JC, Tilby M, et al. Investigation of structure-activity relationships of Oxyntomodulin (Oxm) using Oxm analogs. *Endocrinology*. 2009;150(4):1712-22. Epub 2008/12/17.
265. Jorgensen R, Kubale V, Vrecl M, Schwartz TW, Elling CE. Oxyntomodulin differentially affects glucagon-like peptide-1 receptor beta-arrestin recruitment and signaling through Galpha(s). *The Journal of pharmacology and experimental therapeutics*. 2007;322(1):148-54.
266. Koole C, Wootten D, Simms J, Valant C, Sridhar R, Woodman OL, et al. Allosteric ligands of the glucagon-like peptide 1 receptor (GLP-1R) differentially modulate endogenous and exogenous peptide responses in a pathway-selective manner: implications for drug screening. *Molecular pharmacology*. 2010;78(3):456-65.
267. Chaudhri OB, Parkinson JR, Kuo YT, Druce MR, Herlihy AH, Bell JD, et al. Differential hypothalamic neuronal activation following peripheral injection of GLP-1 and oxyntomodulin in mice detected by manganese-enhanced magnetic resonance imaging. *Biochemical and biophysical research communications*. 2006;350(2):298-306.
268. Parkinson JR, Chaudhri OB, Kuo YT, Field BC, Herlihy AH, Dhillo WS, et al. Differential patterns of neuronal activation in the brainstem and hypothalamus following peripheral injection of GLP-1, oxyntomodulin and lithium chloride in mice detected by manganese-enhanced magnetic resonance imaging (MEMRI). *NeuroImage*. 2009;44(3):1022-31.
269. Pocai A. Unraveling oxyntomodulin, GLP1's enigmatic brother. *The Journal of endocrinology*. 2012;215(3):335-46.
270. Du X, Kosinski JR, Lao J, Shen X, Petrov A, Chicchi GG, et al. Differential effects of oxyntomodulin and GLP-1 on glucose metabolism. *American journal of physiology Endocrinology and metabolism*. 2012;303(2):E265-71.

271. Kosinski JR, Hubert J, Carrington PE, Chicchi GG, Mu J, Miller C, et al. The glucagon receptor is involved in mediating the body weight-lowering effects of oxyntomodulin. *Obesity* (Silver Spring). 2012;20(8):1566-71. Epub 2012/03/17.
272. Schulman JL, Carleton JL, Whitney G, Whitehorn JC. Effect of glucagon on food intake and body weight in man. *J Appl Physiol*. 1957;11(3):419-21.
273. Salter JM, Ezrin C, Laidlaw JC, Gornall AG. Metabolic effects of glucagon in human subjects. *Metabolism: clinical and experimental*. 1960;9:753-68.
274. Penick SB, Hinkle LE, Jr. Depression of food intake induced in healthy subjects by glucagon. *The New England journal of medicine*. 1961;264:893-7.
275. Langhans W, Zeiger U, Scharrer E, Geary N. Stimulation of feeding in rats by intraperitoneal injection of antibodies to glucagon. *Science*. 1982;218(4575):894-6.
276. Habegger KM, Heppner KM, Geary N, Bartness TJ, DiMarchi R, Tschop MH. The metabolic actions of glucagon revisited. *Nature reviews Endocrinology*. 2010;6(12):689-97.
277. Biedzinski TM, Bataille D, Devaux MA, Sarles H. The effect of oxyntomodulin (glucagon-37) and glucagon on exocrine pancreatic secretion in the conscious rat. *Peptides*. 1987;8(6):967-72. Epub 1987/11/01.
278. Parlevliet ET, Heijboer AC, Schroder-van der Elst JP, Havekes LM, Romijn JA, Pijl H, et al. Oxyntomodulin ameliorates glucose intolerance in mice fed a high-fat diet. *American journal of physiology Endocrinology and metabolism*. 2008;294(1):E142-7. Epub 2007/11/01.
279. Shankar S.S. SRR, Mixson L., Pramanik B.S., Stoch A., Steinberg H.O. Oxyntomodulin has significant acute glucoregulatory effects comparable to liraglutide in subjects with type 2 diabetes. *Diabetes*. 2013;62(Suppl. 1):A48.
280. Troke RC, Tan TM, Bloom SR. The future role of gut hormones in the treatment of obesity. *Ther Adv Chronic Dis*. 2014;5(1):4-14. Epub 2014/01/02.
281. Pournaras DJ, Osborne A, Hawkins SC, Mahon D, Ghatei MA, Bloom SR, et al. The gut hormone response following Roux-en-Y gastric bypass: cross-sectional and prospective study. *Obesity surgery*. 2010;20(1):56-60. Epub 2009/10/15.
282. Hruby VJ, Krstenansky J, Gysin B, Pelton JT, Trivedi D, McKee RL. Conformational considerations in the design of glucagon agonists and antagonists: examination using synthetic analogs. *Biopolymers*. 1986;25 Suppl:S135-55. Epub 1986/01/01.
283. Hussin AH, Allan CJ, Hruby VJ, Skett P. The effects of glucagon and TH-glucagon on steroid metabolism in isolated rat hepatocytes. *Molecular and cellular endocrinology*. 1988;55(2-3):203-7. Epub 1988/02/01.

284. Kim YH, Berry AH, Spencer DS, Stites WE. Comparing the effect on protein stability of methionine oxidation versus mutagenesis: steps toward engineering oxidative resistance in proteins. *Protein Eng.* 2001;14(5):343-7. Epub 2001/07/05.
285. Murphy WA, Coy DH, Lance VA. Superactive amidated COOH-terminal glucagon analogues with no methionine or tryptophan. *Peptides.* 1986;7 Suppl 1:69-74. Epub 1986/01/01.
286. Moon MJ, Park S, Kim DK, Cho EB, Hwang JI, Vaudry H, et al. Structural and molecular conservation of glucagon-like Peptide-1 and its receptor confers selective ligand-receptor interaction. *Frontiers in endocrinology.* 2012;3:141. Epub 2012/11/28.
287. McCance RA, Widdowson EM. The absorption and excretion of zinc. *The Biochemical journal.* 1942;36(7-9):692-6. Epub 1942/09/01.
288. Maret W. Zinc coordination environments in proteins determine zinc functions. *J Trace Elem Med Biol.* 2005;19(1):7-12. Epub 2005/10/26.
289. Berg JM, Godwin HA. Lessons from zinc-binding peptides. *Annu Rev Biophys Biomol Struct.* 1997;26:357-71. Epub 1997/01/01.
290. Gavrilova J, Tougu V, Palumaa P. Affinity of zinc and copper ions for insulin monomers. *Metallomics.* 2014;6(7):1296-300. Epub 2014/06/04.
291. Hareter A, Hoffmann E, Bode HP, Goke B, Goke R. The positive charge of the imidazole side chain of histidine7 is crucial for GLP-1 action. *Endocrine journal.* 1997;44(5):701-5. Epub 1998/02/18.
292. Sarkar B, Wigfield Y. The structure of copper(II)-histidine chelate. The question of the involvement of the imidazole group. *The Journal of biological chemistry.* 1967;242(23):5572-7. Epub 1967/12/10.
293. Leader B, Baca QJ, Golan DE. Protein therapeutics: a summary and pharmacological classification. *Nature reviews Drug discovery.* 2008;7(1):21-39.
294. Duncan R. The dawning era of polymer therapeutics. *Nat Rev Drug Discov.* 2003;2(5):347-60. Epub 2003/05/17.
295. Hermeling S, Crommelin DJ, Schellekens H, Jiskoot W. Structure-immunogenicity relationships of therapeutic proteins. *Pharmaceutical research.* 2004;21(6):897-903.
296. Schellekens H. Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clinical therapeutics.* 2002;24(11):1720-40; discussion 19.
297. Mentlein R. Cell-surface peptidases. *International review of cytology.* 2004;235:165-213. Epub 2004/06/29.
298. Mentlein R, Lucius R. Methods for the investigation of neuropeptide catabolism and stability in vitro. *Brain research Brain research protocols.* 1997;1(3):237-46. Epub 1997/08/01.

299. Wood WG, Wachter C, Scriba PC. Experiences using chloramine-T and 1, 3, 4, 6-tetrachloro-3 alpha, 6 alpha-diphenylglycoluril (Iodogen) for radioiodination of materials for radioimmunoassay. *Journal of clinical chemistry and clinical biochemistry Zeitschrift fur klinische Chemie und klinische Biochemie*. 1981;19(10):1051-6.
300. Blache P, Kervran A, Bataille D. Oxyntomodulin and glicentin: brain-gut peptides in the rat. *Endocrinology*. 1988;123(6):2782-7.
301. Shardlow C, Generaux G, Patel A, Tai G, Tran T, Bloomer J. Impact of Physiologically-based Pharmacokinetic Modelling and Simulation in Drug Development. *Drug Metab Dispos*. 2013.
302. Theil FP, Guentert TW, Haddad S, Poulin P. Utility of physiologically based pharmacokinetic models to drug development and rational drug discovery candidate selection. *Toxicology letters*. 2003;138(1-2):29-49.
303. Frei P, Dev KK. Drug dealers: \$20 trillion of in-licensing payments. *Drug discovery today*. 2013.
304. Benet LZ. Pharmacokinetic parameters: which are necessary to define a drug substance? *European journal of respiratory diseases Supplement*. 1984;134:45-61. Epub 1984/01/01.
305. Ruiz-Garcia A, Bermejo M, Moss A, Casabo VG. Pharmacokinetics in drug discovery. *Journal of pharmaceutical sciences*. 2008;97(2):654-90. Epub 2007/07/17.
306. Ritschel WA, Banerjee PS. Physiological pharmacokinetic models: principles, applications, limitations and outlook. *Methods and findings in experimental and clinical pharmacology*. 1986;8(10):603-14. Epub 1986/10/01.
307. Pfeifer HJ, Greenblatt DJ. An introduction to clinical pharmacokinetics. *Comprehensive therapy*. 1979;5(3):33-7. Epub 1979/03/01.
308. Derendorf H, Meibohm B. Modeling of pharmacokinetic/pharmacodynamic (PK/PD) relationships: concepts and perspectives. *Pharmaceutical research*. 1999;16(2):176-85. Epub 1999/04/01.
309. Bellissant E, Sebillé V, Paintaud G. Methodological issues in pharmacokinetic-pharmacodynamic modelling. *Clinical pharmacokinetics*. 1998;35(2):151-66. Epub 1998/09/18.
310. Fitzgerald M. Principles of pharmacokinetics. *Journal of the American Academy of Nurse Practitioners*. 1994;6(12):581-3. Epub 1994/12/01.
311. Takimoto CH. Basic pharmacokinetics and pharmacodynamic principles. *Cancer treatment and research*. 2001;106:85-101. Epub 2001/02/28.
312. Statistics HO. *Statistics of Scientific Procedures on Living Animals* UK 2011
313. Migdalof BH. Methods for obtaining drug time course data from individual small laboratory animals: serial microblood sampling and assay. *Drug metabolism reviews*. 1976;5(2):295-310.

314. Casal M, Haskins M. Large animal models and gene therapy. *European journal of human genetics : EJHG*. 2006;14(3):266-72.
315. Martignoni M, Groothuis GM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert opinion on drug metabolism & toxicology*. 2006;2(6):875-94.
316. Poggesi I. Predicting human pharmacokinetics from preclinical data. *Current opinion in drug discovery & development*. 2004;7(1):100-11.
317. Bennett AF. Allometry: scaling. *Science*. 1984;226(4681):1412-3.
318. Boxenbaum H. Interspecies scaling, allometry, physiological time, and the ground plan of pharmacokinetics. *Journal of pharmacokinetics and biopharmaceutics*. 1982;10(2):201-27.
319. Dedrick RL. Animal scale-up. *Journal of pharmacokinetics and biopharmaceutics*. 1973;1(5):435-61.
320. Visser SA, Smulders CJ, Reijers BP, Van der Graaf PH, Peletier LA, Danhof M. Mechanism-based pharmacokinetic-pharmacodynamic modeling of concentration-dependent hysteresis and biphasic electroencephalogram effects of alphaxalone in rats. *J Pharmacol Exp Ther*. 2002;302(3):1158-67.
321. Rippe A, Rippe C, Sward K, Rippe B. Disproportionally low clearance of macromolecules from the plasma to the peritoneal cavity in a mouse model of peritoneal dialysis. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 2007;22(1):88-95.
322. Kervran A, Dubrasquet M, Blache P, Martinez J, Bataille D. Metabolic clearance rates of oxyntomodulin and glucagon in the rat: contribution of the kidney. *Regulatory peptides*. 1990;31(1):41-52.
323. Oshima I, Hirota M, Ohboshi C, Shima K. Comparison of half-disappearance times, distribution volumes and metabolic clearance rates of exogenous glucagon-like peptide 1 and glucagon in rats. *Regulatory peptides*. 1988;21(1-2):85-93. Epub 1988/05/01.
324. Chiou WL. The phenomenon and rationale of marked dependence of drug concentration on blood sampling site. Implications in pharmacokinetics, pharmacodynamics, toxicology and therapeutics (Part II). *Clinical pharmacokinetics*. 1989;17(4):275-90.
325. Thrivikraman KV, Huot RL, Plotsky PM. Jugular vein catheterization for repeated blood sampling in the unrestrained conscious rat. *Brain research Brain research protocols*. 2002;10(2):84-94.
326. Benet LZ, Zia-Amirhosseini P. Basic principles of pharmacokinetics. *Toxicologic pathology*. 1995;23(2):115-23.

327. Tait JF. Review: The Use of Isotopic Steroids for the Measurement of Production Rates in Vivo. *The Journal of clinical endocrinology and metabolism*. 1963;23:1285-97.
328. Slordal L, Spigset O. [Basic pharmacokinetics--distribution]. *Tidsskrift for den Norske laegeforening : tidsskrift for praktisk medicin, ny raekke*. 2005;125(8):1007-8. Grunnleggende farmakokinetikk--distribusjon.
329. Tozer TN. Concepts basic to pharmacokinetics. *Pharmacology & therapeutics*. 1981;12(1):109-31.
330. Nightingale CH, Carver P. Basic principles of pharmacokinetics. *Clinics in laboratory medicine*. 1987;7(2):267-78.
331. Parkes D JC, Smith P, Nayak S, Rinehart L, Gingerich R, Chen K, Young A. Pharmacokinetic Actions of Exendin-4 in the Rat: Comparison With Glucagon-Like Peptide. *DRUG DEVELOPMENT RESEARCH*. 2001;53:260-7.
332. Cao Y, Gao W, Jusko WJ. Pharmacokinetic/pharmacodynamic modeling of GLP-1 in healthy rats. *Pharmaceutical research*. 2012;29(4):1078-86. Epub 2011/12/20.
333. Gao W, Jusko WJ. Target-mediated pharmacokinetic and pharmacodynamic model of exendin-4 in rats, monkeys, and humans. *Drug Metab Dispos*. 2012;40(5):990-7. Epub 2012/02/18.
334. Gao W, Jusko WJ. Pharmacokinetic and pharmacodynamic modeling of exendin-4 in type 2 diabetic Goto-Kakizaki rats. *J Pharmacol Exp Ther*. 2011;336(3):881-90. Epub 2010/12/16.
335. Holt AW, Tulis DA. Experimental Rat and Mouse Carotid Artery Surgery: Injury & Remodeling Studies. *ISRN minimally invasive surgery*. 2013;2013.
336. Nishihira T, Komatsu H, Endo Y, Shineha R, Sagawa J, Nakano T, et al. New technology for continuous intravenous infusion via the central and portal veins in the rat. *The Tohoku journal of experimental medicine*. 1994;173(2):275-82.
337. Deacon CF, Johnsen AH, Holst JJ. Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo. *The Journal of clinical endocrinology and metabolism*. 1995;80(3):952-7.
338. Mentlein R, Gallwitz B, Schmidt WE. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *European journal of biochemistry / FEBS*. 1993;214(3):829-35.
339. Parkes D, Jodka, C., Smith, P., Nayak, S., Rinehart, L., Gingerich, R., Chen, K. and Young, A. . Pharmacokinetic actions of exendin-4 in the rat: Comparison with glucagon-like peptide-1. . *Drug Dev Res*. 2001;53:260-7.
340. Emmanouel DS, Jaspan JB, Rubenstein AH, Huen AH, Fink E, Katz AI. Glucagon metabolism in the rat. *The Journal of clinical investigation*. 1978;62(1):6-13.

341. Schjoldager BT, Baldissera FG, Mortensen PE, Holst JJ, Christiansen J. Oxyntomodulin: a potential hormone from the distal gut. Pharmacokinetics and effects on gastric acid and insulin secretion in man. *European journal of clinical investigation*. 1988;18(5):499-503.
342. Zhou L, Li S, Su Y, Yi X, Zheng A, Deng F. Interaction between histidine and Zn(II) metal ions over a wide pH as revealed by solid-state NMR spectroscopy and DFT calculations. *J Phys Chem B*. 2013;117(30):8954-65. Epub 2013/07/12.
343. Kenakin TP. Cellular assays as portals to seven-transmembrane receptor-based drug discovery. *Nature reviews Drug discovery*. 2009;8(8):617-26. Epub 2009/07/18.
344. Day JW, Ottaway N, Patterson JT, Gelfanov V, Smiley D, Gidda J, et al. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nature chemical biology*. 2009;5(10):749-57. Epub 2009/07/15.
345. Vilsboll T, Christensen M, Junker AE, Knop FK, Gluud LL. Effects of glucagon-like peptide-1 receptor agonists on weight loss: systematic review and meta-analyses of randomised controlled trials. *BMJ*. 2012;344:d7771. Epub 2012/01/13.
346. Pauling L, Corey RB, Branson HR. The structure of proteins; two hydrogen-bonded helical configurations of the polypeptide chain. *Proceedings of the National Academy of Sciences of the United States of America*. 1951;37(4):205-11. Epub 1951/04/01.
347. Aurora R, Rose GD. Helix capping. *Protein science : a publication of the Protein Society*. 1998;7(1):21-38. Epub 1998/03/26.
348. Richardson JS, Richardson DC. Amino acid preferences for specific locations at the ends of alpha helices. *Science*. 1988;240(4859):1648-52. Epub 1988/06/17.
349. Lyu PC, Liff MI, Marky LA, Kallenbach NR. Side chain contributions to the stability of alpha-helical structure in peptides. *Science*. 1990;250(4981):669-73. Epub 1990/11/02.
350. Sukumar M, Gierasch LM. Local interactions in a Schellman motif dictate interhelical arrangement in a protein fragment. *Folding & design*. 1997;2(4):211-22. Epub 1997/01/01.
351. Chakrabartty A, Kortemme T, Baldwin RL. Helix propensities of the amino acids measured in alanine-based peptides without helix-stabilizing side-chain interactions. *Protein science : a publication of the Protein Society*. 1994;3(5):843-52. Epub 1994/05/01.
352. Doig AJ, Baldwin RL. N- and C-capping preferences for all 20 amino acids in alpha-helical peptides. *Protein science : a publication of the Protein Society*. 1995;4(7):1325-36. Epub 1995/07/01.
353. Kirkpatrick A, Heo J, Abrol R, Goddard WA, 3rd. Predicted structure of agonist-bound glucagon-like peptide 1 receptor, a class B G protein-coupled receptor. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109(49):19988-93. Epub 2012/11/22.

354. Pocai A, Carrington PE, Adams JR, Wright M, Eiermann G, Zhu L, et al. Glucagon-like peptide 1/glucagon receptor dual agonism reverses obesity in mice. *Diabetes*. 2009;58(10):2258-66. Epub 2009/07/16.
355. Takacs BJ, Girard MF. Preparation of clinical grade proteins produced by recombinant DNA technologies. *Journal of immunological methods*. 1991;143(2):231-40. Epub 1991/10/25.
356. Kanoski SE, Rupprecht LE, Fortin SM, De Jonghe BC, Hayes MR. The role of nausea in food intake and body weight suppression by peripheral GLP-1 receptor agonists, exendin-4 and liraglutide. *Neuropharmacology*. 2012;62(5-6):1916-27. Epub 2012/01/10.
357. Sanger GJ, Andrews PL. Treatment of nausea and vomiting: gaps in our knowledge. *Autonomic neuroscience : basic & clinical*. 2006;129(1-2):3-16. Epub 2006/08/29.
358. Elashoff M, Matveyenko AV, Gier B, Elashoff R, Butler PC. Pancreatitis, pancreatic, and thyroid cancer with glucagon-like peptide-1-based therapies. *Gastroenterology*. 2011;141(1):150-6. Epub 2011/02/22.
359. Torgerson JS, Lindroos AK, Naslund I, Peltonen M. Gallstones, gallbladder disease, and pancreatitis: cross-sectional and 2-year data from the Swedish Obese Subjects (SOS) and SOS reference studies. *The American journal of gastroenterology*. 2003;98(5):1032-41. Epub 2003/06/18.
360. Pi-Sunyer X, Astrup A, Fujioka K, Greenway F, Halpern A, Krempf M, et al. A Randomized, Controlled Trial of 3.0 mg of Liraglutide in Weight Management. *New England Journal of Medicine*. 2015;373(1):11-22.
361. Egan AG, Blind E, Dunder K, de Graeff PA, Hummer BT, Bourcier T, et al. Pancreatic safety of incretin-based drugs--FDA and EMA assessment. *The New England journal of medicine*. 2014;370(9):794-7. Epub 2014/02/28.
362. McKay NJ, Kanoski SE, Hayes MR, Daniels D. Glucagon-like peptide-1 receptor agonists suppress water intake independent of effects on food intake. *American journal of physiology Regulatory, integrative and comparative physiology*. 2011;301(6):R1755-64. Epub 2011/10/07.
363. Gutzwiller JP, Hruz P, Huber AR, Hamel C, Zehnder C, Drewe J, et al. Glucagon-like peptide-1 is involved in sodium and water homeostasis in humans. *Digestion*. 2006;73(2-3):142-50. Epub 2006/07/01.
364. Smith DA, Jones BC, Walker DK. Design of drugs involving the concepts and theories of drug metabolism and pharmacokinetics. *Medicinal research reviews*. 1996;16(3):243-66. Epub 1996/05/01.
365. Mahmood I. Interspecies scaling: predicting volumes, mean residence time and elimination half-life. Some suggestions. *The Journal of pharmacy and pharmacology*. 1998;50(5):493-9. Epub 1998/06/27.

366. Caldwell GW, Masucci JA, Yan Z, Hageman W. Allometric scaling of pharmacokinetic parameters in drug discovery: can human CL, Vss and t_{1/2} be predicted from in-vivo rat data? European journal of drug metabolism and pharmacokinetics. 2004;29(2):133-43. Epub 2004/07/03.