

Investigation of the pancreatic safety of proglucagon-derived peptides and the development of novel anti-obesity agents

A thesis submitted for the degree of Doctor of Philosophy from
Imperial College London

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Abstract

GLP-1 is a gut peptide that promotes satiety and insulin secretion. Its agonists are currently licensed and used as a type 2 diabetes treatment. Glucagon is best known for opposing the actions of insulin to increase blood glucose levels in response to hypoglycaemia. Glucagon also increases energy expenditure, but the mechanism for this is poorly understood. A study in humans suggested that co administration of GLP-1 and glucagon can increase appetite inhibition and energy expenditure to reduce body weight, while avoiding glucose excursions. This observation promoted the idea that co administration of the two peptides could act as an anti-obesity agent. However, concerns regarding the safety of GLP-1 receptor agonists have been raised.

Patients taking GLP-1 receptor agonists have reported a higher incidence of pancreatitis and the proposed mechanism is elevated pancreatic ductal replication and increased amylase secretion from acinar cells. However, little is known about the acute effect of GLP-1 and glucagon receptor agonists on the exocrine pancreas. Work in this thesis investigates if GLP-1, glucagon, oxyntomodulin, and exendin-4 could cause pancreatitis. In the studies described, treatment with the peptides did not cause a significant increase in proliferation of biliopancreatic ductal cells or amylase secretion *in vitro* or *in vivo* in rats. Results did not support the proposed link of GLP-1 receptor agonists to pancreatitis, in rat.

A second aim of this thesis was to develop novel analogues of GLP-1 and glucagon with improved efficacy and with enhanced pharmacokinetic profiles to be co administered as a treatment of obesity. Substitution of 14 amino acids in the GLP-1 peptide resulted in an analogue with similar efficacy as GLP-1, as determined by *in vitro* bioactivity, yet a terminal half-life of approximately 7 days. Substitution of residues His²⁰ and Leu²⁷ and addition of a histidine tail at the C-terminal of glucagon resulted in an analogue with almost 2-fold higher efficacy than glucagon, and a terminal half-life of 3 days. Co administration of the two peptides *in vivo* demonstrated a reduction in body weight and increased glucose tolerance, an effect suggested being synergistic when compared to individual agent administration groups.

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, 4 and 5:

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

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Acknowledgements

I would like to thank Professor Steve Bloom for giving me the opportunity to carry out this work and for his supervision and ideas.

Special thanks to Dr James Minnion for his supervision, guidance and advice through the past few years. I would also like to acknowledge the 'G-Series' group in all its different shapes and sizes.

I am incredibly grateful to my family and friends for their support and encouragement throughout my time as a student.

I finally wish to thank all the people I met through the past two years who made this journey worthwhile.

If you can't find spring, then create it.

Odysseus Elytis

Abbreviations

AgRP	Agouti-related peptide
ANOVA	Analysis of variance
BAT	Brown adipose tissue
BDNF	Brain-derived neurotrophic factor
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CCK	Cholecystokinin
CHO	Chinese Hamster Ovary
CNS	Central nervous system
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary
DIO	Diet Induced Obesity
DPP-IV	Dipeptidyl peptidase 4
DMSO	Dimethylsulphoxide
DMEM	Dulbecco's modified Eagle medium
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbant assay
FBS	Foetal Bovine serum
FDA	Food and drug administration (US)
FMOC	Fluorenylmethyloxycarbonyl
GRF	Growth –hormone release factor
GCG	Glucagon
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
GIP	Gastric inhibitory polypeptide
GRP	Gastrin-releasing peptide
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
ICV	Intracerebroventricular
IEGs	Immediate Early Genes
IP	Intraperitoneal
IV	Intravenous
LCDs	Low Calorie Diets
LHA	Lateral hypothalamic area
LLOQs	Lower Limit of Quantification

α-MSH	alpha-melanocortin-stimulating hormone
MCH	Melanin-concentrating hormone
NEAAs	Non-essential amino acids
NPY	Neuropeptide-Y
NTS	Nucleus of the solitary tract
OXM	Oxyntomodulin
PAL	Physical Activity Levels
PanINs	Pancreatic intra-epithelial neoplasia
PYY	Peptide-tyrosine-tyrosine
PP	Pancreatic polypeptide
PVN	Paraventricular nucleus
POMC	Pro-opiomelanocortin
PBS	Phosphate buffered saline
PMSF	Phenylmethanesulphonylfluoride
PKA	Protein kinase A
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
SC	Subcutaneous
SEM	Standard error of the mean
SNS	Sympathetic nervous system
SPA	Spontaneous Physical Activity
TRH	Thyroid Releasing Hormone
UCP-1	Uncoupling protein-1
VLCDs	Very Low Calorie Diets
VMN	Ventromedial nucleus

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CHAPTER 1

General Introduction

1.1. Obesity and its management

Body weight is a balance between food intake and energy expenditure. Food intake and energy expenditure are set in a way so that this balance is maintained, yet a long-term change in either way can result in a change in body weight.

Obesity is defined as Body Mass Index (BMI) of over 30 kg/ m² and a high degree of fat accumulation. It is mainly a result of an imbalance between calories consumed and calories expended and is the causative factor of a number of different health impairments. In 2011 in England, approximately a quarter of the population were obese, and it has been predicted that from 2015 this will cost the National Health System £6.4 billion per year (1), as a result of the wide range of health problems it can cause, such as Type 2 Diabetes, cardiovascular diseases, high blood pressure, cancer, sleep apnoea and infertility. It has therefore been recognised as global epidemic, subsequently a number of treatments to combat obesity have become available over the past two decades.

Dietary interventions, physical activity and cognitive behavioural therapy, individually or in combination, have shown to cause a small reduction of weight loss that can be difficult to maintain. A number of diets, including Liquid Meal Replacements, Low Calorie Diets (LCDs) and Very Low Calorie Diets (VLCDs) are used in order to reduce daily food intake and create a negative energy balance (2). Physical activity is also prompt to tilt the energy balance by increasing the energy expenditure, but it is less effective when not in combination with dietary intervention (3). Finally, behavioural therapy, delivered usually in groups of participants, improves the dedication of the patients to their weight loss goals (4, 5). , a number of studies suggest that weight regain is observed post therapy (6, 7).

A wide range of anti-obesity medication treatments has become available over the past decade, yet at the moment only orlistat (Xenical) is approved by the US Food and Drug Administration (FDA). Orlistat is a gastric lipase inhibitor that reduces intestinal fat absorption by inhibiting pancreatic lipase, therefore it does not have an effect on food intake. It has been shown to decrease body weight by 2% in combination with diet and exercise but its gastrointestinal side effects include steatorrhea and urgent bowel movements. (8, 9). So far, pharmacological agents appear to aid maintenance of weight loss

rather than inducing it. Nevertheless, a new pharmacological agent, known as Saxenda, has recently won the approval of US FDA. Saxenda is an anorectic long lasting analogue of the native peptide Glucagon-Like Peptide 1 (GLP-1), currently administered in a lower dose as a Type 2 Diabetes Mellitus treatment, which aspires to induce weight loss through reduction of appetite and delayed gastric emptying (10).

To date, the most effective treatment for obesity is bariatric surgery, which includes gastric banding, sleeve gastrectomy and different forms of gastric bypass. Bariatric surgery and especially Roux-En-Y gastric bypass, can cause over 75% weight loss within the first 24 months post-operation (11). Nonetheless, high-cost and trained surgeon availability, as well as risks of morbidity and mortality and a need for life-long supplements, have limited this option to patients with a BMI higher than 35kg/ m² (12).

1.2. Energy Homeostasis

Energy balance is a tightly regulated process, achieved through a number of chemical and neural signals which control caloric intake by signalling the brain to regulate the sensation of hunger, in addition to regulating energy flow in response to the organism needs, such as heat. As a balance, energy intake must match the amount of energy expended in order to maintain the appropriate body weight.

Energy expenditure is the sum of physical activity and internal heat. Physical activity includes both the voluntary external work, known as Physical Activity Levels (PAL) and non-voluntary activity, known as Spontaneous Physical Activity (SPA). SPA includes functions such as muscle contractions and posture maintenance which have been linked to the sympathetic nervous system (SNS), especially through the neuropeptide orexin (13, 14). SPA has also been shown to protect against fat mass gain in rodents (15).

Internal heat includes both the thermic effect of food and basal thermogenesis. The thermic effect of food is the energy used in digestion, absorption and distribution of nutrients in the body, as well as their storage (16). Basal thermogenesis is the energy expended to maintain

the organism's temperature and is sufficient only for the basic functioning of the vital organs through maintaining cellular integrity, enzyme activity and protein production. It accounts for approximately 60% of the energy expended daily (17).

A major mediator of basal thermogenesis is the Brown Adipose Tissue (BAT). BAT thermogenesis takes place in numerous, densely-packed mitochondria containing a BAT-specific inner membrane protein UCP-1, in contrast to muscle, which generates heat by reactions within its cytoplasm. UCP-1 activation uncouples aerobic respiration by disintegrating the intermembrane proton-motive force, creating a cycle of ions. This results in generation of heat, instead of the final product of ATP (18). As UCP-1 is only expressed in BAT, it is the primary mediator of thermogenesis (19).

BAT deposits have been observed in small mammals and in new-born humans, usually between the scapulae. Rodent studies pointed that the role of BAT is primarily to increase heat generation in response to cold through shivering, but also to decrease adiposity by increasing energy expenditure (20). Its activation has been therefore investigated by a number of researchers as it could be a mediator of weight loss.

1.3. Food intake regulation

Food intake is regulated by a number of hormones in response to nutrient sensing. These hormones are primarily released from peripheral tissues in response to nutrient detection, but can act both peripherally and through the Central Nervous System (CNS) in order to regulate food intake. Therefore, different pathways are activated upon hormonal binding on receptors expressed on different organs.

1.3.1. Central Control

Regulation of energy intake is highly controlled by specific areas of the brain. In order to do this, the brain receives a number of signals that can either be short-term or long-term and can cause stimulation or inhibition of appetite (Figure 1.1.). Short-term signals include satiety signals from the gastro-intestinal tract in response to food or drink consumed. Different nutrients are sensed once they reach the intestine, and a number of hormones are released in response to induce satiety. Long-term signals are sent in response to the overall proportion of body fat. These are called adiposity signals and mainly include leptin, secreted from the adipose tissue, and insulin, secreted from the endocrine pancreas (21).

These signals are a vital part of the homeostatic system of food intake, as they regulate the needs for nutrients according to the existent energy stores. Nonetheless, obesity is a primary example that this is not the only system controlling appetite. The hedonic system senses the taste and smell of a food, and can increase the homeostatic mechanism by inducing the need to consume more highly palatable food types. The exact interaction between the homeostatic and hedonic systems is still unclear, yet appear to function in a complementary way (22).

1.3.1.1. Hypothalamus and nuclei interactions

During the past decade, our understanding of how the hypothalamus regulates food intake has increased. Numerous studies have pointed at environmental, genetic and hormonal factors affecting hypothalamic signalling and increasing or inhibiting appetite. These factors compose an interconnected network of pathways elaborating and signalling neurons. Therefore, neurons produce orexigenic and anorectic signals, mainly depending on the concentration of hormonal and other biologically active compounds in the extracellular fluid surrounding them. Specific hypothalamic nuclei, linked to neural appetite regulating mechanisms, are recognised as vital for energy homeostasis maintenance. These nuclei include the arcuate nucleus, paraventricular nucleus, ventromedial nucleus, dorsomedial nucleus and lateral hypothalamic area (23, 24).

The arcuate nucleus is located at the base of the hypothalamus. Its role in appetite regulation was clearly demonstrated when mice with hypothalamic lesions developed an obese phenotype (25). Its position is of vital importance, as it is in direct communication with peripherally secreted hormones such as leptin and insulin due to the lack of insulation from the blood-brain barrier. It has a prominent role in appetite regulation, as it contains two major neuron populations. The first population stimulates appetite by co-expressing neuropeptide Y (NPY) and anorectic agouti-related protein (AgRP) (26). The second population expresses the cocaine- and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC), which inhibit appetite (27). Both neuronal populations form projections to different parts of the hypothalamus, such as the closely linked paraventricular nucleus and the lateral hypothalamic area (27). A prominent example of this is the projections of NPY/AgRP neurons to the paraventricular nucleus neurons, which contain thyrotrophin releasing hormone (TRH), a hormone shown to increase energy expenditure and reduce appetite (28).

The paraventricular nucleus is a crucial site for the action of orexigenic signals. It has also been demonstrated to express receptors for the majority of neuropeptides, such as NPY, orexin-A and ghrelin, as microinjecting the nucleus with each one of the peptides caused an increase in food intake. Moreover, leptin and GLP-1 cause a decrease of appetite, potentially pointing at the expression of their receptors as well (29, 30). Notably, the paraventricular nucleus is the only area of the hypothalamus in which release of the most potent orexigenic signal, known as NPY, was increased both when investigated *in vivo* and *in vitro* (31, 32).

Similar to the paraventricular nucleus, the lateral hypothalamic area also receives NPY/AgRP, in addition to melanin concentrating hormone (MCH), and has neuron projections that come in direct contact with orexigenic signalling cells. In addition, MCH receptor knockout mice were shown to have increased energy expenditure and did not appear to have an obese phenotype after a high-fat diet was introduced to them. Unlike the arcuate nucleus, lesions in the LH produced temporary aphagia, adipsia, and loss in body weight (25, 33). Lateral hypothalamic electrical stimulation either inhibited the action of anorexigenic signals or activated those orexigenic pathways, pointing at the fact that it acts as a feeding centre (34, 35).

The ventromedial nucleus also includes NPY/AgrP neuronal projections from the arcuate nucleus. This could be the reason why lesions on the ventromedial nucleus cause hyperphagia, indicating that, along with the arcuate nucleus, it could be functioning as a satiety centre (25). Even though the ventromedial nucleus does not appear to produce any orexigenic or anorectic signals, it does express brain-derived neurotrophic factor (BDNF), which in large doses does reduce appetite (36). It also contains a large population of glucoreponsive neurons, which potentially points at a role in glucose homeostasis (37).

Like the ventromedial nucleus, lesions on the dorsomedial nucleus induce obesity and NPY neuronal projections from the arcuate nucleus are found in its structure (25). Moreover, microinjection of different orexigenic peptides cause increased appetite, pointing at the existence of individual receptors (29, 38-40). Nonetheless, unlike paraventricular and arcuate nuclei, NPY levels in the dorsomedial were not increased in response to fasting (41, 42).

1.3.1.2. Brainstem

The nucleus of the solitary tract (NTS) in the brainstem is the first site of the CNS that receives most gut and adipose tissue signalling in response to nutrient detection. This is potentially due to the fact that the signal transmission is mediated by the afferent vagus nerve, the end of which is on the NTS (43). In addition, the brainstem interacts with a number of hypothalamic sites that have an important part in the neural processing of nutrient derived signals and in stimulating food intake. Moreover, inhibitory peptides leptin and melanocortins appear to affect the neural activity and behavioural potency of the NTS to reduce appetite (44).

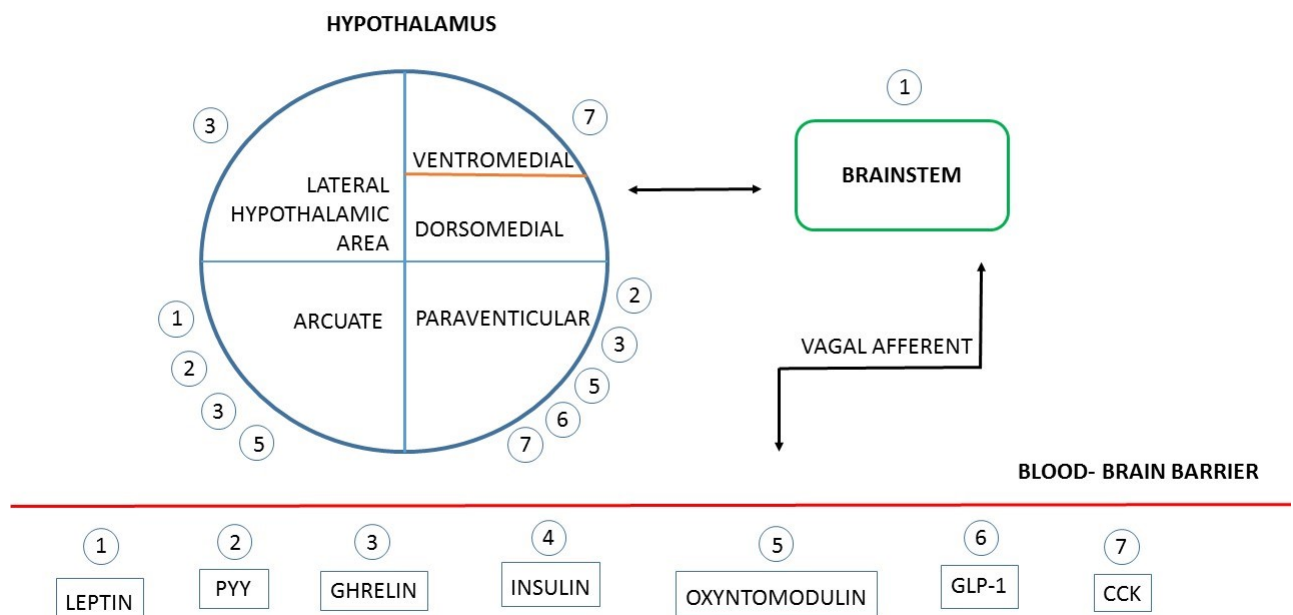


Figure 3.1. Brief overview summarizing appetite regulation through central and peripheral control. The nuclei in the hypothalamus and the peptides they interact with through the blood-brain barrier are presented. Hormones acting via vagal afferents act on the brainstem nuclei which then signals the hypothalamus.

1.4. Peripheral Control

As mentioned before, central control and peripheral control of food intake are two directly linked systems that depend on short term and long term signals. Therefore, peripheral control can be subdivided in two categories. The first category contains long-term signals that are released in response to overall adipose tissue accumulation of the organism, and include mainly the adipokines leptin and adiponectin, and pancreatic β -cell released insulin. The second category are short-term signals which include a higher number of peptides from the gut, the pancreas and the stomach that are released in response to a meal or the lack of a meal in the case of orexigenic ghrelin.

1.4.1. Long-term adiposity signals

One of the main long-term adiposity signal mediators is leptin. The human obese (OB) gene encoding leptin was identified in 1994 in chromosome 7 (45). Despite the fact that OB gene

mRNA is mainly found in human adipose tissue, leptin is also produced in small amounts in other human tissues such as the stomach, mammary epithelium, placenta and heart (46-48). It is released in proportion to adipose tissue energy stores and can decrease body weight both by reducing appetite and by increasing energy expenditure (49).

Leptin activates the leptin receptor, known as Ob-Rb, which is mainly expressed in the hypothalamus. After release by adipocytes, leptin crosses the blood-brain barrier and provides information about the status of adiposity, thereby decreasing body weight. As mentioned previously, the main areas of leptin action are the arcuate, the dorsomedial, the ventromedial nuclei and the lateral hypothalamic area, where orexigenic NPY/AgrP and anorectic POMC/ CART neurons are expressed (50).

Leptin was originally thought to be the answer to obesity, by assuming that a large exogenous injection could inhibit appetite and increase energy expenditure; however it was soon apparent that the majority of obese individuals had already high circulating levels of leptin, to which they were resistant. This occurred potentially due to lower levels of leptin transfer across the blood brain barrier, in addition to reduced activation of signalling in the leptin-functioning hypothalamic nuclei (51).

Another adipokine that is involved in long-term food intake control is adiponectin. It is gaining increasing interest for its insulin-sensitising, anti-inflammatory and anti-atherogenic effects and it is therefore directly linked to the levels of adiposity and glucose accumulation. Adiponectin is suggested to have a vital role in the regulation of glucose and lipid metabolism in humans. Interestingly, its levels appear to drop in obese and insulin-resistant humans and murine models (51, 52). This link between adiponectin, glucose lowering and insulin sensitivity made it a potential marker for metabolic syndrome. Energy expenditure increase is also a function partly credited to adiponectin, as its daily administration to high-fat fed mice did result in weight reduction, yet not food intake reduction (53).

Another vital long-term signalling peptide is insulin. Insulin is secreted from pancreatic β -cells in response to glucose detection in plasma in order to balance the concentration in circulation. It is therefore the most significant protein in maintaining glucose homeostasis. Despite the fact that insulin is acutely secreted in response to a meal, the levels secreted are also relative to the total body adiposity and, like leptin, has an anorectic effect.

Insulin controls appetite primarily through its action of the arcuate nucleus of the hypothalamus, where insulin receptors are located (54). The hypothalamic paraventricular nucleus and the lateral hypothalamic area are also involved in maintaining glucose homeostasis as they contain neurons that express receptors for both NPY and AgRP (55). Insulin not only reduces appetite, but also has a role in energy expenditure. Its importance to thermogenesis was shown in an *in vivo* study where insulin secretion was inhibited and thermogenesis was rapidly reduced following a meal high in carbohydrates (56). Moreover, injecting insulin into the hypothalamic ventromedial and paraventricular nuclei demonstrated an increase in energy expenditure and weight loss (57). As a result, it has been suggested that insulin has an effect on both the central nervous system through which it inhibits appetite, and also to brown adipose tissue as a mechanism for thermogenesis (58).

1.4.2. Short-term signals

The short-term adiposity signals are primarily secreted in response to a nutrient detection or absence. These signals are hormones released from a number of organs, mainly the gut and pancreas, and can be orexigenic or anorectic.

Ghrelin is one of the first orexigenic hormones discovered. Its secretion is vitally increased during fasting periods and decreases after food intake (59). It is secreted from the stomach but lower levels are also found in the gut. It is composed of 28 amino acids from which Ser3 is n-octanoylated. This is the first known case in mammals and it is vital for ghrelin's activity. Therefore, ghrelin is found in two forms in circulation: the n-octanoylated ghrelin, which is the active form, and the des- n-octanoylated form which is unmodified (60). As ghrelin stimulates growth hormone release, it functions through the growth hormone secretagogue receptor (GHS-R).

The slow rate at which stomach-secreted ghrelin passes through the blood-brain barrier forces peripheral ghrelin to activate specific hypothalamic regions through a different,

indirect pathway which involves the vagus nerve, specifically the nodose ganglion, where GHS-R receptors have been located. Therefore, vagotomy inhibits ghrelin action on appetite. Even so, the arcuate nucleus also expresses ghrelin-containing neurons. A number of studies have demonstrated that injecting it with ghrelin increases food intake and decreases energy expenditure (61-63). This is potentially because ghrelin-containing neurons send efferent fibers to NPY/ AgPR neurons to stimulate orexigenic signals, while inhibiting anorectic signals by activating POMC neurons (64).

Cholecystokinin (CCK) was the first peptide discovered to inhibit food intake. It is secreted after a meal by enteroendocrine cells (I cells) located in the mucosa of the duodenum, jejunum, and proximal ileum. It has numerous functions including pancreatic secretion, gastric emptying, gallbladder contraction and intestinal motility. It functions through two main receptors, CCK-1 and CCK-2, found on a number of organs. Its function on appetite inhibition is not completely understood, despite being discovered over 3 decades ago (65). The primary mechanism of its action involves CCK release in circulation in response to food reaching the small intestine. It binds on CCK-1 receptors found on the vagal afferents, which supply the duodenum, and a signal is transmitted to the NTS (66). Moreover, CCK activates the stomach CCK-1 receptors and inhibits gastric emptying. Another possible mechanism involves the peptides orexin A and B, secreted in the lateral hypothalamic area, which increase food consumption. CCK weakens the expression of pre-proorexin mRNA when hypoglycaemia, caused by an insulin level increase, is detected, thereby potentially pointing at a down regulation of orexigenic hormones in the hypothalamus being a mechanism for anorectic effect (67).

Three peptides known as the pancreatic polypeptide-fold family have a vital role in appetite control. These peptides function through G-coupled receptors, named Y1, Y2, Y4, Y5, and Y6, each one of which functions through the formation of the secondary messenger cyclic adenosine monophosphate (cAMP). Each peptide functions through a selection of organs and each have a different function. For example NPY is an orexigenic peptide secreted in various locations in the brain, including the hypothalamus.

In contrast, pancreatic polypeptide (PP), secreted from the islets of Langerhans, has an anorectic effect in response to a meal. PP secretion levels are proportional to the calorific

intake and can remain elevated for up to 6 hours with the vagus nerve being the primary stimulator (68). Apart from food intake control, it has an effect on gastrointestinal motility and metabolism. It also has a main action on the exocrine pancreas, where it inhibits vagal output in order to reduce pancreatic juice secretion (69, 70).

A potential part for PP as a satiety factor was linked with the observation that its levels are eliminated in obese children with Prader-Willi syndrome (71). In addition, an early study showed that morbidly obese patients have been observed to have low PP levels, while intravenous infusion of PP on healthy humans reduced their food intake acutely (72, 73). An *in vivo* study demonstrated that PP reduced body weight in obese ob/ ob mice (74). Subsequently, it was found that overexpressing PP chronically in transgenic mice reduced their body weight for over 6 months, as well as stimulating an increase in energy expenditure (75, 76). Moreover, studies showed that the effect of PP on food intake include the activation of Y4 receptors located in the hypothalamic nuclei. These receptors also mediate the action of Peptide tyrosine-tyrosine (PYY) through a different pathway (77).

PYY is also a member of the pancreatic polypeptide-fold family. It contains 36 amino acids and is produced by the L cells of the gut, with highest concentrations found in the large bowel and the rectum (78). PYY is found in circulation in two forms: its full 36-amino acid form and a 3-36 form caused by cleavage of its N-terminal by the enzyme dipeptidyl peptidase IV (DPP-IV). The cleaved form is primarily found in circulation post-prandially as it appears to have a higher affinity for the Y2 receptor, through which PYY functions (79). Like PP, PYY secretion is proportional to the calorific intake and can remain elevated for up to 6 hours post production. It has also been observed that release of PYY(3-36) is increased when food reaches in the distal gut and is predominantly elevated after a high fat diet consumption (80).

Studies on humans and rodents using intravenous PYY infusions caused a significant decrease in food intake, as well as increased thermogenesis, lipolysis, post-prandial insulin and glucose response (81-83). The effect of PYY on the CNS was initially investigated through single injections of the peptide peripherally, and investigation of the hypothalamic effect. It was shown that PYY caused an increase of expression of the immediate early gene c-fos in the arcuate nucleus, marking a neuronal activation and the local function of the

peptide. It also caused a decrease in expression of the orexigenic hypothalamic NPY mRNA. Following this, direct injection of PYY and Y2 receptor specific agonists directly into the arcuate nucleus of wild type mice caused inhibition of food intake, an effect that was not present of Y2 receptor knockout mice. *Ex vivo* hypothalamic explants treated with PYY further demonstrated that the activity of anorexigenic POMC/ α -MSH neurons was upregulated following peptide treatment, leading to the conclusion that PYY is an anorectic peptide by both increasing satiety and by decreasing orexigenic signals (81). The vagus-brainstem-hypothalamic pathway has also been implicated in PYY anorectic action, as vagal disruption using ligation eliminated this effect, while peripheral infusion of PYY caused afferent vagal discharges (84).

1.5. The Proglucagon Gene

Two main short short-term satiety signal hormones, GLP-1 and oxyntomodulin, are derived from the proglucagon gene along with a number of peptides. The proglucagon gene was discovered in the early 1980s as the gene that encodes glucagon (85). Later on it was discovered that it also produces two glucagon-like peptides known as GLP-1 and GLP-2, as well as glicentin, glicentin related polypeptide and oxyntomodulin (86, 87). These peptides are cell-specific expressed, mainly in the α -cells of the pancreatic islets, the distal ileum, the large intestine and CNS neuronal cells.

1.5.1. Glucagon-like peptide 1

GLP-1 is a 36-amino acid proglucagon-derived peptide, produced by cleavage of the gene by prohormone convertase PC1/3. It is released from the intestine in response to a meal digestion and can induce satiety in addition to insulin stimulation from the pancreas. In

circulation, the first 6 amino acids are cleaved resulting in its two main bioactive forms: GLP-1(7-36) and GLP-1(7-36)-amine, the latter of which is found in higher levels (88, 89).

GLP-1 is secreted by the enteroendocrine L-cells, found in the intestine, in response to nutrient sensing, but also stimulation from the vagus nerve, glucose-dependent insulinotropic polypeptide (GIP) and gastrin releasing peptide (GRP). Its powerful effect on insulin secretion, known as the incretin effect, is limited by its short half-life of only 3 minutes. As with most peptides, this is partly due to the renal clearance rate that filters the peptide. A major reason for its clearance is the effect of the enzyme DPP-IV, a serine exopeptidase that cleaves the first two amino acids of the active form of GLP-1, resulting in GLP-1(9-36) which demonstrates very weak binding to the GLP-1 receptor (90, 91).

The action of GLP-1 was first investigated in the pancreas, as its receptor was initially isolated from rat pancreatic islets (92). GLP-1 receptor is a G-protein-coupled receptor containing 7 transmembrane regions and can be found in pancreatic α -cells and β -cells, as well as brain, heart (93), stomach and intestine (94). In some species, unlike humans, it can also be found in lung (95). The GLP-1 receptor is coupled to adenylyl cyclase and therefore triggers the cAMP pathway.

The most prominent feature of GLP-1 is its ability to stimulate insulin secretion from the pancreatic β -cells. The incretin effect was observed when insulin secretion was enhanced following an oral glucose load in humans, compared to an intravenous glucose infusion, pointing that something located in the gastrointestinal tract enhanced insulin secretion. Moreover, human studies have also demonstrated that GLP-1 infusion increases insulin secretion and suppresses endogenous glucose production (96). Studies have shown that GLP-1 receptor activation stimulates insulin gene transcription, biosynthesis and exocytosis, as well as β -cell inhibition of apoptosis and can increase in proliferation (97, 98). As a result, β -cell mass is increased and the incretin effect is elevated.

An equally vital effect is that of GLP-1 on gastric emptying. It has been demonstrated that GLP-1 is a strong regulator of gastric secretion and motility (99). A GLP-1 dose-dependent inhibition of gastric emptying following peptide infusion to type 2 diabetic patients was observed, resulting in a significant decrease of glycemic load following a meal, unconnected to the existent insulin levels (100). A great focus on GLP-1 was also given because when

released in response to nutrient digestion, it stimulates satiety. Preclinical data suggested that exogenous GLP-1 reduced food intake, in addition to gastric emptying delay, an effect weakened after vagotomy, indicating that this function is regulated by the CNS (101, 102).

A number of studies investigated the effect of GLP-1 on the CNS in order to shed light on the pathways responsible for feeding regulation. Intracerebroventricular (ICV) injections of GLP-1 in rats demonstrated a decrease of food intake and body weight (101), while inhibition of GLP-1 binding using an antagonist injection to the hypothalamus significantly increased food intake (103). Later on, a number of ICV injection studies on specific nuclei of the hypothalamus demonstrated that GLP-1 action is focused on multiple brain regions such as the arcuate, the periventricular, the ventromedial, the dorsomedial nuclei and the lateral hypothalamic area (103, 104).

Further studies that employed different techniques, such as nerve lesions and ex vivo electrophysiological studies were used to understand the mechanism through which GLP-1 achieves satiety. Administration of a fluorescent liraglutide, a long lasting GLP-1 analogue, demonstrated that the anorectic POMC/ CART neurons of the arcuate nucleus internalized the peptide, pointing to increased receptor affinity, while the activity of NPY/ AgRP orexigenic neurons activity was inhibited (105).

GLP-1 demonstrated some contradictory results in terms of energy expenditure. A number of studies have also demonstrated a potential inhibiting effect of GLP-1 on energy expenditure in mice and rats, in addition to a decrease in food intake (106, 107). Nonetheless, further research demonstrated that ICV liraglutide injection increased brown adipose tissue temperature and stimulated a number of genes implicated in thermogenesis (108).

1.5.2. Exendin peptides

A breakthrough in diabetes drug discovery came when exendin-3 and exendin-4 peptides were isolated from the venom of the lizard *Heloderma horridum* and *Heloderma suspectum* (109). Among the two, exendin-4 was found to be a potent agonist for GLP-1 receptor in mammals and that it shared 53% of GLP-1 sequence. The specificity to the receptor was

coupled by a similar function as GLP-1, including gastric emptying, insulin secretion and inhibition of appetite (110).

The exendin-4 sequence has been widely investigated through different types of studies, including truncation and alanine scanning. Through early truncation studies, it was found that exendin(9-39) could bind to the GLP-1 receptor with high affinity but failed to activate it (111). It was therefore named a GLP-1 receptor antagonist and had been used in a number of *in vivo* and *in vitro* studies, as well as clinical experiments investigating the normal function of GLP-1.

The exendin-4 sequence forms a central helical region, and has 9 additional amino acids in the C-terminal, which form a structure called the 'Trp-cage'. A vital difference between GLP-1 and exendin-4 is the length of the peptide's half-life, found to be up to 10 times longer in exendin-4 than GLP-1 when given as an intravenous bolus infusion (112). This was partly attributed to the fact that exendin-4 sequence includes a glycine in position 2, compared to GLP-1 which has an alanine. This is important as Ala² serves as a recognition site for DPP-IV to cleave and deactivate the peptide (113, 114). Another reason for the longer half-life observed in exendin-4 is the rate in which the enzyme neprilysin cleaves exendin-4, which is hundred times slower than the rate of GLP-1 (115). This was attributed to a number of factors, such as the population of cleavage sites found in each peptide, as well as exendin's helical structure which is thought to cause steric hindrance to the proteases (116). Moreover, glomerular filtration is also a potential factor for the peptide's half-life differences, as it is similar to its metabolic rate in exendin-4 (117).

The advantage of the extended pharmacokinetic profile of exendin-4 was used in order to create an anti-diabetic drug as it was found to increase insulin secretion and, as a result, balance blood glucose concentration. Its commercial name, Byetta, was approved in April 2005 as a twice-daily subcutaneous injection to treat type 2 diabetes, followed by Bydureon, a once-weekly injection, which was approved in 2012. Its GLP-1-like function also included food intake inhibition, a fact that made it a target peptide for obesity treatment.

1.5.3. Glucagon

Glucagon is a 29 amino acid peptide, also derived from proglucagon gene translation, mainly in the α -cells of the islets of Langerhans in the pancreas. Its secretion is mediated by the prohormone convertase enzymes which process proglucagon to glucagon and is stimulated by hypoglycaemia (118).

Glucagon action is primarily to oppose the action of insulin in order to increase blood glucose concentration when low levels are detected. To achieve this, it controls gluconeogenesis and glycogenolysis, in which glucose is liberated from glycogen stores in perivenous hepatocytes, and consequently its action is mainly on the liver. It is therefore inhibited by glucose detection, insulin and somatostatin (119). Interestingly, GLP-1 also appears to inhibit its secretion (120). Due to this function, a number of therapeutics to neutralise excess circulating glucagon or to antagonise glucagon receptors have been designed in order to reduce hyperglycaemia in diabetic individuals who have dysregulated glucagon release and imbalanced glucose homeostasis.

Glucagon functions through the glucagon receptor, which is a G-coupled receptor found in a number of tissues including the liver, kidneys, CNS, muscle, pancreatic β -cells and adipose tissue (121). The glucagon receptor signals through activation of adenylate cyclase, but also through upregulation of intracellular calcium (122). GABA receptor subunits were found to be expressed in the α -cells and lack of their activation resulted in failure of glucose to suppress glucagon release (123). Insulin has been shown to activate these GABA receptors through an Akt kinase-dependent pathway, which ultimately hyperpolarised the α -cell membrane resulting in glucagon release inhibition (124).

The action of glucagon is not only focused on the liver. It has also been found to increase cAMP in many regions of the CNS. Hypothalamic infusion of glucagon in rats demonstrated an increase in c-Fos expression while inhibiting the breakdown of hepatic glucose and in order for that to occur, the hepatic branch of the vagus nerve was required (125).

A number of studies also demonstrated a glucagon effect on lipolysis *in vivo* and *in vitro*. A primary example is the activation of the glucagon receptor in mice which increased the Fibroblast Growth Factor 21 (FGF-21) plasma levels as well as its mRNA transcripts in liver,

causing a change in hepatic lipid levels, an observation not found in FGF-21 knockout mice (126). However, human *in vivo* experiments produced contradicting results, pointing at a less intense effect of glucagon on adipocytes.

Even so, what became a key area of research was the effect of glucagon on satiety and increase in energy expenditure. Short-term satiety was observed postprandially after glucagon infusion, an effect considered to be through the liver, as increased level of glucagon concentration in plasma is found in the hepatic portal vein compared with the systemic circulation (127), and hepatic vagotomy abolishes any satiety effect it may cause (128, 129).

An initial study demonstrated a significant decrease of food intake and body weight after glucagon infusion in man (130), while an increase in resting metabolic rate during hyperglucagonaemia was also observed (131). In addition, co administration of glucagon and GLP-1 in overweight individuals demonstrated an increase in resting energy expenditure and a decrease in non-esterified fatty acids and ghrelin levels (132). *In vivo* studies in diabetic Zucker rats also demonstrated this effect, as exogenous glucagon caused a reduction in body weight without reducing food intake, compared to a control group (133). These observations triggered the idea of the use of glucagon as a potential therapeutic in obesity while maintaining glucose homeostasis.

1.5.4. Oxyntomodulin

Oxyntomodulin is a 37 amino acid peptide, the sequence of which includes the 29 amino acid sequence of glucagon and an octapeptide extension in the C-terminal. Initially known as enteroglucagon, it is secreted from the intestinal L-cells in response to food intake. As GLP-1, it is rapidly degraded by DPP-IV and neprilysin through cleavage, resulting in a short half-life. An interesting characteristic of the peptide is its affinity for both glucagon and GLP-1 receptors, despite its sequence similarity to glucagon, while an oxyntomodulin receptor has not yet been discovered (134).

This characteristic made it a target for a number of studies that investigated its effect as both a satiety signal, due to its GLP-1 function, and as a stimulant to energy expenditure

increase through glucagon receptor action. Oxyntomodulin mimics the GLP-1 function of gastric emptying and gut motility while also inhibiting food intake (135). Direct administration of oxyntomodulin to the paraventricular nucleus of the hypothalamus resulted in reduced food intake, an effect that was not observed when a GLP-1 antagonist was administered (136). Moreover, peripheral administration presented similar effects, yet not gastric emptying (137), a result also observed in humans acutely after a 90 minute peptide infusion (138). Its differentiated function on GLP-1 and glucagon receptors was researched through knockout models that pointed to an abolished satiety effect in mice that did not express the GLP-1 receptor (107). The effect of the peptide in energy expenditure was also investigated in obese humans that received a three times a day administration before a meal. Four days later, food intake was reduced significantly compared to the control group, and total energy expenditure was increased (139).

1.6. Obesity Therapeutics

Through these investigations, it became clear that both GLP-1 and glucagon agonism could result in a potent anti-obesity treatment. In order to achieve this, a functional ratio between the two has to be achieved to ensure an effect in both satiety and energy expenditure, while maintaining glucose homeostasis and avoiding nausea. In addition, longer lasting agents would be an advantage to achieve increased duration of action and subsequently less frequent administration which would eventually lead to a promising therapeutic.

1.7. Hypothesis

The overarching hypothesis of this thesis is that GLP-1 and glucagon long-lasting agonists can be created through structural domain investigation and amino acid substitution. The co-administration of the two agonists at an optimal ratio can cause a significant weight reduction without inducing hyperglycemia.

CHAPTER 2

Materials and Methods

2. 1. Peptides

Human GLP-1 and exendin-4 were purchased from Bachem Ltd. (Merseyside, UK). Synthetic human sequence GLP-1 and glucagon were used in all experiments, unless otherwise stated. The form used in all experiments was GLP-1(7-36)NH₂ and will now be referred to simply as GLP-1.

2.2. Custom Synthesis of Peptides

All peptides were synthesised and purchased from Insight Biotechnology Ltd (Middlesex). Peptides were produced using an automated fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis method, where each amino acid is sequentially added from the C to the N terminus.

Peptides were cleaved from the resin, and then purified using reverse phase preparative high performance liquid chromatography (HPLC), followed by lyophilisation. All peptides supplied had a purity of >95%. All amino acid sequences are presented in the 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 Diet Induced Obese (DIO) mice

Adult male Diet Induced Obese (DIO) C57/BL6J mice (Harlan, Oxfordshire, UK) weighing 30-45g were maintained in individual cages under controlled temperature (21-23°C) and light

(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 a 60% of kcal by fat diet (Research Diets Inc., New Brunswick, USA), unless otherwise stated. To minimise any non-specific stress effects, the animals were regularly handled and acclimatised to subcutaneous (SC) injections.

2.3.1.2. Rats

Adult male Wistar rats (Charles River Ltd., Margate, Kent, UK) weighing 300-500 g were double housed in cages under controlled temperature (21-23°C) and light (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, the animals were regularly handled and acclimatised to SC injections.

2.4. Acute animal feeding studies

Experiments were initiated in the early light-phase (0800-0900). Compounds were expected to show an anorectic effect and were therefore administered to mice fasted from 1400 on the preceding day in order to demonstrate the effects successfully.

DIO C57BL/6J mice were injected SC (at time 0) with the peptide or vehicle (0.9% weight/volume NaCl) in 1ml/ kg, using a 0.5ml insulin syringe with a 29 gauge needle. Post injection, the mice were returned to their home cage and provided with a known amount of food. The remains of food were then weighted at specific time points for the next 48 hours. Food weight was measured to three decimal places. The cage was inspected for spilled food at each reweigh. All studies were conducted under conditions in which all external disturbances were minimised.

2.5. Rat pharmacokinetics

In order to investigate the plasma clearance of the peptide after a SC injection, rat pharmacokinetic studies were carried out. Male Wistar rats were randomly allocated to weight matched groups of 4. Each animal was injected SC with 1mg of peptide in 20µl vehicle (0.9% weight/ volume NaCl). Blood was collected by superficial venesection of the lateral tail vein at 6 time points following injection. Each blood sample (200 µl maximum) was placed into lithium heparin flushed microtubes containing 0.1ul protease inhibitors (Sigma manufacturer). In order to separate the sample plasma, tubes were centrifuged for 8 minutes at 3,000 x g at 4°C (Sigma Laboratory centrifuges 3 K18, Rotor No. 19777-H). The upper plasma was isolated and stored at -20°C. Plasma peptide concentrations were determined by radioimmunoassay (section 2.9).

2.6. Human GLP-1 Receptor and Human Glucagon Receptor Overexpressing Cell Lines

For chapter 3, a human GLP-1 receptor overexpressing cell line was used to assess the efficacy of GLP-1 agonists to the GLP-1 receptor. Accordingly, for chapter 4, a glucagon receptor overexpressing cell line was used. Both cell lines were developed using naïve and recombinant Chinese Hamster Ovary (CHO) cell cultures and GLP-1 and glucagon receptor expressing plasmids. Human GLP-1 receptor (CHO-hGLP-1r) and human glucagon receptor (CHO-hGCGr) overexpressing cell lines were kindly provided by Dr. James Minnion.

2.6.1. Maintenance of cells

Materials:

Dulbecco's modified medium (DMEM) without sodium pyruvate containing 4.5g/l glucose (Invitrogen)

Dialysed foetal bovine serum (DFBS) (Invitrogen)

MEM Non-Essential Amino Acids Solution (NEAAS) 10 mM (Sigma)

100 units/ml penicillin 100µg/ml streptomycin (Sigma)

HEPES_4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma)

Non-enzyme cell dissociation buffer (Sigma)

Method:

CHO-hGLP-1r and CHO-hGCGr cells were cultured in DMEM supplemented with 10% DFBS, 2% NEAAS (vol/ vol), 2% HEPES (vol/ vol) and 1% antibiotics (vol/ vol). Cells on passage number 25-36 were used for all studies. The medium was changed every 2-3 days and the cells were sub-cultured when they reached 70% confluence. When required, the medium was aspirated from the vessel and non-enzyme cell dissociation buffer was added (2ml/50cm² flask surface area). The cells were then briefly incubated at room temperature until they detached from the flask and their mobility could be visualised under a microscope. Medium, equal to the amount of cell dissociation buffer, was then added to the vessel and the cells were centrifuged at 1000g. The supernatant was aspirated and the cell pellet was re-suspended in fresh complete medium and transferred to a new flask at a 1:5 dilution.

2.7. Receptor Binding Assays

Membranes from both cell membrane suspension and rodent tissue were obtained using the method of homogenisation and differential centrifugation.

2.7.1. Preparation of membrane from tissues

Tissues were obtained from C57BL/6J mice maintained in the conditions described above. A minimum of 40 mice were used per membrane preparation. Rodents were killed by cervical dislocation in the early light phase, between 0900 and 1200, and the tissues were snap frozen in liquid nitrogen and stored at -80°C.

Frozen tissues were homogenised (Ultra-Turrax T25; IKA Labortechnik, Staufen, Germany) in ice-cold homogenisation buffer (50mM HEPES buffer pH 7.4 containing 0.25 M sucrose, 10µg/ml soybean trypsin inhibitor (Sigma, Dorset, UK), 0.5µg/ml pepstatin (Sigma) 0.5µg/ml antipain (Sigma), 0.5µg/ml leupeptin (Sigma), 0.1mg/ml benzamidine (Sigma), 1ml 100KIU/ml Trasylol® aprotinin (Bayer, Haywards Heath, UK) at 4°C.

The homogenates were centrifuged at 1000 x g (Beckman J2-21, rotor JS-13.1) for 15 minutes at 4°C; supernatants were then centrifuged at 100000 x g (Sorvall Ultracentrifuge OTD55B, rotor A-841; DuPont, St.Neots, Cambridgeshire, UK) for 60 minutes at 4°C. Pellets were removed and re-suspended 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 re-suspended to a final protein concentration of 2-3mg/ml and stored at -80°C. Protein concentration was measured by the Bradford assay (Section 2.7.3.).

2.7.2. Preparation of membrane from cells

2.7.2.1. Maintenance of cells

CHO-hGLP-1r and CHO-hGCGr cells were cultured as described above.

2.7.2.2. Receptor purification

The culture medium was removed and the cells were detached from the flasks using ice-cold 0.02 M phosphate buffered saline (PBS; Sigma- Aldrich) and scrapping. A minimum of 30 T175 cm² flasks were used for each membrane preparation. Cells were then centrifuged at 1000 x g for 5 minutes (Sigma Laboratory Centrifuge 3, K18, rotor No. 19777-H), the supernatants discarded, and the pellets placed on ice. Pellets were then re-suspended in 20 ml of ice-cold homogenisation buffer (section 2.7.1.) and homogenised for 1 minute using an Ultra Turrax homogeniser (IKA Labortechnik). Homogenates were further centrifuged for 30 minutes at 4°C 100000 x g (Sorvall OTD 55B ultra-centrifuge; DuPont). The supernatants were discarded and the pellets were re-suspended in homogenisation buffer without

sucrose using the hand-held/Teflon homogeniser (Jencons). Finally, the pellets were re-suspended to a final protein concentration of 1-2mg/ml and stored at -80°C. Protein concentration was measured by the Bradford assay (Section 2.7.3).

2.7.3. Bradford assay

0.1ml of cell membrane solution (purified from cells overexpressing the GLP-1 receptor or glucagon receptor) was added to 3ml of Bradford reagent (Sigma-Aldrich). A standard curve was also determined using known concentrations of bovine serum albumen (BSA; Sigma-Aldrich), where 0.1ml of 0, 0.25, 0.5, 1.0, 1.5 mg/ml solution was added to 3ml 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 595nm using a spectrophotometer (WPA UV 1101) ((Biochrom Ltd. Cambridge, UK)) Standard curve was used to determine the unknown concentration of protein in the membrane samples.

2.7.4. Iodination of peptides

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

2.7.4.1. The direct iodogen method

The direct iodination method, previously described by Owji et al (140) was used in this study to label GLP-1, glucagon and exendin-4. 3nmols (15 μg) of peptide in 10 μl of 0.2M phosphate buffer (pH 7.2) was reacted with 37MBq of Na^{125}I (Amersham) and 23nM (10 μg) of 1,3,4,6,-tetrachloro-3 α , 6 α -diphenylglycoluril (iodogen reagent; Pierce Chemical Co, Perbio Science UK Ltd., Cramlington, UK) for 4 minutes at 22°C. The ^{125}I -peptide was purified by reversed-phase high performance liquid chromatography using an acetonitrile (AcN)/ H_2O /0.05% TFA gradient.

2.7.5. GLP-1 receptor binding studies

Cell membranes from either mouse lung or from GLP-1 receptor overexpressing cell lines (CHO cells overexpressing hGLP-1 receptor) were used for these studies.

The assays were performed in 1.5ml siliconised microtubes, with a total volume of 500µl. The assay buffer comprised of 20mM HEPES (pH 7.4), 1mM MgCl₂, 5mM CaCl₂, 1% BSA, 0.05% Tween 20, 0.1mM diprotin A and 0.2mM phenylmethanesulfonylfluoride (PMSF) ((Sigma)). An amount of 50µl of ¹²⁵I-GLP-1 dissolved in assay buffer, at 1000 counts per second, was incubated with 10µl of the membrane suspension (at a protein concentration of 1-2µg/ml). Microtubes contained increasing concentrations of unlabelled peptide. Each peptide concentration was assayed in duplicate or triplicate. Microtubes were each vortexed and incubated at room temperature for 90 minutes. These were then centrifuged at 15781 x g at 4°C for 3 minutes. The supernatant was removed and discarded, and then another 500µl of assay buffer was 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:

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

2.7.6. Glucagon receptor binding studies

The assay was completed as above, except for the following differences: The assay buffer used was 25mM HEPES (pH 7.4), 2mM MgCl₂, 1% BSA, 0.05% Tween20, 0.1mM diprotin A and 0.2mM PMSF. The radiolabel used was ¹²⁵I-glucagon. Cell membranes from either mouse liver or from glucagon receptor overexpressing cell lines (CHO cells overexpressing the human glucagon receptor) were used for these studies.

2.7.7. Whole cell binding assay

The whole cell binding assay follows the same principle as the receptor binding assay using cell membrane preparations (Section 2.7.2).

Competition between binding of unlabelled and labelled ligand was assessed in live whole cells in culture. Cells were plated at a concentration of 100,000 cells/ ml on 12 well plates at 500µL per well and incubated at 37°C in 5% CO² overnight. For the experiment, the media was removed and replaced with 500µl of assay buffer (as in section 2.7.5. or 2.7.6.). Assay buffer contained 1000 counts per second of ¹²⁵I-labelled ligand and a known concentration of the unlabelled peptide whose affinity was to be examined. Each peptide concentration was assayed in duplicate or triplicate and the assay was incubated for 90 minutes at room temperature. At 90 minutes, cells were scraped from the surface of each well, and the cells along with the assay buffer were transferred into siliconised microtubes. These tubes were then centrifuged, washed and centrifuged again, as described in section 2.7.2.2. Measurement of γ-radiation, analysis and calculation of IC₅₀ values was subsequently performed, as in section 2.7.5.

2.8. cAMP accumulation assay

CHO cells overexpressing the human GLP-1 receptor or the human glucagon receptor were plated onto 48 well plates one day prior to assay. These were aliquoted at concentration of 100,000 cells/ ml in 250µl of standard media (DMEM 6429, with L-glutamine, 4.5 g glucose, sodium pyruvate; Sigma-Aldrich, UK) supplemented with 10% dialysed foetal bovine serum (DFBS) and 1% antibiotic v/v (100 units/ml Penicillin and 0.1mg/ ml Streptomycin; Sigma). The following day, the media was removed and replaced with DFBS-free DMEM media and cells were incubated for 1 hour. Concentration range of peptides to be tested was prepared in serum-free DMEM. Media was aspirated from each well containing cells and replaced with incubation media containing the test peptide. These were incubated for exactly 30 minutes. After incubation, the media was removed and replaced with 110ul lysis buffer (0.1M HCl with 0.5% Triton-X) and incubated for a further 10 minutes. The lysate, which contained cAMP, was stored at -20°C prior to analysis.

Cell lysate was diluted at a ratio of 1:10 in lysis buffer and assayed using a 'Direct cyclic AMP Enzyme-linked Immunosorbant assay (ELISA) kit (ADI-900-066; EnzoLifeSciences, UK) according to the manufacturer's protocol. Briefly, 50µl of Neutralising Reagent (provided) was added to each reaction well, except the total and blank wells. Provided standards (100µl) and samples (100µl) were aliquoted into the corresponding wells before adding 50 µl of conjugate and 50µl of antibody into each well. The plate was then sealed and incubated at room temperature for 2 hours on a plate shaker (~500 rpm). The contents were emptied from the wells and washed three times with 400µl of wash buffer. 'Substrate solution' (200µl) was added to each well and left to incubate at room temperature for 1 hour. After this, 50µl 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 the 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:

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

2.9 Radioimmunoassay (RIA)

2.9.1. Exendin-4 RIA

Exendin-4-like immunoreactivity was measured with a specific and sensitive radioimmunoassay, as described in Appendix C. The assay was performed in a total volume of 0.7 ml of 0.06 M phosphate EDTA buffer (0.05M Na₂HPO₄·2H₂O, 0.0006M KH₂PO₄, 0.01M disodium-EDTA·2H₂O, 0.008M NaN₃) at pH 7.4, containing 0.3% bovine serum albumin (Sigma, UK). The antibody R4 was produced in rabbits against purified exendin-4 coupled to bovine serum albumin by glutaraldehyde and used at a final dilution of 1:3500. Antibody R4 cross-reacts with exendin-4 and does not cross-react with any other known gastrointestinal hormones. A radio-labelled analogue of exendin-4, exendin-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 a peptide standard of the peptide being quantified and were prepared in assay buffer at 2pmol/ml. Standards were added in duplicate, at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl. Separation of the bound from the free antigen is achieved by addition of dextrancoated charcoal. The assay was incubated for 3 days at 4°C before charcoal separation of the free and antibody-bound label (free label is contained in the charcoal pellet following centrifugation). Both the pellet and supernatant were counted for 180 seconds in a γ-counter (model NE1600, Thermo Electron Corporation). Exendin-4 concentrations in the samples were calculated using a non-linear plot (GraphPad Software Inc. San Diego Version 5.01, USA) and the results were calculated in terms of interpolated values from the standard curve.

2.9.2. GLP-1 RIA

GLP-1 N-terminal like concentration was measured with a specific and sensitive radioimmunoassay, as described in Appendix C. The antibody Af1 cross-reacted by 100% with all amidated forms of GLP-1 but does not cross react with glycine extended forms GLP(1-37) and GLP(7-37) or any other known gastrointestinal peptides. As above, the assay was performed in a total volume of 0.7 ml and incubated for 3 days at 4°C before charcoal separation of the free and antibody-bound label. Both the pellet and supernatant were counted for 180 seconds in a γ-counter. ¹²⁵I-GLP-1 was prepared by the direct iodination method and purified by reverse phase high-pressure liquid chromatography. All samples were assayed in duplicate. Standard curves for each assay used the purified peptide to be tested and were prepared in assay buffer at 0.25pmol/ ml, then added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl. GLP-1 concentrations in the samples were calculated using a non-linear plot (GraphPad Software Inc. San Diego Version 5.01, USA) and the results were calculated in terms of interpolated values from the standard curve.

2.9.3. Glucagon RIA

Glucagon N-terminal-like immunoreactivity was measured with a sensitive radioimmunoassay, as described in Appendix C. The assay was performed in a total volume of 0.7 ml in 0.06 M phosphate EDTA buffer (as above) at pH 7.4, containing 0.3% bovine

serum albumin. The antibody R2641 was produced in rabbits against the purified glucagon(1-16) fragment coupled to bovine serum albumin by glutaraldehyde. This antibody cross reacts to the N-terminal sequence of glucagon and oxyntomodulin present in the peptide analogues and does not cross-react with other known gastrointestinal hormones. ¹²⁵I-glucagon was prepared by the direct iodination method and purified by reverse phase high-pressure liquid chromatography. All samples were assayed in duplicate. Standard curves for each assay used the purified peptide to be tested and were prepared in assay buffer at 1pmol/ ml, then added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl. 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 were counted for 180 seconds in a γ-counter (Section 2.9.1.). Peptide concentrations in the samples were calculated using a non-linear plot (GraphPad Software Inc. San Diego, USA) and the results were calculated in terms of the standard.

2.10. Statistical Analysis

All data are expressed as the mean value ± S.E.M. In the presented *in vitro* studies of the native peptides, coefficient of variation was also calculated. Interval food intake and peptide hormone levels were compared by one-way 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. 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

Investigation of GLP-1 analogues as anorectic agents
for treatment of obesity

3.1. Introduction

3.1.1. Ligand – receptor interactions

Understanding the relationship between ligands and receptors is vital in both research and drug discovery. A number of theoretical models that determine a ligand's interaction with a potential receptor, in order to engineer a ligand with the high binding and cellular activation have been developed over the past decades (141-144).

Crystallographic techniques can be used to indicate the relationship between ligands and receptors by isolating the complex that is formed once bound, and investigate resulting structural changes. Major breakthroughs in crystallography have aided the holistic understanding of the molecular organisation of receptors, an example of which is the structure of G-protein coupled receptors that enabled the design of GLP-1 ligands used as a type 2 diabetes treatment (145). Alanine scanning is used to define the function and contribution of specific amino acids in a peptide sequence. Each residue of interest is systematically replaced by alanine, a small uncharged amino acid that can mimic the secondary structure preferences of many other amino acids while maintaining a neutral function, and is then examined for any functional alterations (146). Alanine scanning can pose limitations as its substitution may alter the structure of the peptide investigated instead of having a neutral effect (147). Truncation of peptides is another technique that has been widely used in both peptide function research and drug discovery, as it can determine the importance of protein residues by eliminating them and analysing the change in function (148-150).

These techniques have been extremely useful in designing analogues with a structure similar to the structure of a native peptide so as to increase the stability between the ligand and the receptor, thus achieving higher affinity. By understanding the peptide structure, its activity can be altered in order to achieve long-lasting receptor binding to enhance their activation, resulting in a greater physiological effect, an important factor in treatment design.

3.1.2. Structure and properties of GLP-1 and exendin- 4:

Selected cleavage of the proglucagon gene transcript results in different peptides. One of the transcription products, GLP-1, is a 30 amino acid peptide produced in intestinal L- cells, secreted in response to nutrient intake (151). Its full non-truncated form appears to be completely inactive, yet there are two main active products that are naturally found in circulation, GLP-1(7-37) and GLP-1(7-36), where the latter differs by the presence of an amine residue in the N-terminus. Both forms present identical incretin effect, yet GLP-1(7-36)NH₂ appears to cause a more rapid decrease of blood glucose levels following a meal (152).

The amino acid residues of critical biological importance were investigated through studies based on synthetic truncated forms of GLP-1, as well as alanine substitutions of each amino acid. Affinity of the truncated and substituted peptides to the GLP-1 receptor was frequently used to assess neutral and detrimental changes (153).

NMR structural analysis demonstrated three main domains of the peptide (153). Amino acids 1-7 form a coiled structure, followed by amino acids 7-14 which form a short alpha-helix. A second alpha-helix is present (a.a. 18-29), which is linked to the first one through a short ligand region (a.a. 15-17). This region, more specifically a glycine located at position 16, is suggested to serve as an N-cap for the C-terminal helix (154-157). An arginine and the amino group form the end of the molecule in positions 30-31.

The N terminal truncation, GLP-1(9-36) has 25-fold less affinity and activity than the full length sequence, confirming the importance of histidine and alanine found in positions 7 and 8. Furthermore, C-terminal truncation resulting in GLP-1(7-30) leads to poor affinity in addition to low bioactivity (158). It has also been suggested that the link that connects the two helices allows the aromatic residues of all the amino acids to lay on one side, forming a structure that enables membrane association, a fact that could be vital for receptor binding affinity as the residues displayed are hydrophobic (159).

Exendin-4 is a 39-amino acid GLP-1 receptor agonist which shares 53% homology to GLP-1, found in the venom of the Gila monster. It has an addition of nine amino acids at the

C-terminal. Exendin-4's long action is due to its reduced rate of degradation by proteases and neutral endopeptidases, perhaps due to steric hindrance, as its compact helical structure is thought to restrict access of endoproteases (116).

The sequence of exendin-4 and GLP-1 are very similar at the N-terminus, with the main distinction being a glycine instead of alanine at position 2. Unlike GLP-1, which has two separate helices, exendin-4 forms a continuous helix in positions 7-27, in a micelle environment. The helical structure of exendin-4 is promoted by the glutamine residue at position 16. The additional 9 amino acids found in the C-terminal of exendin-4 have been shown to form a structure called the 'Trp-cage'. This structure has been found to interact with the N-terminal extracellular domain of the GLP-1 receptor as it increases receptor affinity, a fact that can explain the greater bioactivity of exendin-4 (160). Early attempts to assign a pattern of binding and activity of GLP-1 across its sequence provided indefinite results, yet showed that C-terminal truncated forms of GLP-1 stimulated insulin release from the pancreas more effectively than the full length sequence (161). GLP-1 was found to be degraded by DPP-IV, in a similar process as previously shown for growth –hormone release factor (GRF) (90). As the routes through which GLP-1 is deactivated and cleared have been established, GLP-1 analogues which provide protection from these, such as DPP-IV resistance, have been invented. These analogues mainly included substitutions of dipeptidase target residues, such as Ala², or individual amino acids.

These studies also provided an insight into the role of certain regions that could affect both receptor binding and activation. N-terminal truncation of exendin-4 (9-39) resulted in a reduction of affinity, while C-terminal (1-30) truncation caused 25-fold reduction of affinity, pointing at the importance of the Trp cage (156). These results indicate that, contrary to GLP-1, exendin-4 binds to a receptor primarily through the C-terminal. In addition, N-terminal truncation pointed at an increase in DPP-IV resistance, as alanine is replaced by glycine in position 2, which increased *in vivo* efficacy.

3.1.3. Structure and properties of the GLP-1 receptor

GLP-1 and exendin-4 both act through the GLP-1 receptor, a G protein-coupled receptor. G protein-coupled receptors are a group of membranous receptors, characterised by their molecular form of seven transmembrane alpha-helices that are linked together through peptide loops. They establish a superfamily that triggers different cellular pathways in response to the binding of ligands such as hormones and neurotransmitters. The family includes over 400 receptors in humans and mice (162). Elucidation of their structure and function has been key to advancements in drug development, leading to the 2012 Nobel Prize in Chemistry being awarded to Kobika and Lefkowitz for pivotal work on these receptors (163). The GPCR superfamily has been divided into 6 different classes of receptors based on sequence, structure and function. Class A, the largest, is subdivided in 19 groups and includes somatostatin and CCK receptors. Class B (secretin-like) is divided in 3 groups that include glucagon, GLP-1, GLP-2 and GIP receptors (164, 165).

The function of GLP-1 receptor is based on the interplay of its structural molecules that allow it to fold, bind and initiate intracellular signalling. It forms three different structures during activation and stimulates the adenylyl cyclase pathway, resulting in increased cAMP production and triggering of insulin secretion when expressed in the islets of Langerhans (Figure 3.1.). Despite the number of structural related peptides such as glucagon, VIP and secretin, the GLP-1 receptor achieves a high degree of ligand specificity to GLP-1 (92, 97, 166). As well as high levels of expression in the pancreas, the receptor has also been found in the CNS and heart and the GLP-1 is thought to have act through these receptor populations (167).

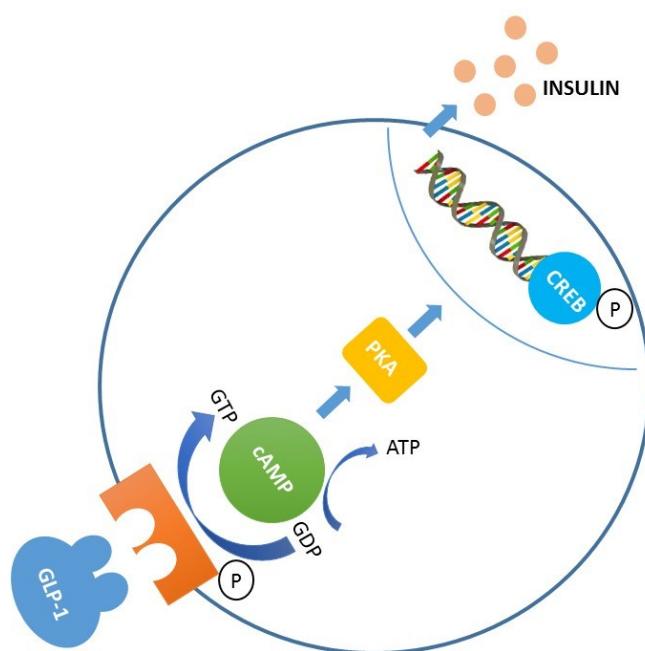


Figure 4.1. Summary of GLP-1 receptor signalling in β -cells. GLP-1 binds on GLP-1 receptor, increasing intracellular concentration of cAMP, which induces PKA-mediated activation of the transcription factor CREB.

3.1.4. Analogues as pharmacological agents

Studies of the structure and function of GLP-1 and the GLP-1 receptor have enabled the design and development of several effective treatments of type 2 diabetes by creating potent and longer lasting analogues. In rodent studies, treatment with GLP-1 receptor agonists, which are satiety factors, can cause significant weight loss. However, the effective weight loss seen in rodent studies has not been fully translated to humans mostly due to their tolerability, as the pharmacological plasma levels required to suppress appetite may cause nausea and vomiting in a number of cases. Rodents do not have the physiological reflex to vomit; therefore this most common adverse side effect of GLP-1 is difficult to assess when high doses are investigated. This allows much higher plasma levels and greater weight loss to be achieved in rodents which is not achievable in man.

It has recently been demonstrated in short term infusion studies that combining GLP-1 and glucagon administration in man provides an increase in energy expenditure and a reduction in appetite (132). This is potentially due to the combination of the GLP-1 anorectic effect

and glucagon's ability to increase energy expenditure. Despite glucagon's effect on energy expenditure, it is not possible to administer it on its own as it increases glucose plasma levels. Creating this agent by combining two separate compounds has the advantage of adjusting the ratio of GLP-1 and glucagon receptor agonism, which could be essential in order to personalise a treatment to maximise weight loss while avoiding glucose excursions or nausea, as GLP-1 plasma levels would not need to reach a high enough level to induce nausea yet would still inhibit appetite.

Nonetheless, native peptides have the disadvantage of a short half-life and that would require frequent and high doses within the day. It is therefore essential to create a peptide analogue that will present long duration of action, as well as being potent and highly efficacious to the target. To achieve this, compounds designed through amino acid substitutions were screened to evaluate their pharmacokinetic (PK) and pharmacodynamic profiles using cell-based assays and animal models.

3.1.5. Generation of GLP-1 analogues

Structure investigations on GLP-1 have shown that the N-terminal is mainly responsible for receptor binding as it is the part that enters the receptor binding site, while the C-terminal aids both binding and activation. Even so, the whole sequence is thought to be synergistically involved in the binding activity, with the linker region (positions 16-17) between the peptide's two helices being of vital importance for receptor binding (159).

Generation 1:

Previous studies showed that truncation of residues 1-15 resulted in 90% loss of binding, mainly due to the loss of the first two amino acids (168, 169). Substitutions in the N-terminal part of the GLP-1 molecule, with the corresponding glucagon residues, moderately reduced the affinity for the GLP-1 receptor (170). In addition, substitution of alanine in position 2 decreased GLP-1 degradation, as it is the residue targeted by dipeptidase, and most specifically DPP-IV. Therefore, the first generation had the purpose of reducing

enzymatic degradation by investigating position 2 of the native sequence and to increase receptor binding by focusing on the N-terminal, specifically positions 10 and 12.

Generation 2:

The second generation mainly investigated the linker region in residues 16-17 to assess the importance of flexibility between the two helices as well as the initial part of the second helix. As the linker region is the main difference between the GLP-1 and the glucagon structure, in a micelle environment, investigating the loss of flexibility by replacing glycine could result in a more glucagon-like single helix instead of two separate ones.

Generation 3:

Studies have shown that truncation of the C-terminus resulted in loss of activity (169, 171, 172), while exchanges in the C-terminal portion of GLP-1 with glucagon reduced the affinity for the GLP-1 receptor more than 100-fold (170). The third generation focused on the C-terminal to evaluate the effect on receptor activation through positions 21, 24, 27, 28, 29 and 30.

3.1.6. Aims

A potential therapeutic agent must present high efficacy, as well as a slow release into the circulation following administration and/or a long circulatory half-life to maintain a steadily elevated state and avoid burst release which may cause nausea.

In this study, three generations of GLP-1 analogues were designed according to the peptides' structure and function and investigated for receptor affinity, efficacy and PK properties. Using the evidence of each peptide's modifications, further knowledge of specific residue function was achieved. Subsequently, the properties of the analogue were examined *in vitro*. In addition, *in vivo* studies to investigate the anorectic effect and the plasma concentration of the agent were performed.

Summary:

The aim of this chapter is to investigate the importance of selected amino acid residues of GLP-1 for receptor affinity, efficacy and pharmacokinetics.

3.2. Materials and Methods

3.2.1. Design and synthesis of GLP-1 analogues

Three generations of GLP-1 analogues were designed and synthesised, the sequences of which are shown in table 1. The first generation consisted of 5 analogues, followed by the second generation of 7 analogues and the final third generation of 4 analogues. Peptides were manufactured by Insight Biotechnology Ltd (Middlesex) ((Section 2.2.)).

3.2.2. GLP-1 competitive receptor binding assays

Binding studies were performed using preparations of CHO-hGLP-1r cell membranes and mouse lung tissue cell membranes (section 2.7.5). ^{125}I - GLP-1 was used as the competing peptide for all studies (Section 2.7.4). Binding affinities were calculated from a minimum of two separate experiments, where each concentration duplicate or triplicate. Results shown as IC_{50} , errors shown are $\pm$ SEM.

3.2.3. GLP-1 cAMP accumulation assays

CHO-hGLP-1r cells (section 2.6.1) were treated with a GLP-1 receptor agonist at a range of doses as described in section 2.8. Accumulation of cAMP was quantified using an ELISA (ADI-900-066, EnzoLifeSciences, UK). Bioactivity was calculated from a minimum of three separate experiments, where each concentration was tested in duplicate or triplicate. Results expressed as EC_{50} , errors shown are $\pm$ SEM.

	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	38	39	40
GLP-1 (7-36 NH ₂)	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	NH ₂									
Exendin-4	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	P	S	NH ₂
GL1	H	A	Q	G	T	F	T	S	D	Y	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL2	H	G	E	G	T	F	T	S	D	Y	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL3	H	G	E	G	T	F	T	S	D	L	S	H	Y	L	E	G	Q	A	A	Q	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL4	H	G	Q	G	T	F	T	S	D	L	S	H	Y	L	E	S	Q	A	A	Q	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL5	H	S	Q	G	T	F	T	S	D	L	S	K	Y	L	E	G	E	A	A	Q	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL6	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	E	Q	A	V	Q	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL7	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	S	Q	A	V	H	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL8	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	E	Q	A	V	R	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL9	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	S	Q	A	V	R	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL10	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	S	E	A	V	R	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL11	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	E	E	A	V	R	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL12	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	E	E	A	V	H	E	F	I	A	W	L	V	K	G	R	NH ₂									
GL13	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	E	E	A	V	H	L	F	I	E	W	L	V	K	G	R	NH ₂									
GL14	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	E	E	A	V	H	L	F	I	E	W	L	K	K	G	R	NH ₂									
GL15	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	E	E	A	V	H	L	F	I	E	W	L	K	N	T	G	NH ₂									
GL16	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	E	E	E	A	V	H	L	F	I	E	W	L	K	N	T	H	NH ₂									

Table 3. 1: Amino acid sequences of peptides used in Chapters 3. Red filled residues are conserved residues across the GLP-1 peptide family; Green filled residues are derived from the exendin-4 sequence; Blue filled residues represent point substitutions.

3.2.4. Acute feeding studies in mice

The effect of GLP-1 analogues on food intake was investigated using C57BL/6J mice fasted for 16 hours, as described in section 2.4. Mice were injected SC at time 0 with peptide or vehicle (0.9% weight/volume NaCl). Dose and injection volume were adjusted according to body weight, with a maximal injection volume of 0.05ml.

Dose selection was based on earlier studies (data not shown). All analogues were tested at 50 nmol/ kg. Exendin-4 was used as a positive control and was administered at a 10 nmol/ kg. Food intake was measured at 1, 2, 4, 8, 24, 48 hours post administration.

3.2.5. *In vivo* pharmacokinetic studies

In vivo pharmacokinetics studies were performed in male Wister rats as described in section 2.5. Each animal received a 1mg SC injection of peptide (50mg/ml) reconstituted in 1:1 molar ratio Zn:NaCl (saline). Blood was collected into lithium heparin tubes by superficial venesection of the lateral tail vein at 6 time points following peptide administration. In order to separate the sample plasma, tubes were centrifuged for 8 minutes at 3,000 x g at 4°C. The upper plasma was isolated and stored at -20°C. Peptide hormone levels in plasma were measured by GLP-1 radioimmunoassay as described in section 2.9.3.

3.2.5.1. Radioimmunoassays

Plasma peptide levels were determined by GLP-1 radioimmunoassays as described in section 2.9.3. The GLP-1 antibody Af1 was raised in rabbits against GLP-1(1-16) and coupled to bovine serum albumin by glutaraldehyde. ¹²⁵I- GLP-1 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, prepared in assay buffer at 0.25pmol/ ml, and then added in duplicate. Peptide concentrations in the samples were calculated using a non-linear plot and results were calculated in terms of the standard.

3.3. Results

3.3.1. *In vitro* characterisation of first generation of GLP-1 analogues

3.3.1.1. Affinity of the GLP-1 analogues to the human GLP-1 receptor

Binding assays with ^{125}I -GLP-1 resulted in greater than 70% specific binding to a cell membrane preparation of CHO-hGLP-1r cells. GLP-1 and exendin-4 bound with similar affinities to the human GLP-1 receptor (Figure 3.3.1.1). In generation 1 of analogues (GL1 to GL5), GL2 had a 15-fold, GL5 a 13-fold and GL4 an 8-fold lower affinity than GLP-1, while GL1 and GL3 appeared to bind at the same affinity (Figure 3.3.1.2).

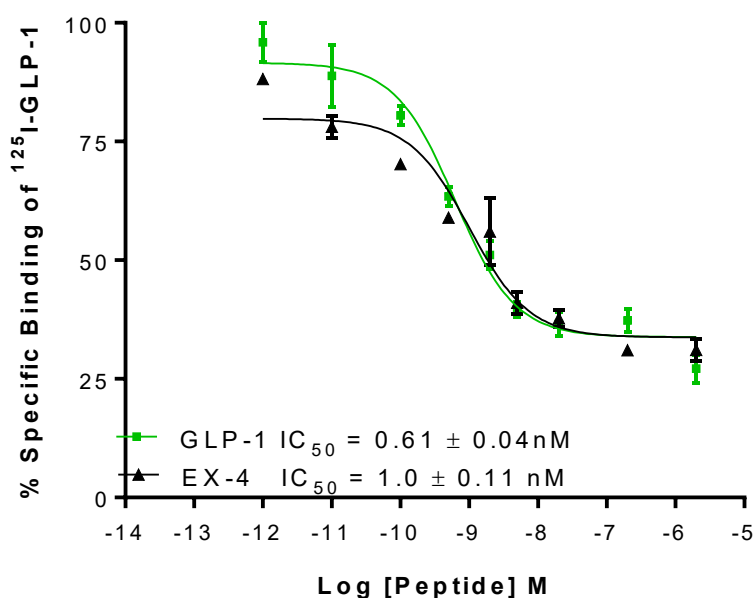


Figure 3.3.1. 1. Binding affinity of GLP-1 and exendin-4 to the human GLP-1 receptor. Human GLP-1 receptor over-expressing CHO cell membranes was the source of the GLP-1 receptors. Peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm \text{SEM}$. Coefficient of variation was calculated GLP-1: 0.04, Ex-4: 0.56

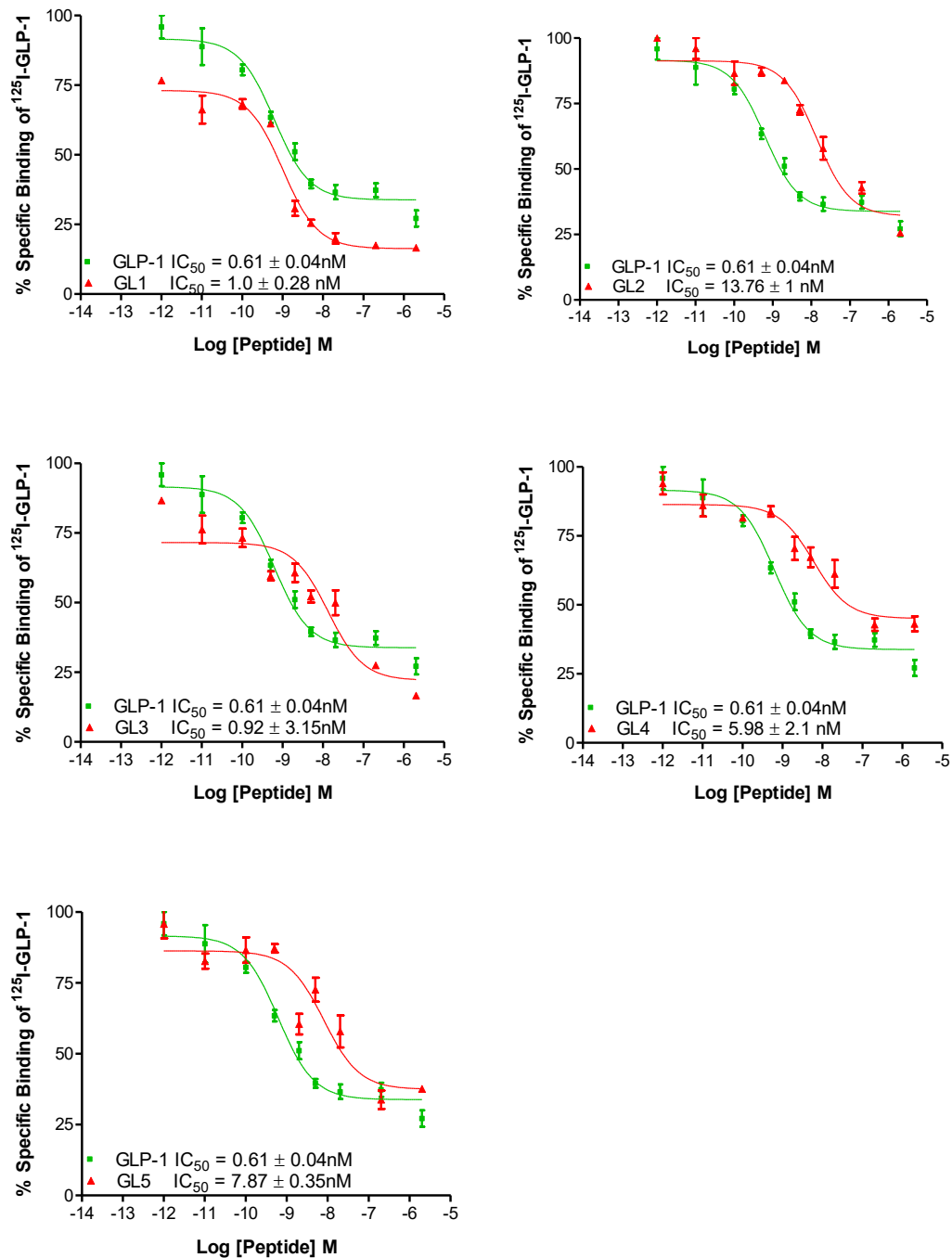


Figure 3.3.1. 2. Binding affinity of GLP-1 analogues to the human GLP-1 receptor. Human GLP-1 receptor over-expressing CHO cell membranes was the source of GLP-1 receptors. Peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm$ SEM.

3.3.1.2. Affinity of the GLP-1 analogues to the mouse GLP-1 receptor

Binding assays with ^{125}I -GLP-1 resulted in greater than 90% specific binding to a preparation of cell membrane extracted from mouse lung. Exendin-4 bound with an almost 2-fold higher affinity to the mouse GLP-1 receptor than GLP-1 (Figure 3.3.1.3). Binding affinities of the first generations of GLP-1 analogues at the mouse GLP-1 receptor were tested and compared with endogenous GLP-1. GL1 had a 25-fold, GL2 a 0.6-fold, while GL3, GL4 and GL5 had an approximately 2-fold lower affinity than GLP-1 (Figure 3.3.1.4).

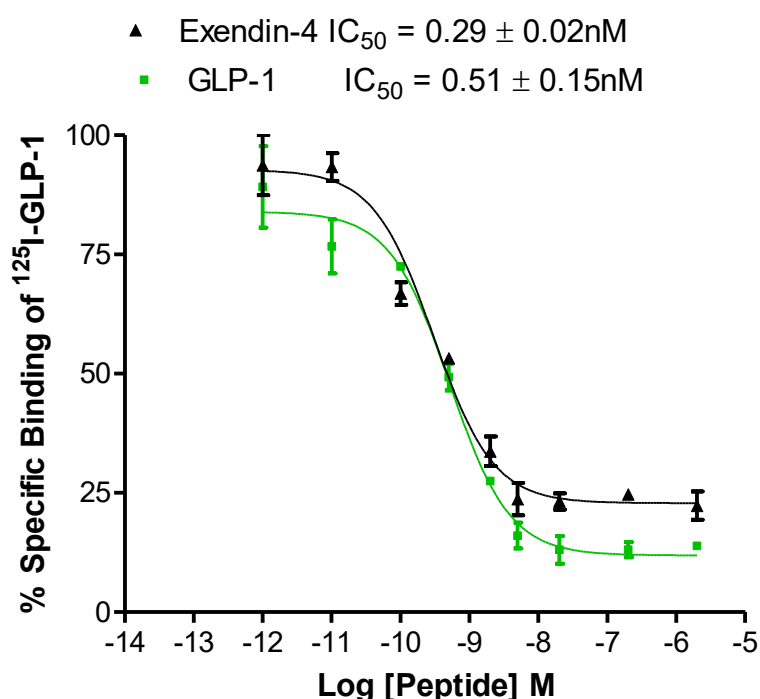


Figure 3.3.1. 3. Binding affinity of GLP-1 and exendin-4 to the mouse GLP-1 receptor. Mouse lung was the source of GLP-1 receptors. Peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm$ SEM. Coefficient of variation was calculated GLP-1: 0.42, Ex-4: 0.10

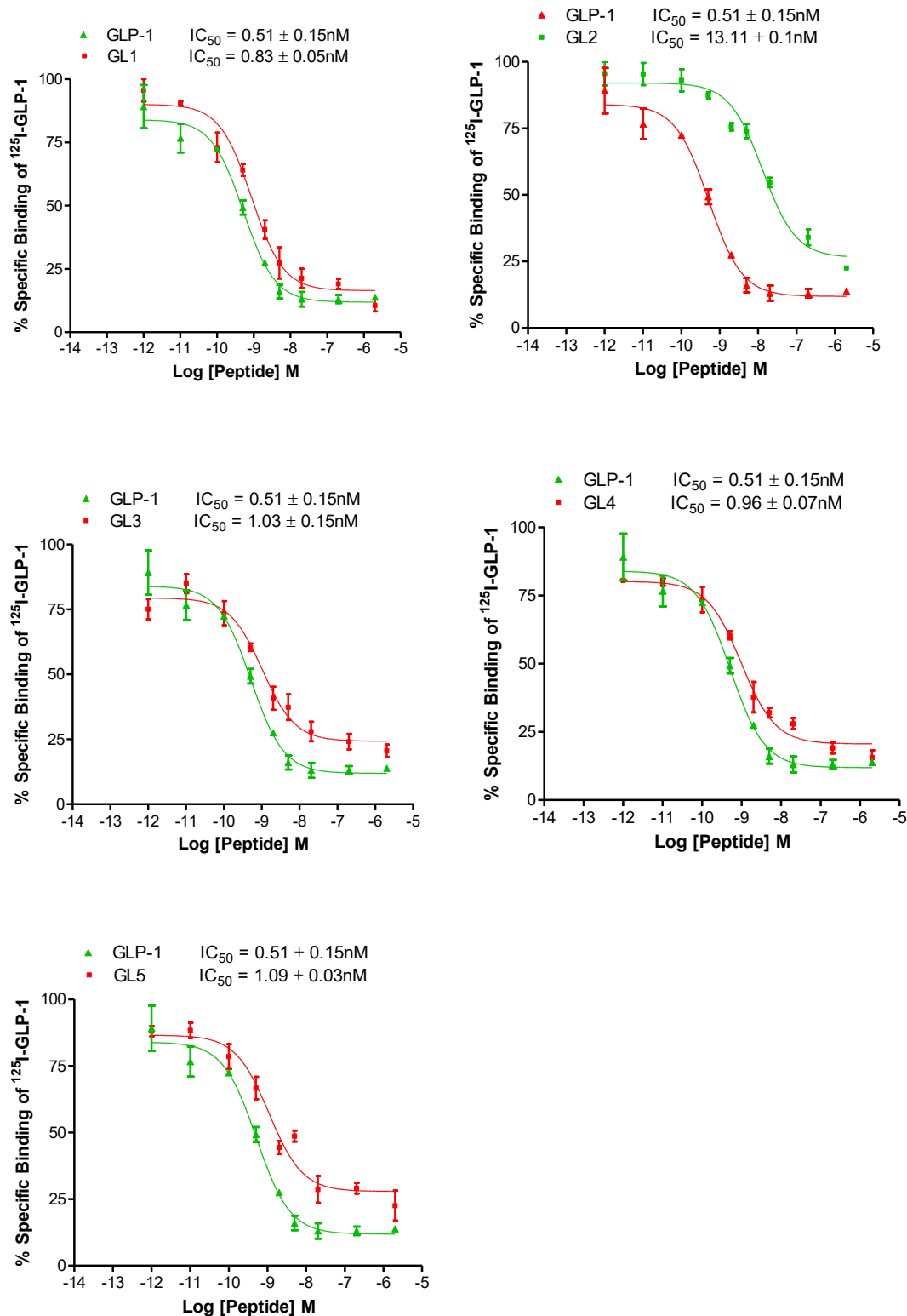


Figure 3.3.1. 4. Binding affinity of GLP-1 analogues to the mouse GLP-1 receptor. Mouse lung was the source of GLP-1 receptors. Peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm$ SEM.

3.3.1.3. Effect of GLP-1 analogues on receptor-mediated cAMP accumulation

The ability of GLP-1 and exendin-4 to stimulate cAMP accumulation in CHO-hGLP-1r cells was investigated. The potencies of GLP-1 and exendin-4 were similar, with exendin-4 having a 2-fold higher potency than GLP-1 (Figure 3.3.1.5). The bioactivity of the first generations of GLP-1 analogues at cAMP accumulation was compared with endogenous GLP-1. GL2 was over 70-fold lower, GL3, GL4 and GL5 did not reach a level that was high enough to obtain an EC_{50} , and GL1 had 3-fold lower potency than GLP-1 (Figure 3.3.1.6).

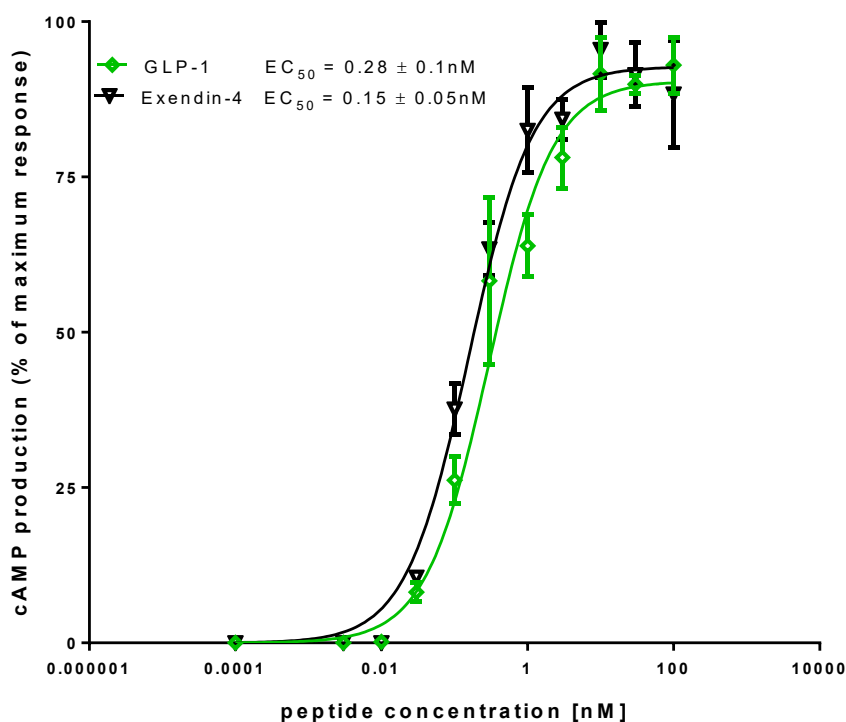


Figure 3.3.1. 5. Human GLP-1 receptor-mediated cAMP accumulation in CHO cells overexpressing the human GLP-1 receptor, by GLP-1 and exendin-4. Peptide concentrations were tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments, errors shown are $\pm$ SEM. Coefficient of variation was calculated GLP-1: 0.42, Ex-4: 0.51

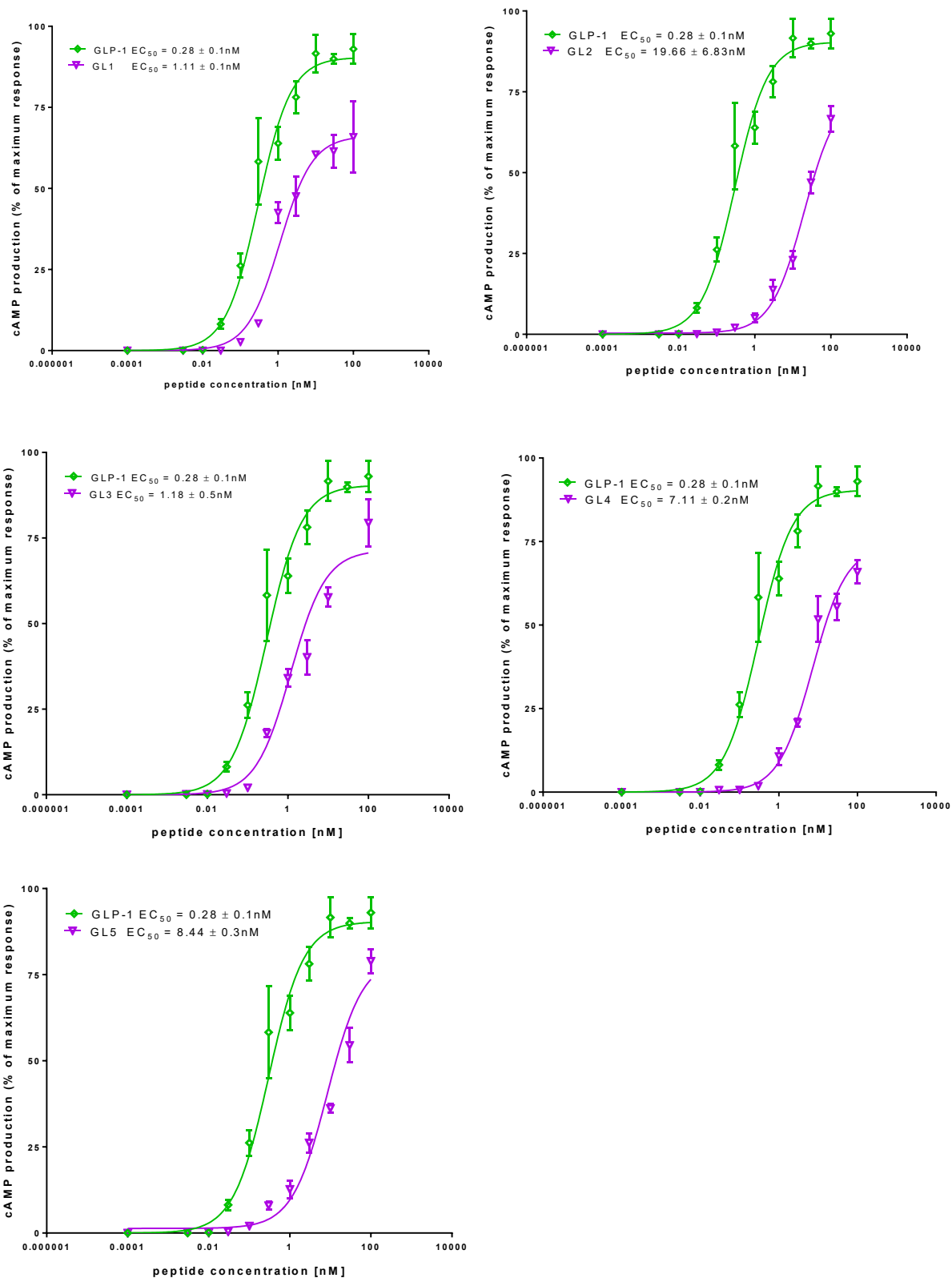


Figure 3.3.1. 6. Human GLP-1 receptor-mediated cAMP accumulation in CHO cells overexpressing the human GLP-1 receptor, by GLP-1 and generation 1 analogues. Peptide concentrations were tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments, errors shown are $\pm$ SEM.

3.3.2. *In vitro* characterisation of second generation of GLP-1 analogues

3.3.2.1. Affinity of the GLP-1 analogues to the human GLP-1 receptor

In the second generation (analogues GL6 to GL12), GL12 had the receptor affinity reaching 18-fold, GL7 a 15-fold, GL8 and GL9 an 8-fold, GL6 and GL10 a 3-fold lower affinity to the human GLP-1 receptor than GLP-1. Analogue GL11 presented the highest affinity with an IC_{50} of 1.2 ± 0.24 nM (Figures 3.3.2.1, 2).

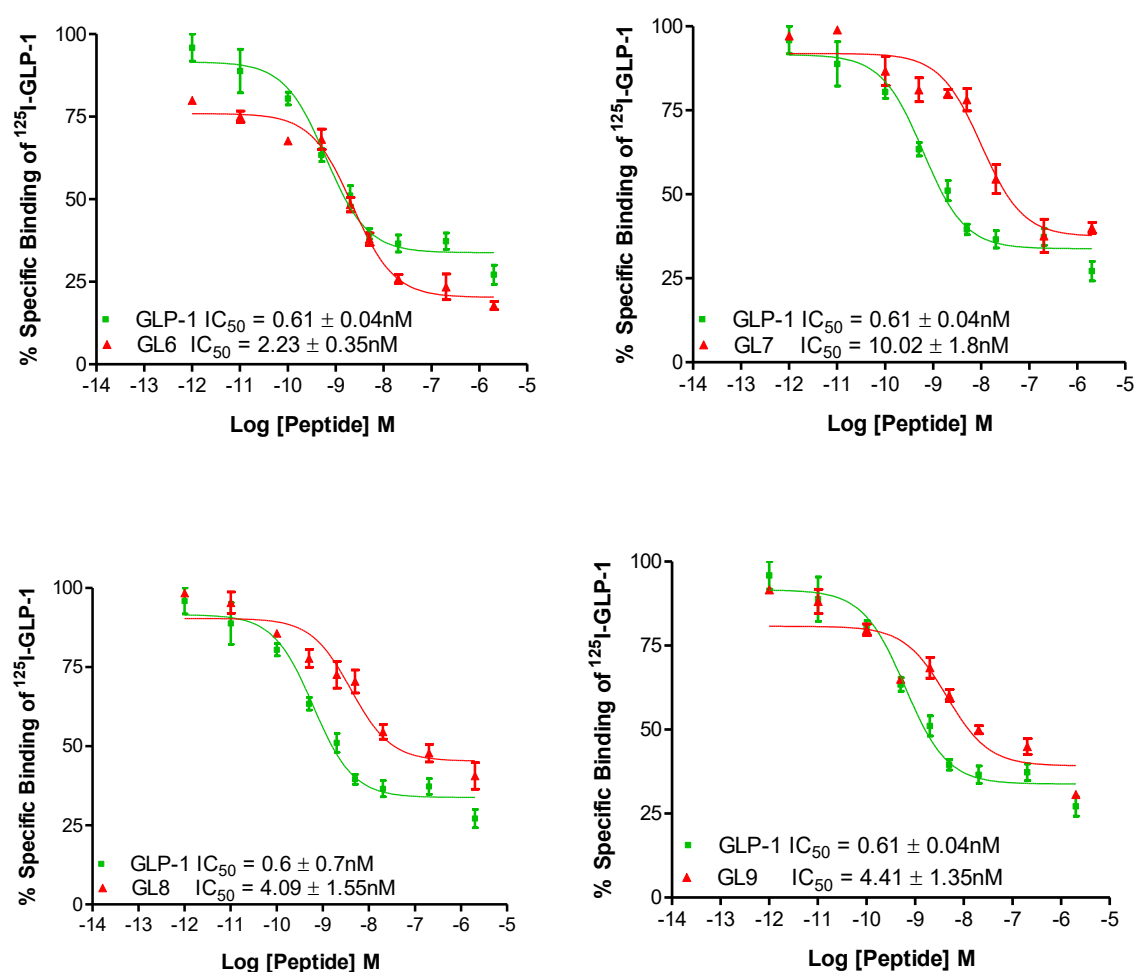


Figure 3.3.2. 1. Binding affinity of GLP-1 analogues to the human GLP-1 receptor. Human GLP-1 receptor over-expressing CHO cell membranes was the source of GLP-1 receptors. Peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm$ SEM.

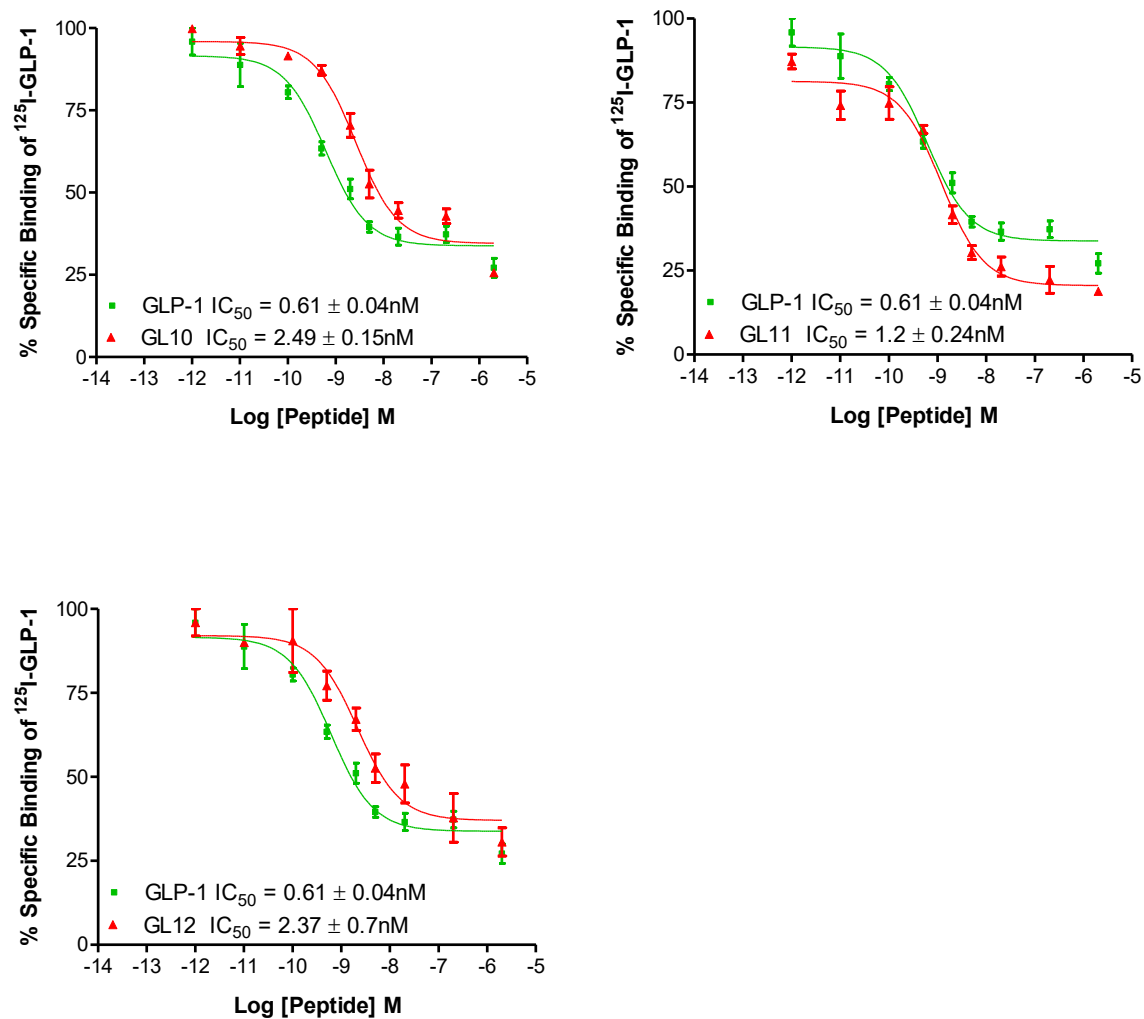


Figure 3.3.2. 2. Binding affinity of GLP-1 analogues to the human GLP-1 receptor. Human GLP-1 receptor over-expressing CHO cell membranes was the source of GLP-1 receptors. Peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm \text{SEM}$.

3.3.2.2. Affinity of the GLP-1 analogues to the mouse GLP-1 receptor

At the mouse GLP-1 receptor testing generation 2 analogues, GL7 had the lowest affinity with an IC_{50} of $7.06 \pm 0.2nM$ while GL9 had a 12-fold, GL10 a 6.8-fold, GL12 a 3.7-fold, and finally GL6, GL8 and GL11 approximately 2-fold lower affinities than GLP-1 (Figure 3.3.2.3, 4). GL11 presented a similarly high affinity to the mouse receptor, as it did on the human receptor ($1.37 \pm 0.2nM$).

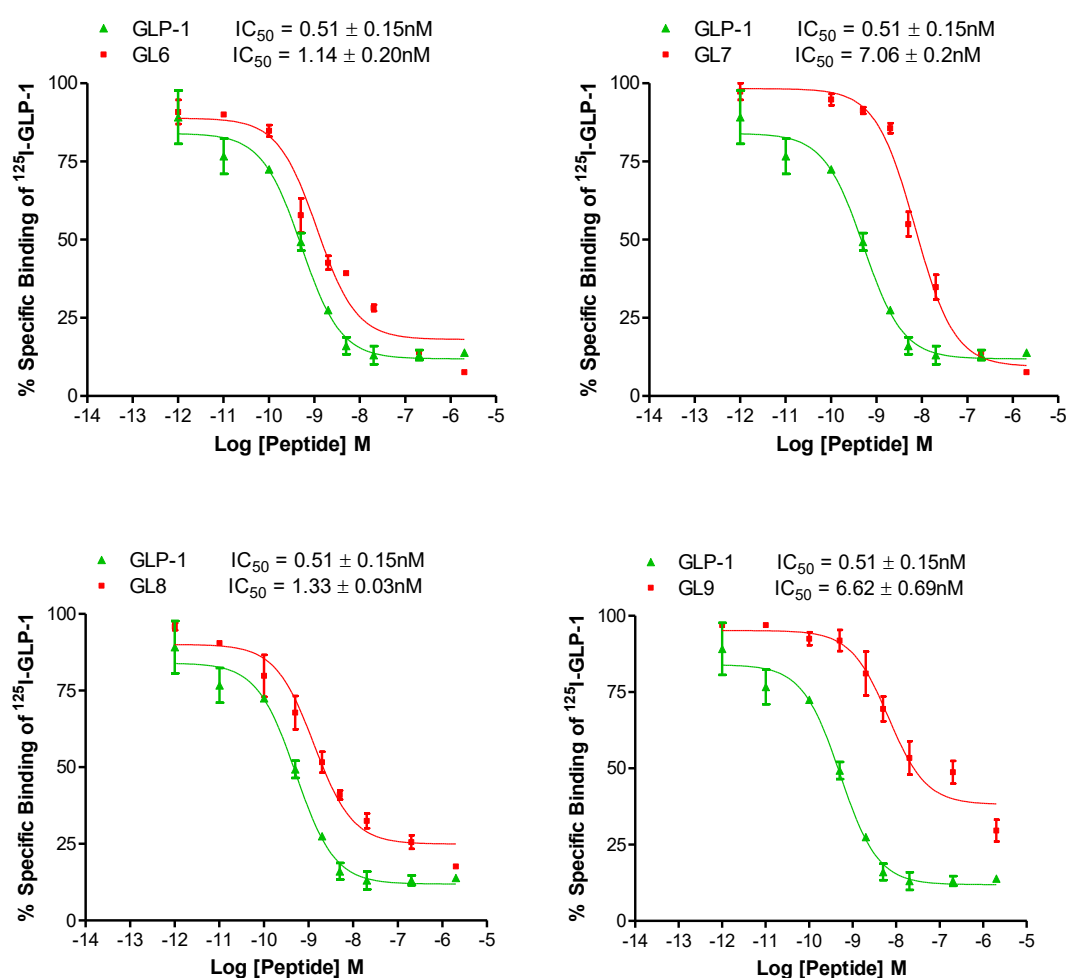


Figure 3.3.2. 3. Binding affinity of GLP-1 analogues to the mouse GLP-1 receptor. Mouse lung was the source of GLP-1 receptors. Peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm$ SEM.

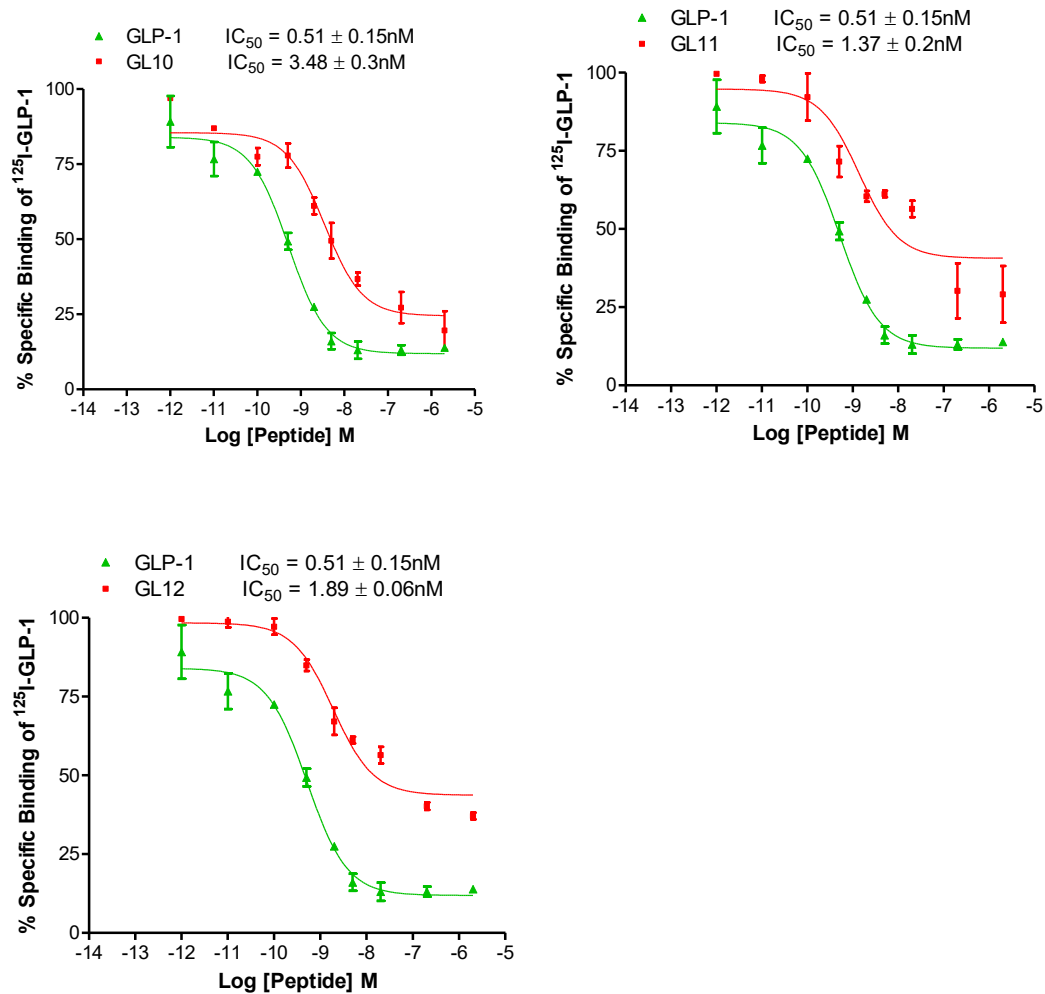


Figure 3.3.2. 4. Binding affinity of GLP-1 analogues to the mouse GLP-1 receptor. Mouse lung was the source of GLP-1 receptors. Peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm \text{SEM}$.

3.3.2.3. Effect of GLP-1 analogues on receptor-mediated cAMP accumulation

The ability of GLP-1 analogues to stimulate cAMP accumulation in CHO-hGLP-1r cells was investigated. In generation 2, GL12 had 4-fold, GL7 and GL9 had a 16-fold; GL8 and GL10 a 10-fold; GL11 a 4-fold; and GL6 a 2-fold lower potency than GLP-1 presenting an EC_{50} of $0.55\text{nM} \pm 0.05\text{nM}$ (Figure 3.3.2.5, 6).

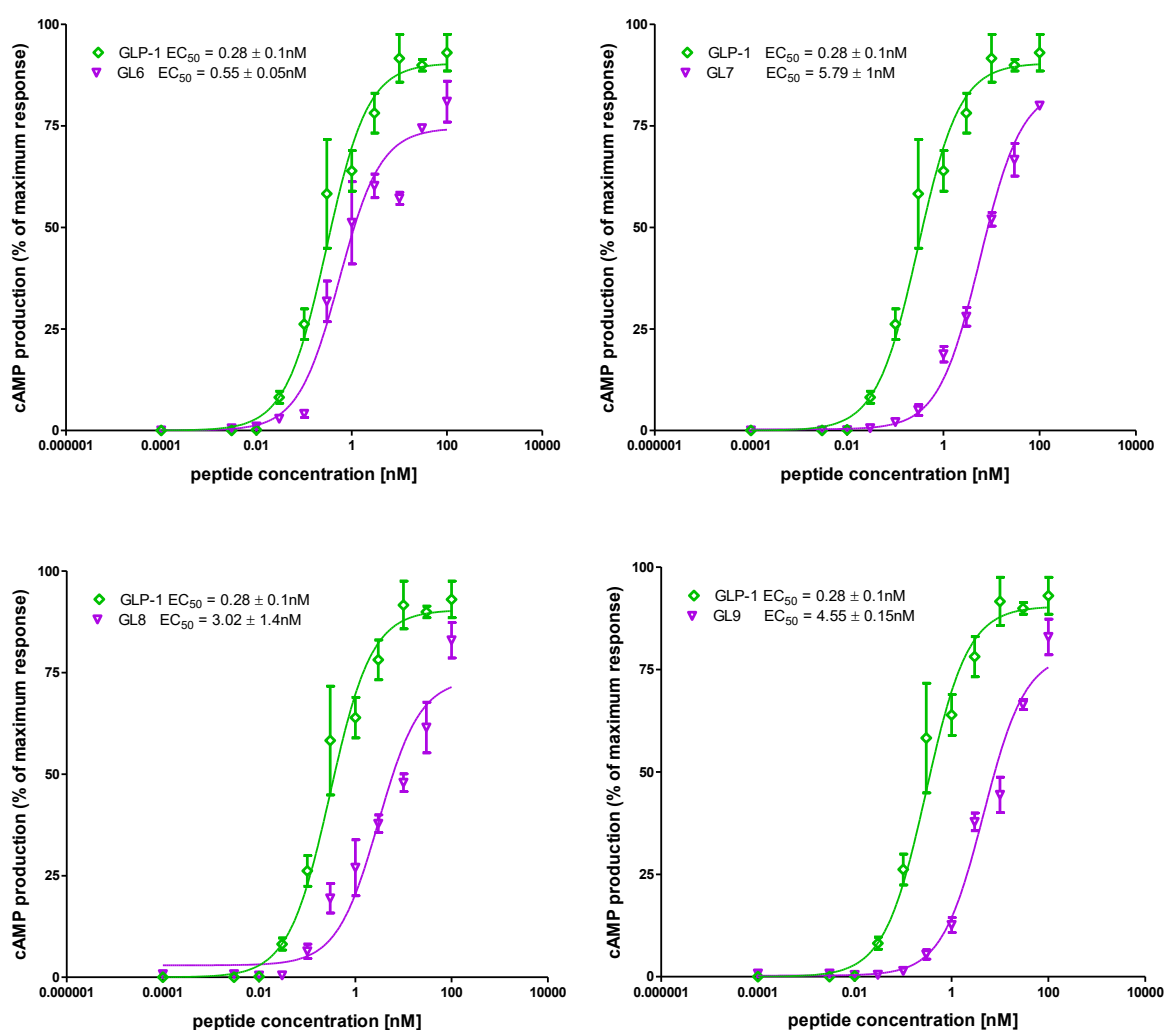


Figure 3.3.2. 5. Human GLP-1 receptor-mediated cAMP accumulation in CHO cells overexpressing the human GLP-1 receptor by GLP-1 and generation 2 analogues. Peptide concentrations were tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments, errors shown are $\pm$ SEM.

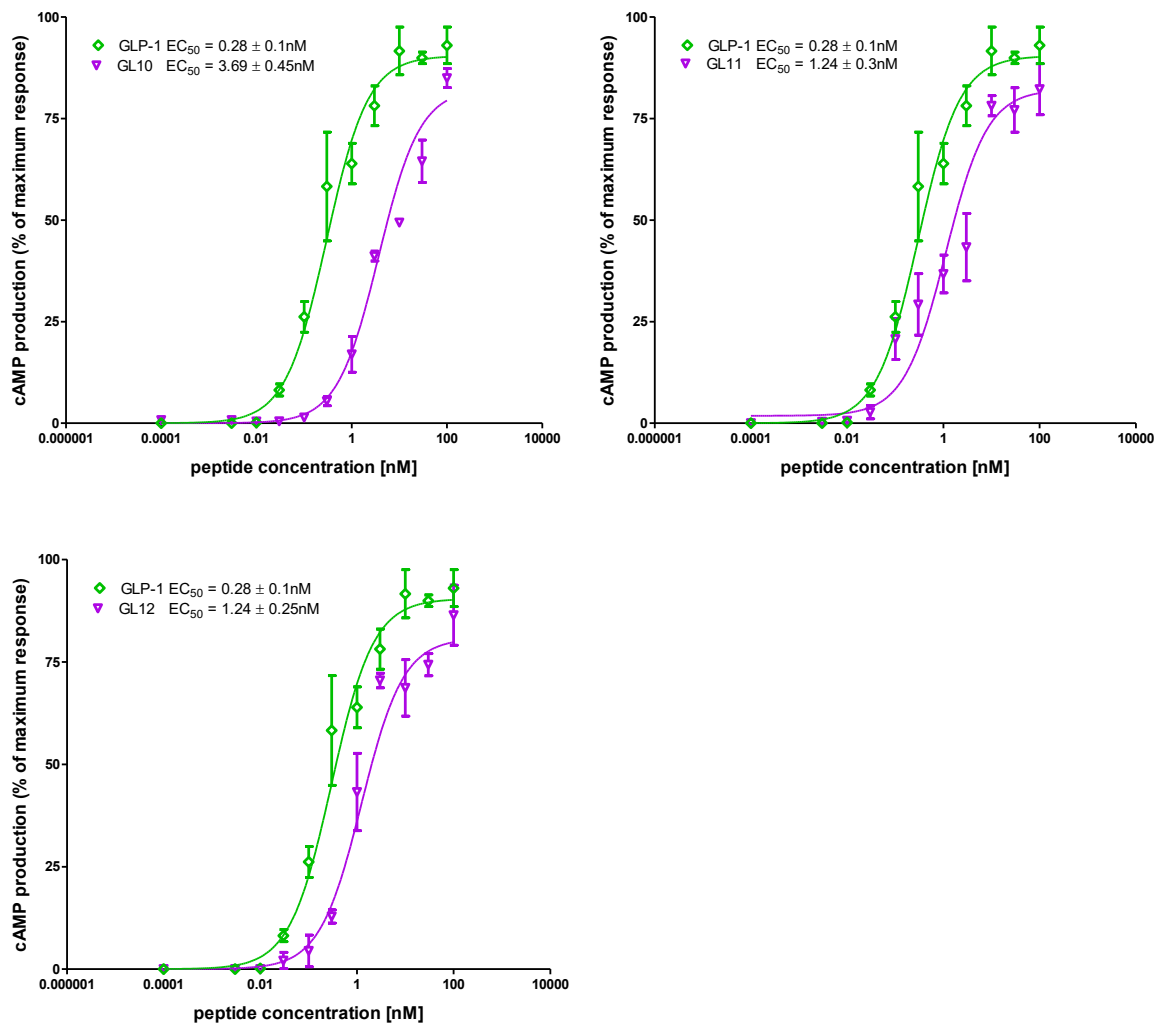


Figure 3.3.2. 6. Human GLP-1 receptor-mediated cAMP accumulation in CHO cells overexpressing the human GLP-1 receptor by GLP-1 and a generation 2 analogues. Peptide concentrations were tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments, errors shown are $\pm$ SEM.

3.3.3. *In vitro* characterisation of third generation of GLP-1 analogues

3.3.3.1. Affinity of the GLP-1 analogues to the human GLP-1 receptor

The majority of the final generation analogues (GL14-16) had similar affinity as native GLP-1 to the human GLP-1 receptor, all binding within 3 fold of native GLP-1 ($0.61 \pm 0.04\text{nM}$), with the exception of GL13 that bound with 8-fold lower affinity, reaching an IC_{50} of $5.02 \pm 0.15\text{nM}$ (Figures 3.3.3.1).

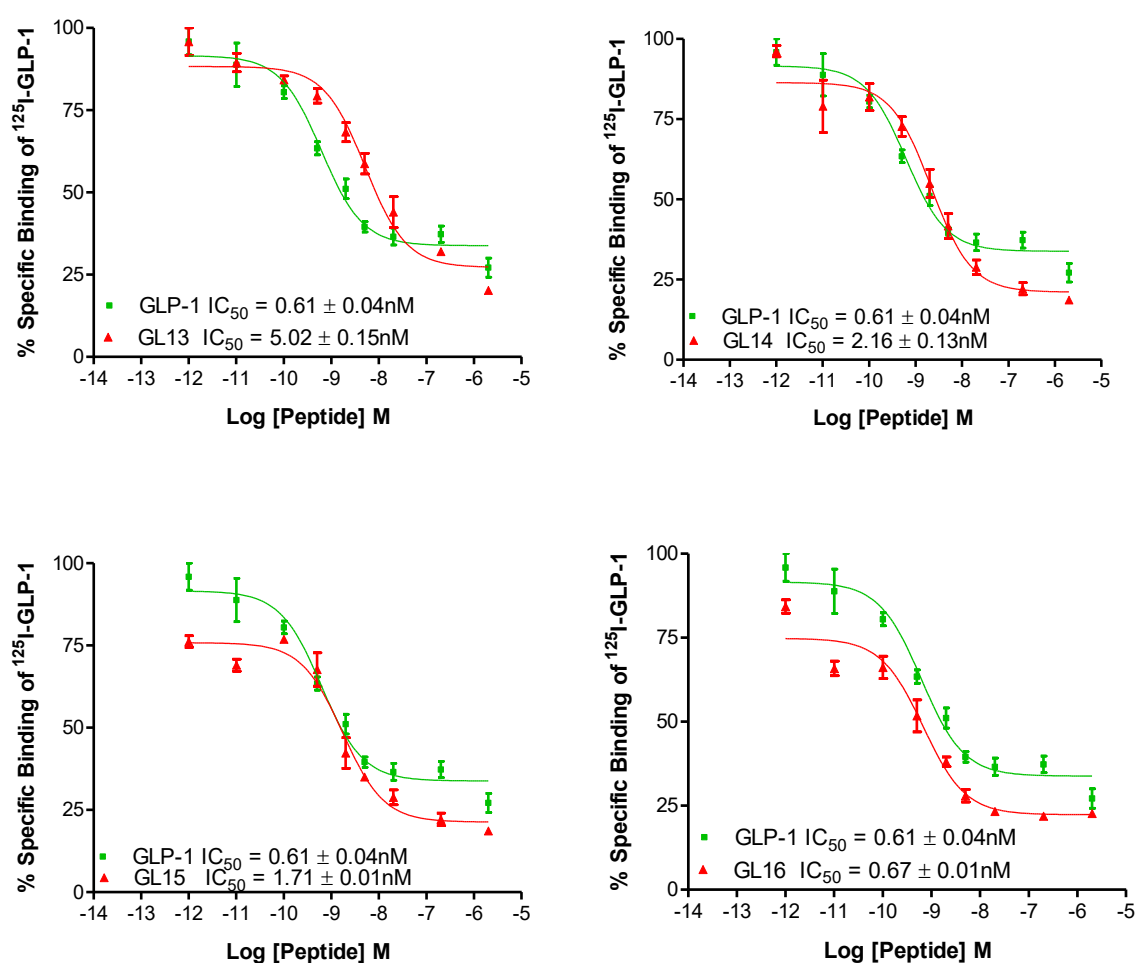


Figure 3.3.3. 1. Binding affinity of GLP-1 analogues to the human GLP-1 receptor. Human GLP-1 receptor over-expressing CHO cell membranes was the source of GLP-1 receptors. Each peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm \text{SEM}$.

3.3.3.2. Affinity of the GLP-1 analogues to the mouse GLP-1 receptor

In the final generation, analogues GL13, GL14 and GL15 had similar affinity as GLP1 to the mouse GLP-1 receptor (0.51 ± 0.15 nM). GL16 appeared to bind at a slightly higher level than GLP-1 (0.48 ± 0.05 nM) (Figure 3.3.3.2).

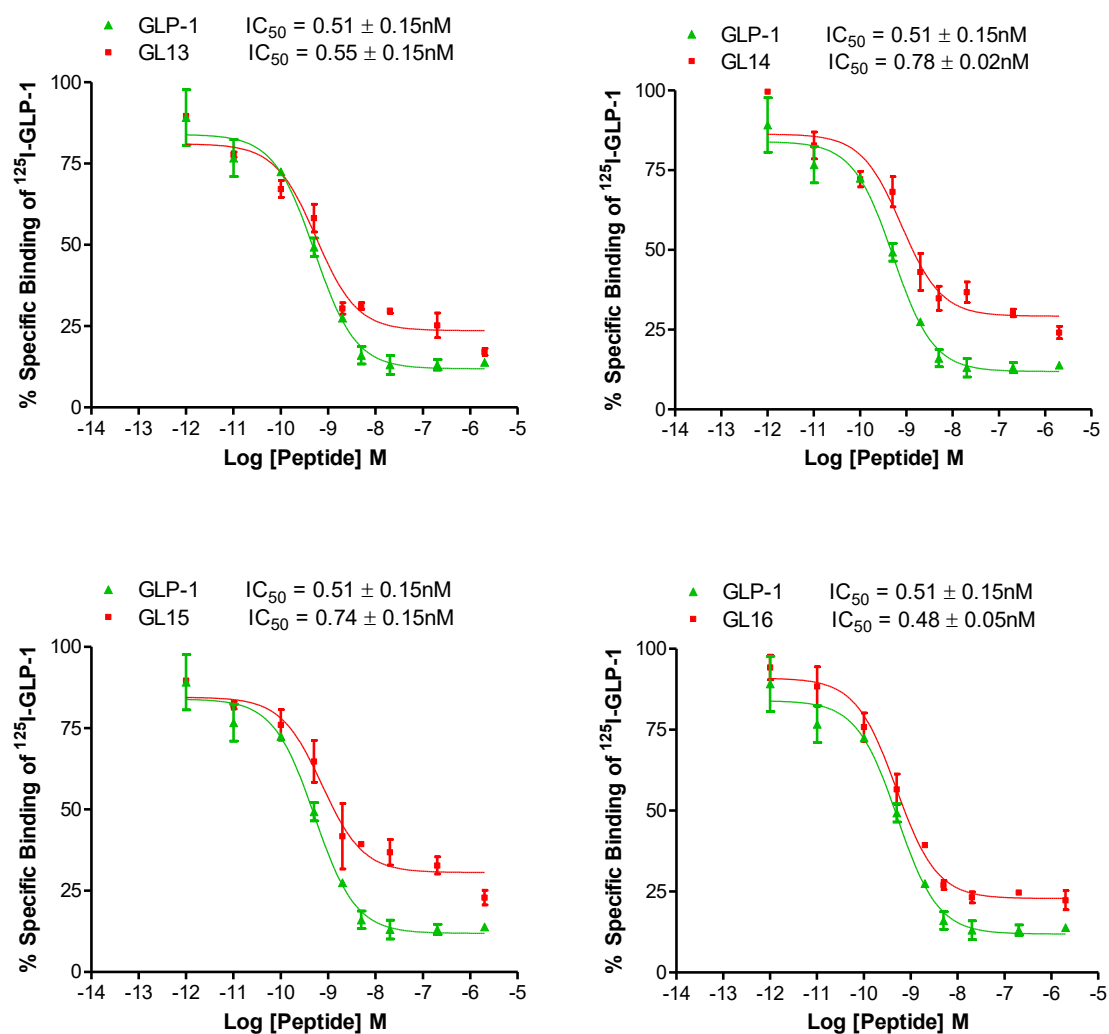


Figure 3.3.3. 2. Binding affinity of GLP-1 analogues to the mouse GLP-1 receptor. Mouse lung was the source of GLP-1 receptors. Peptide concentrations were tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm$ SEM.

3.3.3.3. Effect of GLP-1 analogues on receptor-mediated cAMP accumulation

The ability of GLP-1 analogues to stimulate cAMP accumulation in CHO-hGLP-1r cells was investigated. In generation 3, GL13 had 4-fold, GL15 had 2-fold lower potency, but GL14 and GL16 had similar potency as GLP-1 (0.37 ± 0.3 nM and 0.30 ± 0.4 nM) as shown in figure 3.3.3.3. The values for affinity and *in vitro* potency are tabulated and summarised in table 3.1.

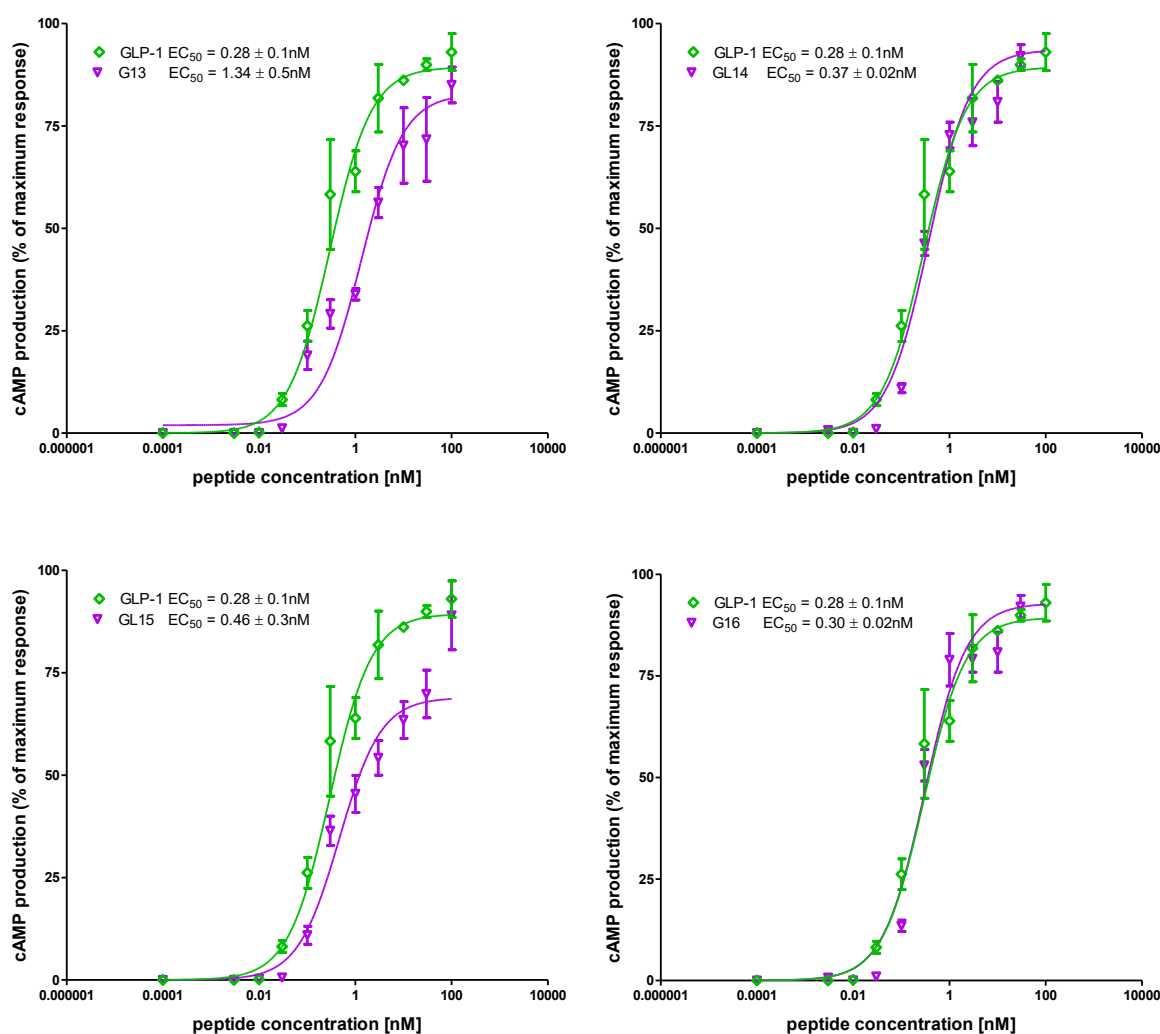


Figure 3.3.3. 3. Human GLP-1 receptor-mediated cAMP accumulation in CHO cells overexpressing the human GLP-1 receptor by GLP-1 and generation 3 analogues. Peptide concentrations were tested in duplicate or triplicate in each experiment. EC_{50} values calculated from a minimum of three separate experiments, errors shown are $\pm$ SEM.

	Mouse GCG receptor	Human GLP-1 receptor	
	Receptor binding affinity	Receptor binding affinity	cAMP accumulation
	IC ₅₀ (nM) ± SEM	IC ₅₀ (nM) ± SEM	EC ₅₀ (nM) ± SEM
GLP-1	0.51 ± 0.15	0.61 ± 0.04	0.28 ± 0.10
Exendin-4	0.29 ± 0.02	1.00 ± 0.11	0.15 ± 0.05
GL1	13.11 ± 0.05	1.00 ± 0.28	1.11 ± 0.10
GL2	0.83 ± 0.10	8.79 ± 1.05	19.66 ± 6.83
GL3	1.03 ± 0.15	0.92 ± 3.15	1.18 ± 0.50
GL4	0.96 ± 0.07	5.98 ± 0.21	7.11 ± 0.20
GL5	1.09 ± 0.03	8.05 ± 0.35	8.44 ± 0.30
GL6	1.14 ± 0.20	2.23 ± 0.35	0.55 ± 0.05
GL7	7.06 ± 0.20	10.02 ± 1.80	5.79 ± 1.00
GL8	1.33 ± 0.03	4.09 ± 1.55	3.02 ± 1.4
GL9	6.62 ± 0.69	4.41 ± 1.35	4.55 ± 0.15
GL10	3.48 ± 0.3	2.49 ± 0.15	3.69 ± 0.45
GL11	1.38 ± 0.20	1.20 ± 0.24	1.24 ± 0.30
GL12	1.89 ± 0.06	2.37 ± 0.70	1.24 ± 0.25
GL13	0.55 ± 0.15	5.02 ± 0.15	1.34 ± 0.50
GL14	0.78 ± 0.02	2.16 ± 0.13	0.37 ± 0.025
GL15	0.74 ± 0.15	1.13 ± 0.01	0.46 ± 0.30
GL16	0.48 ± 0.05	0.67 ± 0.01	0.3 ± 0.02

Table 3.2. A summary of receptor affinity (IC₅₀) to the human and mouse GLP-1r and receptor activity (EC₅₀) at the human GLP-1 receptor for the natural ligands and GLP-1 analogues. Half-maximal concentration values are shown as mean ± SEM.

3.3.4. Effect of GLP-1 analogues on food intake in fasted mice

SC administration of exendin-4 (10nmol/ kg) and all GLP-1 analogues except GL1 (50nmol/ kg) reduced food intake in fasted C57BL/6J mice over the first eight hours post injection compared to vehicle control treated mice. In the first generation, GL2 to GL5 reduced food intake from 2 to 8 hours, while GL2, GL4 and GL5 preserved the food intake inhibition up to 24 hours (Figure 3.3.4.1.). Analysis of cumulative food intake data showed exendin-4 (10nmol/ kg) significantly reduced food intake 0-4 hours post-injection compared to vehicle control treated mice. Cumulative food intake at 0-8 and 0-24 hours post-injection was significantly reduced in exendin-4 and GL2, GL3, GL4 and GL5 compared to the vehicle control group (Figure 3.3.4.2). GL2, GL3 and GL5 maintained this effect until 48hr, however this translated to significant weight loss only in groups GL2 and GL5 (Figure 3.3.4.3.).

Generation 1:

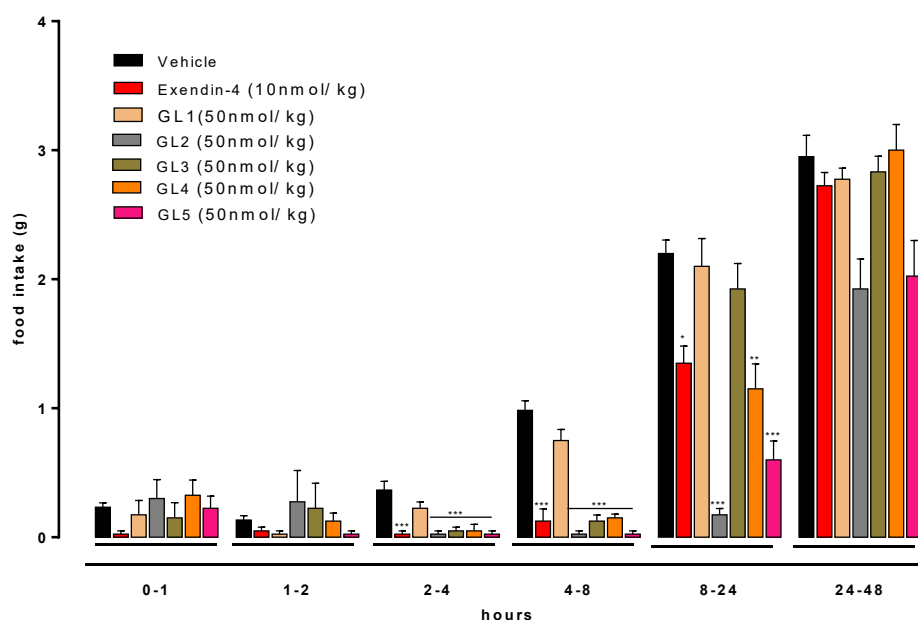


Figure 3.3.4. 1. Effect of a single SC injection of exendin-4 (10nmol/ kg) and generation 1 GLP-1 analogues (50nmol/ kg) on acute food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice (n=7-8 per group), for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour, 8-24 hour and 24-48 hour intervals. Statistics: One-way ANOVA with Dunnett's post-hoc test *=p<0.05, **=p<0.01, ***=p<0.001 vs. vehicle.

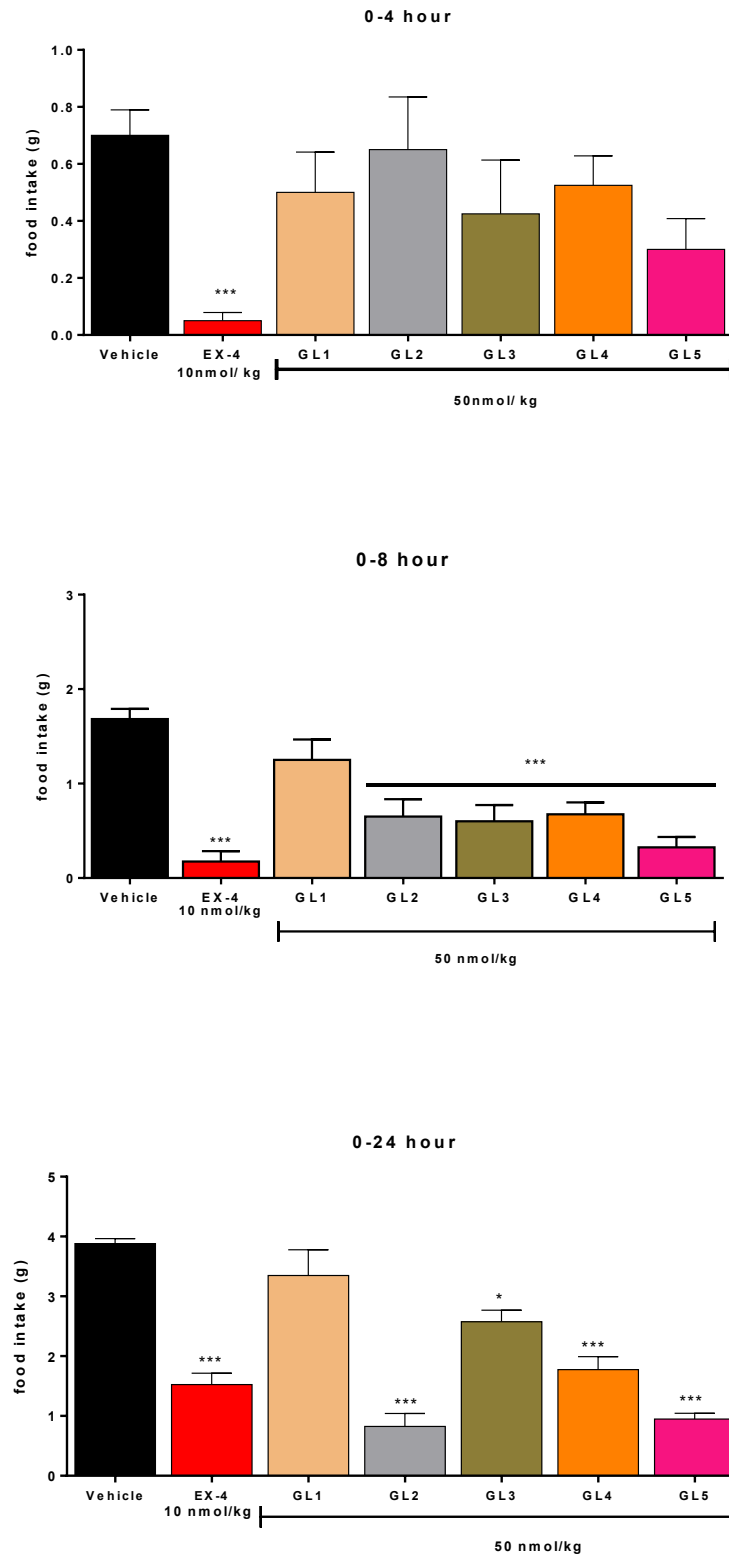


Figure 3.3.4. 2. Effect of a single SC injection of exendin-4 (10nmol/ kg) and generation 1 GLP-1 analogues (50nmol/ kg) on cumulative food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice (n=5-6 mice per group) for 0-4, 0-8 and 0-24 hours. Statistics: One-way ANOVA with Tukey's post-hoc test. *= p <0.05, *= p <0.001 vs. vehicle.**

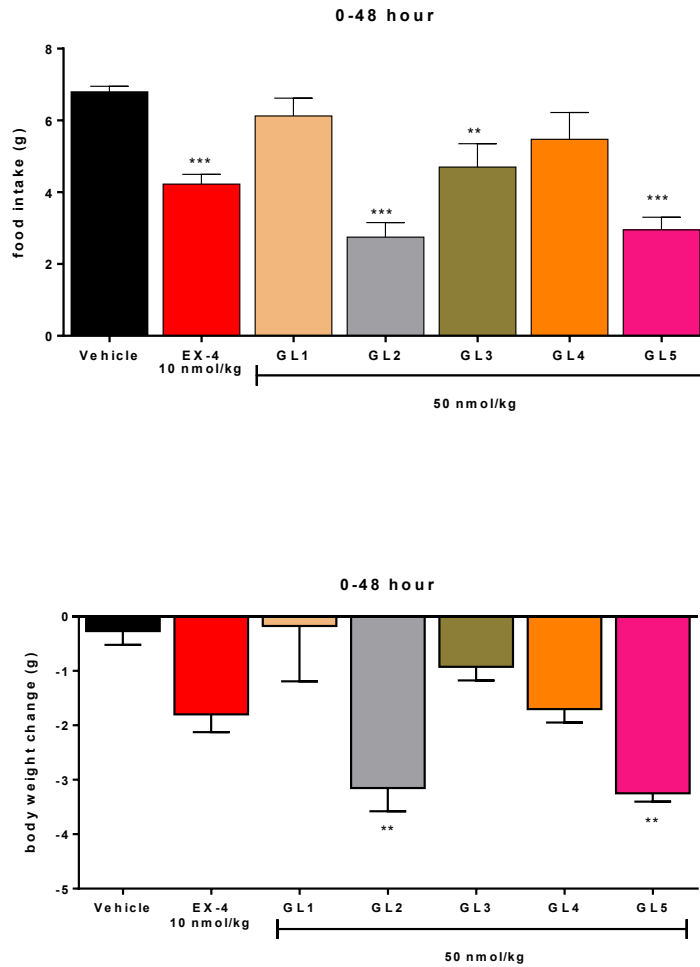


Figure 3.3.4. 3. Effect of a single SC injection of exendin-4 (10nmol/ kg) and generation 1 GLP-1 analogues (50nmol/ kg) on cumulative food intake and body weight change in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake and body weight change in mice (n=5-6 mice per group) for 0-48 hours. Statistics: One-way ANOVA with Tukey's post-hoc test. **= $p < 0.01$, *= $p < 0.001$ vs. vehicle.**

Generation 2:

In the second generation, exendin-4, GL6, GL8 and GL9 significantly reduced food intake from 2 to 8 hours post injection. GL10 also reduced food intake between 4 and 8 hours (Figure 3.3.4.4.). Cumulative food intake at 0-4 hours showed significant inhibition in exendin-4 and GL6 groups compared to vehicle controls, while at 0-8 hours the effect was extended to GL8, GL9, GL10 and GL11 (Figure 3.3.4.5.). GL6, GL8, GL9 and GL10 maintained the effect until 48 hours, yet they did not cause a significant weight loss, compared to the vehicle control group (Figure 3.3.4.6).

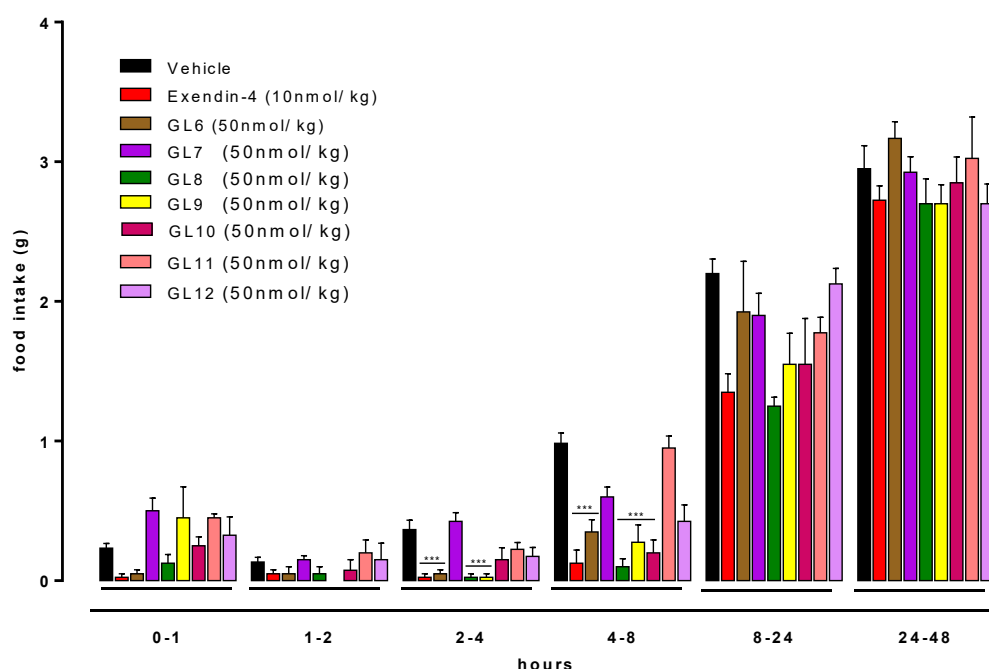


Figure 3.3.4. 4. Effect of a single SC injection of exendin-4 (10nmol/ kg) and generation 2 GLP-1 analogues (50nmol/ kg) on acute food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice (n=7-8 per group) for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour, 8-24 hour and 24-48 hour intervals. Statistics: One-way ANOVA with Dunnett's post-hoc test ***=p<0.001 vs. vehicle.

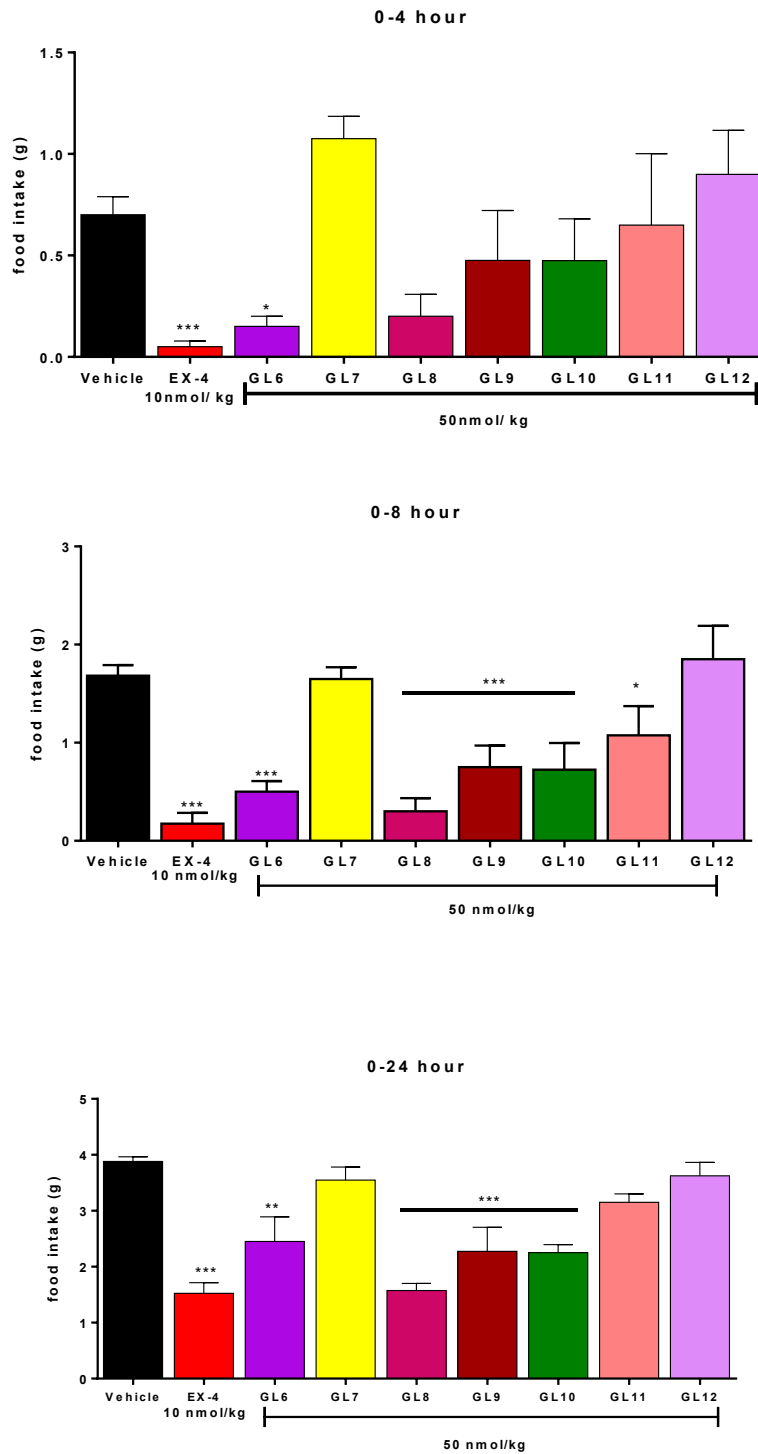


Figure 3.3.4. 5. Effect of a single SC injection of exendin-4 (10nmol/ kg) and generation 2 GLP-1 analogues (50nmol/ kg) on cumulative food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice (n=5-6 mice per group) for 0-4, 0-8 and 0-24 hours. Statistics: One-way ANOVA with Tukey's post-hoc test. *=p<0.05, ***=p<0.001 vs. vehicle.

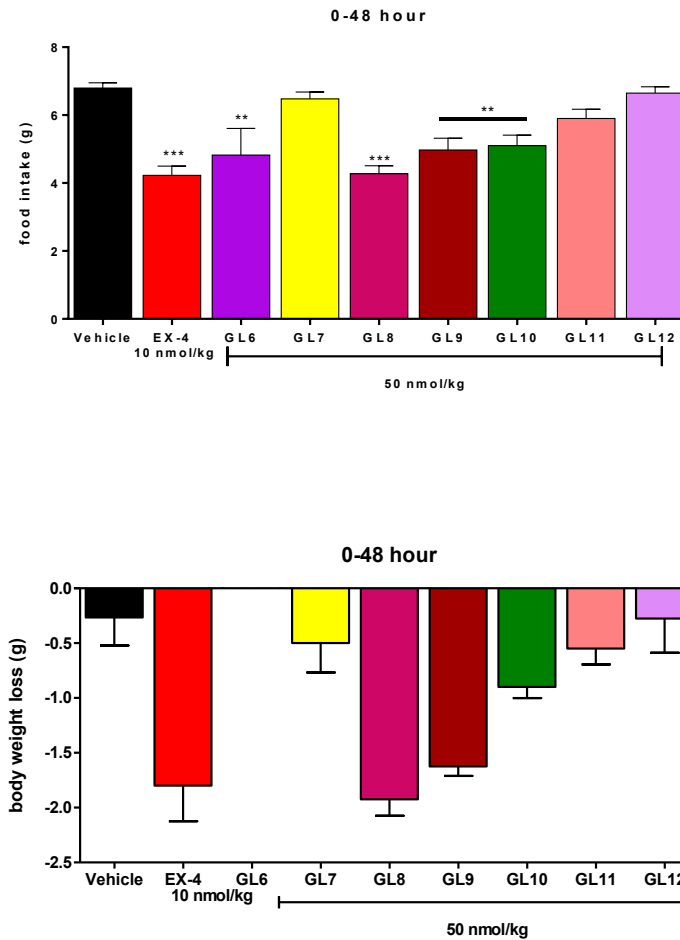


Figure 3.3.4. 6. Effect of a single SC injection of exendin-4 (10nmol/ kg) and generation 2 GLP-1 analogues (50nmol/ kg) on cumulative food intake and body weight change in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake and body weight change in mice (n=5-6 mice per group) for 0-48hours. Statistics: One way ANOVA with Tukey's post-hoc test. **= $p < 0.01$, ***= $p < 0.001$ vs. vehicle.

Generation 3:

In generation 3, food intake was significantly inhibited in mice, up to the 4-8h interval with analogues GL14, GL15 and GL16 (50nmol/ kg), compared to vehicle controls. GL13 (50nmol/ kg) inhibited food intake in the 8-24h interval while no other group, including exendin-4 did, when compared to vehicle controls (Figure 3.3.4.7.). Cumulative data presents significant food intake inhibition in all peptide groups up until 8 hours. GL13, GL14 and GL16 maintained this at 24 hours (Figure 3.3.4.8.), and GL13 and GL16 by 48 hours. , none caused a significant weight loss at 48 hours, when compared to the vehicle control group (Figure 3.3.4.9.).

Analysis of cumulative body weight change data showed no significance between the majority of the analogues and the control group, with the exception of GL2 and GL3 which both caused an average of 3 g body of weight loss (Figure 3.3.4.3.).

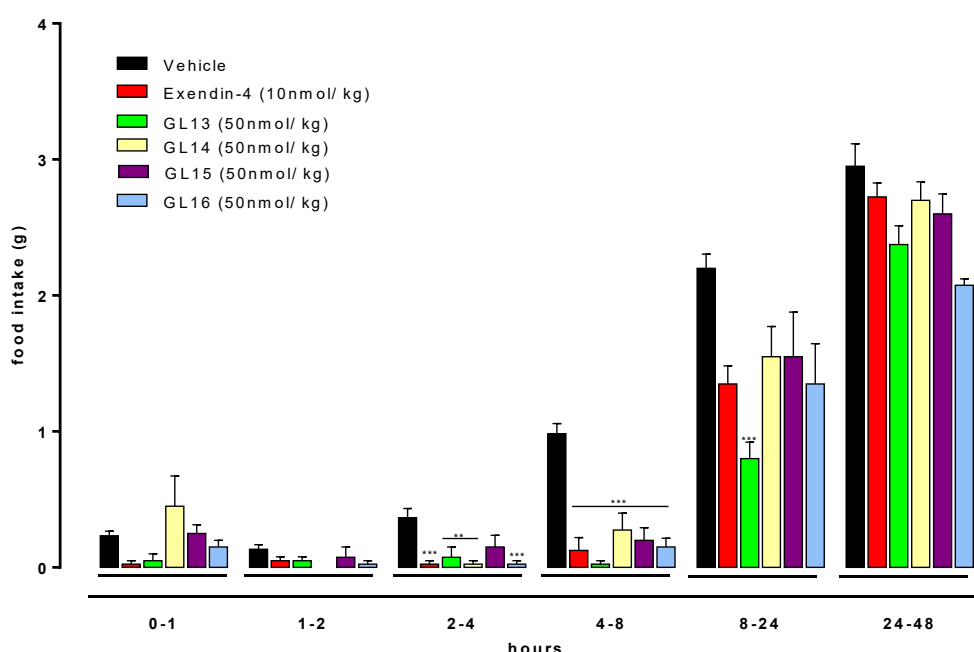


Figure 3.3.4. 7. Effect of a single SC injection of exendin-4 (10nmol/ kg) and generation 3 GLP-1 analogues (50nmol/ kg) on acute food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice (n=7-8 per group) for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour, 8-24 hour and 24-48 hour intervals. Statistics: One-way ANOVA with Dunnett's post-hoc test **= $p < 0.01$, *= $p < 0.001$ vs. vehicle.**

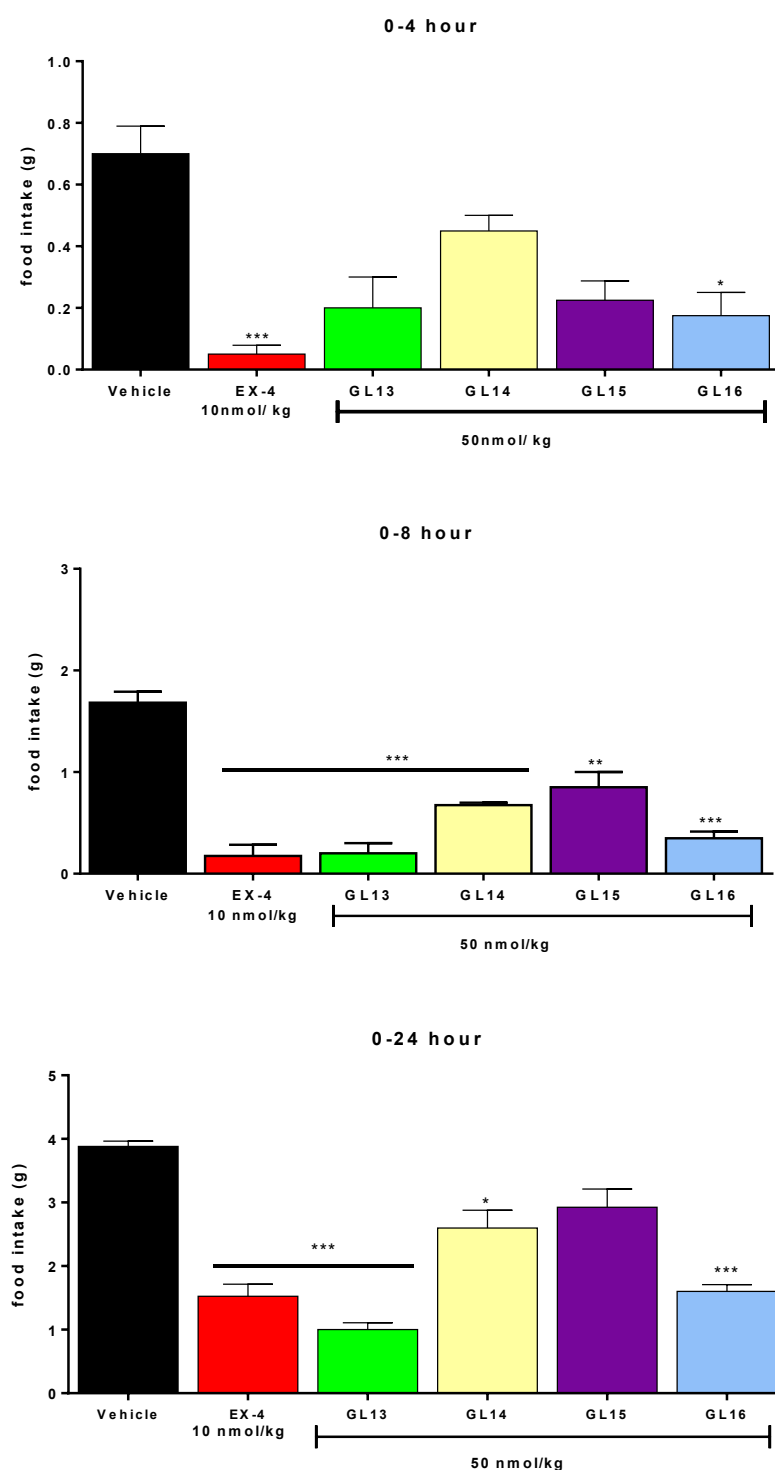


Figure 3.3.4. 8. Effect of a single SC injection of exendin-4 (10nmol/ kg) and generation 3 GLP-1 analogues (50nmol/ kg) on cumulative food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice (n=5-6 mice per group) for 0-4, 0-8 and 0-24 hours. Statistics: One-way ANOVA with Tukey's post-hoc test. *=p<0.05, **=p<0.01, *=p<0.001 vs. vehicle.**

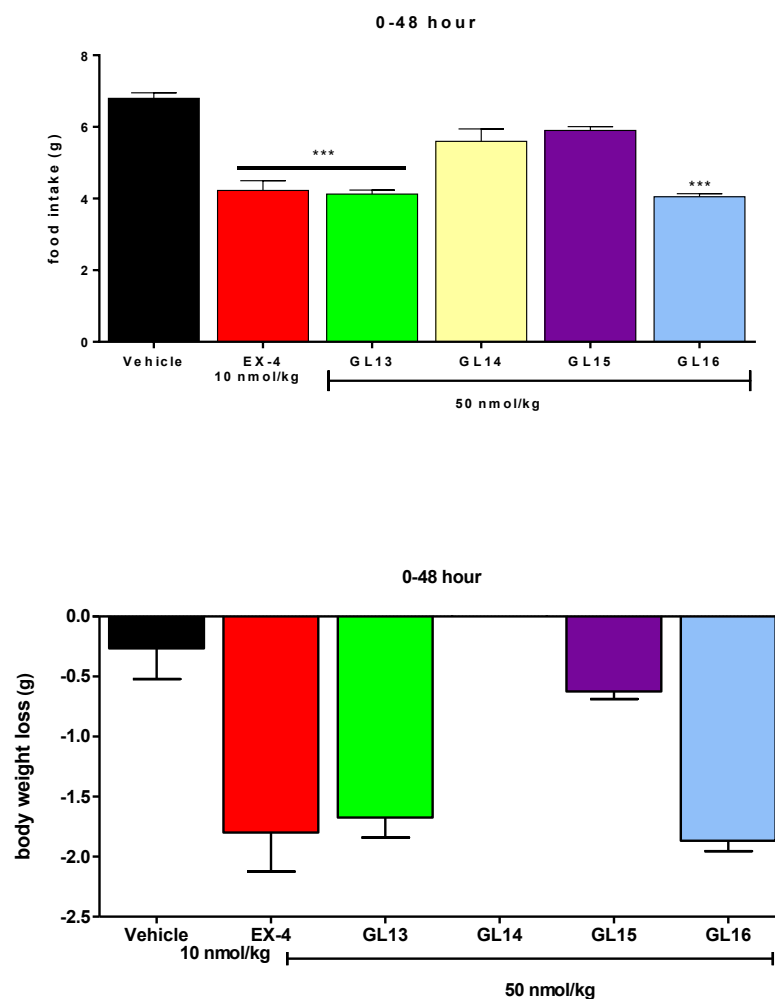


Figure 3.3.4. 9.Effect of a single SC injection of exendin-4 (10nmol/ kg) and generation 3 GLP-1 analogues (50nmol/ kg) on cumulative food intake and body weight change in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake and body weight change in mice (n=5-6 mice per group) for 0-48hours. Statistics: One-way ANOVA with Tukey's post-hoc test. ***=p<0.001 vs. vehicle.

3.3.5. Study of *in vivo* pharmacokinetics of GLP-1, exendin-4 and GLP-1 analogues in male Wistar rats

The pharmacokinetic profiles of the GLP-1 analogues, when administered as a 1mg 1:1 molar ratio Zn:NaCl (saline) SC injection to male Wistar rats, were investigated. In generation 1, T_{max} for GL1 was recorded at 4h yet the peptide concentration dropped at approximately half C_{max} at 24h and became undetectable by 7 days. GL2 peaked at 4h and dropped by 75% by 24h. GL3 T_{max} was observed at 4h and peptide concentration remained approximately stable until 24h, yet could no longer be detected past 2 days (Figure 3.3.5.1). Of note, GL4 T_{max} was at 24h and peptide concentration dropped by 50% in less than 2 days, while GL5 peaked at 4h and reached its half maximal concentration by 24hr (Figure 3.3.5.2.).

Similarly, in generation 2, GL6 recorder T_{max} was at 4h and peptide concentration remained stable until 24h but was not detectable at 2 days. GL7 peaked at 4h and reached its half maximal concentration by 2 days but levels remained high until 7 days. GL8 recorded T_{max} was at 4h and peptide concentration dropped by 50% at 24h (Figure 3.3.5.3.). Similarly, GL9 and GL10 observed T_{max} were at 4h and half maximal levels lasted almost 2 days but were not detectable past that point. GL11 maintained the peak concentration between 4hr and 24hr and only dropped after 2 days (Figure 3.3.5.4.), while GL12 T_{max} was recorded at 24h and reached its half maximal concentration at approximately 2 days. Both peptides were not detectable by 7 days (Figure 3.3.5.5.).

In generation 3, GL13 T_{max} was at 24h and was maintained until approximately 2 days with a small clearance at 3 days, while GL14 T_{max} was observed between 4 and 24 hr, yet peptide levels remained stable between 3 and 7 days. GL15 T_{max} was recorded at 4h and half maximal concentration was observed at 2 days (Figure 3.3.5.6.). GL16 T_{max} was stable between 4 and 24h and peptide levels only dropped by 30% until 7 days post injection (Figure 3.3.5.7.).

Generation 1:

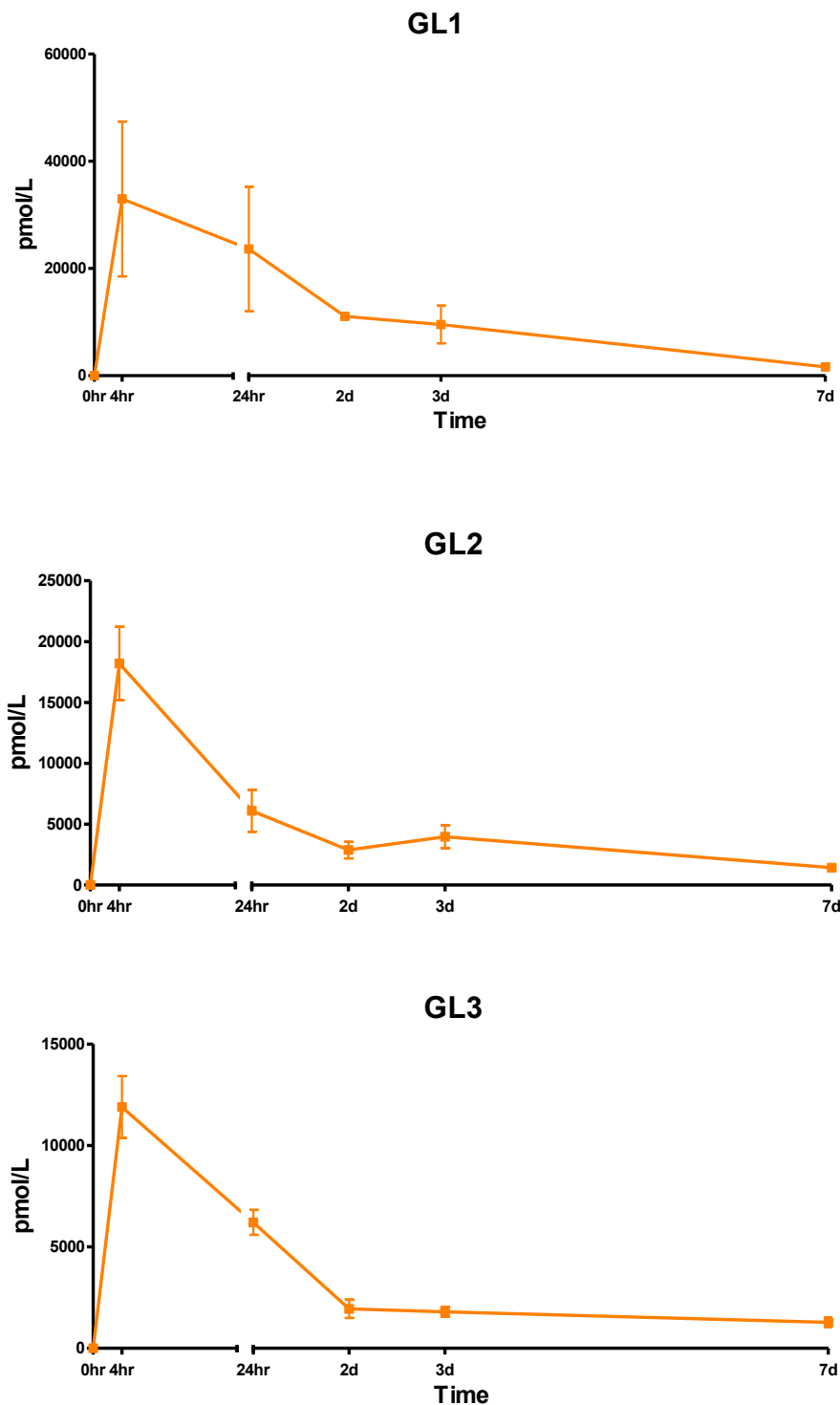


Figure 3.3.5. 1. Plasma levels of GLP-1 analogues GL1-3 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection. Plasma concentrations recorded in pmol/L with error bars denoting $\pm$ SEM. Values were corrected according to baseline sample and Lower Levels Of Quantifications (LLOQs) for RIA.

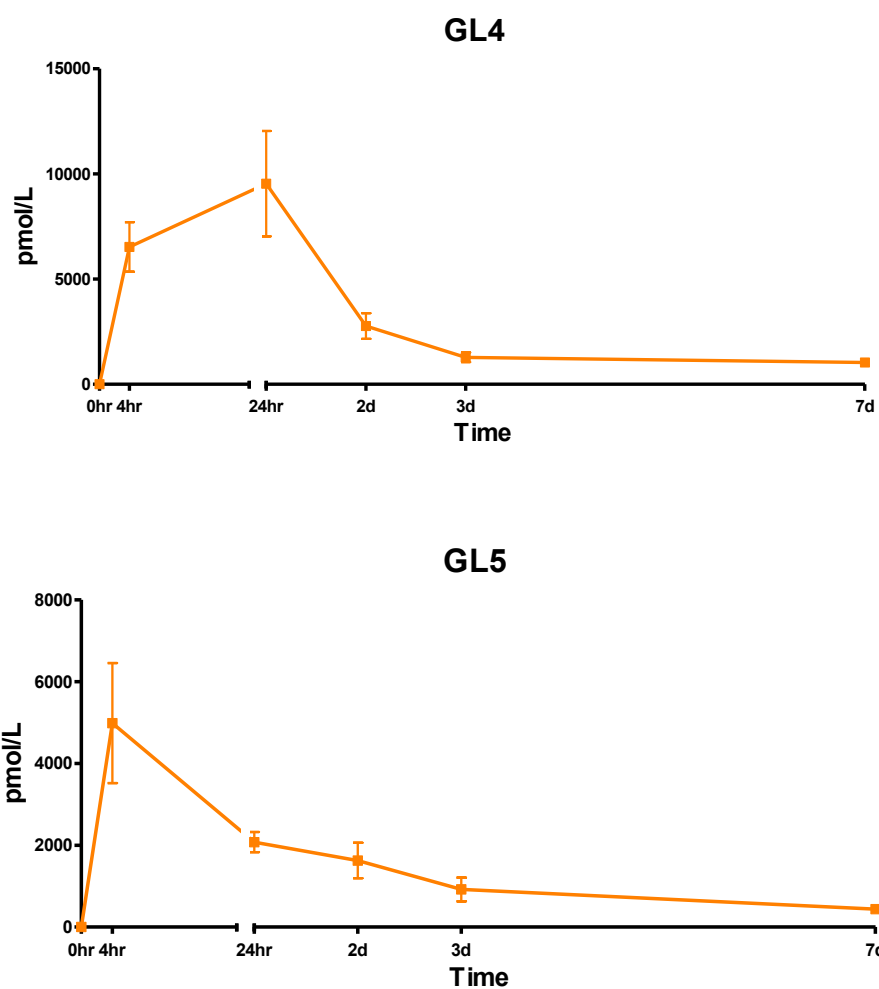


Figure 3.3.5. 2. Plasma levels of GLP-1 analogues GL4-5 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection. Plasma concentrations recorded in pmol/L with error bars denoting $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for RIA.

Generation 2:

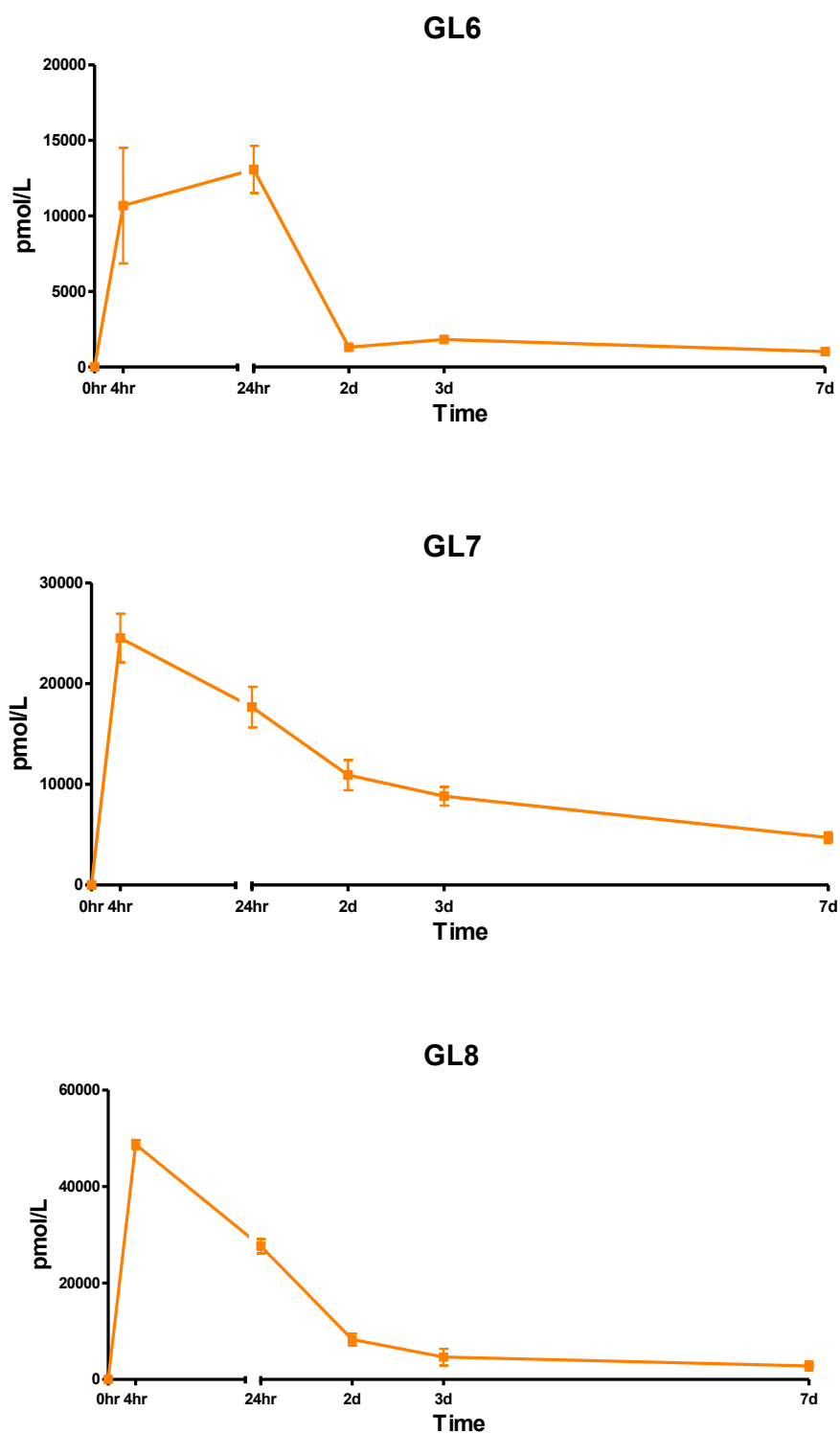


Figure 3.3.5. 3 .Plasma levels of GLP-1 analogues GL6-8 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection. Plasma concentrations recorded in pmol/L with error bars denoting $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for RIA.

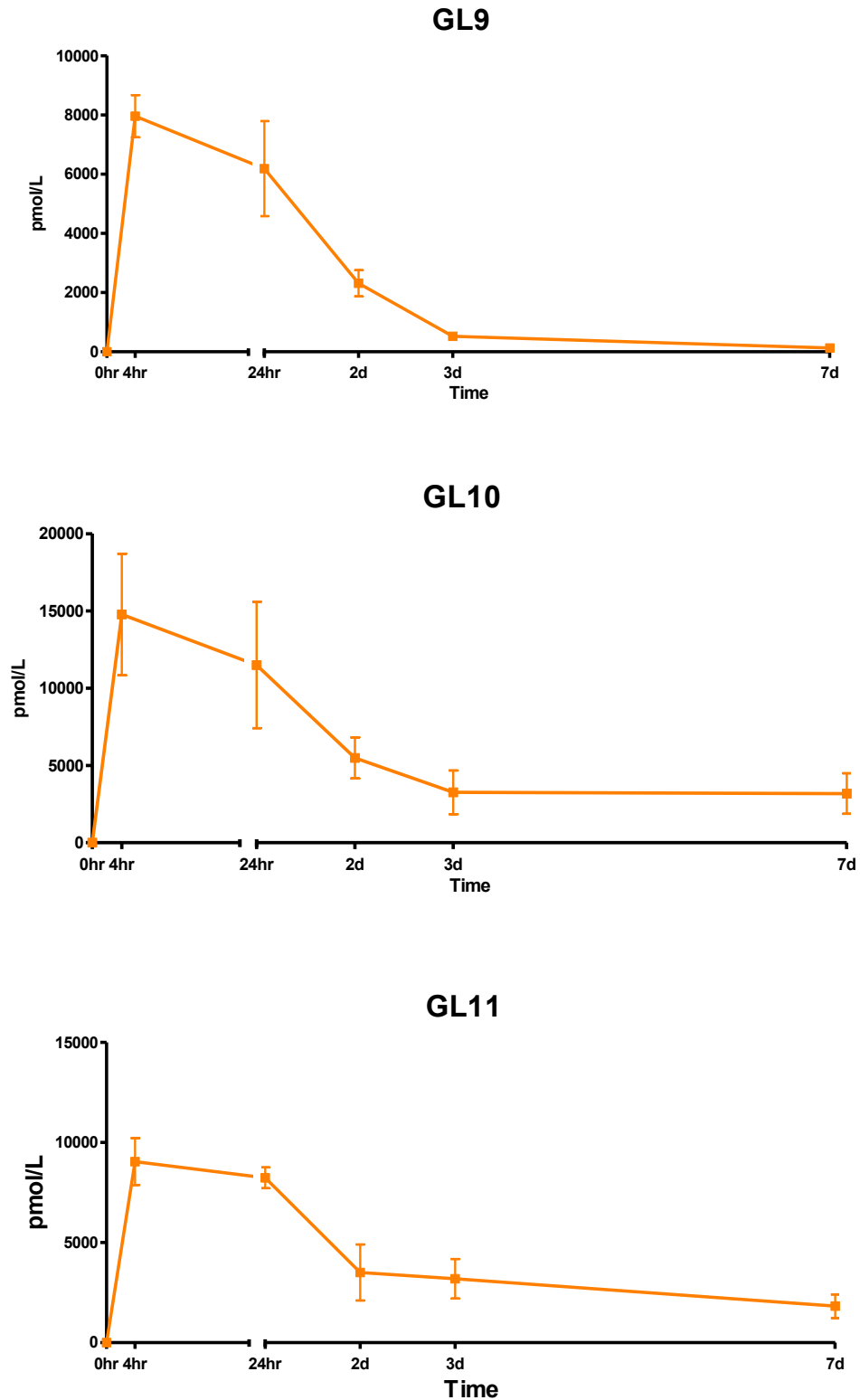


Figure 3.3.5. 4. Plasma levels of GLP-1 analogues GL9-11 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection. Plasma concentrations recorded in pmol/L with error bars denoting $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for RIA.

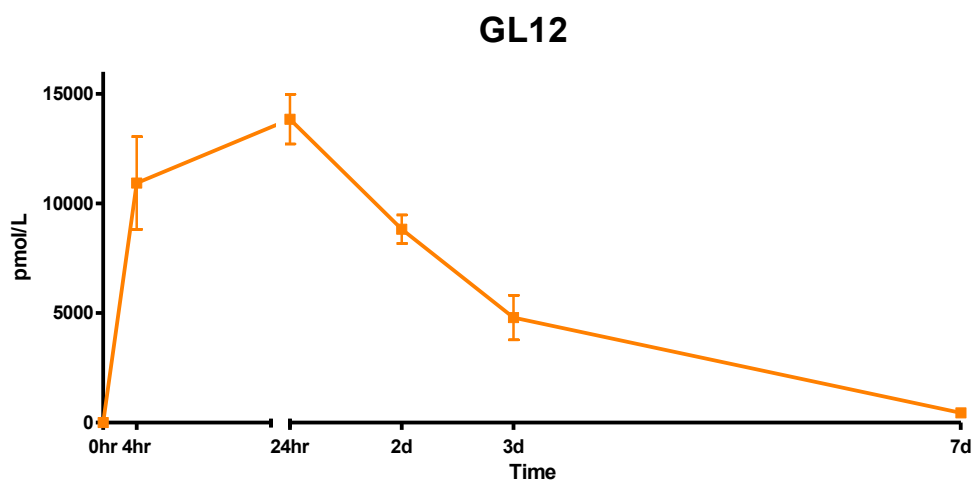


Figure 3.3.5. 5. Plasma levels of GLP-1 analogue GL12 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection. Plasma concentrations recorded in pmol/L with error bars denoting $\pm$ SEM. The LLOQs for each peptide in the RIA are shown as horizontal red dashed lines. Interpolated values which fall below this level are still plotted.

Generation 3:

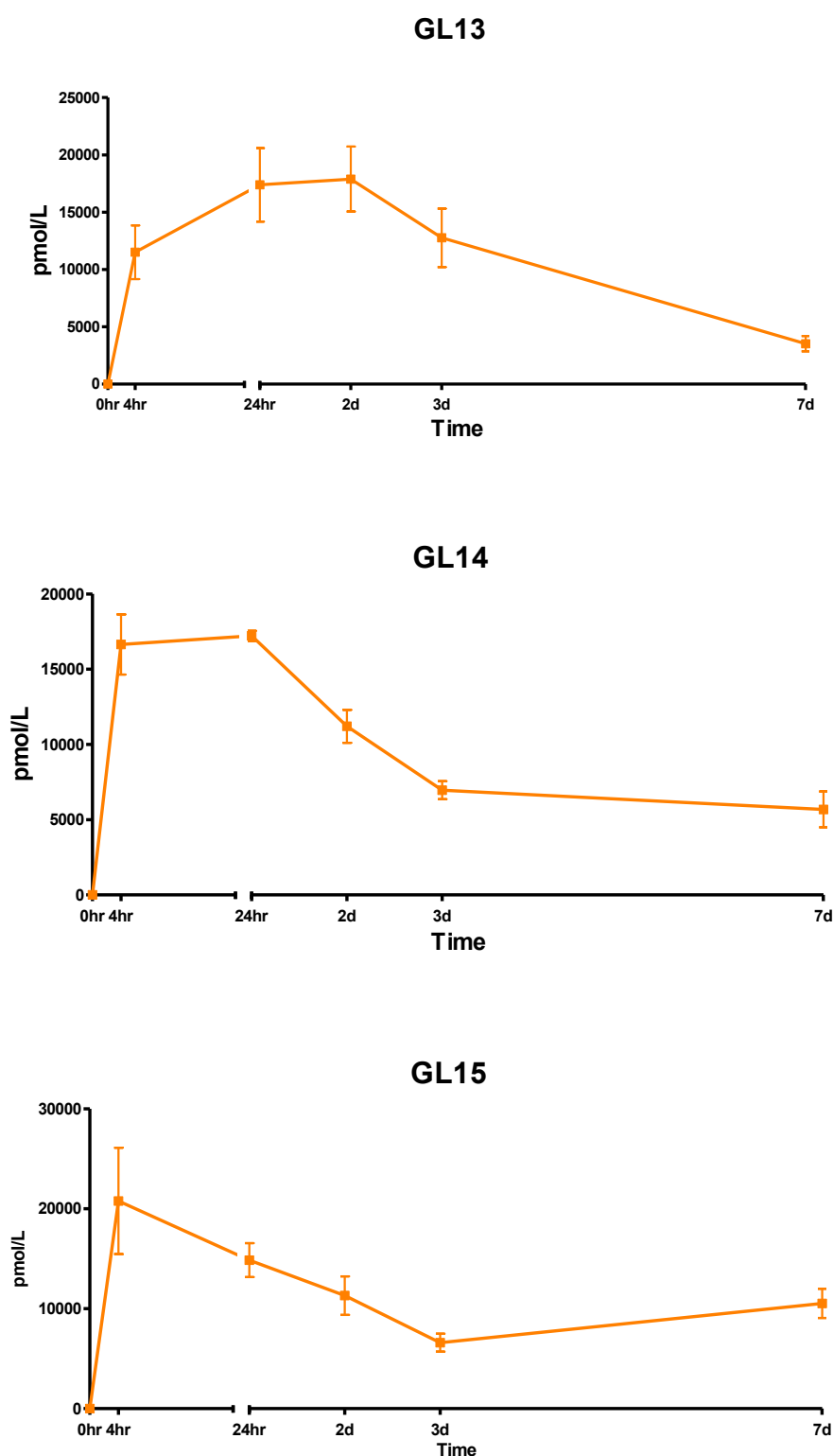


Figure 3.3.5. 6. Plasma levels of GLP-1 analogues GL 13-15 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection. Plasma concentrations recorded in pmol/L with error bars denoting $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for RIA.

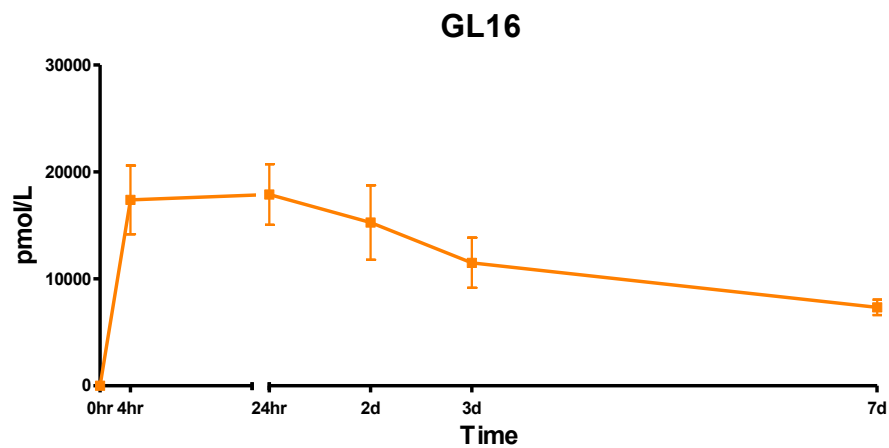


Figure 3.3.5. 7. Plasma levels of GLP-1 analogue GL16 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection. Plasma concentrations recorded in pmol/L with error bars denoting $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for RIA.

3.4. Discussion

The aim of the work described in this chapter was to design an analogue of GLP-1 which was potent and has a prolonged period of action. In order to achieve this, a number of screening tests were used. As the overall project aim is to develop a treatment of obesity, the key study of a GLP-1 agonist is to assess its property as a satiety signal and therefore monitoring inhibition of food intake in acute feeding studies. As these studies were conducted in mice, it is of significant importance to confirm that the actions seen in mouse would translate into man and therefore receptor affinity studies were conducted at both the human and mouse GLP-1 receptor. It was not anticipated that there would be differences between the human and mouse receptor affinity, as the human and mouse GLP-1 receptor sequences are similar differing in only 42 positions out of 463.

While receptor affinity is a good screening tool for likely species differences, it has limitations as a testing system as it is unable to distinguish agonists from antagonists, and cannot give any indication of efficacy (173). It was therefore considered necessary to also test novel analogues for their bioactivity using the secondary messenger system associated with the function of food intake inhibition, adenylate cyclic AMPase activity (cAMP) (174). As the GLP-1 receptor system is complex, with a number of endogenous peptide ligands exhibiting different signalling preferences at the receptor, and evidence that further small molecule ligands act as receptor modulator factors and alter the signalling pathway activation (175), the cAMP data had to be analysed with some caution and in parallel to the mouse *in vivo* feeding study.

Finally, to understand the PK properties of the novel analogues, a further test system is required as simply comparing the length of action in a feeding study is not sufficient. Due to the limited sampling blood volume of mice, PK studies were conducted in rats which allow for multiple blood samples to be collected from each animal. Only when considering all the parameters is it possible to understand the impact and likely reason for the modified biological effect and to get some confidence that the modification may translate into man.

When assessing if there was a bias of the analogues to the human or mouse receptor, no clear trend was observed. For the majority of peptides across all three generations, the ratio

of affinity to that of GLP-1 at the same receptor was within three-fold. However, an example is the first generation of analogues, which demonstrated that four out of the five analogues had a species bias. GL1 was biased to the human GLP-1 receptor with noticeably lower affinity to the mouse GLP-1 receptor. For exendin-4 and analogues GL2, GL4 and GL5 there was higher affinity to the mouse receptor when compared to affinity to the human GLP-1 receptor. Analysis of the sequences in these analogues did not find a particular sequence element which could be attributed to these differences. Therefore, it was considered important to continue screening at both the human and mouse GLP-1 receptor to avoid selecting an analogue such as GL3 where the affinity data suggests will be highly effective in mouse compared to man. From the second and third generation, only GL13 was species biased and was approximately eight times stronger at the human than mouse GLP-1 receptor.

It has been demonstrated through the spare receptor theory that a ligand only needs to activate a small percentage of receptors to get full response (176). Therefore, a peptides' affinity and efficacy, as well as *in vitro* and *in vivo* results, will not necessarily correlate, as different ligands with similar affinities can induce downstream signalling cascades with different potencies. Since the major effector of GLP-1 receptor-mediated action is the second messenger cAMP, peptides were tested for their potency at inducing intracellular accumulation of cAMP in a cell line overexpressing the human GLP-1 receptor. In the studies described in this chapter, it was demonstrated that the binding affinity of GLP-1 to the human GLP-1 receptor resulted in over 70% specific binding. The highest value of cAMP accumulation as a response to GLP-1 cellular treatment was used as maximal secretion.

The experiments described focused at designing a GLP-1 receptor agonist which had an extended duration of action following a single SC administration. Therefore, the analogues were also tested *in vivo* to evaluate the effect on food intake and pharmacokinetic properties.

Circulating levels of GLP-1 agonists have a short half-life and are only transiently in the required therapeutic range; they were therefore administered through a SC injection to reduce degradation prior to absorption into the circulatory system. In order to assess the impact of the analogues *in vivo*, food intake and body weight change in fasted mice were

measured post-analogue administration. Exendin-4 was used as a positive comparison peptide and was administered at a dose of 10nmol/ kg, a dose known to routinely cause a significant acute inhibition of food intake within the first 4 hours post-administration. The prolonged pharmacokinetic profile of most compounds is complex to achieve due to the high rate of clearance from the blood. For example, in clinical settings, exendin-4 requires twice daily injections due to its short half-life. Consequently, it is essential to administer the compound in a slow release formulation. A number of compounds, such as semaglutide and insulin, have managed to maintain a longer half-life by achieving slower release in circulation. Therefore, in order to assess the pharmacokinetics of the analogues *in vivo*, the peptides were administered through a subcutaneous injection in rats with a divalent metal ion, in this case zinc. Then, the burst release, followed by the slower release and then the total clearance was recorded for each peptide and compared.

The first generation of GLP-1 peptides focused on the N-terminal, mainly to examine the effect of amino acid substitutions on receptor binding. Positions 2, 3, 10, 12, 16 and 17 were investigated through 5 analogues. None of the analogues demonstrated a higher affinity than GLP-1 to the GLP-1 receptor. In receptor binding assays using the human GLP-1 receptor, GL1 and GL3 presented similar affinities yet bound almost 2-fold less than native GLP-1, while the rest of the peptides bound with a much lower affinity. Comparably, GL1 and GL3 both had 3-fold lower efficacy in cAMP accumulation, while GL2, GL4, GL5 and GL6 were much lower than native GLP-1. GL2 had a high affinity to the mouse receptor, and GL5 had similar affinity to native GLP-1, and they both caused greater weight loss of approximately 3 g. GL5 demonstrated the most appropriate pharmacokinetic profile as it did not cause a burst but sustained peptide concentration levels within the first 24 hours.

For generation 1, there was good correlation between the analogues with the highest affinity to the mouse GLP-1 receptor and causing the greatest inhibition of food intake in the first 24h post administration in mouse acute feeding studies. For example, GL1, which had at least 13 fold lower affinity to the mouse GLP-1 receptor than GL2 – GL5, was significantly weaker than other analogues over 24h, while GL2 caused a significant food intake inhibition and weight loss reduction.

The N-terminal region of GLP-1 and exendin-4 has been shown to be almost identical but with a crucial difference in the second amino acid residue, Ala² in GLP-1 and Gly² in exendin-4. Past studies showed that exendin-4 has a greater impact on insulin secretion and weight loss than GLP-1 due to its longer half-life which is mainly attributed to its resistance to DPP-IV degradation. Exendin-4 derived Gly² has been demonstrated to give almost complete protection both *in vitro* and *in vivo* from DPP-IV. Exendin-4 derived Gly² has been used in a number of analogues in order to assess whether it could have the same effect in GLP-1. In addition, previous studies have shown that substitution of Ala² enhanced the potency of Growth Hormone, which is also degraded by DPP-IV, pointing at a potential role of the substitution in breakdown prevention (177).

In this study, Ala² was substituted in order to avoid DPP-IV degradation, as residue 2 is recognised by the enzyme. The exendin-4 derived Gly² substitution caused significant food reduction *in vivo*, yet Ser² demonstrated a better sustained pharmacokinetic profile with a longer half-life and a more balanced effect in the initial concentration peak. The Glu – Gln substitution at position 3 did not interfere greatly with binding in the human receptor, but did not improve the *in vivo* effect, pointing that it is position 2 specifically which causes the effect.

The first α -helix of GLP-1 is thought to affect the interaction between ligand and receptor, especially through the hydrophilic side at positions 4 and 6, 7 and 9, which are directly involved with receptor interactions (160, 178-181). Additionally, through two substitutions in positions 10 and 12, it became apparent that the positive charge of the imidazole side chain of the histidine residue, as well as the presence of a ring structure and a basic function and correct positioning of both appears to be crucial for GLP-1 action (179).

Further in position 12 substitutions, I observed that replacement of polar Ser¹² with His¹² in the GL3 analogue showed an improvement in receptor affinity and peptide half-life, even though their size and charge are different. Residues 12-16 have been suggested to be a recognition determinant at the GLP-1 receptor for exendin-4, yet this has not been fully established on the GLP-1 sequence (170, 182). Positions 16 and 17 were further investigated in generation 2.

In generation 2, Ser², Gln³, Tyr¹⁰ and His¹² were maintained, as the peptides containing them previously demonstrated the best pharmacokinetic and pharmacodynamic profiles *in vivo*. Residues 15-17 serve as the N-cap for the C-terminal helix (159, 178) and was further investigated here, in addition to positions 19 and 20. Affinity to the human receptor was similar for GL6, GL10 and GL12, yet was 3-fold lower than native GLP-1, while GL8 and GL9 were approximately 6-fold lower. GL6 affinity did translate into efficacy, as cAMP accumulation post-GL6 treatment was 2-fold lower, whereas the rest of the peptides had an even lower efficacy compared to GLP-1. GL6, GL8, GL11 and GL12 had 2-fold lower affinity to the mouse receptor, yet almost all analogues caused significant food inhibition up until 8hr post-administration in mice. Nonetheless, no significant weight loss was observed post peptide treatment, as food intake levels increased after 24hr. Of note, all peptides sustained a stable plasma concentration when administered with zinc in rats. GL11 and GL12 demonstrated the most promising profile, as they did not cause a high concentration peak, and were still detectable 3 days post-injection. Therefore, Glu¹⁶, Glu¹⁷, Val¹⁹ and His²⁰ were maintained in generation 3.

The most important observation during the second generation concerned the central linker region. The importance of this region, particularly Gly¹⁶ in its receptor interaction is interesting, as it was reported that the presence of the small, helix-destabilizing, Gly¹⁶ has an important role in membrane and receptor binding because it provides high flexibility to the peptide (159, 180) and its removal has shown to reduce GLP-1 binding affinity (178). This is potentially because the N-terminal helix in GLP-1 is positioned in opposition to the hydrophobic face of the C-terminal α -helix. Gly¹⁶ breaks the helix in order for the peptide to bend and bind to the membrane receptor. This glycine residue is possibly the reason why GLP-1 has two separate helices, while glucagon forms a large helix within approximately the same residues. In this investigation, Gly¹⁶ was replaced by Glu and Ser in order for a continuous α -helix to be formed. As suggested by the results, binding was reduced when compared to native GLP-1. Despite the affinity reduction, significant food intake reduction was observed, as well as a longer-lasting pharmacokinetic profile, potentially pointing at a higher stability of the molecule in circulation.

In the third and final generation, four peptides were designed to evaluate the C-terminal helix of the peptide with substitutions being introduced in positions 21, 24, 27, 28, 29 and

30. GL16 was the only analogue that had similar affinity to native GLP-1, potentially pointing at the importance of His³⁰. Despite this, GL14, GL15 and GL16 presented similar cAMP accumulation as GLP-1. These results point to the fact that potentially the C-terminal does alter receptor binding, in addition to activation. Affinity to the mouse receptor was similar in all four peptides and GLP-1, but the effect did not translate *in vivo*, as no analogue caused a significant weight loss. None-the-less, GL13 and GL16 maintained a significant food intake inhibition at 48 hours post-injection, which agrees with the *in vivo* pharmacokinetic data, as all three analogues maintained a stable profile within the first 3 days and then plasma concentration declined. GL16 presented the best sustained plasma concentration of the last generation of analogues, as it remained detectable at high levels for almost 7 days.

C-terminal amidation has been shown to be less important for GLP-1 action when compared to the main acids in the N-terminus (178). NMR analysis has shown residues 10-30 in the central region of exendin-4 is helical in structure and that it has 9 amino acids at the C-terminal which have been shown to form a Trp-cage (159, 183). Even though GLP-1 and exendin-4 only share 8 residues in this area, it has been shown that they lie on the same side of the helix which is assumed to be the face that interacts with the receptor (154, 156, 184). This information has been used in the design of generation 3 of analogues.

In the analogues presented, amino acids at positions 27 and 28 of GLP-1 were substituted according to exendin-4 in order to achieve higher receptor affinity, while the His³⁰ to Arg³⁰ substitution was chosen to improve the peptide's half-life. GL16 had a total of 3 histidine substitutions, which could be a reason of its balanced peptide concentration observed post-injection in rats.

It has previously been demonstrated through insulin that the pharmacokinetic stability includes histidine's imidazole ring and charge (179). Histidine becomes iodinated at pH 6, as it gains protons and changes the amino group from NH to NH²⁺. While diluted in the acidic environment of zinc (pH 6), positively charged histidine will repel, yet once injected, body fluids buffer the injection solution to reach histidine's isoelectric point of pH 7.4. At this point, histidine is half-negatively and half-positively charged. The negative part attracts positively charged Zinc, causing the peptide and the metal to create coordinate bonds between histidine amino acids and Zinc atoms, a process known as chelation which induces a slower release (185).

As a whole, a number of amino acid substitutions for a more potent GLP-1 analogue were derived from the exendin-4 sequence, while others were mainly chosen due to similar size and side chain polarity. GL16 appeared to have an agonistic effect to the human receptor, while presenting a prolonged half-life and a stable concentration through the time range of 7 days.

3.5. Conclusion

GL16 was the successful GLP-1 analogue that demonstrated both high affinity and efficacy to the human receptor, in addition to a balanced peak of concentration *in vivo* that was maintained for 3 days. It reduced food intake significantly until 8 hours post injection, yet demonstrated a similar weight loss to that of exendin-4 at 48 hours. It is a result of 14 amino acid changes: Ser², Gln³, Tyr¹⁰, His¹², Glu¹⁶, Glu¹⁷, Val¹⁹, His²⁰, Leu²¹, Glu²⁴, Lys²⁷, Asn²⁸, Thr²⁹ and His³⁰ which focused on the N-terminal α -helix, the linker region and the C-terminal α -helix.

GL16 will be used as a co-compound with an equally potent glucagon analogue, with which it will be injected in a chronic study to investigate its effect on body weight and food intake reduction.

CHAPTER 4

Investigation of glucagon analogues and co-administration of GLP-1
and glucagon analogues as therapeutic agents for treatment of
obesity

4.1. Introduction

4.1.1. Structure and properties of glucagon

Glucagon is a proglucagon-derived peptide consisting of 29 amino acid residues with approximately 50% sequence homology to GLP-1. It is released by the pancreatic α -cells in response to low serum glucose concentration. In a state of low glucose, glucagon triggers a reaction through which liver glycogen storage is broken down, and therefore glucose concentration is increased (186). This procedure is called glycogenolysis, and occurs in addition to gluconeogenesis in hepatocytes (187, 188). Initial studies in 1980 suggested that glucagon also has a potential physiological role in stimulating lipolysis in adipose tissue and increase energy expenditure (189, 190). There are divergent schools of thought as the potential therapeutic use of glucagon analogues. Several companies are developing glucagon receptor antagonists for the treatment of diabetes while other research is focused on the use of glucagon agonists for the treatment of obesity.

Studies investigating the structure-function relationship of glucagon have been conducted over the last 40 years. Data on glucagon structure have been contradictory due to its structural polymorphism, which is marked by the fact that it exists in a number of different conformational states. A detailed structure of the peptide in a crystalized form was achieved on an early breakthrough study (191). In aqueous solution, glucagon is very unstructured and flexible as it is largely unfolded with few stable intramolecular bonds (192). However, when in an organic solvent, it forms a more helical structure (193). A number of techniques were employed to build a model for glucagon structure, mainly based on a hierarchy of intramolecular interactions where hydrophobic bonding is before hydrogen bonding (194), and it has been established that upon binding, glucagon forms an α -helix between residues 5-27 (195).

In the pursuit of a glucagon receptor antagonist, either competitive or non-competitive, for the treatment of diabetes, agonists and partial antagonists have been identified. These glucagon analogues have provided structure function information and an insight to ligand

receptor interactions (196). The first generation of antagonists were mainly desHis¹ glucagon and variations of the peptide. Predicted structure pointed that, residues 2- 5 stick out the polypeptide like a key, catalysing the adenyl cyclase. Removing the histidine distorts the chain, creating a competitive antagonist, which binds to the receptor but fails to stimulate adenyl cyclase activation (197-200). It has therefore been concluded that the N-terminal is important for signal transduction, while other evidence highlights the importance of the C-terminal for receptor binding (22-29).

4.1.2. Structure and properties of glucagon receptor

As for GLP-1, the functions of glucagon are considered to be facilitated primarily through GPCR and the cAMP signalling mechanism system, as binding of glucagon to its transmembrane receptor activates the receptor's α -subunit which then activates adenylate cyclase, catalysing ATP into cAMP (Figure 4.1.). In super physiological concentrations, cAMP stimulates alternative pathways which result in activation of several other processes such as proliferation, apoptosis and transcription factor phosphorylation (201).

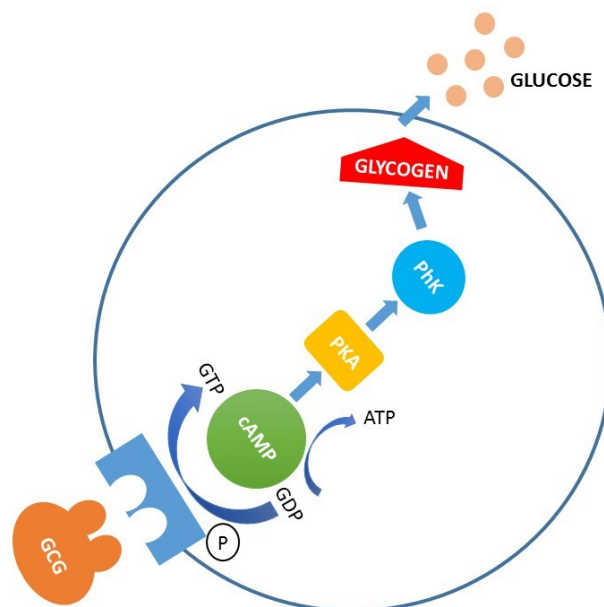


Figure 4. 2. Summary of glucagon receptor signalling. Glucagon binds on glucagon receptor, increasing intracellular concentration of cAMP, which culminates in activation of phosphorylase kinase a, the enzyme that catalyzes the first step in the glycogenolitic pathway.

The glucagon receptor is a member of the class B of G-protein coupled receptor family. It activates a number of G protein mediated intracellular pathways, the majority of which result in adenylyl cyclase stimulation (202) and phosphoinositol turnover (122). Activation occurs when a ligand diffuses into an unligated receptor. Receptor's function has been described by a two-state model, in which an unbound receptor is inactive (R) but has a basal activity which increases to full activity once an agonist binds (R*). The efficacy of ligands is a reflection of their capacity to change the equilibrium between R and R*. Full agonists are capable of stabilising the R* state while partial agonists balance between R and R* (203, 204).

It has been suggested that the G-protein coupled receptors follow a 2-step binding mechanism, where the ligand binds non-specifically to the cell membrane, causing a two-dimensional diffusion of the membrane before specific binding to the target receptor occurs. The C-terminal of glucagon interacts with the hydrophobic regions of the extracellular N-terminal receptor domain and extracellular loops (205). This induces the formation of an α -helix in residues 5 to 27, thus allowing those residues to bind to the receptor. The hydrophobic interactions stabilize the protein-receptor complex (206).

A deletion study on the rat glucagon receptor established that the N-terminal extracellular portion of the receptor is vital for ligand binding, while most of the C-terminal tail is not as truncation does not significantly affect affinity of a ligand. In addition, it was demonstrated that the absence of the tail may slightly increase the receptor binding affinity for glucagon. The C-terminal tail is not required for adenylyl cyclase coupling and therefore does not appear to play a direct role in receptor activation (207).

In a further investigation of the different domains of the glucagon receptor, region specific antibodies were used for N-terminal residue 126-137 and extracellular loop residue 206-219. The antibodies blocked peptide binding to the receptor, suggesting these regions are potentially important in the procedure of binding. To further investigate the role of the extracellular N-terminal and ligand binding, amino acid substitutions were made in this region and also caused a reduction in receptor affinity (208).

4.1.3. Glucagon and GLP-1 analogue infusion

Glucagon is best known for its glycogenolytic properties and its role on the feedback loop of insulin secretion. Despite this, a number of researches have demonstrated an additional action on increasing energy expenditure (209). As a result, a glucagon receptor agonist that can improve glucagon's affinity and efficacy could be having a more potent effect on weight loss and be a potential drug for treatment of obesity.

The effect of glucagon on energy expenditure has initiated an investigation on its possible use as an obesity treatment in combination with GLP-1, as a co-administration therapy of both peptides could potentially be promising in obesity treatment. However, the hyperglycemic property of glucagon would make treating a diabetic population, which many obese people are, dangerous. It was therefore postulated that an oxyntomodulin-like compound which combines GLP-1 and glucagon receptor agonism as a potentially enhanced obesity treatment than a GLP-1 receptor agonist alone. It is unknown what the optimal ratio of GLP-1 and glucagon receptor agonism is in man to produce maximal weight loss while avoiding nausea, consequence of GLP-1 receptor agonists, and hyperglycemia, consequence of glucagon receptor agonism. Therefore, two separate agents which can be co-administered provide the option to modify the ratio depending on the efficacy and adverse effects. This approach has been partially validated in a human study.

Administration of both peptides in healthy human volunteers demonstrated an increase in energy expenditure and improvement of hyperglycaemia, in addition to a decrease of orexigenic ghrelin (132). These results indicate that, other than a single GLP-1/ glucagon co agonist, GLP-1 and glucagon analogues could also be beneficial to investigate weight loss and glycaemic control while avoiding side effects such as nausea and vomiting. It is important that the two analogues have similar pharmacokinetic profiles in order to achieve a similar half-life.

4.1.4. Generation of glucagon analogues

A total of three generations of glucagon analogues were designed for the purpose of developing a glucagon analogue for co-administration with a GLP-1 analogue.

Generation 1:

The first generation consists of 6 peptides with modifications in residues 12, 15, 17 and 18, residues which have all been shown to have an effect on receptor binding. Position 12 is also believed to aid secondary structure stabilisation. Previous research showed that an important topographical relationship exists between the region near Lys¹² and Ser¹⁸ (200) as it seems to act as a hinge region that contributes to the appropriate orientation of the C-terminal in order to achieve receptor recognition. In the same region, the electrostatic interactions of the negatively charged side chain groups of aspartic acid at positions 9, 15 and 21 were reported to be essential in glucagon function (210, 211).

Generation 2:

Previous reports suggest that the ϵ -amino group of positively charged Lys¹⁸ forms a salt bridge with the carboxyl of Glu²¹, which potentially stabilizes the turn of a potential α -helix between them, which could enhance a super agonist activity (212). In addition, Met²⁷ replacement seems to increase glucagon activation 5-fold pointing at a link between the C and the N-terminal (213). Therefore, the second generation focused on substitutions in positions 21, 27 and 29 in order to improve receptor efficacy.

Generation 3:

The third generation of a total of 5 peptides, utilised the results of positions 21 and 27 obtained by generation 2, and investigated position 20. The main focus of the generation was the addition of a histidine tail, which pharmacologically has been shown to achieve a longer half-life when co administered with zinc.

4.1.5. Aims

It has been shown that infusion studies that combined GLP-1 and glucagon administration in man increased energy expenditure and reduced appetite without causing nausea and hyperglycaemia. Therefore, two analogues that present a longer half-life than the native peptides could offer a stable and balanced action as an anti-obesity treatment.

The GLP-1 analogue selected in Chapter 3 is GL16, which had higher efficacy compared to native GLP-1 in addition to its longer lasting concentration in plasma. In order to create the second part of the treatment, a glucagon analogue with similar properties must be designed. Specifically, it must present a pharmacokinetic profile with a similar half-life, in order to achieve a balanced blood glucose concentration. I therefore designed three groups of glucagon analogues according to the peptide's structure and function and investigate the substitutions in relation to receptor affinity and efficacy. I then performed *in vivo* studies that investigated the anorectic effect and the pharmacokinetic profile through plasma concentration of the agent at different time points.

Once a glucagon analogue with the appropriate properties was designed, I co administered both GLP-1 and glucagon analogues as a single daily injection in different doses to investigate the optimal ratio, in a chronic study in mice. In addition, a Glucose Tolerance Test (GTT) was performed to ensure that a glucagon injection does not increase glucose blood levels.

Summary:

1. To identify amino acid residues in glucagon which are important for receptor binding and receptor activation by use of analogues in receptor binding and efficacy studies *in vitro*.
2. To investigate the effect of glucagon amino acid substitutions on degradation and stability using *in vivo* studies in laboratory rodents.
3. To assess chronic effects of GLP-1 and glucagon analogues co administration *in vivo* in laboratory rodents.

4. To evaluate whether GLP-1 and glucagon analogues co administration causes an increase in blood glucose in laboratory rodents.

4.2. Materials and Methods

4.2.1. Design and synthesis of GCG analogues

Three generations of glucagon analogues were designed and synthesised, the sequences of which are shown in table 4.1. The first generation consisted of 6 analogues, followed by the second generation of 6 analogues and the final third generation of 5 analogues. Peptides were manufactured by Insight Biotechnology Ltd (Middlesex) ((Section 2.2.)).

4.2.2. Glucagon competitive receptor binding assays

Binding studies were performed as described in section 2, using CHO-hGCGr cell membrane (cells overexpressing the human glucagon receptor) and mouse liver membrane (Section 2.7.1). ^{125}I -GCG was used as the competing peptide for all studies (Section 2.7.4). Binding affinities were calculated from a minimum of three separate experiments, where each concentration was tested in duplicate or triplicate. Errors shown are $\pm$ SEM.

4.2.3. Glucagon cAMP accumulation assays

Glucagon receptor agonist dose response studies were completed. CHO-hGCGr cells (cells overexpressing the human GCG receptor (section 2.6.1) were treated with glucagon receptor agonists at a range of doses as described in section 2.8. Accumulation of cAMP, a marker of cAMPase activity, a surrogate of activity was quantified using an ELISA (ADI-900-066, EnzoLifeSciences, UK). Bioactivities were calculated from a minimum of three separate experiments, where each concentration was tested in triplicate. Errors shown are $\pm$ SEM.

	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
Glucagon	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								
Oxyntomodulin	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
GC1	H	S	Q	G	T	F	T	S	D	Y	S	H	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T								
GC2	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	E	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T								
GC3	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	Q	R	A	Q	D	F	V	Q	W	L	M	N	T								
GC4	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	K	R	A	Q	D	F	V	Q	W	L	M	N	T								
GC5	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	K	K	A	Q	D	F	V	Q	W	L	M	N	T								
GC6	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	H	A	Q	D	F	V	Q	W	L	M	N	T								
GC7	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	E	F	V	Q	W	L	M	N	T								
GC8	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	H	F	V	Q	W	L	M	N	T								
GC9	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	H	F	V	Q	W	L	L	N	T								
GC10	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	H	F	V	Q	W	L	L	N	G								
GC11	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	E	S	R	R	A	Q	E	F	V	Q	W	L	L	N	G								
GC12	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	L	N	T								
GC13	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	T								
GC14	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	H	D	F	V	Q	W	L	L	N	T								
GC15	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	H	D	F	V	Q	W	L	L	N	T	H							
GC16	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	H	D	F	V	Q	W	L	L	N	T	H	H						
GC17	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	H	D	F	V	Q	W	L	L	N	T	H	H	NH2					

Table 4. 1. Amino acid sequences of peptides used in Chapters 4. Red filled residues are conserved residues across the glucagon peptide family; Blue filled residues represent point substitutions.

4.2.4. Acute feeding studies in mice

The effect of glucagon analogues on food intake was investigated using DIO C57BL/6J mice on high fat diet, fasted for 16 hours, as described in section 2.3.1. Mice were injected SC at time 0 with the peptide or vehicle (0.9% weight/ volume NaCl).

Dose selection was based on earlier studies (data not shown). All analogues were administered via a SC injection at a dose of 50nmol/ kg. Glucagon was administered at a 300nmol/ kg dose, as this dose suffices to achieve an inhibition of food intake. Food intake was measured at 1, 2, 4, 8, 24, 48 hours and body weight was measured 48 hours post administration.

4.2.5. Chronic feeding studies in mice

The effects of analogues GL16 and GC17 were investigated in a 20 day chronic study. C57BL/6J DIO mice (average body weight 40gr) were used for this repeat dosing study. Each peptide was administered individually and in combination (n=10 for each group). Saline was used as vehicle and control (0.9% weight/ volume NaCl) in 0.05ml volume.

GLP-1 analogue GL16 was administered daily by SC injections at 5nmol/kg for days 1-6 and 10nmol/ kg for days 7-20. Glucagon analogue GC17 was daily administered through SC injection at a higher dose of 20 nmol/ kg for the first 6 days of the study, and later on increased at 40nmol/ kg for days 6 to 20.

Co administration of both peptides GL16 and GC17 was given at different doses for each peptide, as a single injection of 0.05ml volume. Food intake and body weight were measured daily, prior to treatment at 1530h.

4.2.6. Glucose Tolerance Test in mice

A glucose tolerance study was performed on the final day of the chronic study (section 4.2.5.). Ad libitum fed mice were SC injected with GL16, GC17 and a co-administration of

GL16/ GCG17 at 0700 on the day of study and then fasted until time 0 at 14.00 when bolus glucose was administered.

Tail tip samples of blood were obtained 30mins before glucose administration. At T0, each mouse was injected IP with 2g/ kg glucose solution. Tail tip samples of blood were then obtained at time points: 0 min, 30 mins, 60 mins, 90 mins and 120 mins 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 provided glucose measuring strips, and the blood glucose concentration was shown and recorded.

4.2.7. *In vivo* pharmacokinetic studies in rat

In vivo pharmacokinetics studies were performed in male Wistar rat as described in section 2.5. Each animal was injected SC with 1mg 1:1 molar ratio Zn:NaCl (saline). Blood was collected by superficial venesection of the lateral tail vein, at 6 time points following injection. In order to separate the sample plasma, tubes were centrifuged for 8 minutes at 10,000 x g at 4°C the upper plasma was isolated and stored at -20°C. Peptide hormone levels in plasma were measured by glucagon radioimmunoassay as described in section 2.9.3.

4.2.7.1. Radioimmunoassays

Plasma peptide levels were determined by glucagon radioimmunoassays as described in section 2.9.3. The antiserum was produced in rabbits against the purified glucagon(1-16) fragment, coupled to bovine serum albumin by glutaraldehyde. 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 1pmol/ ml and added in duplicate. Peptide concentrations in the samples were calculated using a non-linear plot and results were calculated in terms of the standard.

4.3. Results

4.3.1. *In vitro* characterisation of first generation of glucagon analogues

4.3.1.1. Affinity of the glucagon analogues to the human glucagon receptor

Binding assays with ^{125}I -GCG resulted in greater than 80% specific binding to a cell membrane preparation of CHO-hGCGr cells (Figure 4.3.1.1.). Binding affinities of three generations of a total of 17 glucagon analogues at the human glucagon receptor were tested and compared to glucagon. GC3, GC4, and GC5 had similar affinities, while GC6 had approximately 1.5-fold and GC1 and GC2 had 2.2 fold lower affinities to human glucagon receptor compared to glucagon (Figure 4.3.1.1.2.).

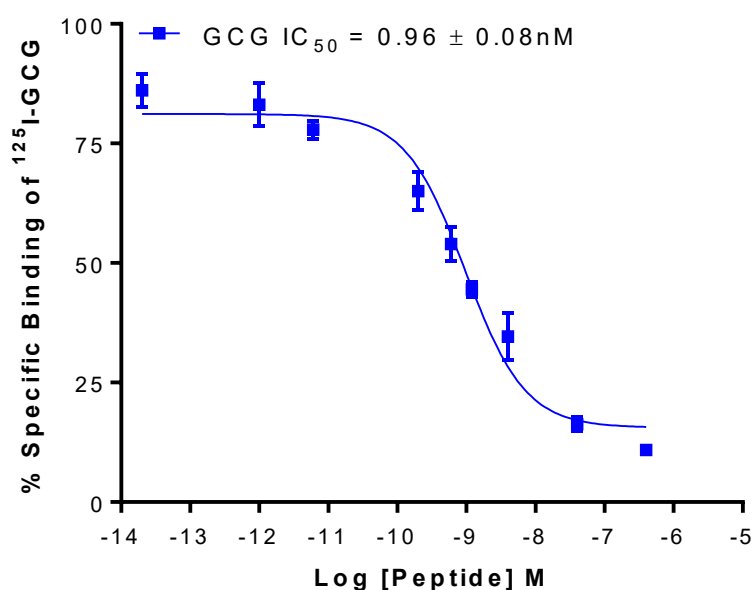


Figure 4.3.1. 1. Binding affinity of glucagon to the human glucagon receptor. Human glucagon receptor over-expressing CHO cell membrane was the source of glucagon receptor. Each peptide concentrations was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments $\pm$ SEM. Coefficient of variation was calculated GCG: 0.16

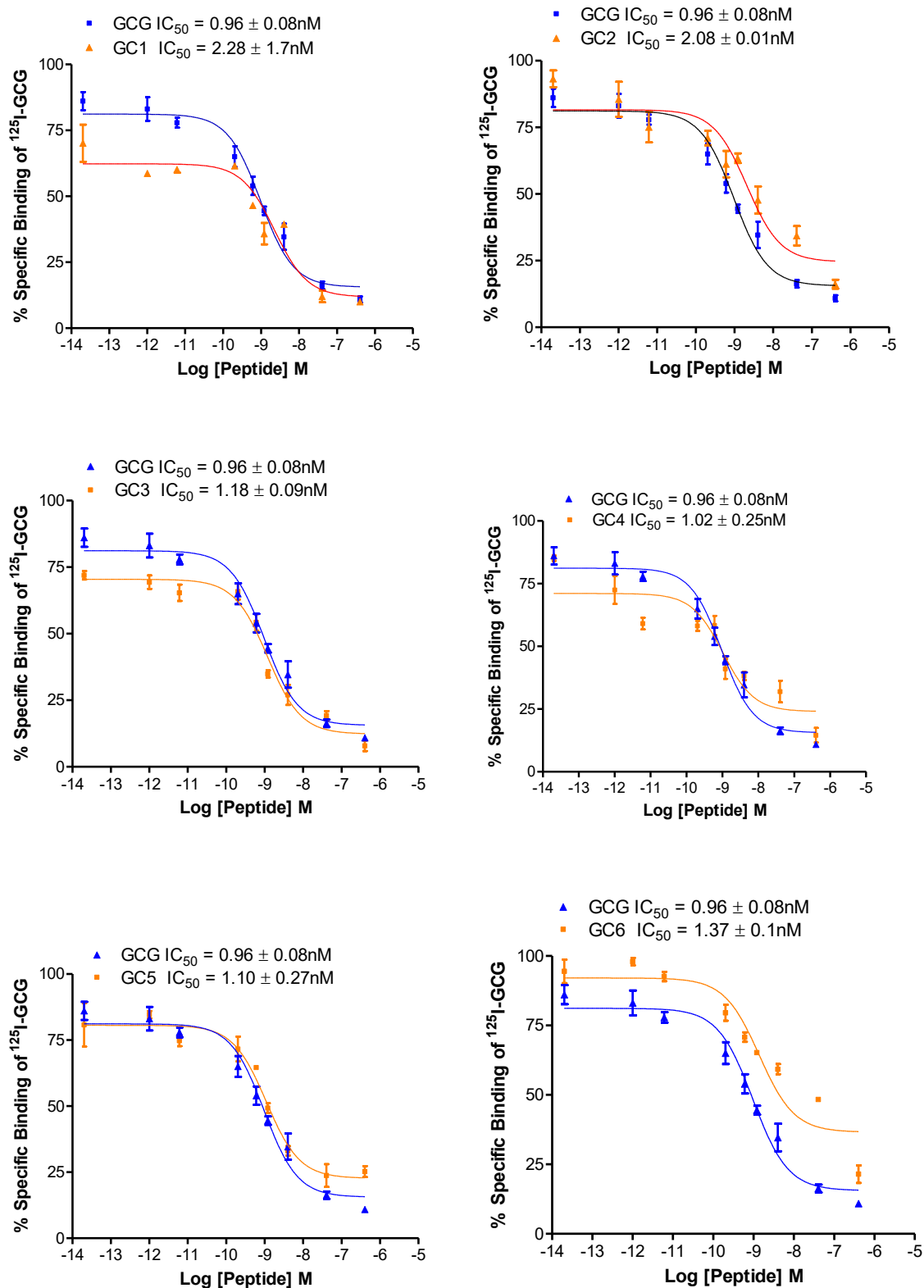


Figure 4.3.1. 2. Binding affinity of glucagon analogues to the human glucagon receptor. Human glucagon receptor over-expressing CHO cell membrane was the source of glucagon receptor. ^{125}I -GCG was used as the competing peptide. Each peptide concentrations was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of three separate experiments $\pm$ SEM.

4.3.1.2. Affinity of the glucagon analogues to the mouse glucagon receptor

Binding assays with ^{125}I -GCG resulted in greater than 80% specific binding to a membrane preparation of mouse liver (Figure 4.3.1.3.). Binding affinities of three generations of a total of 17 glucagon analogues at the mouse glucagon receptor were tested and compared with endogenous glucagon. In the first generation, GC1 had similar affinity, GC3 had 10-fold and GC2 had 8-fold lower affinity. GC4 had approximately 6-fold, while GC5 and GC6 had 2-fold lower affinity than glucagon (Figure 4.3.1.4.).

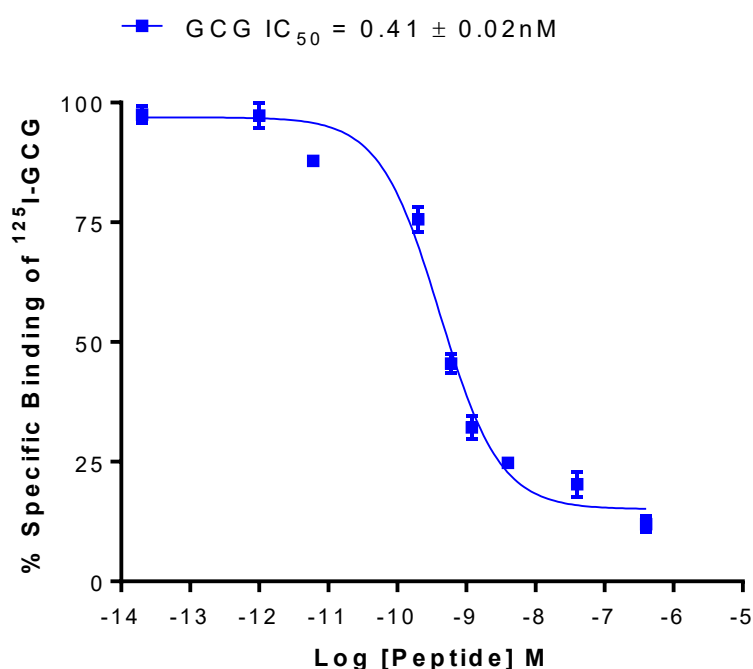


Figure 4.3.1. 3. Binding affinity of glucagon to the mouse glucagon receptor. Human glucagon receptor over-expressing CHO cell membrane was the source of glucagon receptor. Each peptide concentrations was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of two separate experiments. Errors shown are $\pm \text{SEM}$. Coefficient of variation was calculated GCG: 0.09

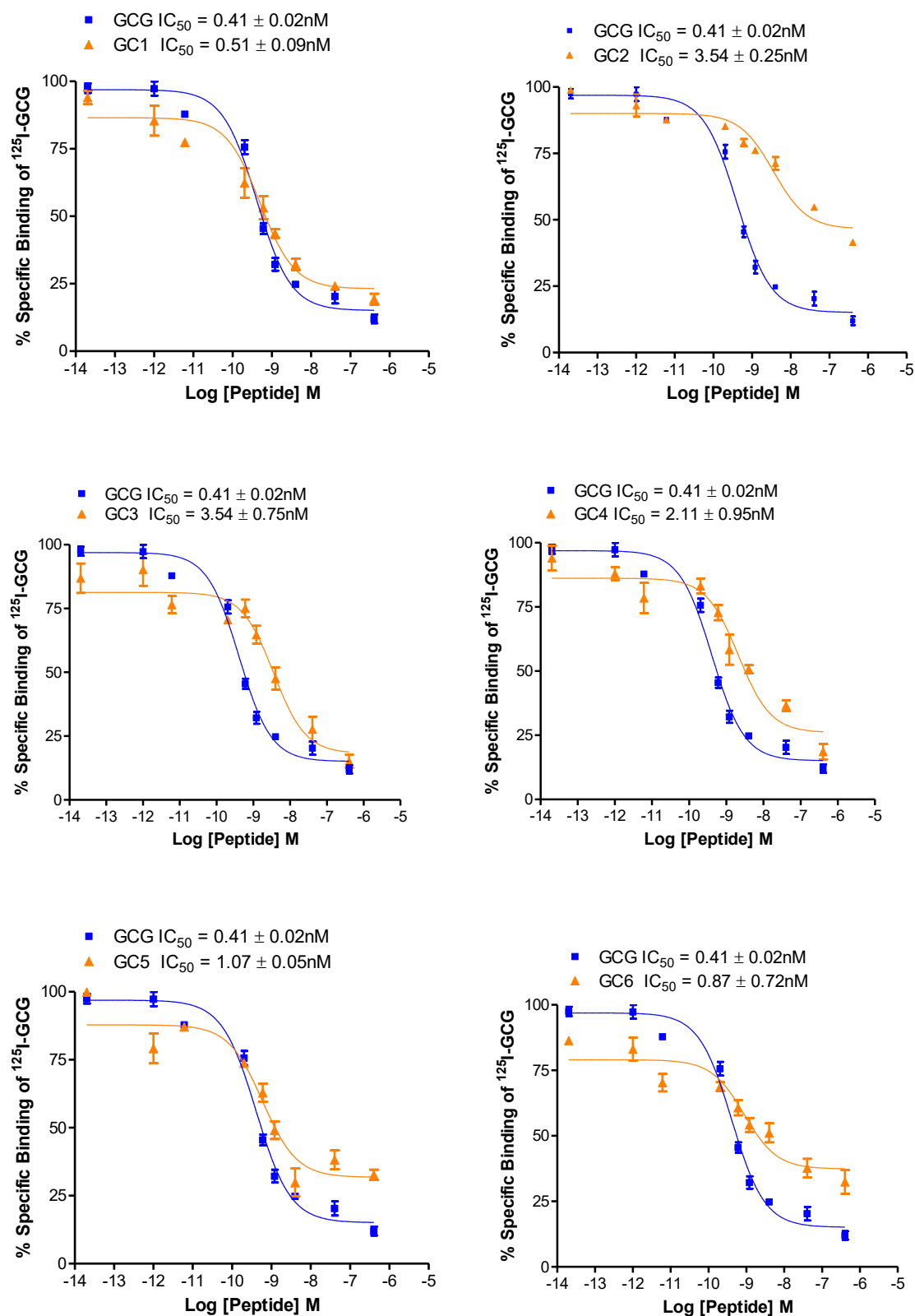


Figure 4.3.1. 4. Binding affinity of glucagon analogues to the mouse glucagon receptor. Mouse liver was the source of glucagon receptor. ^{125}I -GCG was used as the competing peptide. Each peptide concentrations was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of three separate experiments $\pm \text{SEM}$.

4.3.1.3. Effect of glucagon analogues on receptor-mediated cAMP accumulation

The ability of glucagon to stimulate cAMP accumulation in CHO-hGCGr cells was investigated. The potency of glucagon is shown graphically in figure 4.3.1.5. The bioactivity of three generations of a total of 17 glucagon analogues at cAMP accumulation was compared to endogenous glucagon. In the first generation, GC3, GC4, GC5 and GC6 had only 0.1-fold lower potency whether GC1 and GC2 had approximately 0.5-fold higher potencies than glucagon (Figure 4.3.1.6).

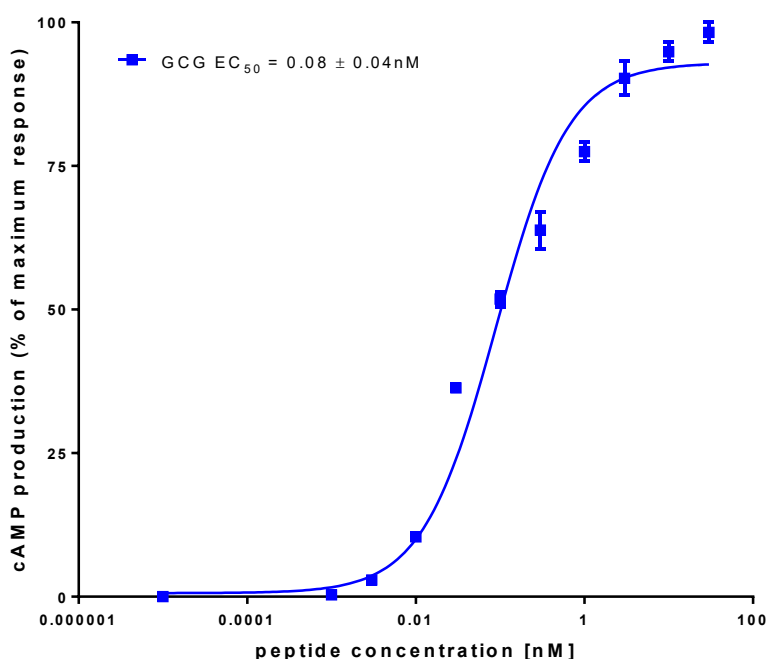


Figure 4.3.1. 5. Human glucagon receptor-mediated cAMP accumulation in CHO cells overexpressing the human glucagon receptor by glucagon. Each peptide concentrations was tested in duplicate or triplicate in each experiment. EC₅₀ values calculated from a minimum of three separate experiments, errors shown are $\pm$ SEM. Coefficient of variation was calculated GCG: 0.47

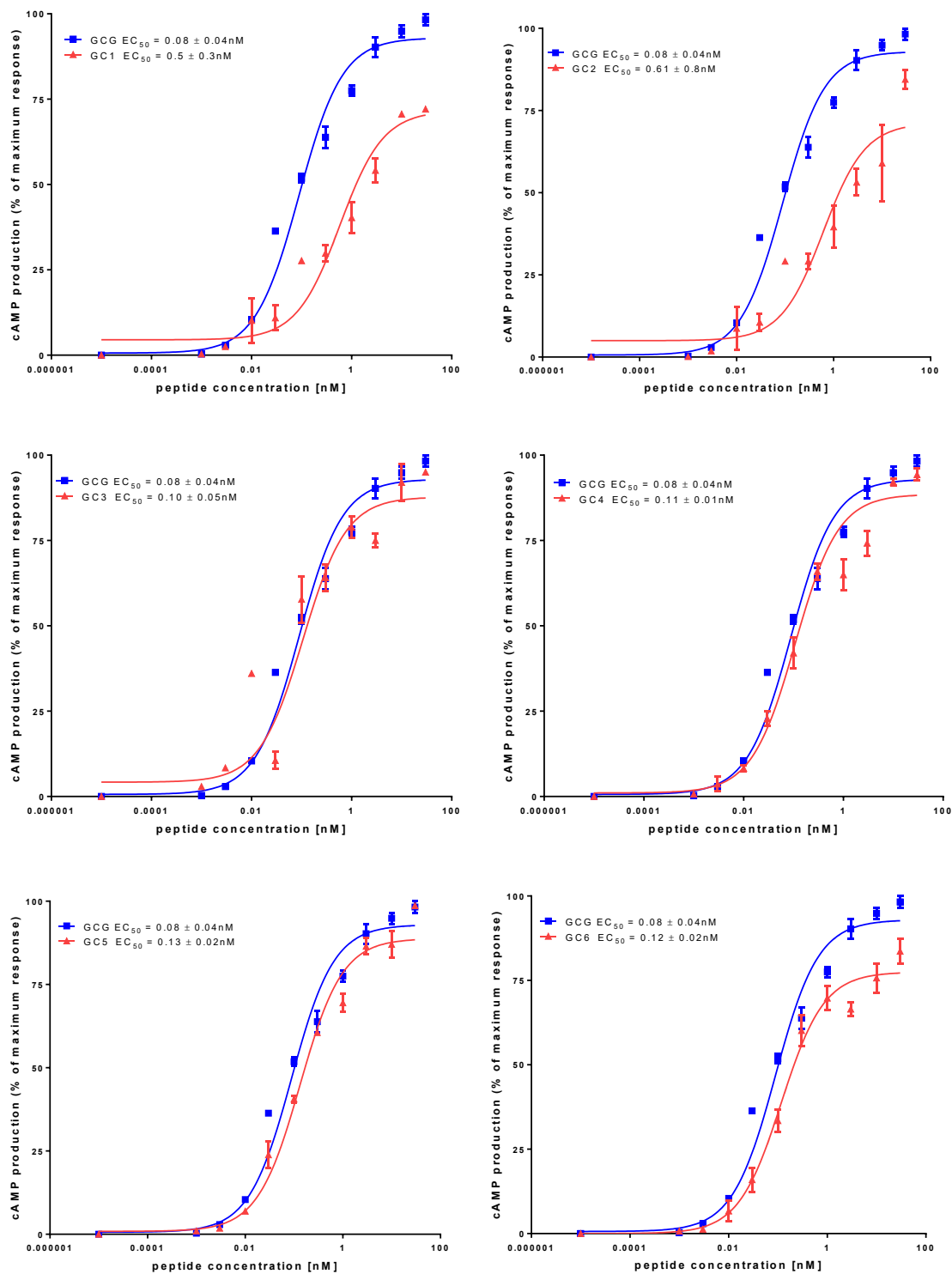


Figure 4.3.1. 6. Human glucagon receptor-mediated cAMP accumulation in CHO cells overexpressing the human glucagon receptor by glucagon and generation 1 glucagon analogues. Each peptide concentrations was tested in duplicate or triplicate in each experiment. EC₅₀ values calculated from a minimum of three separate experiments, errors shown are ±SEM.

4.3.2. *In vitro* characterisation of second generation of glucagon analogues

4.3.2.1. Affinity of the glucagon analogues to the human glucagon receptor

In the second generation consisting of analogues GC7 - GC12, analogues GC7, GC9, GC10 and GC12 had similar affinity to glucagon, to the human glucagon receptor. Analogue GC11 presented the lowest affinity compared to glucagon, with 1.5-fold lower affinity to human glucagon receptor and an EC₅₀ of $1.52 \pm 0.05\text{nM}$, while GC8 was 0.7-fold lower (EC₅₀: $1.30 \pm 0.35\text{nM}$) (Figure 4.3.2.1).

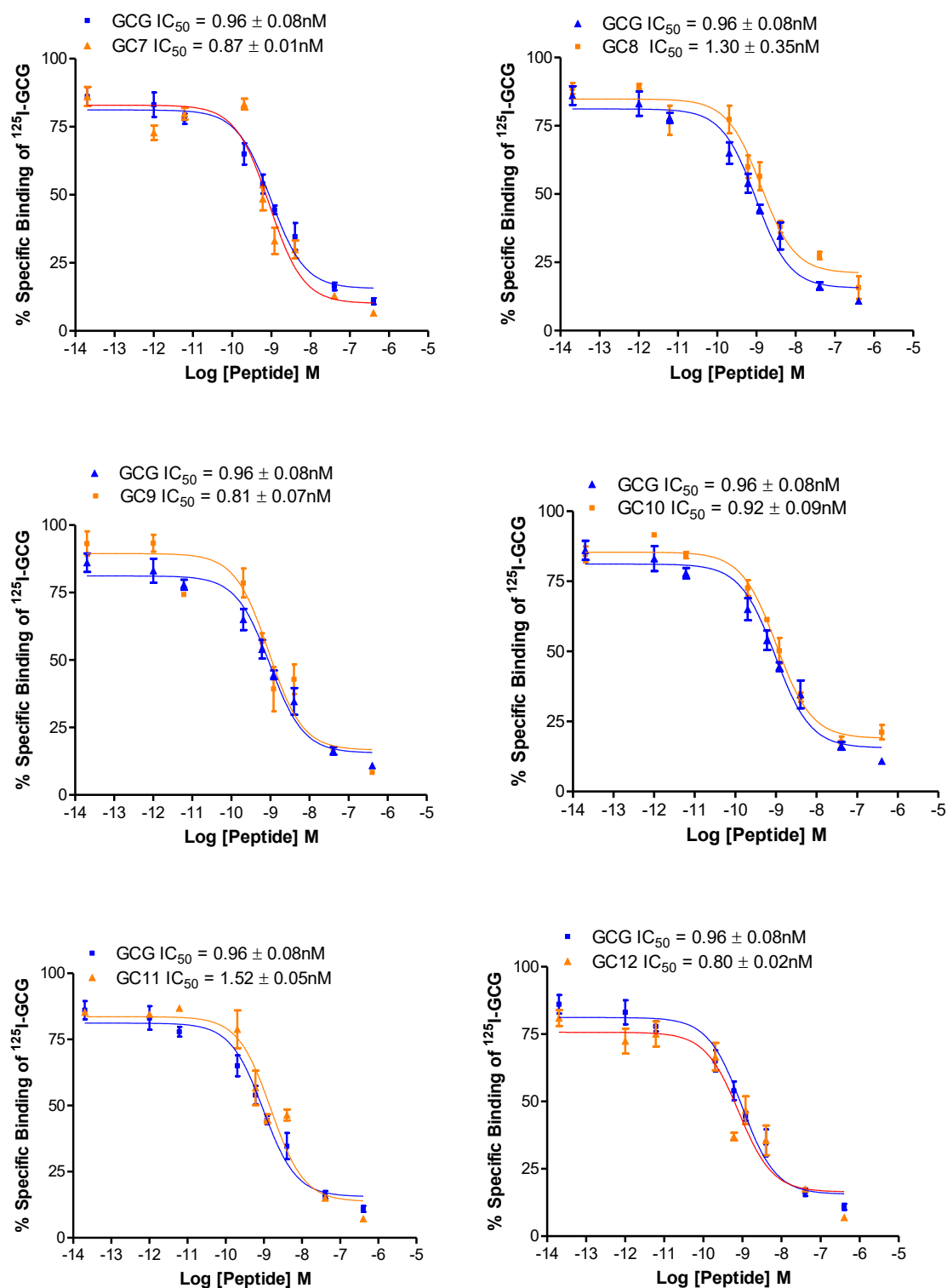


Figure 4.3.2. 1. Binding affinity of glucagon analogues to the human glucagon receptor. Human glucagon receptor over-expressing CHO cell membrane was the source of glucagon receptor. ^{125}I -GCG was used as the competing peptide. Each peptide concentrations was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of three separate experiments $\pm$ SEM.

4.3.2.2. Affinity of the glucagon analogues to the mouse glucagon receptor

On the second generation, analogues demonstrated a small improvement to the mouse receptor, when compared to the first generation of analogues. At the mouse glucagon receptor, GC8 had approximately 6-fold (EC_{50} : 2.28 ± 0.45 nM), GC7 had 3-fold and GC9 and GC10 had 2-fold lower affinity affinities to glucagon (Figure 4.3.2.2). Analogues GC11 and GC12 were the only two that had similar affinities to glucagon, with approximately 0.55 ± 0.04 nM.

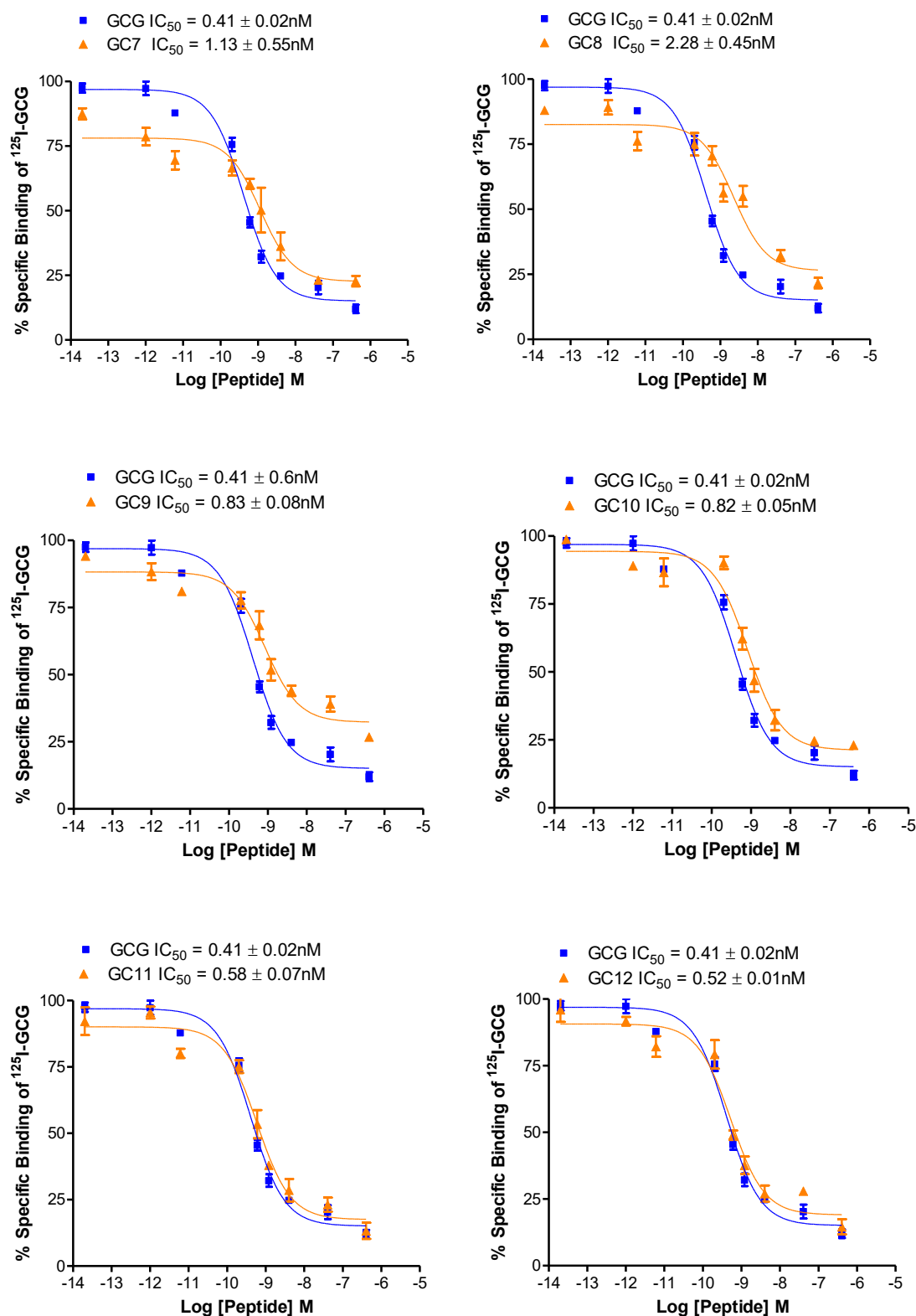


Figure 4.3.2. 2. Binding affinity of glucagon analogues to the mouse glucagon receptor. Mouse liver was the source of glucagon receptor. ^{125}I -GCG was used as the competing peptide. Each peptide concentrations was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of three separate experiments $\pm$ SEM.

4.3.2.3. Effect of glucagon analogues on receptor-mediated cAMP accumulation

In the second generation of glucagon analogues, GC7, GC9 and GC10 had similar bioactivity EC_{50} as glucagon with an average EC_{50} of 0.08 ± 0.04 nM. Analogue GC8 was 19-fold less potent than glucagon, followed by GC11 which presented almost 2-fold lower efficacy than with an EC_{50} of 0.13 ± 0.01 nM. Of note, analogue GC12 had approximately 0.5-fold higher potency than glucagon at the human glucagon receptor, presenting an EC_{50} of 0.05 ± 0.01 nM (Figure 4.3.2.3).

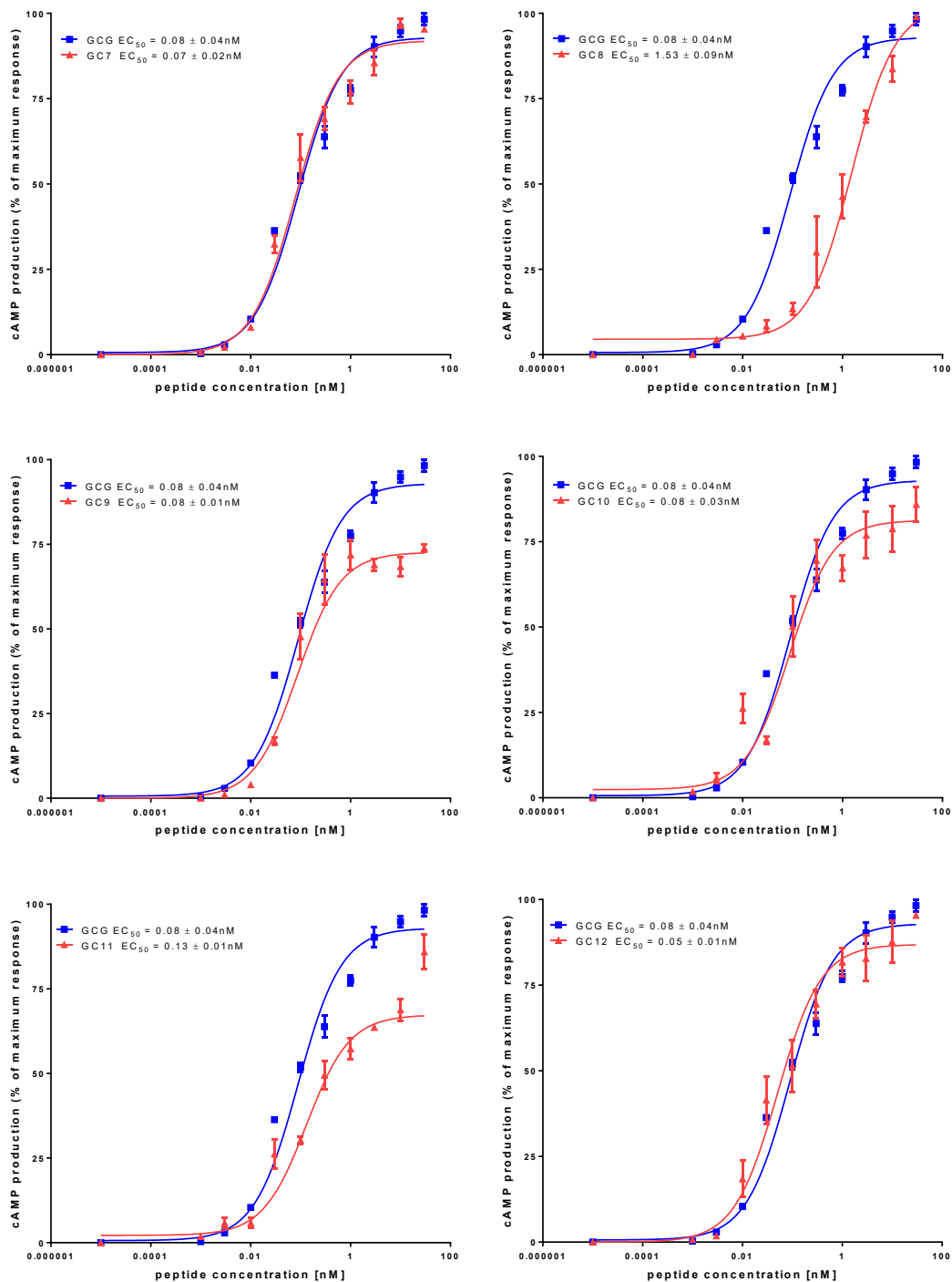


Figure 4.3.2. 3. Human glucagon receptor-mediated cAMP accumulation in CHO cells overexpressing the human glucagon receptor by glucagon and generation 2 glucagon analogues. Each peptide concentrations was tested in duplicate or triplicate in each experiment. EC₅₀ values calculated from a minimum of three separate experiments, errors shown are ±SEM.

4.3.3. *In vitro* characterisation of third generation of glucagon analogues

4.3.3.1. Affinity of the glucagon analogues to the human glucagon receptor

Within the third generation of glucagon analogues, all peptides demonstrated an increased potency to the human glucagon receptor, when compared to native glucagon. More specifically, GC13, GC14, GC15 and GC16 had 0.5 fold higher affinities than glucagon in the human glucagon receptor, and GC17 demonstrated the highest IC_{50} of 0.47 ± 0.01 nM (Figure 4.3.3.1).

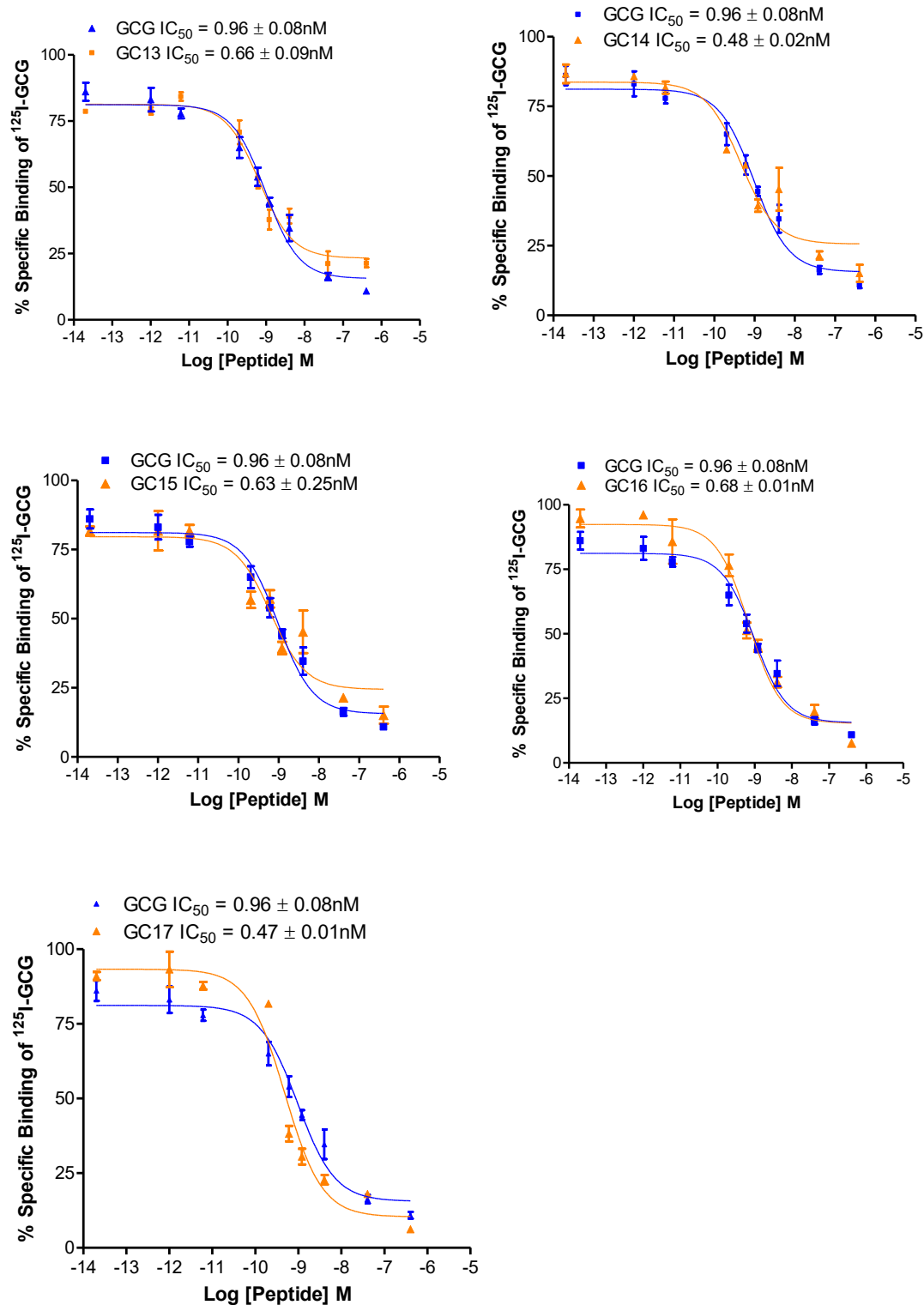


Figure 4.3.3. 1. Binding affinity of glucagon analogies to the human glucagon receptor. ^{125}I -GCG was used as the competing peptide. Each peptide concentrations was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of three separate experiments $\pm$ SEM.

4.3.3.2. Affinity of the glucagon analogues to the mouse glucagon receptor

In the third generation of glucagon analogues, all five peptides demonstrated a similar affinity to the mouse receptor as glucagon. The analogue that presented the highest affinity was GC17 with an IC_{50} of 0.34 ± 0.05 nM, which is in agreement with the human glucagon receptor results (Figure 4.3.2.2).

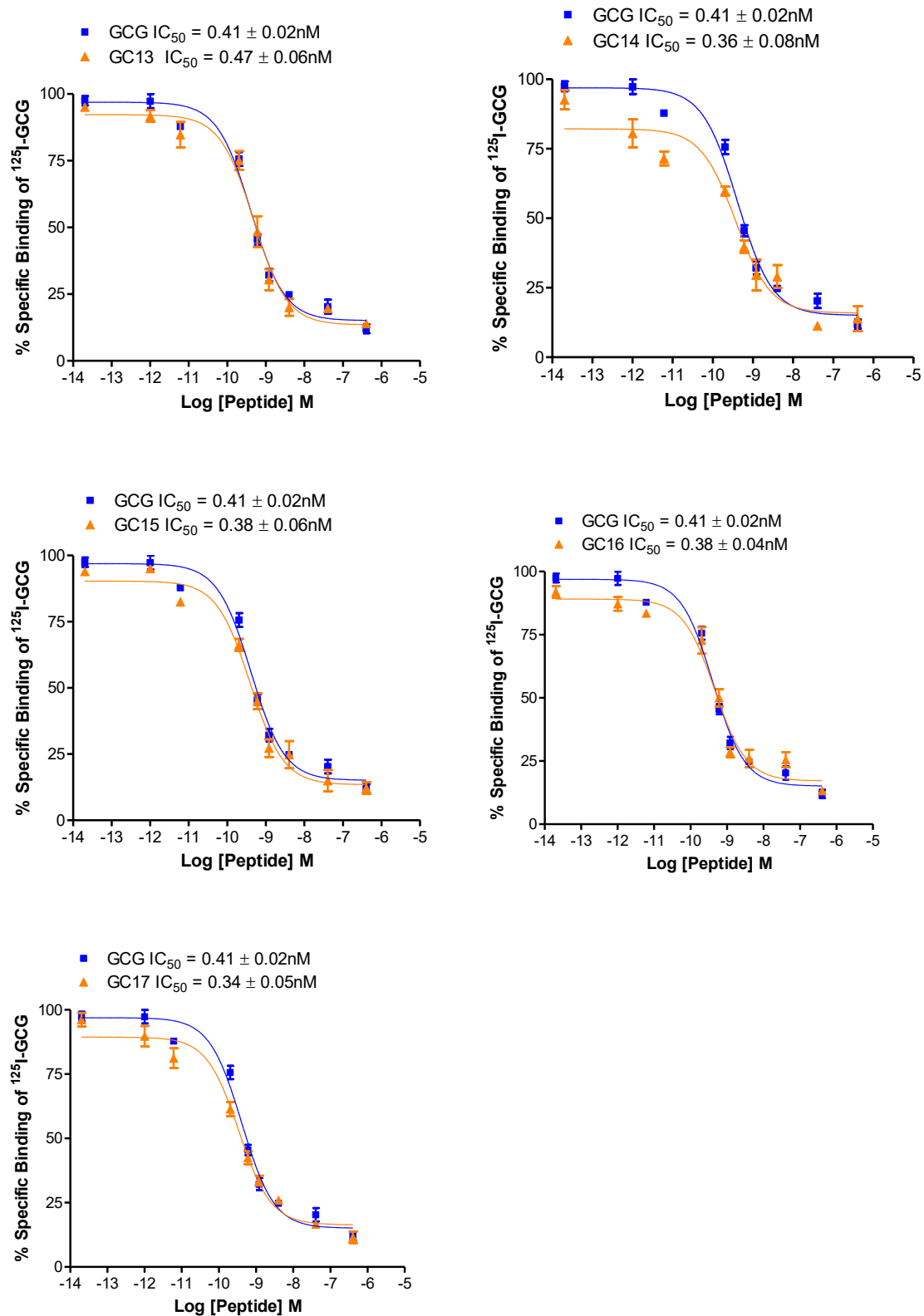


Figure 4.3.3. 2. Binding affinity of glucagon to the mouse glucagon receptor. Mouse liver was the source of glucagon receptor. ^{125}I -GCG was used as the competing peptide. Each peptide concentrations was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from a minimum of three separate experiments $\pm\text{SEM}$.

4.3.3.3. Effect of glucagon analogues on receptor-mediated cAMP accumulation

In the third generation of glucagon analogues, GC13, GC14, GC15, GC16 and GC17 had nearly 0.1-fold higher EC_{50} than glucagon, with an approximate value of 0.05 ± 0.02 nM. The values for affinity and *in vitro* potency are tabulated and summarised in table 4.1 (Figure 4.3.3.3.).

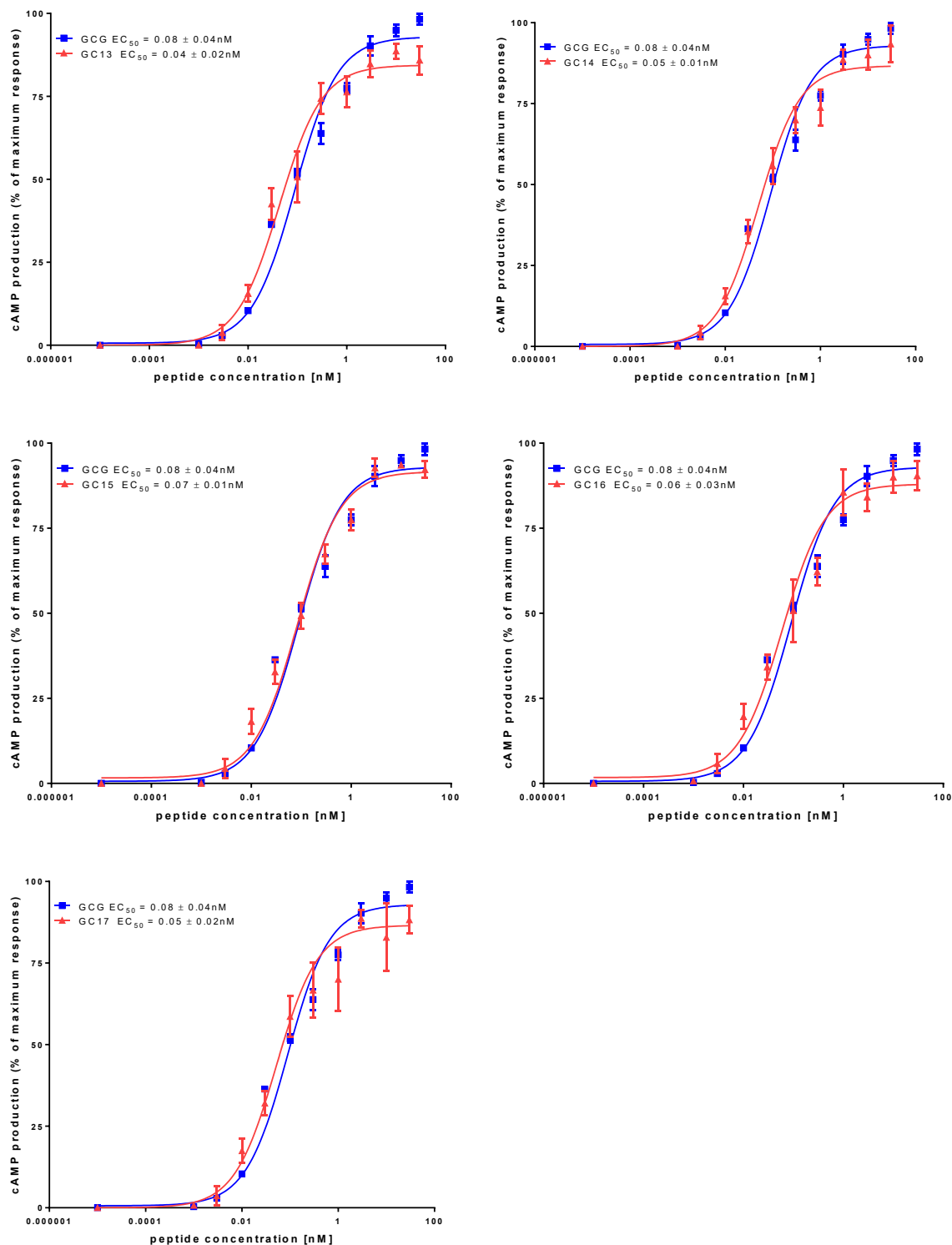


Figure 4.3.3. 3. Human glucagon receptor-mediated cAMP accumulation in CHO cells overexpressing the human glucagon receptor by glucagon and generation 3 glucagon analogues. Each peptide concentrations was tested in duplicate or triplicate in each experiment. EC₅₀ values calculated from a minimum of three separate experiments, errors shown are ± SEM.

	Mouse GCG receptor	Human GCG receptor	
	Receptor binding affinity	Receptor binding affinity	cAMP accumulation
	IC ₅₀ (nM) ± SEM	IC ₅₀ (nM) ± SEM	EC ₅₀ (nM) ± SEM
GCG	0.41 ± 0.02	0.96 ± 0.08	0.08 ± 0.04
GC1	0.51 ± 0.09	2.28 ± 1.70	0.58 ± 0.30
GC2	3.54 ± 0.25	2.08 ± 0.01	0.61 ± 0.80
GC3	3.54 ± 0.75	1.18 ± 0.09	0.10 ± 0.05
GC4	2.11 ± 0.95	1.02 ± 0.25	0.11 ± 0.01
GC5	1.07 ± 1.30	1.10 ± 0.27	0.13 ± 0.02
GC6	0.87 ± 0.72	1.37 ± 0.10	0.12 ± 0.02
GC7	1.13 ± 0.55	0.87 ± 0.04	0.07 ± 0.02
GC8	2.28 ± 0.45	1.30 ± 0.01	1.53 ± 0.09
GC9	0.83 ± 0.08	0.81 ± 0.07	0.08 ± 0.01
GC10	0.82 ± 0.05	0.92 ± 0.09	0.08 ± 0.01
GC11	0.58 ± 0.07	1.52 ± 0.05	0.13 ± 0.03
GC12	0.52 ± 0.01	0.80 ± 0.02	0.05 ± 0.01
GC13	0.47 ± 0.06	0.66 ± 0.09	0.06 ± 0.02
GC14	0.36 ± 0.08	0.48 ± 0.02	0.06 ± 0.01
GC15	0.38 ± 0.06	0.63 ± 0.25	0.05 ± 0.01
GC16	0.38 ± 0.04	0.68 ± 0.01	0.06 ± 0.03
GC17	0.34 ± 0.05	0.47 ± 0.01	0.05 ± 0.02

Table 4. 2. A summary of IC₅₀ and EC₅₀ values for glucagon and glucagon analogues at the glucagon human and mouse receptors. Half-maximal concentration values are shown as mean ± SEM.

4.3.4. Effect of glucagon analogues on food intake in fasted mice

Assessment of the effect of SC administration of glucagon (300nmol/ kg) and the three generations of glucagon analogues (50nmol/ kg) on food intake in mice was completed. Peptides were administered in the early light phase in overnight fasted mice. Analogues GC1, GC2, GC4 and GC5 did not significantly inhibit food intake compared to saline controls in any interval tested, generation 1 peptide GC3 significantly reduced food intake over the first hour post injection compared with vehicle control treated mice (Figure 4.3.4.1). GC4 (50nmol/ kg) had a significant inhibition of cumulative food intake when compared to glucagon at 0-24hr (Figure 4.3.4.2.). No significant body weight change was observed at 0-48hr (Figure 4.3.4.2.).

Generation 1:

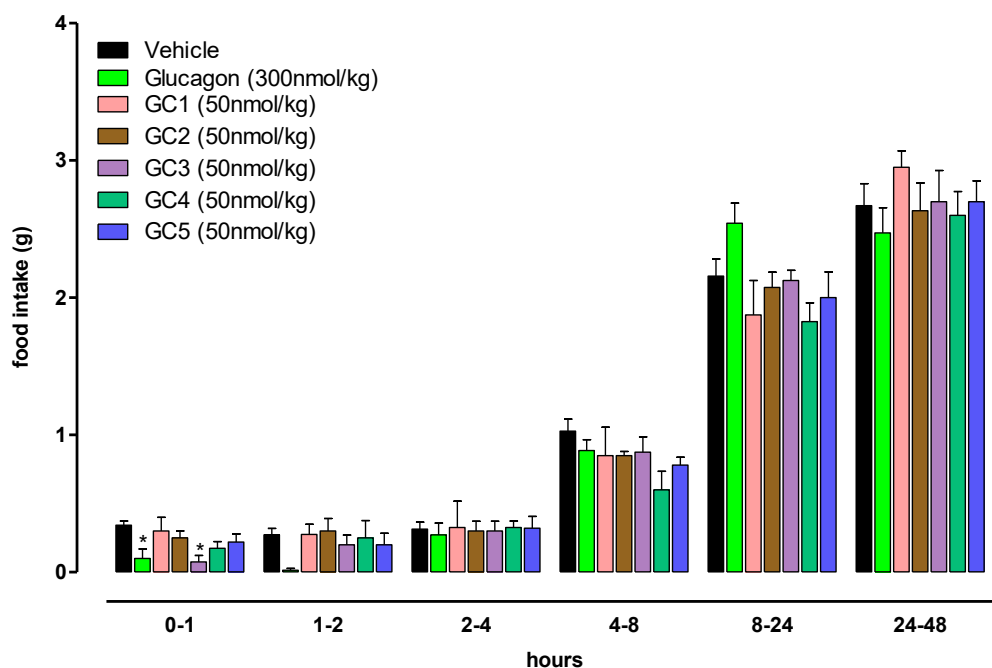


Figure 4.3.4. 1. Effect of a single SC injection of glucagon (300nmol/kg) and glucagon analogues GC1-5 (50nmol/ kg) on acute food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake, for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour, 8-24 hour and 24-48hour intervals (n=7-8 per group). Statistics: One way ANOVA with Dunnett's post-hoc test *=p<0.05 vs. vehicle

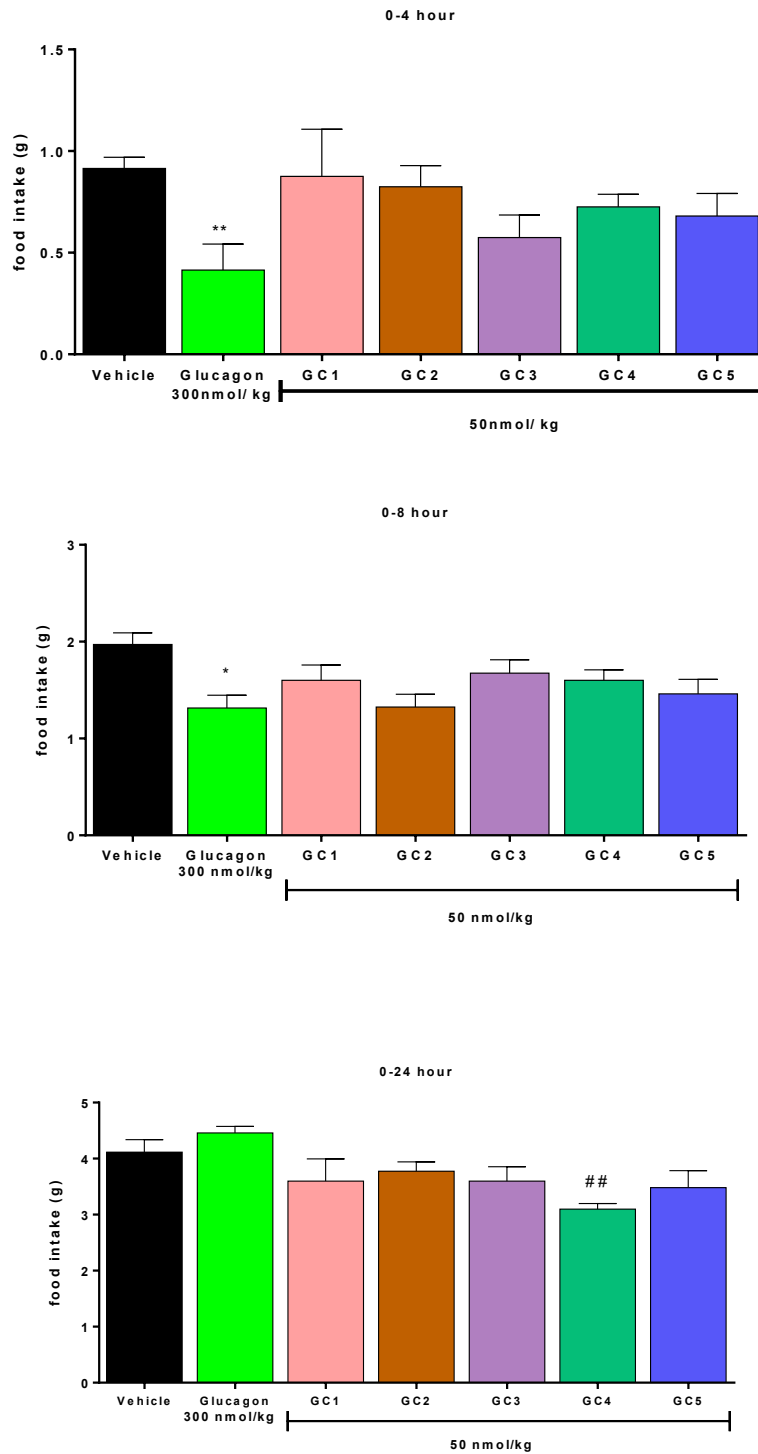


Figure 4.3.4. 2. Effect of a single SC injection of glucagon (300nmol/ kg) and glucagon analogues GC1-5 (50nmol/kg), on cumulative food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-4, 0-8, 0-24 hours. n=5-6 mice per group Statistics: One way ANOVA with Tukey's post-hoc test. *= $p < 0.05$, **= $p < 0.01$ vs. vehicle, $p < 0.01$ vs. glucagon

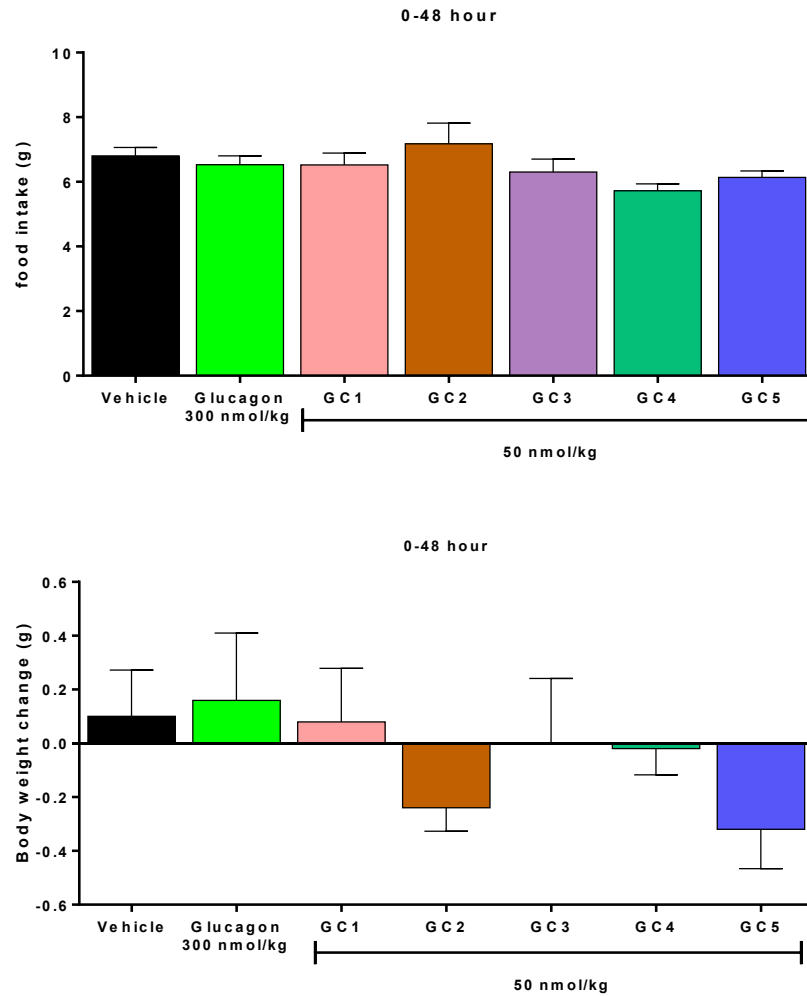


Figure 4.3.4. 3. Effect of a single SC injection of glucagon (300nmol/ kg) and glucagon analogues GC1-5 (50nmol/kg), on cumulative food intake and body weight change in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake and body weight change in mice for 0-48 hours. n=5-6 mice per group Statistics: One way ANOVA with Tukey's post-hoc test.

Generation 2:

In generation 2, GC6, GC7 and GC9 caused a significant inhibition of food intake in the 0-1 hour interval. A trend of continued food intake inhibition was observed in the 1-2 and 2-4 hr intervals for analogues GC6, GC7 and GC9. Despite causing a mean greater food intake than vehicle controls in the 0-1h interval, analogue GC10 showed a trend of food intake inhibition compared to vehicle controls in the 1-2 and 2-4 hr intervals. At 4-8 hours post injections all 6 analogues significantly inhibited food intake, while at 8-24 hours only GC6, GC9 and GC10 maintained it (Figure 4.3.4.4). Cumulative food intake at 8 hours post-injection was significantly reduced in glucagon and GC6 (50nmol/ kg) compared to the vehicle control group ($p<0.05$) (Figure 4.3.4.5.). No significant body weight change was observed at 0-48hr (Figure 4.3.4.6.).

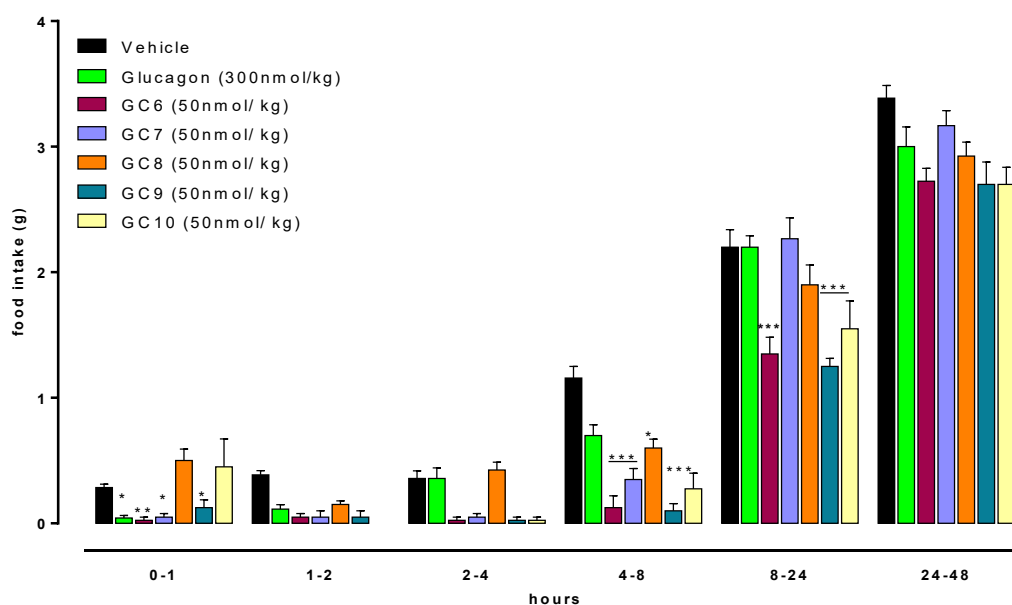


Figure 4.3.4. 4. Effect of a single SC injection of glucagon (300nmol/ kg) and glucagon analogues GC6-12 (50nmol/ kg on acute food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake, for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour, 8-24 hour and 24-48hour intervals (n=7-8 per group). Statistics: One way ANOVA with Dunnett's post-hoc test $*=p<0.05$, $=p<0.01$, $***=p<0.001$ vs. vehicle**

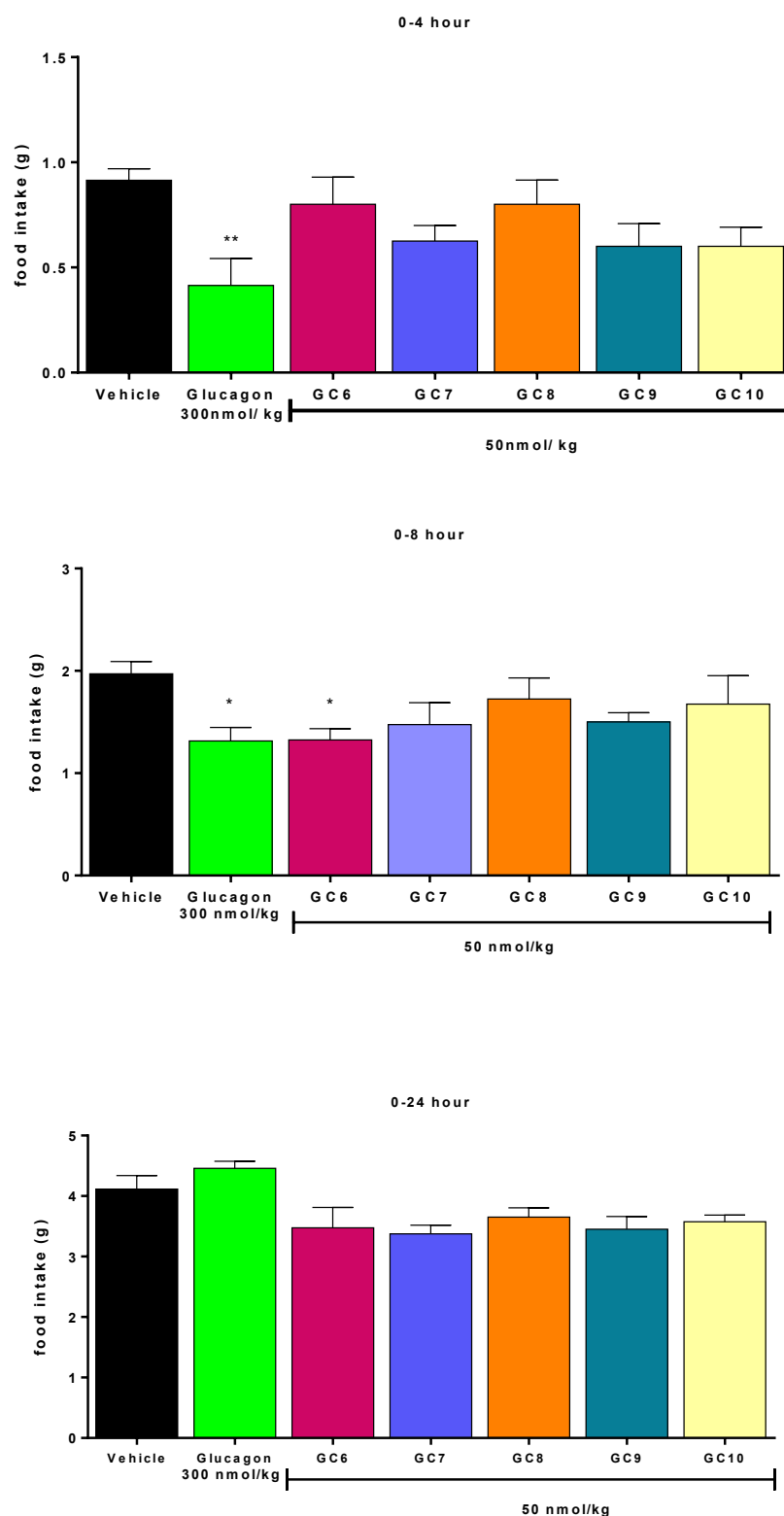


Figure 4.3.4. 5. Effect of a single SC injection of glucagon (300nmol/ kg) and glucagon analogues GC6-10 (50nmol/ kg), on cumulative food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-4, 0-8, 0-24 hours. n=5-6 mice per group Statistics: One way ANOVA with Tukey's post-hoc test. *= $p < 0.05$, **= $p < 0.01$ vs. vehicle

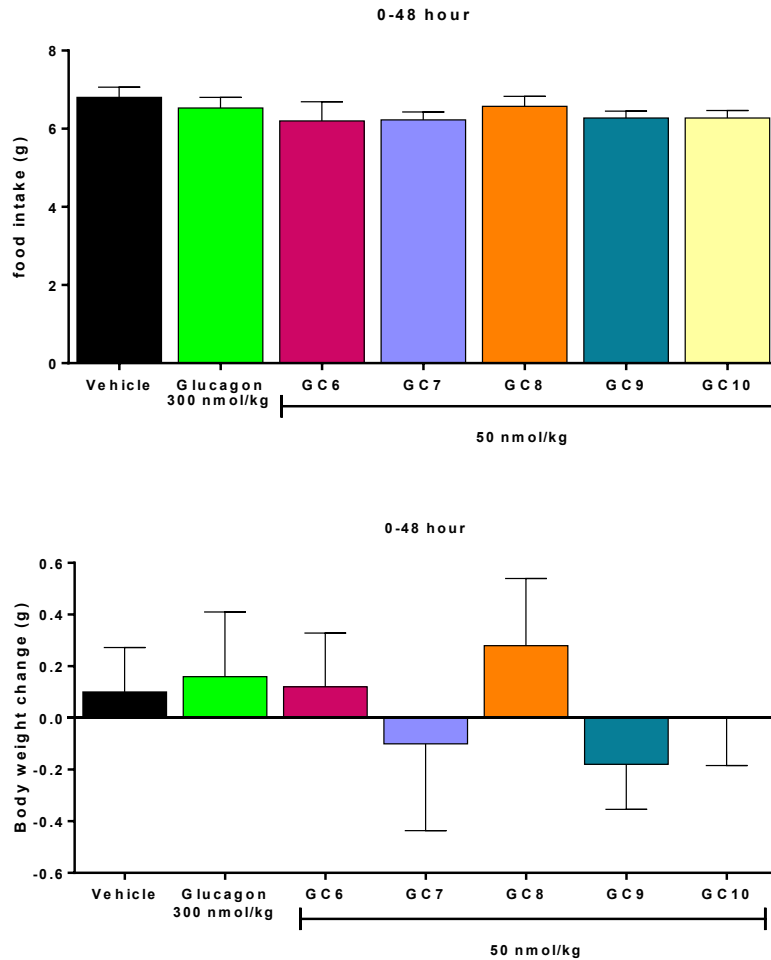


Figure 4.3.4. 6. Effect of a single SC injection of glucagon (300nmol/ kg) and glucagon analogues GC6-10 (50nmol/ kg), on cumulative food intake and body weight change in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake and body weight change in mice for 0-48 hours. n=5-6 mice per group Statistics: One way ANOVA with Tukey's post-hoc test.

Generation 3:

In generation 3, GC14 and GC16 significantly inhibited food intake over the first hour, yet GC12, GC14, GC15 and GC17 had a significant effect at 8-24 hours. At 24-48 hours, GC15 and GC17 with the addition of GC16 still significantly reduced food intake (Figure 4.3.4.7). GC12 and GC14 groups (50nmol/ kg) had a significant inhibition of food intake when compared to glucagon at 24hr (Figure 4.3.4.8.). Of note, at 48hr GC16 and GC17 groups of mice ate significantly less than the glucagon group. Analysis of cumulative body weight change data showed a significant weight loss of 0.8 g between the analogue GC17 and the control group (Figure 4.3.4.1.9.).

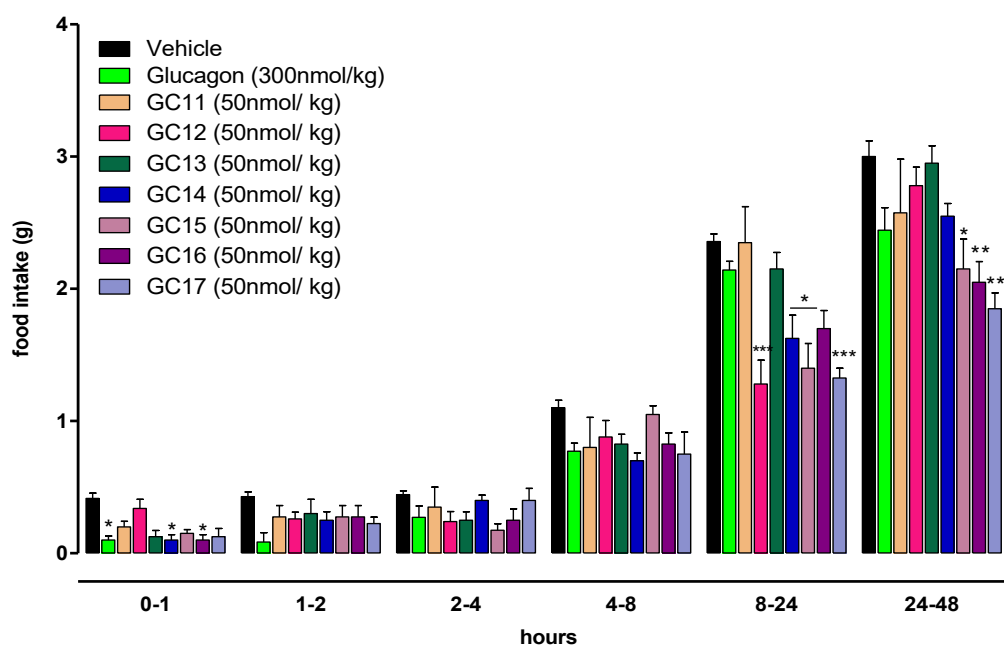


Figure 4.3.4. 7.Effect of a single SC injection of glucagon (300nmol/ kg) and glucagon analogues GC11-17 (50nmol/ kg on acute food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake, for 0-1 hour, 1-2 hour, 2-4 hour, 4-8 hour, 8-24 hour and 24-48hour intervals (n=5-6 per group). Statistics: One way ANOVA with Dunnett's post-hoc test *=p<0.05, **=p<0.01, ***=p<0.001 vs. vehicle

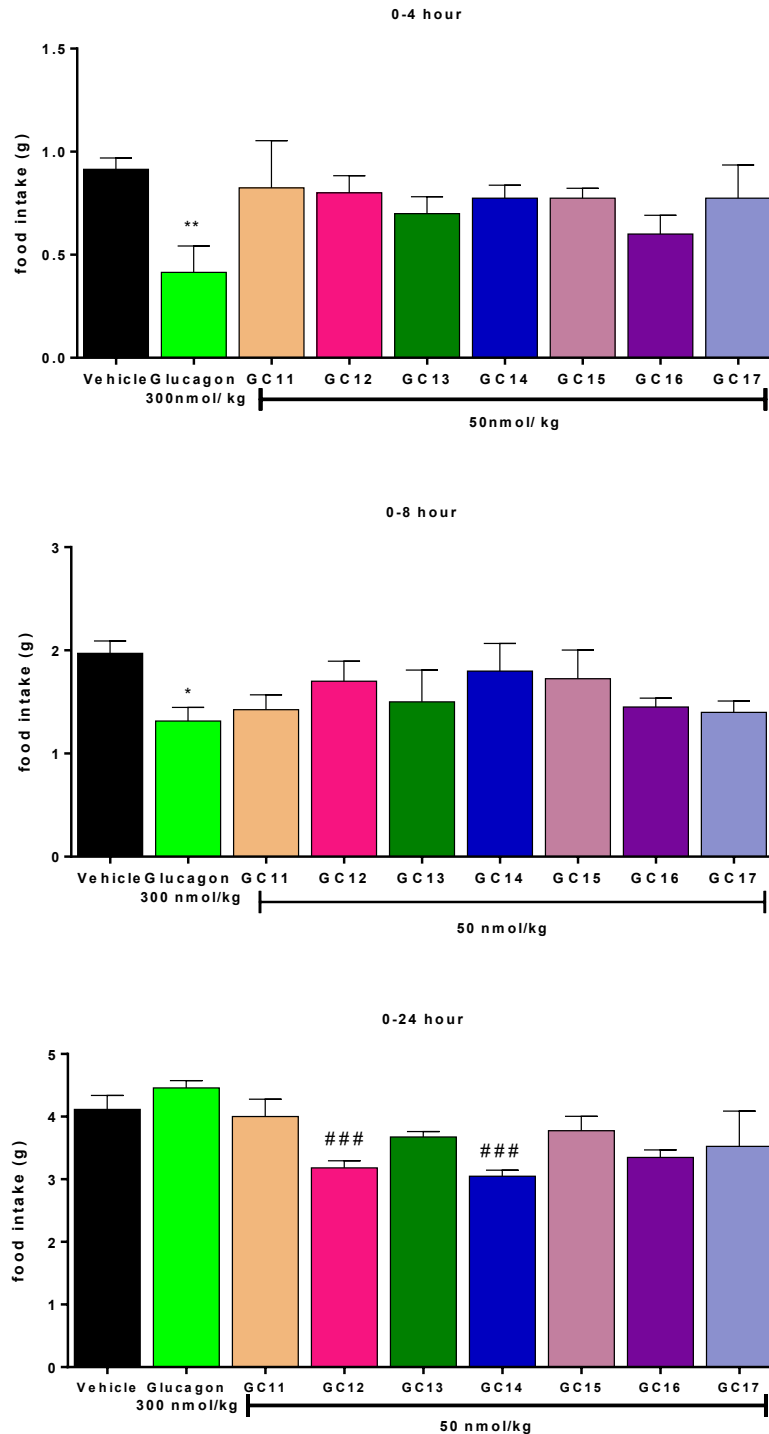


Figure 4.3.4. 8. Effect of a single SC injection of glucagon (300nmol/ kg) and glucagon analogues GC11-17 (50nmol/ kg), on cumulative food intake in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake in mice for 0-4, 0-8, 0-24 hours. n=5-6 mice per group Statistics: One way ANOVA with Tukey's post-hoc test. *=p<0.05, **=p<0.01 vs. vehicle, ### p<0.001 vs. glucagon

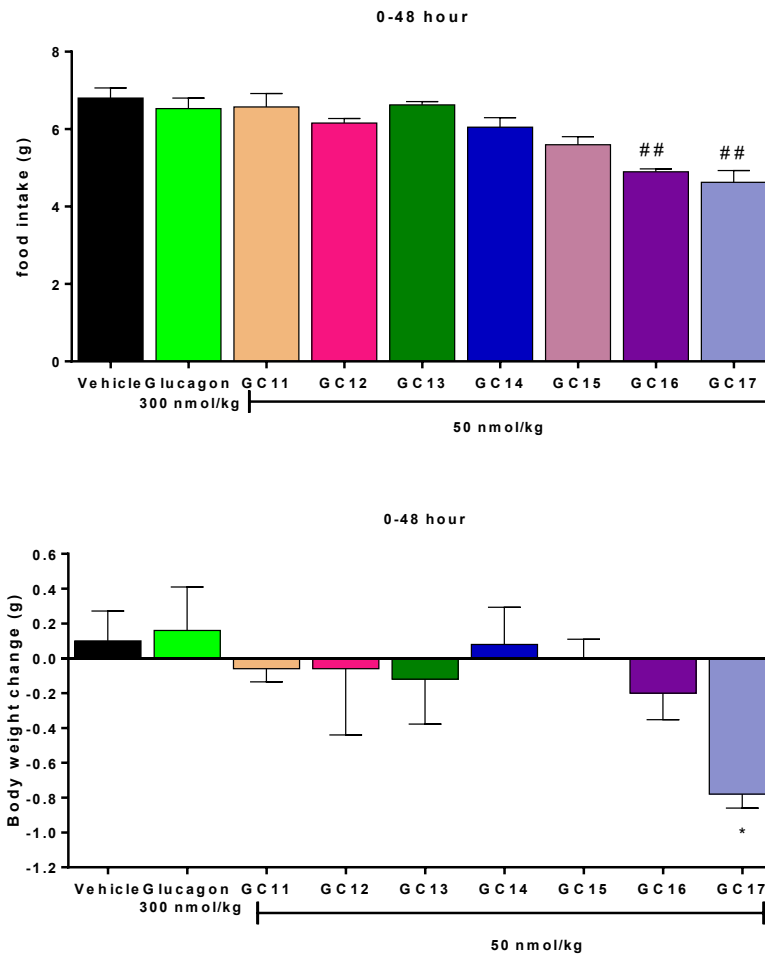


Figure 4.3.4. 9. Effect of a single SC injection of glucagon (300nmol/ kg) and glucagon analogues GC11-17 (50nmol/ kg), on cumulative food intake and body weight change in overnight fasted C57BL/6J mice. Data shown as mean $\pm$ SEM food intake and body weight change in mice for 0-48 hours. n=5-6 mice per group Statistics: One way ANOVA with Tukey's post-hoc test. *=p<0.05 vs. vehicle, ## =p<0.01 vs. glucagon

4.3.5. Study of *in vivo* pharmacokinetics glucagon analogues in male Wistar rats

The three generations of analogues were administered SC as 1 mg of peptide, formulated in a slow release formulation containing 1:1 molar ratio Zn:NaCl (saline), to male Wistar rats (Fig. 4.3.5). Peptide plasma levels were then measured using RIAs. In the first generation, peptides GC4 and GC5 reached their peak recorded plasma levels (C_{max}) at 4hr (T_{max}) and were rapidly cleared by 24hr, while GC1, GC2, GC3 and GC6 sustained release with plasma levels of peptide above 40% of 4hr T_{max} at 24hr, and were cleared by 48hr (Fig. 4.3.5.1, 2). Within the second generation of peptides GC10, GC11, and GC12 also maintained 50% of 4hr T_{max} at 24hr, but dropped by 60% at 48hr (Fig. 4.3.5. 4). GC8 and GC9 peaked at 4hr and rapidly dropped by 60% but maintained 24hr and 48hr peptide plasma concentration at similar levels (Fig. 4.3.5.3.). Similar to GC8 and GC9, third generations' GC14 and GC15 reached their C_{max} at 4hr but sustained release between 24hr and 48hr (Fig. 4.3.5.5.). GC16 also peaked at 4hr yet C_{max} was almost fully constant by 24hr before it dropped by 60% at 48hr. GC17 reached the same C_{max} as GC16 but had a different T_{max} at 24 hr, which was sustained by 64% by 48hr (Fig. 4.3.5.6.).

Generation 1:

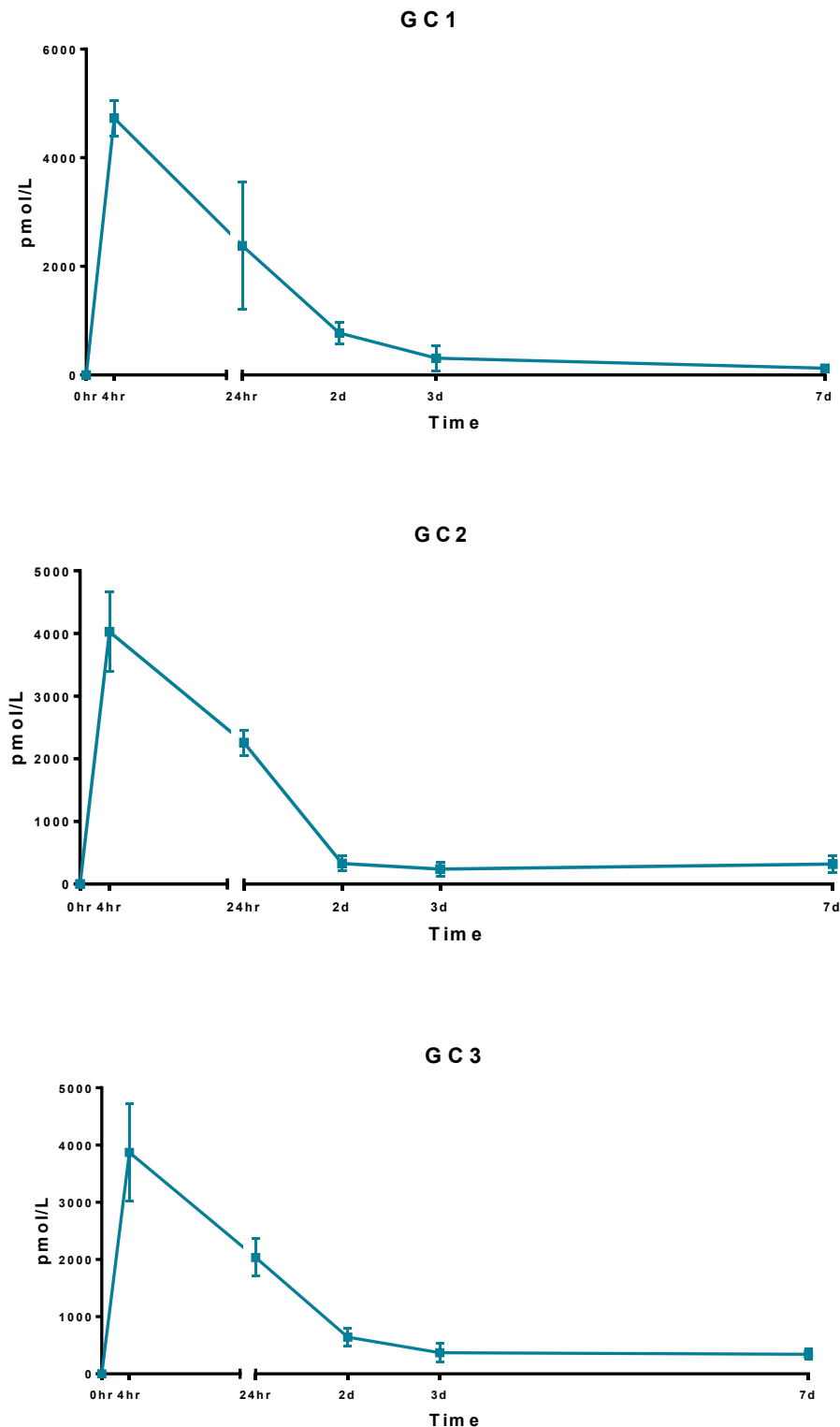


Figure 4.3.5. 1. Plasma levels of glucagon analogues GC1-3 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection, to male Wistar rats (n=6). Plasma concentrations were quantified by RIA and shown as mean pmol/L $\pm$ SEM. Values were corrected according to baseline sample and Lower Levels of Quantification (LLOQs) for RIA.

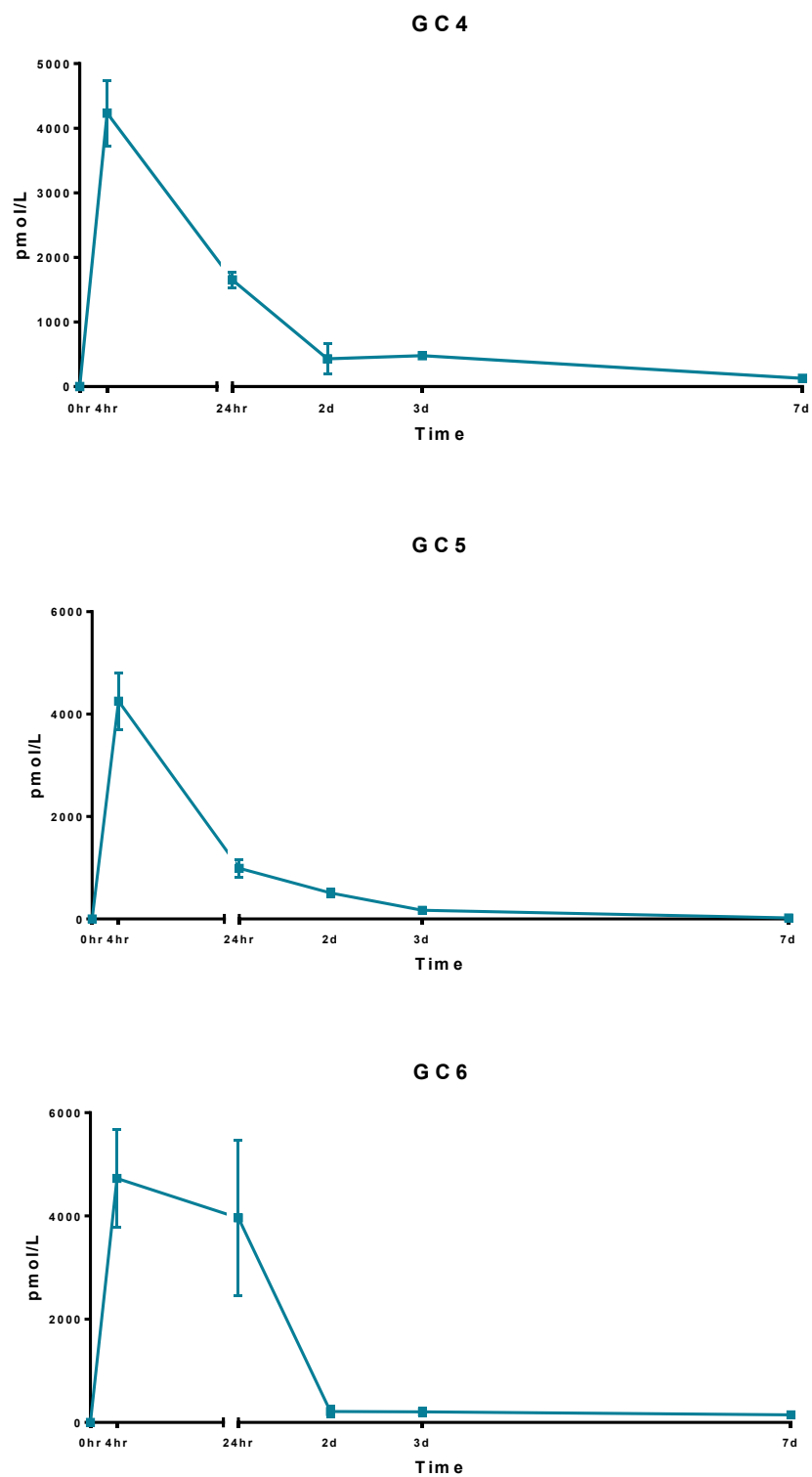


Figure 4.3.5. 2. Plasma levels of glucagon analogues GC4-6 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection, to male Wistar rats (n=6). Plasma concentrations were quantified by RIA and shown as mean pmol/L $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for RIA.

Generation 2:

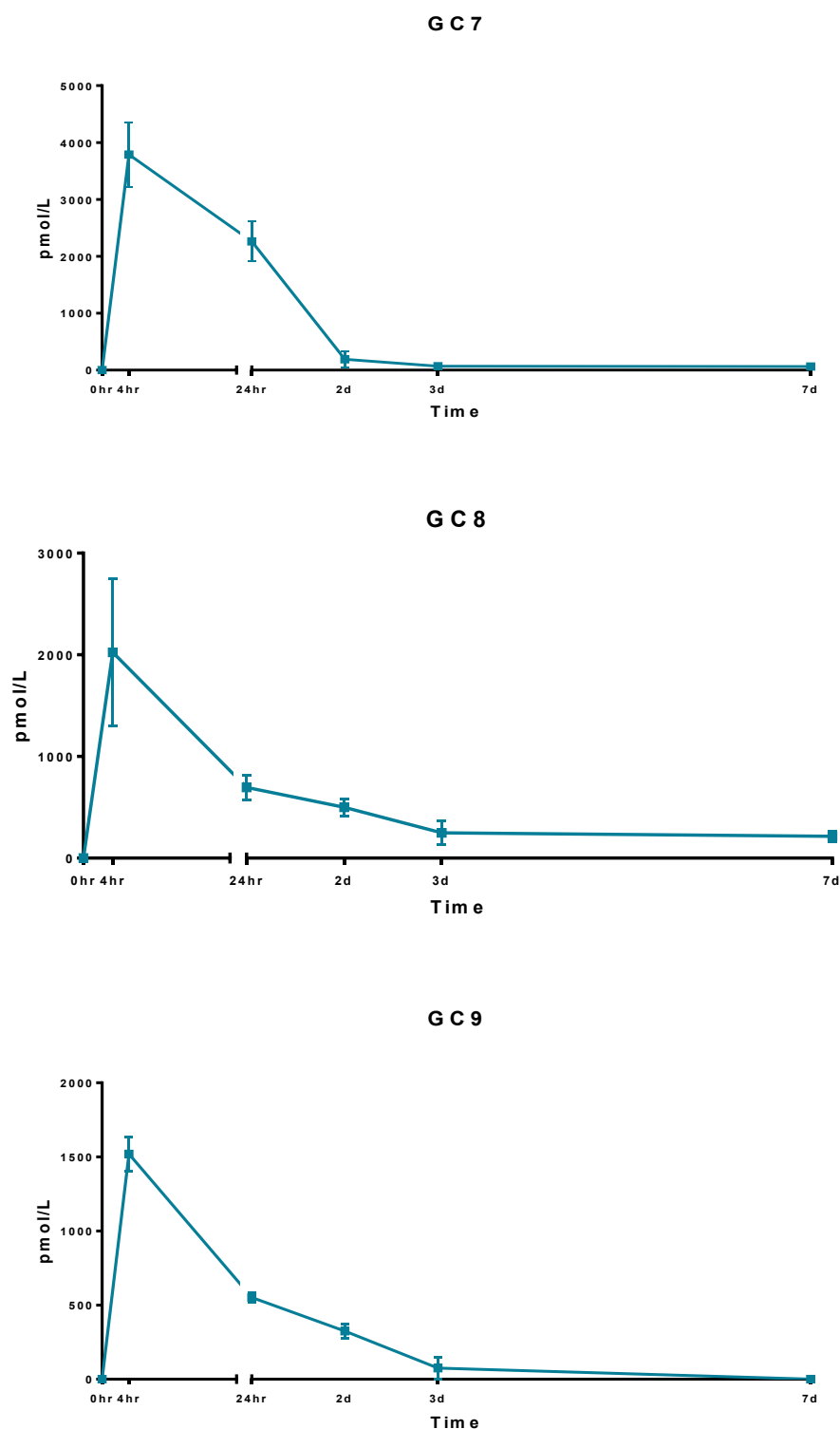


Figure 4.3.5. 3. Plasma levels of glucagon analogues GC7-9 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection, to male Wistar rats (n=6). Plasma concentrations were quantified by RIA and shown as mean pmol/L $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for RIA.

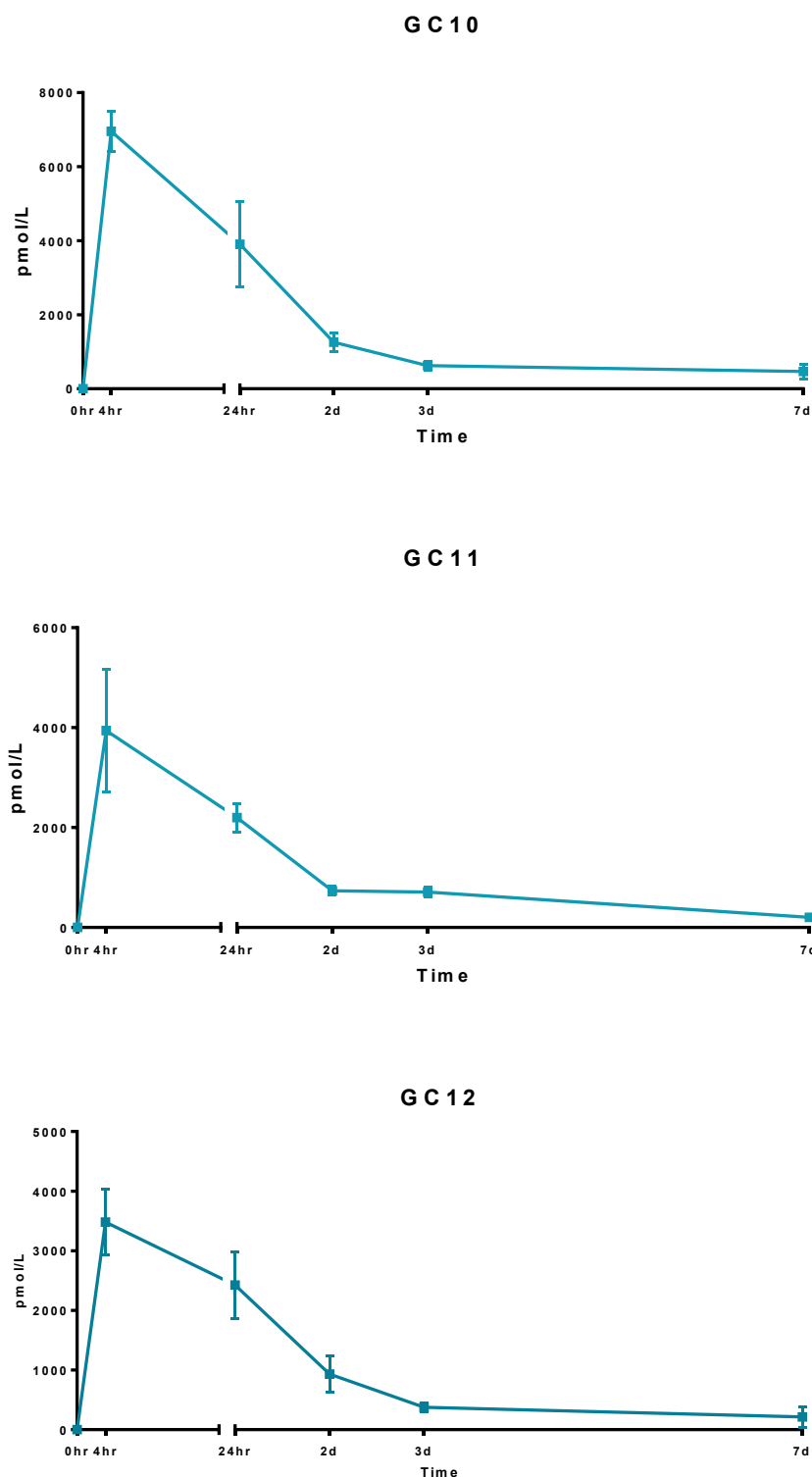


Figure 4.3.5. 4. Plasma levels of glucagon analogues GC10-12 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection, to male Wistar rats (n=6). Plasma concentrations were quantified by RIA and shown as mean pmol/L $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for assay.

Generation 3:

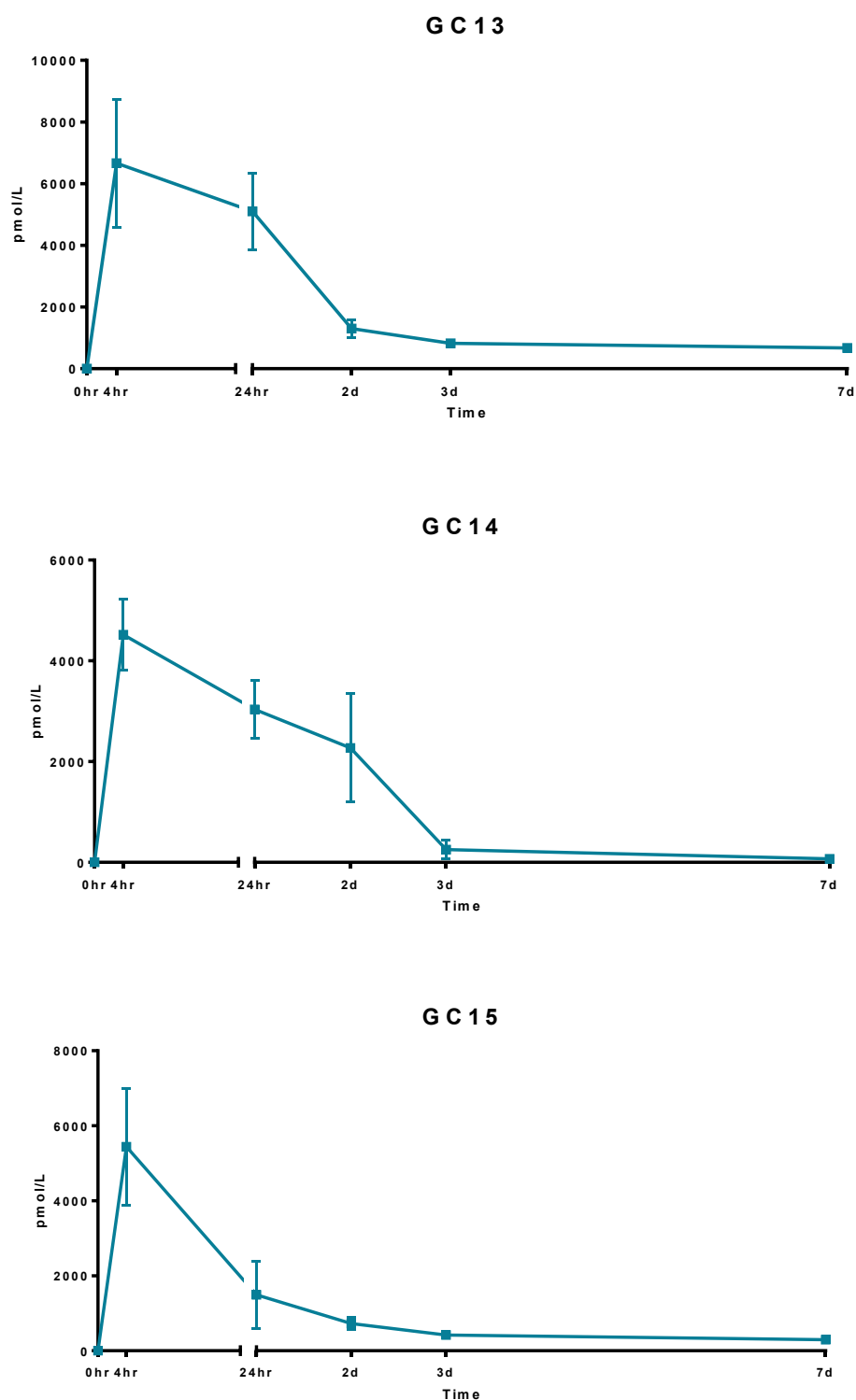


Figure 4.3.5. 5. Plasma levels of glucagon analogues GC13-15 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection, to male Wistar rats (n=6). Plasma concentrations were quantified by RIA and shown as mean pmol/L $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for RIA.

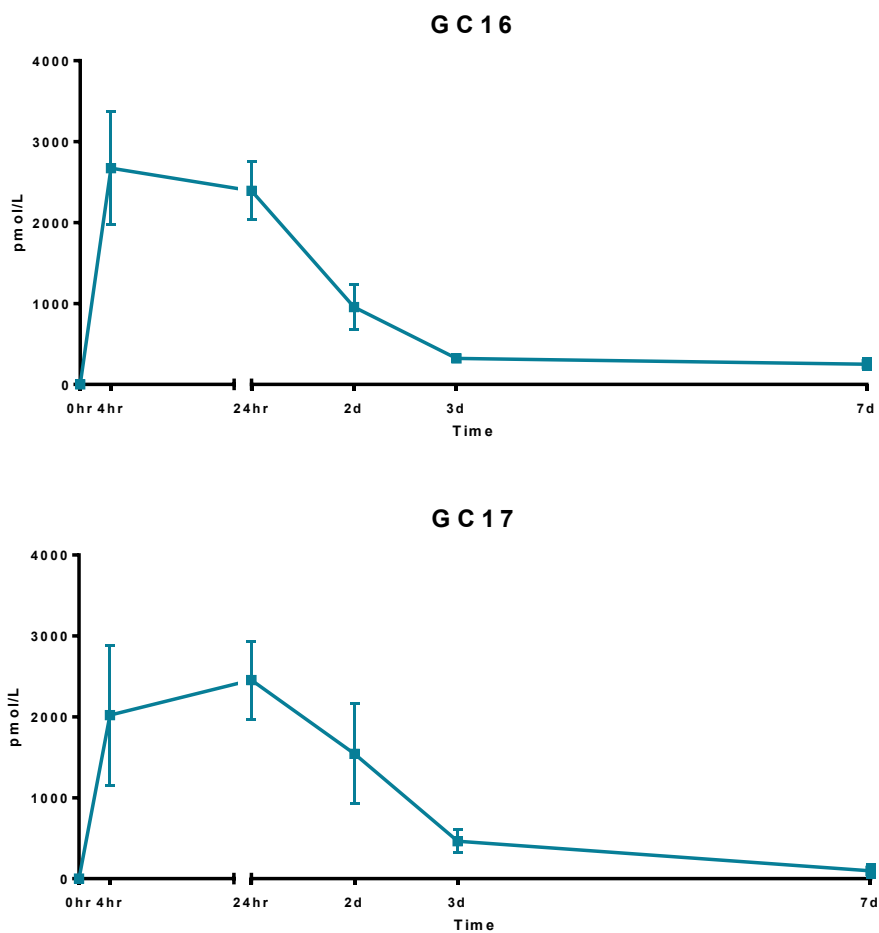
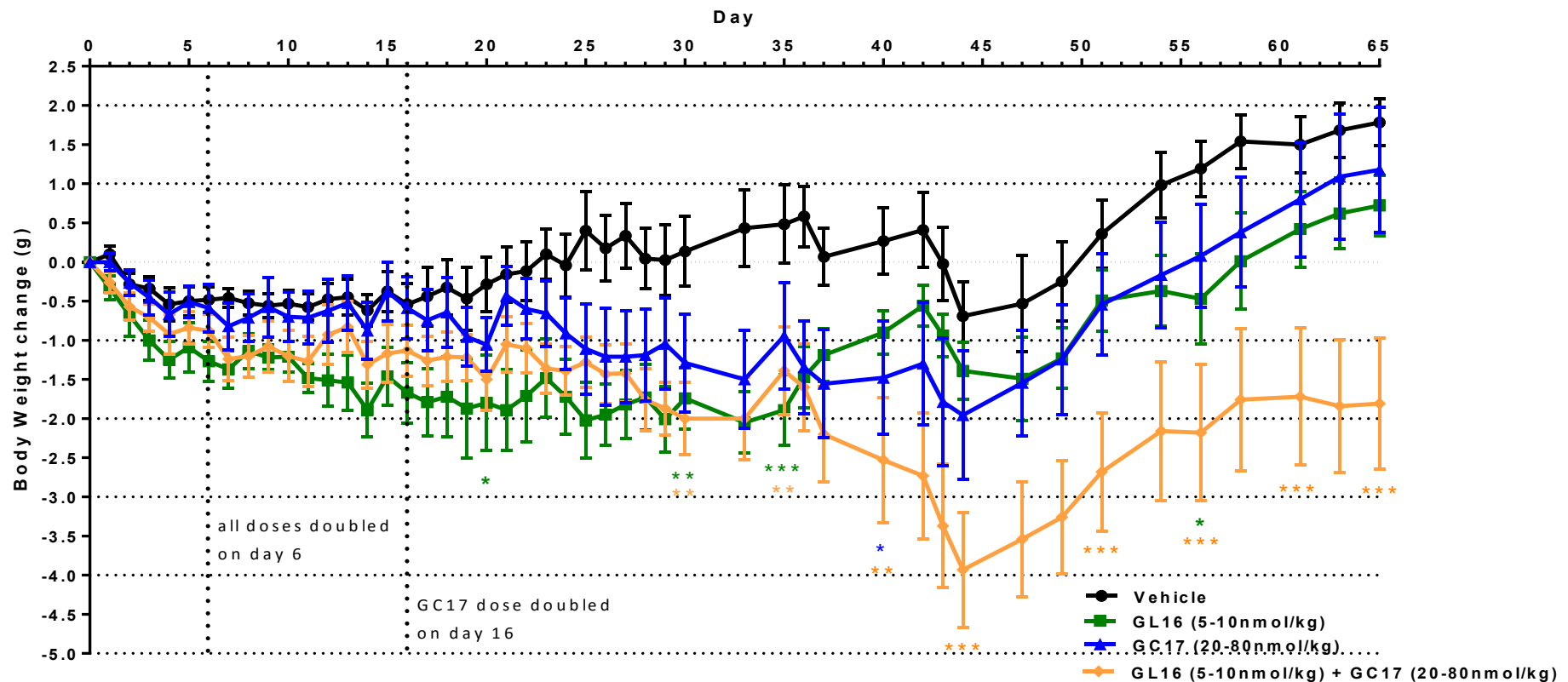


Figure 4.3.5. 6. Plasma levels of glucagon analogues GC16-17 after a 1mg 1:1 molar ratio Zn:saline peptide SC injection, to male Wistar rats (n=6). Plasma concentrations were quantified by RIA and shown as mean pmol/L $\pm$ SEM. Values were corrected according to baseline sample and LLOQs for RIA.

4.3.6. The effect of chronic daily co- administration of GLP-1 and glucagon receptor agonists on food intake and body weight in C57BL/6J mice

The effect of chronic co-administration of GL16 and GC17 was assessed by daily SC injections of each peptide individually and as a co administration as a single agent, in 1:1 Zn:NaCl (saline). In accordance to the principles of drug discovery, the initial dose of the two peptides was low and then both were doubled at the same time until an effect was observed (214). GLP-1 analogue GL16 was administered at a dose of 5nmol/ kg for days 1-5, which was doubled at day 6 to 10nmol/ kg. Glucagon analogue GC17 was administered at a dose 20nmol/ kg for days 1-5, and then doubled at day 6 to 40nmol/ kg. Upon observation of minimal body weight change, GC17 dose was doubled to 80nmol/ kg at day 16 until the end of the study at day 65. Co administration doses were doubled at the same time points.

Cumulative results presenting body weight change does not show significance for any of the treatment groups until day 20 (Figure 4.3.6.1.). On day 20, GL16 (10nmol/ kg) group showed a significant decrease of body weight. At day 30, GL16 and GL16/GC17 co administration at 10/ 80 nmol/ kg doses, caused significant body weight reduction of approximately 2g. All three treatment groups maintained a stable body weight reduction until day 35, yet only groups administered with GL16 and GL16/ GC17 presented significance against the vehicle group. On day 37, a single time point Glucose Tolerance Test was performed to all mice groups. In total, group GC17 demonstrated a significant weight loss only at day 40. From day 40 onwards, a body weight increase is observed in groups treated with analogues GL16 and GC17. Of note, co administration group presented a highly significant weight reduction, reaching an average of 4g weight loss at day 44, which remained stable until the end of the study at day 65.



4.3.7. Glucose Tolerance Test study in mice chronically treated with GLP-1 and glucagon analogues

In order to assess the effect of the analogues on glucose levels, Glucose Tolerance Test was completed on day 65 of the chronic feeding study (Figure 4.3.7.1.). Both analogues presented anti-hyperglycaemic properties, as GLP-1 analogue GL16 and glucagon analogue GC17 sustained significantly lower glucose levels in blood at 30 min post glucose IP injection (≈ 8 mmol/ L), compared to vehicle (≈ 19 mmol/ L), and maintained the levels throughout the 120 min of measurements. Of note, co administration of GL16 and GC17 maintained an even lower and more stable glucose concentration (≈ 4 mmol/ L) when compared to individually administered analogues, in particular GL16, compared to which it presented a significant reduction at 60, 90 and 120 minutes post treatment (≈ 11 mmol/ L).

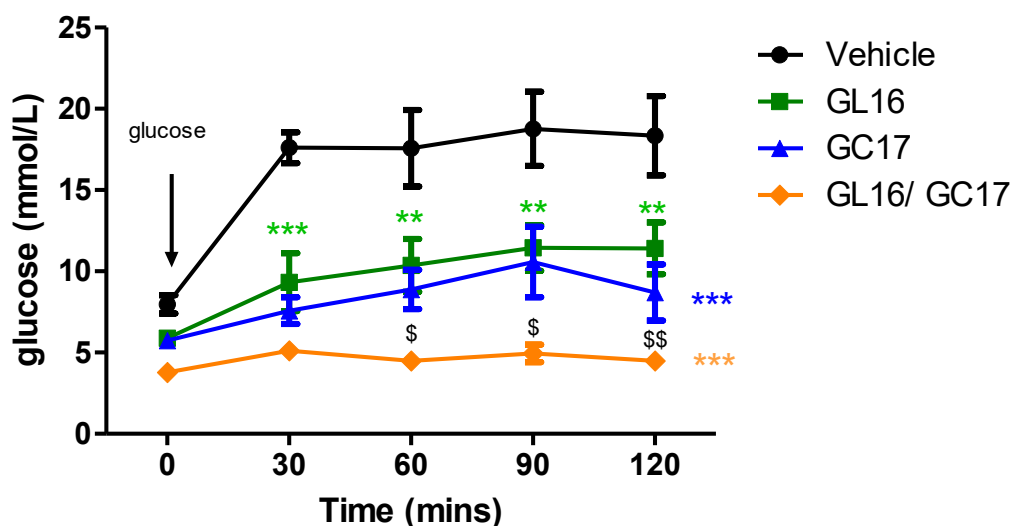


Figure 4.3.7. 1. Glucose measurement (mmol/ L) of tail tip samples of blood at times 0, 30, 60, 90 and 120, after 2g/ kg glucose IP injection with GL16, GC17, GL16/GC17 at time 0. Statistical analysis: 2 way ANOVA of 120 minute time point with Bonferroni correction ** < 0.01, *** P < 0.001 vs. vehicle, \$ < 0.5, \$\$ < 0.01 vs. GL16.

4.4. Discussion

The studies described in this chapter investigated the possibility that a glucagon analogue could function as a long-lasting agonist of the glucagon receptor. As with GLP-1 analogues, the effect of the designed analogues was assessed on food intake and their pharmacokinetic properties in order to investigate their putative viability as treatments for obesity and/or diabetes. As infusion studies co administering GLP-1 and glucagon in man increased energy expenditure and reduced appetite without causing nausea and hyperglycaemia, the aim of this study was to design and co administer a GLP-1 and a glucagon analogue that present a longer half-life than the native peptides could offer a stable and balanced action as an anti-obesity treatment. As with GLP-1 analogue, glucagon analogues were investigated through receptor binding assays, receptor activation assays, feeding studies in mice and pharmacokinetic studies in rats.

Glucagon was used as a comparison peptide and was administered *in vivo* in mice at the dose of 300nmol/ kg. Glucagon inhibited food intake up until 8 hours post administration, and past this point mice were observed to consume more food than control animals, which was also observed in the GLP-1 treatment group of Chapter 3. As glucagon has been shown to be a partial agonist of the GLP-1 receptor, this anorectic effect could be a result of GLP-1 receptor activation at the highest concentration of the peptide in circulation. Nonetheless, it could also be a direct effect of glucagon to its own receptor, although previous research has produced contradicting effects on whether glucagon succeeds in inhibiting food intake in rodents. Despite this, results from glucagon or glucagon antagonist administration suggest that, pancreatic glucagon secreted during meals acts in the liver to initiate a neural signal that is transported by vagal afferents to the brain, where it acts as an anorectic signal (215, 216).

Glucagon analogues were firstly investigated through receptor binding assays. The first generation of analogues focused primary on investigating receptor affinity and contained 6 peptides with substitutions on positions 12, 15, 17 and 18. None of the analogues had higher affinity than glucagon. In receptor binding assays using human glucagon receptor membrane, GC1 and GC2 presented similar affinities, yet were 2-fold worse than native

glucagon, while the rest of the peptides GC3, GC4, GC5 and GC6 bound similarly to glucagon. Comparably, GC1 and GC2 both had 6-fold lower efficacy in cAMP accumulation while GC3, GC4, GC5 and GC6 were approximately 1.5-fold lower than native glucagon. Interestingly, in receptor binding assays using mouse receptor membrane, GC1 had a similar IC_{50} to native glucagon, while GC2 and GC3 were over 8-times worse in comparison. Despite this, feeding studies in mice showed no significant food intake inhibition or body weight loss. Of note, stability of analogues, which was investigated *in vivo* through a 1mg 1:1 Zinc saline SC injection in rats, showed that GC6 sustained 70% of its C_{max} for up to 24 hr.

The importance of the residue at position 12 for receptor affinity was investigated by substitution of lysine for histidine, which resulted in GC1. Histidine was chosen as it is also hydrophilic and has positively charged side chains, therefore would not distort the helix.

Earlier work pointed out that replacement or deletion of aspartic acid at position 9 resulted in high affinity for glucagon receptor yet very low efficacy (210). In analogues that lacked aspartic acid but had a histidine replacement at position 9 receptor activation was present, suggesting that histidine probably compensated for the absence of negative charge by interacting with other carboxylic acids present in the near sequence, potentially Asp¹⁵. It has been suggested that Asp¹⁵ donates an essential feature for receptor recognition (217). The second analogue GC2 had a single amino acid substitution of aspartic acid to glutamate in position 15, in order to check whether it can maintain receptor recognition, as well as the negative charge and potentially improve the helical structure, as glutamate has a higher helix forming propensity.

Within the region 15-21, position 17 was the next one to be investigated. As suggested before, negative charges may line the glucagon binding region in the receptor; therefore positively charged regions may counteract this effect. Past studies tried to replace them with neutral or negatively charged amino acids, yet the result pointed that they contribute to the stabilisation of the hormone-receptor complex and therefore secure the binding (218). To evaluate this, positively charged Arg¹⁷ was initially replaced by negatively charged Gln¹⁷ in GC3. Results indicate that replacement of a positive with a negative amino acid possibly reverses charge and accounts for the retention of substantial binding and consequently activation. Lysine is a positive amino acid of similar size to arginine, the

replacement of which did maintain the affinity and efficacy of glucagon, yet did not improve it in GC4.

Previous research showed that amino acids in positions 12, 17 and 18 have a strong effect on ligand- receptor binding (218, 219). In order to investigate the effect of the positively charged region, Arg¹⁸ was substituted with Lys¹⁸ in GC5 in addition to the substitution of Arg¹⁷ to Lys¹⁷. Affinity and efficacy did not improve compared to glucagon.

As none of the substitutions of the first generation improved receptor binding, native glucagon residues were maintained. The second generation focused on positions 21, 27 and 29, which are thought to have an impact on receptor efficacy. Affinity on the human receptor of GC7, GC9, GC10 and GC12 was similar to native glucagon, while GC8 and GC11 was 1.5-fold worse. Notably, human receptor activation of GC7, GC9 and GC10 was similar to glucagon, while GC12 efficacy was 0.6-fold better. Mouse receptor analogue affinity was 2 to 5 fold worse than glucagon, with the exception of GC11 and GC12 that reached similar levels, while acute feeding studies on mice did not produce a significant decrease of food intake. *In vivo* analogue stability of generation 2 is noteworthy, as GC7, GC10, GC11 and GC12 maintained over 60% of their C_{max} at 24hr.

Previous research has shown that the existence of a salt bridge in residues 18-21 in the C-terminus of glucagon, might facilitate induction of an alpha-helix upon initial hormone association with the membrane bilayer (212). Maintenance of charge by substitution of Asp²¹ to either negative Glu²¹ or positive His²¹ in GC7 and GC8, could improve helical stability by using an amino acid with higher propensity, but did not appear to increase receptor activation when compared to glucagon. Met²⁷ replacement has been proposed to increase glucagon activation 5-fold, indicating C-terminal modifications have an unexpected effect on N-terminal conformation (213). In order to assess this, it was replaced with Leu²⁷ at GC9, while maintaining His²¹ to evaluate their action together. In addition, Thr²⁹ was replaced with Gly²⁹, which could potentially add flexibility to the end of the C-terminal and improve receptor binding and activation. Substitution with glycine, present in the GLP-1 and exendin-4 sequence, would not affect receptor affinity greatly yet could potentially enhance receptor activation due to its small size and increased flexibility compared to threonine, it doesn't appear to increase efficacy compared to native glucagon. In total, individual

substitution with Leu²⁷ in GC12 showed the first higher effect in the human glucagon receptor, when compared to glucagon, in addition to long lasting *in vivo* stability.

In the third and final generation, five analogues were designed. Leu²⁷ which was found to improve affinity and efficacy was maintained from generation 2, and further changes on residues 20 and 21 took place. The main focus of this generation was to prolong the pharmacokinetic profile of the peptide; therefore a histidine tail was introduced on the final three analogues.

Affinity to the human receptor was similar for GC13, GC15 and GC16, and approximately 0.6-fold better than native glucagon, while GC14 and GC17 were 2-fold better. Interestingly, efficacy to the receptor was similar for all five peptides and native glucagon. Similarly in the mouse receptor, peptide affinities were also all similar to glucagon. Of note, GC17 was the only one that caused significant weight loss at 48hr compared to the vehicle group. *In vivo* pharmacokinetics showed that GC14 maintained 40% of its C_{max} at 48hr, while GC16 was more stable between T_{max} at 4hr and 24hr, yet had a rapid concentration drop at 48hr. The most interesting result was obtained by GC17, which was the only analogue with at T_{max} of 24hr, which was 50% maintained at 48hr.

While Leu²⁷ was maintained and Glu²¹ was returned, Gln²⁰ was replaced by His²⁰ in GC13. Histidine was assumed to form more favourable interactions along the hydrophilic face and stabilise the α -helix of the peptide leading to an improvement in affinity and potency (220). As to as improve the analogue's stability, in addition to its bioactivity, the sequence that appeared to cause the highest affinity and efficacy *in vitro* was maintained (His²⁰, Leu²⁷) and three different histidine tails were added after position 29. The final peptide GC17 maintained the GC16 sequence with an amine group addition at position 32.

Mouse and human glucagon receptor have approximately the same size but do differ in most of their sequence, mainly in the N-terminus, thus comparison of affinities was vital. Considering that most analogues had a high EC₅₀, it would be reasonable to assume that they would induce a similarly high effect *in vivo*. Specifically, in analogue GC17, mouse IC₅₀ would point at a similar *in vivo* effect as GC13, GC14, GC15 and GC16, yet only GC17 resulted in significant food intake reduction. However, *in vivo* results always include a number of different factors not present *in vitro*, as a biological response is determined by

different cellular and molecular interactions. An example of this is the different processing of the peptide and the rate of its renal clearance. Moreover, glucagon is mainly known to increase energy expenditure rather than inhibiting appetite. It is therefore difficult to assess major weight loss due to energy expenditure in an acute feeding study, as it requires a longer period of time to present an observable result.

Overall, the initial glucagon analogues designed had individual substitutions at key regions of the peptide. Changes at the N-terminus would mostly affect binding, whereas C-terminus changes would mainly affect activation and stability. The substitution that caused the greatest impact on receptor affinity was Leu²⁷ instead of Met²⁷. Previous investigations have shown that methionine in glucagon is often the reason for loss of synthetic yield, as it is too sensitive to oxidation, a situation not observed as often in GIP (213). Moreover, histidine in position 20 potentially stabilises the α -helix that initiates at position 5, offering higher receptor affinity. Finally, histidine tails increased the long-term stability of the peptide, but not the efficacy. This is potentially due to the imidazole side chain of the amino acid, which at physiologically relevant pH values is able to change its charge, a feature of great value if injected with Zinc. Positively charged Zinc chelates with half negatively charged histidine, forming complexes between the peptide and Zinc molecules which stabilise the molecule and prolong its half-life.

GC17 appeared to reduce food intake at 24 to 48 hours, in addition to a small body weight loss, which could point to a minor anorectic effect. Furthermore, it appeared to peak at 24h and maintained high levels of plasma concentration in rats when administered with Zinc, presenting a pharmacokinetic profile similar to that of GL16. The histidine tail did not affect the peptide's receptor binding and activation negatively, as it maintained similar levels as 29 amino acid analogues. It was therefore the peptide chosen to be co-administered with the GLP-1 analogue chosen in Chapter 3 in a chronic feeding study.

Upon daily injections of the two analogues individually and as a co-administration, GL16 initially caused a greater weight loss than both GC17 and their combination. By day 20, GL16 had caused a body weight loss of almost 2 g, whereas GC17 was only approaching 1 g and the co-administration resulted in approximately 1.5 g weight loss. In day 37, a single time point Glucose Tolerance Test was performed to all mice groups, potentially resulting in high

stress and affecting food intake and probably an increase in energy expenditure, as this resulted in a rapid weight loss, especially in the co-administration group, followed by a burst weight gain from day 45 until the end of the study in day 65. At the end of the study, a Glucose Tolerance Test was performed in all four mice groups. Results showed a highly significant difference between the control group and the three treatment groups. Of note, the GL16/GC17 co-administration group glucose concentrations were also significantly lower than GL16 treatment group.

The chronic study results indicate an effect of GL16/GC17 co-administration in energy expenditure. When compared to the individually treated groups, GC17 alone is initially similar to the control group, as an increase in energy expenditure is not anticipated acutely, and later on decreased body weight up to 2 g. GL16 was expected to decrease body weight, mainly due to its anorectic effect that would inhibit food intake. Upon day 45, GL16/GC17 group loses up to 4 g and maintains the highest body weight loss until the end of the study, pointing at a combinational effect of GL16 anorectic effect and gastric emptying inhibition, and a GC17 energy expenditure increase. Moreover, co-administration group seems to improve carbohydrate tolerance as Glucose Tolerance Test showed that GL16/GC17 maintained stable glucose levels in blood after a glucose bolus injection, surprisingly lower than GL16 group.

As a whole, co administration of GL16 and GC17 increased body weight loss when compared to the control group but also the individually treated groups, pointing at both an anorectic effect derived from GL16 and a potential increase in energy expenditure from GC17. In addition, it appeared to balance glucose blood concentration more effectively than GL16 alone, a fact that demonstrates that co administration maintained and possibly improved the incretin effect.

The series of GLP-1 and glucagon analogues investigated provide a basis for further development of a suitable chimeric compound that could combine the GLP-1 anorectic effect without the potential side effects of nausea and vomiting, in addition to the energy expenditure enhancement effect of glucagon, without the glycogenolytic effect. Further investigations into the effects of specific amino acid substitutions on affinity and potency at

the GLP-1 and glucagon receptors may provide insight into how to improve the characteristics of drug candidates.

4.5. Conclusion

GC17 was the glucagon analogue selected to be co-administered with GLP-1 analogue GL16. It was the result of two amino acid substitutions of His²⁰ and Leu²⁷, with an addition of two histidines and the C-terminal and amino group tail. It had 2-fold higher affinity to the human receptor and 0.7-fold higher efficacy, compared to native glucagon. It caused significant food intake reduction in fasted mice until 48hr past administration and consequently the greatest weight loss of all glucagon analogues. In addition, it presented the highest stability in pharmacokinetic studies *in vivo*.

When administered with GL16 it caused body weight reduction through inhibition of food intake and another mechanism thought to be increased energy expenditure. Moreover, it managed to maintain significantly lower levels of glucose during a Glucose Tolerance Test when compared to the control group.

CHAPTER 5

The effect of GLP-1, glucagon, oxyntomodulin and exendin-4
on the exocrine pancreas

5.1. Introduction

5.1.1. GLP-1 and adverse effects

The pancreas is separated in endocrine and exocrine function. The endocrine function includes the secretion of insulin from β -cells and glucagon from α -cells. The exocrine pancreas secretes digestive juices into the duodenum to assist food breakdown, through the biliopancreatic duct. Pancreatic enzymes are not activated while in duct, but become functional in the small intestine through cleavage, in order to avoid organ digestion. When the pancreas is inflamed, these enzymes become activated while in the duct, causing great damage to the surrounding tissue, a condition known as pancreatitis (221).

During pancreatitis, the digestive enzymes amylase and lipase present a 3-fold increase in serum, compared to normal levels, while the abdomen appears to be swollen. Therefore, diagnosis includes blood tests and CT scan of the abdomen (221). The symptoms vary depending on whether pancreatitis is acute or chronic. They often include abdominal pain, nausea, vomiting and fever. The two most common causes of pancreatitis are gallstones and alcohol, which account for 80% of the cases. Nonetheless, various types of medications are also linked to the disease onset, most frequently corticosteroids (222).

Despite the antihyperglycaemic effects of GLP-1 receptor agonists and the promising future of their use as an anti-obesity drug, adverse effects that could be linked to their use have been reported (223). These effects include nausea, diarrhoea, thyroid carcinoma and cardiovascular effects, and most recently, pancreatitis (224).

The first case that linked antihyperglycemic agents to pancreatitis was observed in 2001 in an Intensive care unit in Tunisia, where poisoning caused by the oral antidiabetic drug metformin resulted in acute pancreatitis (225). Following this, another case that linked antidiabetic agents, this time exendin-4, to acute pancreatitis was reported in 2008. During this case, the use of exendin-4 was initiated to a 44-year old woman with a history of type 1 diabetes, resulting in an acute pancreatitis attack. The treatment was suspended for 1 week and reassessed at a lower dose. Three months later, the patient was re admitted presenting the same symptoms, and exendin-4 was discontinued (226).

Subsequent to this case, a number of similar incidents were reported, linking exendin-4 to the possibility of rare adverse effects (227). As a result, the U.S FDA announced a warning for acute pancreatitis and exendin-4 in 2007, which later on was expanded to all GLP-1 based agents and DPP-IV inhibitors on the market. The FDA called the companies to carry out studies to assess the relationship between the agents and the exocrine pancreas, and therefore risk to patients (228).

The initial argument to the signal claimed that pancreatitis incidence is 2.83-fold higher among people with type 2 diabetes, based on a retrospective cohort study in U.S health care database (229) and therefore, GLP-1 receptor agonists were guilty by association. In addition, no mechanism of GLP-1 action could be linked to the exocrine pancreas, pointing that the association was accidental. In response to this, a series of *in vivo* studies were conducted from both the pharmaceutical companies involved and academia to examine the claims (230).

5.1.2. GLP-1 and exocrine pancreas investigation

The exocrine pancreas contains two populations of cells: the ductal and the acinar cells. The acinar cells secrete the inactive pancreatic enzymes that pass through the pancreatic duct to reach the duodenum. The pancreatic ductal cells line the duct.

A hypothesis linking incretin treatment and acute pancreatitis is the increased replication rate of the pancreatic ductal cells in response to GLP-1 stimulation, resulting in blockages of the pancreatic duct. The potential mechanism has been investigated. The results indicate that the activation of GLP-1 receptor can promote cell proliferation through phosphorylation of CREB and stimulation of MAPK pathways (231). Furthermore, it has been observed that exendin-4 increases the pancreatic levels of the Immediate Early Genes (IEGs) *egr-1* and *c-Fos* which have a vital part in cell proliferation as they encode transcription factors. In respect to *egr-1*, it appears that its up regulation was focused exclusively to exocrine pancreas cells rather than islets. Nonetheless, exendin-4 seemed to have no effect on the regulation of pancreatitis associated pro-inflammatory mediators such

as STAT3 and mcp-1. On the contrary, it increased the pancreatic levels of anti-inflammatory genes PP and Reg IIIa (232).

Assessment of this hypothesis was of vital importance, as ductal hyperplasia is not only associated with acute pancreatitis, but is also the first stage of chronic pancreatitis. Chronic pancreatitis can result in intraductal neoplasia, a morphological state known as pancreatic intra-epithelial neoplasia (PanINs), as well as pancreatic adenocarcinoma, a condition that has a 5 year life expectancy and 10% survival rate (233). Pancreatic ductal adenocarcinoma cells express the GLP-1 receptor, providing a potential direct mechanism by which GLP-1 receptor agonists might increase the risk of acute pancreatitis and pancreatic cancer onset. Epidemiological indications show that around 1% of over 50 year-old diabetic adults will be diagnosed with pancreatic cancer (234) within 3 years of the disease onset, it is therefore essential to determine if administering GLP-1 receptor agonists will enhance this risk.

However, it is not only GLP-1 receptor agonists which have been linked to increase risk of pancreatitis and pancreatic cancer, DPP-IV inhibitors are also under investigation. While no direct mechanism has been proposed, it is considered that elevating levels of endogenous DPP-IV could have the same risk as giving exogenous GLP-1 receptor agonists for pancreatitis onset (235).

An examination on pancreata of organ donors diagnosed with type 2 diabetes mellitus who had been treated with incretin therapy was completed. During the examination of human tissue, a 40% increase in pancreatic mass, elevated exocrine cell replication and elevated PanINs were observed. Glucagon expressing microadenomas and α -cell hyperplasia were also present in 3 out of 7 specimens, in addition to a single neuroendocrine tumour observed in only one patient (236). However, firm conclusions from this paper are hard to ascertain due to limited sample number and incomplete patient background data being provided. Despite the large number of pancreas-associated disease cases that have been linked to GLP-1, the neuroendocrine tumour reported from this study remains the only case of cancer associated with incretin therapy to date.

The first *in vivo* study completed after the raised concern about the link of GLP-1 to pancreatitis included daily exendin-4 injections to rats, for 75 days with follow-up biochemical and histological studies of the pancreas. It demonstrated that, compared to the control group, exendin-4 treated rats presented pancreatic acinar inflammation and

pycnotic nuclei. In addition, higher concentration of the pancreatic marker of serum lipase was observed (237).

Based on a similar protocol, a number of studies were conducted, including more GLP-1 receptor agonists and different animal phenotypes. Initially, a diabetic rat model was used to test the effect of sitagliptin, metformin and their combination, and demonstrated a 3-fold increase in ductal cell replication and ductal metaplasia. Pancreatitis was observed in the sitagliptin group (238). An additional set of studies was completed two years later, the first in a diabetic model of mice, a second in rats. In both studies animals were dosed with exendin-4 for a period of 13 weeks. Exendin-4 treated mice presented extensive chronic pancreatitis, stromal fibrosis and increased lipase activity, in addition to loss of acini replaced by metaplastic duct profiles. In the rat, expansion of the pancreatic duct glands and 4-fold pancreatic duct cells replication, as well as nuclear pseudostratification and loss of polarity in epithelium, was observed in the exendin-4 treated group (231).

As a response to the FDA request, Novo Nordisk, the licence holder of liraglutide, conducted a series of studies in three species on increasingly high doses of liraglutide, from 0.05 to 5 mg/ kg/ day. The studies included rats, mice and monkeys and lasted from 4 to 104 weeks. The results of the study varied, as general observations of focal minimal acinal cell atrophy and inflammatory cell foci were present up to the 26th week of research in both rats and mice. The 104-week study in mice showed moderate focal inflammatory cell infiltration and acinal cell degranulation, while rats appeared to have pale pancreas. , no significant differences to the control group were noticed. The findings were similar to the 13-week monkey study, where minimal focal inflammatory cell infiltration was noticed in some animals (239).

In order to evaluate whether diabetes has an effect on pancreatitis development, Novo Nordisk conducted a 13-week liraglutide and exendin-4 treatment study in obese, diabetic and lean rats on doses varying from 0.4 to 1mg/ kg/ day. No significant changes in duct or acinal cell mass were observed in the treatment groups, when compared to an untreated group. Furthermore, no effect of the treatments on lipase activity was found. Nonetheless, one exendin-4 treated obese rat presented pancreatic oedema, apoptosis-like necrosis and minimal inflammatory infiltration (240). Amylin Pharmaceuticals another company

marketing GLP-1 receptor agonists, repeated the study in diabetic rats using the same protocol as Novo Nordisk. Amylin results were in agreement with Novo Nordisk, a minor amylase increase but no adverse effects on acinal and ductal cells (241).

Novartis assessed the effect of their product, vildagliptin, a DPP-IV inhibitor on pancreatitis by undertaking 104-week studies in mice and rats. Results showed no vildagliptin-related findings were found in the pancreas, as no acinal or ductal neoplasia or inflammation was observed (242).

5.1.4. Glucagon and oxyntomodulin biological mechanism on exocrine pancreas

Like GLP-1, glucagon and oxyntomodulin are currently investigated as possible obesity treatments. Therefore, the effect of the two peptides on the exocrine pancreas is also to be investigated. So far, it has been observed that intravenous glucagon administration in a number of patients caused a decrease in the bicarbonate and protein content of pancreatic juice, as well as a reduction in plasma amylase levels and relief of epigastric pain (243). This suggests that glucagon might have an inhibitory effect on pancreatic exocrine secretion when digestive action is not needed. This has been replicated *in vivo*, in rats with closed duodenal loop induced pancreatitis, where glucagon treatment appeared to significantly inhibit pancreatic injuries such as interstitial oedema and acinal cell vacuolization (244).

Oxyntomodulin has also been shown to decrease gastric acid and exocrine pancreatic secretion (245, 246) possibly through the activity of gastropancreatic intrinsic nerves, or by preganglionic inhibition of excitatory vagal fibres via the CNS.

5.1.5. Aims

Proglucagon derived peptide GLP-1 receptor agonists are widely used as a treatment of type 2 diabetes, and, along with glucagon and oxyntomodulin, they are investigated as a potential obesity therapy. Therefore, the target group of the patients to receive a peptide based therapy is going to increase rapidly in a number of years. All three peptides have been

studied for their effect on the endocrine pancreas, yet very little is known about their potential mechanism of action on the exocrine pancreas.

Recent reports linked GLP-1 to increased pancreatic ductal and acinar replication, relating the peptide to pancreatitis and potentially pancreatic cancer. Even though these reports were investigated by the FDA and GLP-1 receptor agonist and DPP-IV inhibitors are currently considered to be safe compounds, it has become apparent that more research on the exocrine effects of proglucagon derived peptides that target the pancreas is essential. I therefore investigated the possible effect of GLP-1, exendin-4, glucagon and oxyntomodulin *in vitro* using rat pancreatic acinar (AR42J) and primary isolated epithelial biliopancreatic ductal cell lines. The cells were tested for peptide receptor binding, replication numbers and amylase secretion. In addition, I studied the effects of the peptides *in vivo* on the rat pancreas post peptide bolus infusion, to examine the response amylase and insulin secretion in plasma in comparison to the peptide concentration.

Summary:

1. To investigate the mechanisms by which GLP-1, exendin-4, glucagon and oxyntomodulin can cause pancreatitis by a direct action on the exocrine pancreas, by increasing cell proliferation of rat biliopancreatic ductal and acinar cell numbers and amylase secretion from acinar cells.
2. To investigate insulin and amylase secretion in plasma post GLP-1, exendin-4, glucagon and oxyntomodulin bolus infusion in rat surgical model.

5.2. Materials and Methods

5.2.1. Biliopancreatic duct epithelial cell primary isolation

Rat Biliopancreatic Duct isolation

Primary rat pancreatic duct epithelial cells were isolated from naive adult male Wistar rats. Rats were euthanized by exposure to CO₂ for at least 8 minutes and death was certified by neck dislocation prior to initiating dissection. Using straight dissecting scissors, a midline incision was performed through skin and peritoneal muscle in order to expose the abdominal cavity. By stabilising the liver over the cavity and pulling the intestines, the duodenum was reached. The head of the pancreas stretches from the duodenum to the tail, located to the spleen.

The main pancreatic duct is located at the head of the pancreas and runs through the length of the pancreas in branches to the tail. Before it reaches the duodenum it joins the bile duct, forming the biliopancreatic duct. In order to isolate it, curved dissecting scissors were used to carefully cut across the body of the pancreas at the duodenum. The organ was transferred to a 6-cm tissue culture dish with ice-cold pancreatic medium (1:1 Hams F12/DMEM, 100ng/ ml cholera toxin, 100µg/ml soybean trypsin inhibitor, 20% FBS, penicillin 100 units/ ml and streptomycin 100 mg/ ml) and washed.

Using a dissecting microscope, the pancreas was stretched carefully in order to visualise the biliopancreatic duct. Once located, the surrounding pancreatic tissue was cleaned away and the duct was transferred in a tube containing 5ml of collagenase XI solution (Sigma-Aldrich) for 12 minutes. Following incubation, the remaining fibroblasts were removed and the treatment was repeated.

Dissociation of the Main Pancreatic Duct

Following the collagenase digestion reaction, two washes with ice-cold PBS were performed. In order to dissociate ductal cells from the extracellular matrix, the ducts were incubated with dispase I (Sigma-Aldrich, Dorset, UK) solution for 12 minutes and washed twice with PBS (Sigma-Aldrich, Dorset, UK). So that the duct is dissociated into cellular aggregates, it was subjected to a series of 6 trypsinization steps. Each trypsin treatment was stopped by adding ice-cold FBS and the duct was transferred to the next treatment. Before the last treatment, the duct was minced using microscissors and broken down using a sterile Pasteur pipette. The collected fractions were then centrifuged at 700g for 5 minutes at 4°C. The result pellet was then resuspended in pancreatic medium, plated in MaxGel Extracellular Matrix components (ECM) coated wells (Sigma-Aldrich, UK) in 12 well-plates and incubated at 37°C and at 5% CO₂ (247-251).

Primary rat pancreatic duct epithelial cells were maintained in MaxGel ECM coated wells in 12 well plates in pancreatic medium for 48 hours before assay.

5.2.2. Cell culture and maintenance

Rat exocrine pancreatic tumour cells AR42J (Sigma-Aldrich, UK) (passage number 10 to 17) were maintained routinely in RPMI 1640 medium containing 2mmol/l glutamine and supplemented with 10% FBS (v/v) and antibiotics (penicillin 100 IU/ml and streptomycin 100 mg/ml) (Sigma-Aldrich, Dorset, UK). Medium was changed every 48 hours. Cells predominately grew in suspension and were passaged by both EDTA cell dissociation agent and medium collection and centrifuge when they reached 70% confluence (approximately 7 days),

Primary rat biliopancreatic duct epithelial cells maintained in MaxGel ECM matrix (Sigma-Aldrich, Dorset, UK) coated 12 well plates in pancreatic medium (described previously) for 48 hours before assay.

5.2.3. Whole Cell binding assay

Binding studies on the cell membrane were performed as described in Section 2.7.7. ¹²⁵I–GLP-1 and ¹²⁵I–GCG was used as the competing peptide for all studies. Cells were plated and incubated for 2hr in standard conditions before cell media was removed and replaced with assay buffer, label and varying concentrations of peptide of interest. Plates were incubated for 90 min at room temperature before cells were scraped off, transferred to 1.5 mL microtubes and washed prior to counting bound radiation. Binding affinities were calculated from a minimum of two separate experiments, where each concentration was tested in triplicate. Errors shown are $\pm$ SEM.

5.2.5. Cell proliferation markers

Acinar and ductal cells were cultured in the presence or absence of 10nM of each peptide for 18 hours. The medium containing peptide was changed every 6 hours. Peptides were diluted in medium, supplemented with 2% FBS. Total RNA was isolated from AR42J cells using TRI Reagent (Sigma-Aldrich, Dorset, UK) by referring to the manufacturer's protocol. 1-Bromo-3-chloropropane (Sigma-Aldrich, Dorset, UK) was added to precipitate the total RNA and the extracted RNA pellet was then washed with 75% ethanol. RNA was purified using PureLink RNA kit and DNA was digested from total RNA using PureLink DNase (Thermo Fisher Scientific, Paisley, UK). The purified RNA was dissolved in RNase and DNase free distilled water (Thermo Fisher Scientific, Paisley, UK) and was immediately stored at -80°C until further analysis.

Complementary DNA was synthesized from 100 ng of total RNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Paisley, UK) according to the protocol recommended by the manufacturer. The protocol conditions included incubation for 10 minutes at 25°C for primer annealing, for 120 minutes at 37°C for reverse transcription, and for 5 minutes at 85°C for reaction termination. The cDNA was stored at -20°C until further analysis.

Quantitative PCR analysis was used to quantify the expression level of the proliferation marker Ki-67. For the detection of the Ki-67 detection primers were designed (Table 1) using

Primer Express (Invitrogen, Paisley, UK). Primers pairs were designed to cross a splice junction to avoid amplification of genomic DNA. The expression levels were measured by a 7500 Fast Real-Time PCR System (Applied Biosystems, Paisley, UK). The reaction mixture contained 6 µl of Fast SYBR Green Master Mix (Invitrogen, Paisley, UK), 0.35 µl of each primer (10 µM; Thermo Fisher Scientific, Paisley, UK), 3 µl water and 2 µl of cDNA in a final volume of 12 µl. Samples were amplified in duplicates. The reaction was started with incubation for 2 min at 50°C followed by the activation and pre-denaturation step at 95°C for 10 min. The reaction run consisted of 40 cycles comprising 15 s at 95°C and 1 min at 60°C. B-actin was used as an endogenous control according to which statistical analysis was performed, as its levels are not expected to change with the treatment.

Analysed transcript	Sequence of forward primer	Sequence of reverse primer
Ki-67	TTGACCGCTCCTTTAGGTATGAA	GGTATCTTGACCTTCCCCATCA
β-actin	CGAGTCGCGTCCACCC	CATCCATGGCGAACTGGTG

Table 5. 1. Primers used for the quantitative detection of Ki-67, β-actin

5.2.6. Bolus IV injection and IV sampling on rats

Adult Male Wistar rats weighing between 400-600 g were double housed in cages and maintained under controlled temperature (21–23 C) and lights (12-h light, 12-h dark cycle, lights on at 0700 h). Animals were fasted 18h prior procedure.

Animals were anesthetized with the use of isoflurane, via a cone mask, at 2% with an appropriate gas scavenging system. The operating procedure began with the catheterization of the right external jugular vein. Upwards dissection was performed in order to free the jugular from its medial and lateral branches. Once this was completed, a baseline sample was drawn. The left femoral vein was used as an access route for intravenous injection of

the peptide. A baseline sample was also drawn post femoral catheterization. Polyethylene tubing (internal diameter 0.45 mm, outside diameter 0.91 mm) was cut to 10 - 12 cm length for the jugular and the femoral vein respectively.

Each peptide was reconstituted with 1 ml of Gelofusine at a concentration of 30 nmol/ml. CCK was used at a lower concentration of 2.4 nmol/ ml, as any higher dose would cause rapid drop in blood pressure. Once the catheter was correctly positioned and its patency was verified a bolus dose of peptide was administered. Blood samples (100µl) were taken from the jugular catheter during regular intervals throughout the entire procedure (Figure 5.2.1). All samples were tested for glucose concentration to assess surgical stress. Once all the necessary samples had been collected the animals were culled with a Schedule 1 method. All adverse effects and unexpected deaths occurring during the experiments were recorded prospectively in a logbook.

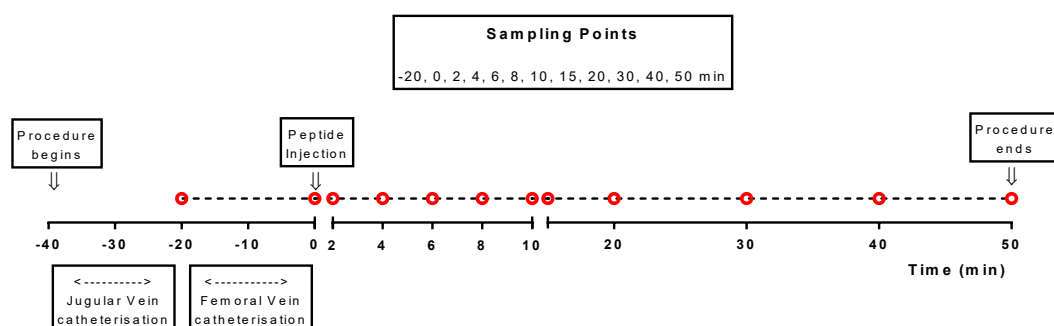


Figure 5.2. 1. Graphical representation of the study design of continuous intravenous infusion in anaesthetised male Wistar rats. Once the animal is anaesthetised, the right jugular vein is cannulated. The left femoral vein is then cannulated and a baseline blood sample is taken. Peptide was injected at time 0. Sampling points are displayed in red circles over time in minutes.

5.2.7. Amylase secretion in AR42J acinar cells

The rat pancreatic acinar cell line AR42J was obtained from Sigma- Aldrich (Dorset, UK). AR42J cells were routinely maintained in RPMI 1640, supplemented with 10% FBS, 100 IU/ ml penicillin, 100 µg/ ml streptomycin and 2mM glutamine (Sigma-Aldrich, Dorset, UK). Cells were incubated with 20nM dexamethasone for 48 h before secretion assay to induce CCK responsivity, as described by Logsdon et al (252). Cells from passage 12-20 were used

throughout this study and were routinely incubated in a humidified incubator at 37°C with 95% air and 5% CO₂.

To assess amylase secretion, cells were plated at approximately 10⁵ cells/ ml onto 24-well plates (500µl/ well). Before peptide treatment, cells were washed with PBS. Cells were treated for 50 min at 37°C with ascending concentrations of the peptides diluted in serum-free RPMI 1640. The medium of each well was aspirated and collected in individual labelled tubes and 100µl of amylase assay lysis buffer was added in each well to lyse the cells and the lysate was individually collected in labelled tubes. All lysate tubes were centrifuged at 16,000 x g for 10 minutes. The clear extract was separated from the pellet and collected in individual tubes.

5.2.8. Amylase detection assay

Medium, lysate supernatants and blood plasma samples were assayed using an Amylase Detection Assay (Abcam, Cambridge, UK). Samples were diluted at 1: 100 for blood plasma, 1:8 for cell lysate and 1:4 for medium. Once samples were added to a clear flat bottom 96-well plate, the wells were supplemented with a reaction mix that included ethylidene-pNP-G7. Each unit of amylase detected would cleave ethylidene-pNP-G7 to generate 1.0 µmol of nitrophenol at pH 7.20 at 25 °C. Nitrophenol generates colour that is measured using a colorimetric plate reader (Multiskan RC 381, Labsystems, MA, USA). The plate was read at OD 405nm at time 0 to obtain basal colour change, and then at 10, 20, 30, 40, 50, 60 min to assess the rate of the enzyme reaction. The plates were incubated in room temperature on an orbital shaker between the time points. Results were calculated using time 60 colour change results.

All data are expressed as the mean value ±S.E.M. Linear regression was used to obtain values according to the Standard curve. 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.

5.3. Results

5.3.1. Biliopancreatic duct epithelial whole cell receptor binding assay

The binding affinities of GLP-1, glucagon and oxyntomodulin in primary isolated rat biliopancreatic duct epithelial cells (≈ 8 rats/ assay) were determined using ^{125}I - GLP-1 to test GLP-1 and exendin-4 and ^{125}I - glucagon to test glucagon and oxyntomodulin. Exendin-4 ($2.97 \pm 0.2\text{nM}$) had 2-fold higher affinity compared GLP-1 ($7.34 \pm 0.9\text{nM}$) (Figure 5.3.1.). Binding of ^{125}I - GCG in the presence of increasing concentrations of glucagon had approximately 4-fold lower affinity than oxyntomodulin (glucagon: $\text{IC}_{50} = 8.84 \pm 1.3\text{nM}$, oxyntomodulin: $\text{IC}_{50} = 2.77 \pm 0.7\text{nM}$) (Figure 5.3.2.).

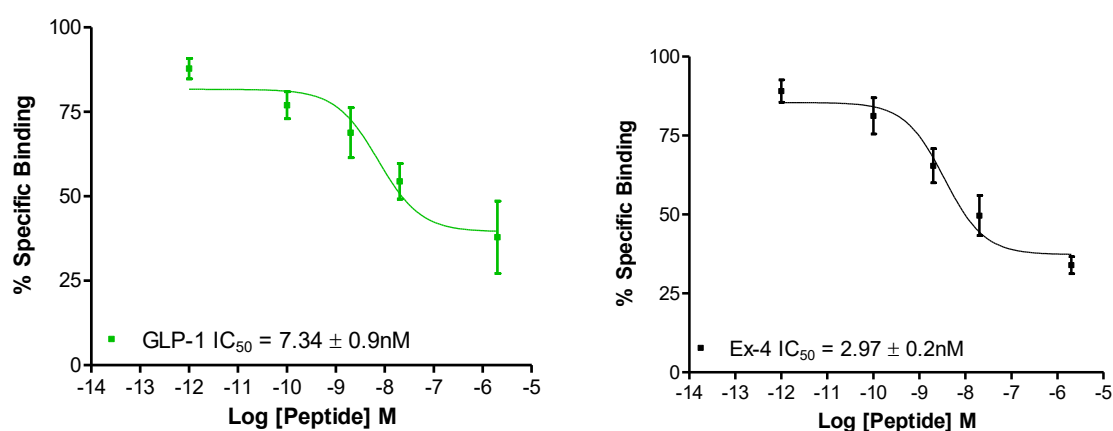


Figure 5.3. 1. Binding affinity of GLP-1 and exendin-4 to the rat biliopancreatic duct. Rat biliopancreatic duct cells were the source of receptors. Each peptide concentration was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from two separate experiments $\pm \text{SEM}$.

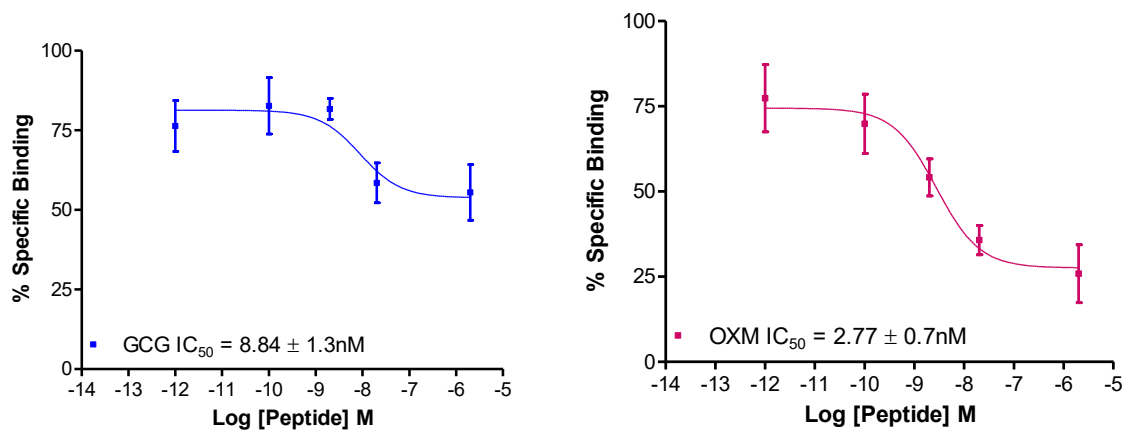


Figure 5.3. 2. Binding affinity of glucagon and oxyntomodulin to the rat biliopancreatic duct. Rat biliopancreatic duct cells were the source of receptors. Each peptide concentration was tested in duplicate or triplicate in each experiment. IC_{50} values were calculated as a mean from two separate experiments $\pm$ SEM.

5.3.2. *In vivo* amylase secretion post IV bolus peptide administration

A small part of amylase that is secreted in the gut leaks into the bloodstream and its concentration can be used as indicator of pancreatitis. To assess any pathogenic effect of peptides on the exocrine pancreas rats were infused with GLP-1, exendin-4, glucagon or oxyntomodulin and plasma amylase level were determined at regular time intervals. CCK was used as a positive control. Plasma amylase concentration did not increase post peptide intravenous infusion *in vivo*, compared to basal amylase and vehicle. Figure 1 shows a comparison between GLP-1, exendin-4, glucagon, oxyntomodulin, positive control CCK and vehicle. Average basal amylase was 10.5 ± 0.39 unit/ l for all groups (n= 4-5). CCK increased amylase secretion significantly from 30 min onwards, reaching approximately 18 unit/ l while the four peptides and the vehicle remain stable but slightly elevated at approximately 12 ± 0.21 unit/ l throughout the 50 min of sampling.

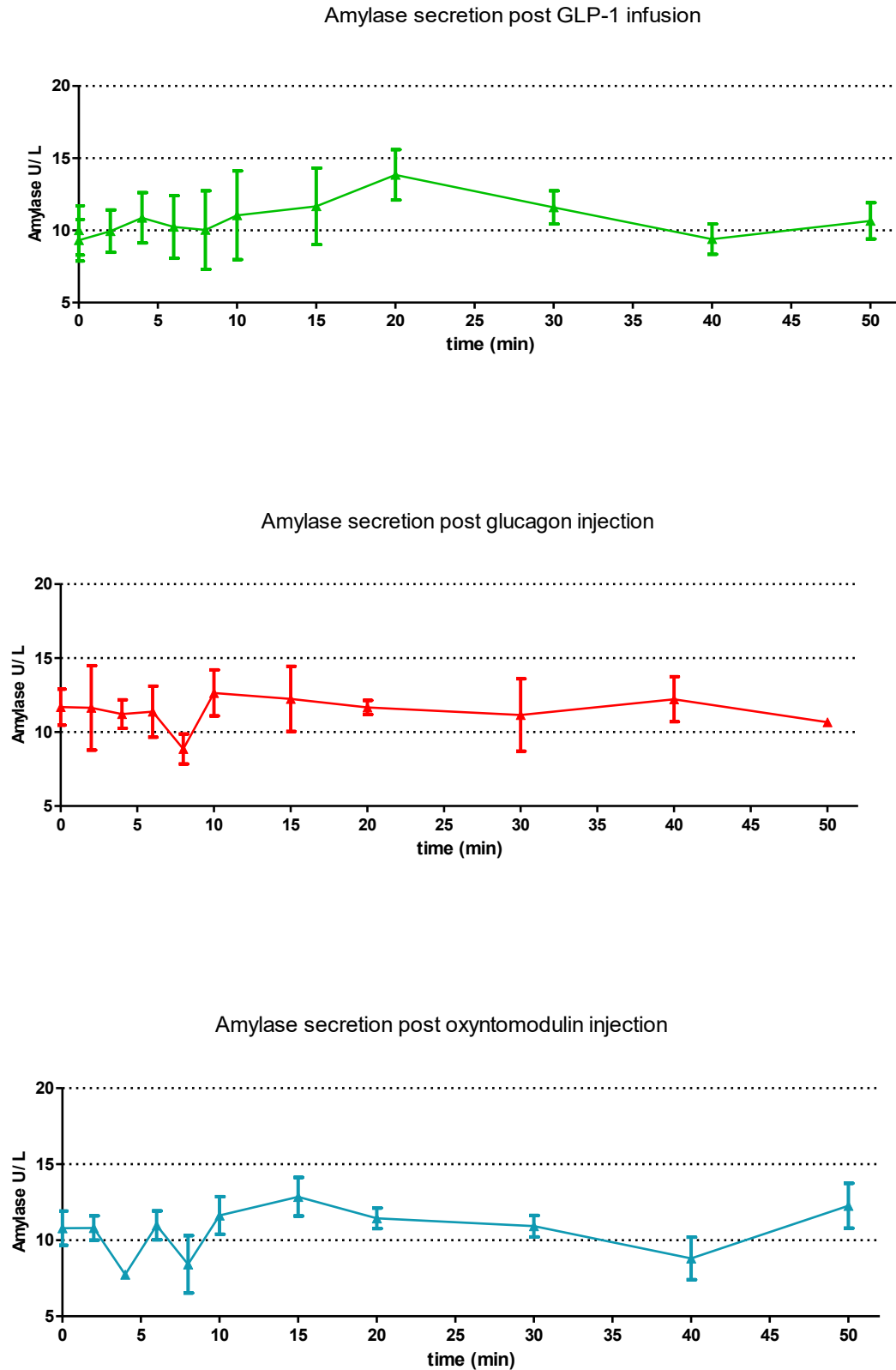
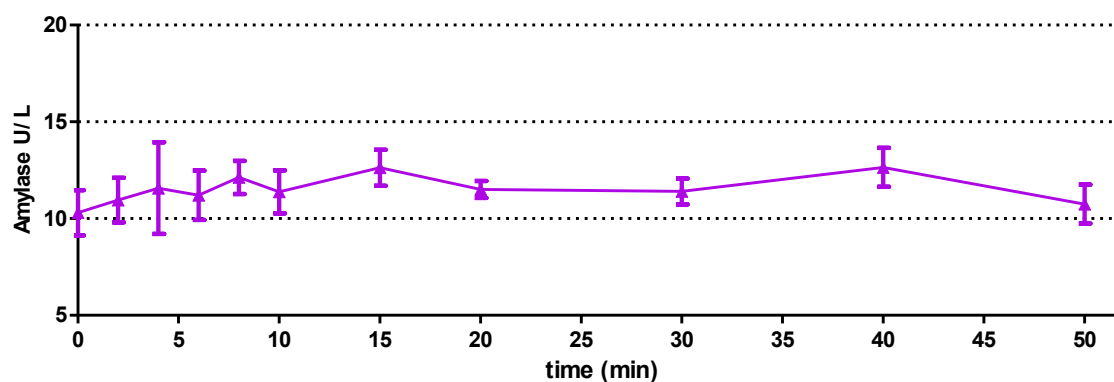
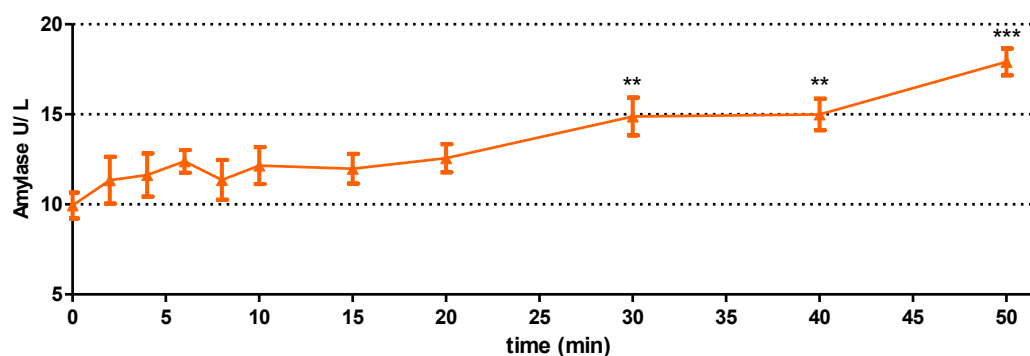


Figure 5.3. 3. Amylase secretion (unit/ l) post peptide bolus 30nmol intravenous injection of a. GLP-1, b. glucagon and c. oxyntomodulin. Gelofusine was used as a vehicle. One way ANOVA with Dunnett's post-hoc test was used. Values are shown as mean $\pm$ S.E.M (n= 4-5).

Amylase secretion post exendin-4 injection



Amylase secretion post CCK injection



Amylase secretion post gelofusine injection

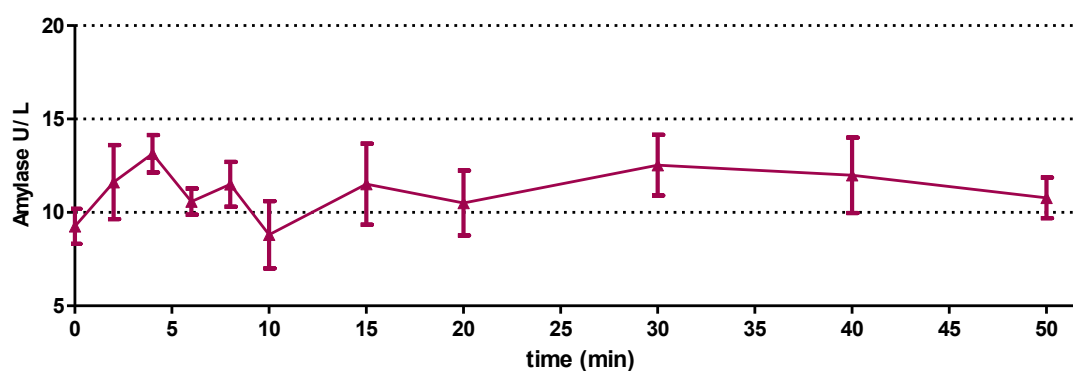


Figure 5.3. 4. Amylase secretion (unit/ l) post peptide bolus 30nmol intravenous injection of exendin-4. CCK was administered at 2.4nmol. Gelofusine was used as a vehicle. One way ANOVA with Dunnett's post-hoc test was used. Values are shown as mean $\pm$ S.E.M. ** $p < 0.01$, *** $p < 0.001$ vs. time 0 basal sample ($n = 4-5$).

5.3.3. *In vivo* amylase secretion post IV bolus CCK-peptide co administration

GLP-1, exendin-4 and glucagon intravenous co administration with CCK showed no significant inhibition of amylase secretion compared to CCK alone. Figure 5.3.4a shows a comparison between GLP-1, exendin-4 and glucagon individually co administered with CCK. GLP-1 increased amylase secretion within the first 10 min while exendin-4 and glucagon reached similar levels more gradually. Average basal amylase was 9.8 ± 0.37 unit/ l for all groups (n= 4-5). Upon bolus infusion, amylase levels increased in a similar rate as CCK.

Of note, oxyntomodulin suppressed amylase release by approximately $3.4 \text{ unit/ l} \pm 0.5$ when co administered with CCK (Figure 5.3.6). Significant inhibition was achieved both in the beginning of sampling at 6 and 8 min, and the end at 30 and 50 min.

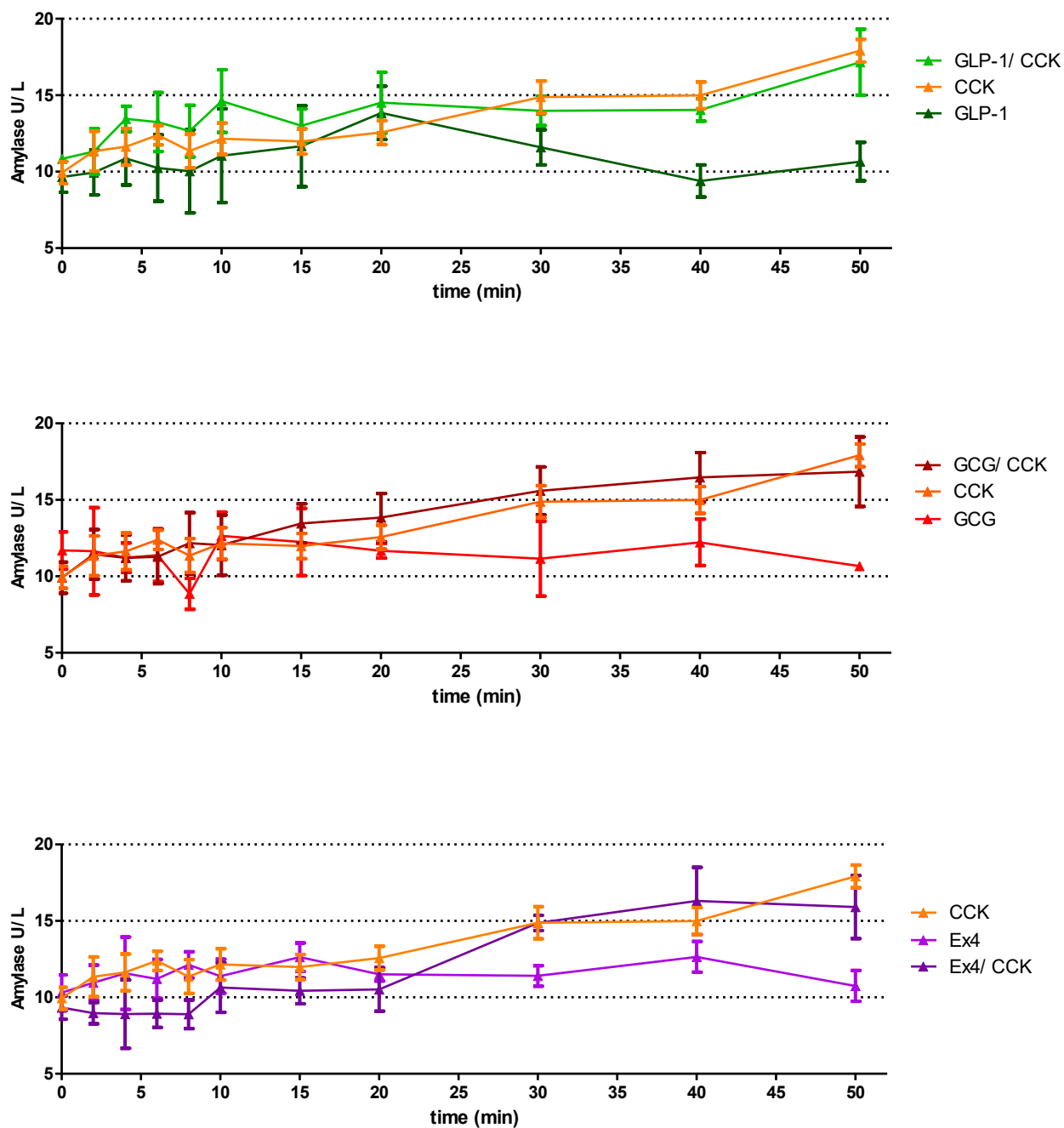


Figure 5.3. 5. Amylase secretion (unit/ l) post GLP-1, glucagon, exendin-4 and CCK intravenous co administration as a single bolus infusion. Each peptide was administered at 30nmol and CCK was administered at 2.4nmol. One way ANOVA with Dunnett's post-hoc test was used. Student t-test were used between individual time points. Values are shown as mean $\pm$ S.E.M. (n= 4-5)

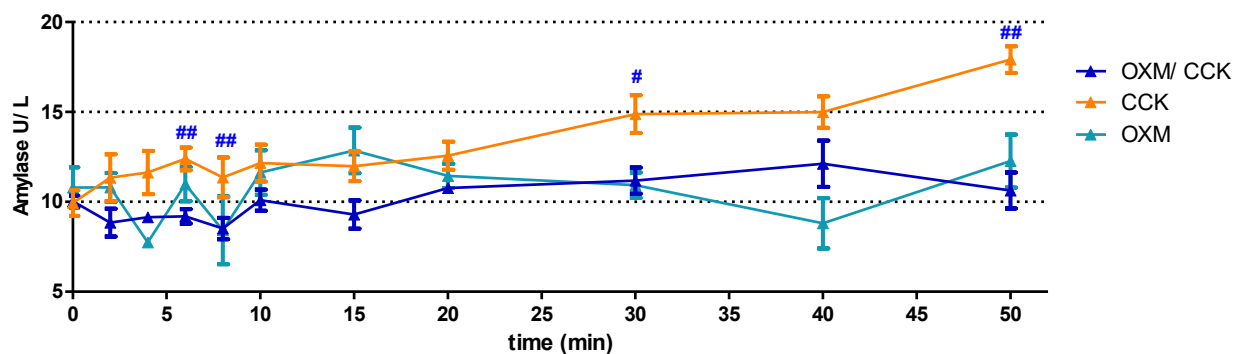


Figure 5.3. 6. . Amylase secretion (unit/ l) post oxyntomodulin and CCK intravenous co administration as a single bolus infusion. Each peptide was administered at 30nmol and CCK was administered at 2.4nmol. One way ANOVA with Dunnett's post-hoc test was used. Student t-test were used between individual time points. Values are shown as mean $\pm$ S.E.M. (n= 4-5)

5.3.4. *In vitro* amylase secretion in AR42J rat acinar cells

To further investigate the effects of proglucagon-derived peptides on the exocrine pancreas *in vitro*, AR42J cells were treated with varying concentrations of the peptides and total amylase and secreted amylase levels were determined. Amylase release was not increased post GLP-1, exendin-4, glucagon and oxyntomodulin treatment at ascending doses. CCK was used as a stimulus of amylase release, doing so in a dose dependent manner on AR42J cells, reaching 28% of total amylase activity in 50 min (Figure 5.3.3.7, 8). Treatment concentrations ranged from 10pM to 100nM for all four peptides and positive control CCK. GLP-1 and exendin-4 did not increase amylase release from AR42J cells compared to untreated cells as secretion remained stable at $13.8 \pm 0.45\%$. Glucagon and oxyntomodulin, either alone or combined with CCK, did not significantly inhibit amylase release compared to untreated cells and CCK accordingly. Despite this, a trend was observed of inhibition of amylase release with all four peptides treated with CCK at a 1/ 10 concentration, when compared to CCK alone (Figure 5.3.3.9).

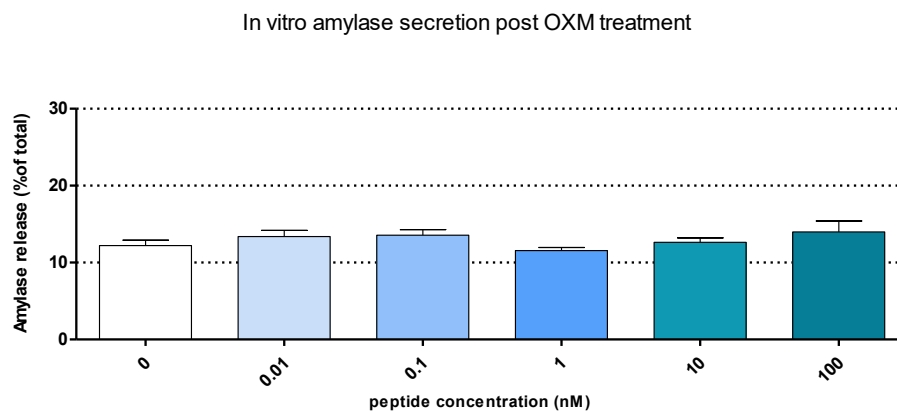
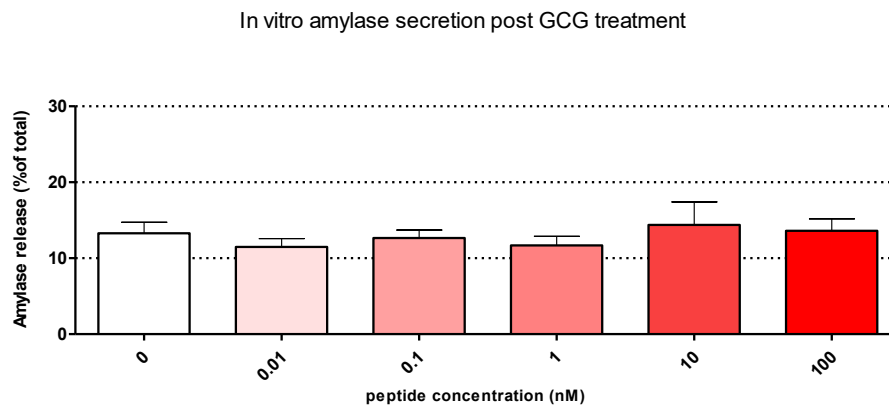
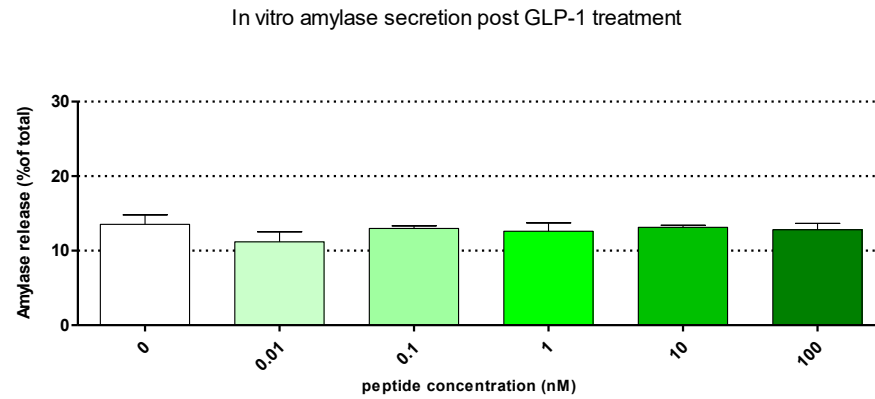


Figure 5.3. 7. Concentration response of amylase release from AR42J cells post peptide treatment at ascending concentrations for 50 min (n=5). Amylase is expressed as a percentage of the secreted amylase into the medium over the total activity of the cells. One way ANOVA with Dunnett's post-hoc test was used. Values are shown as mean $\pm$ S.E.M.

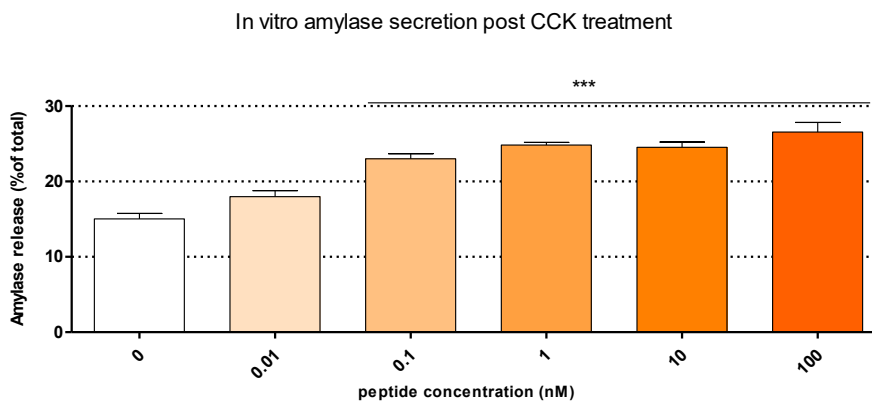
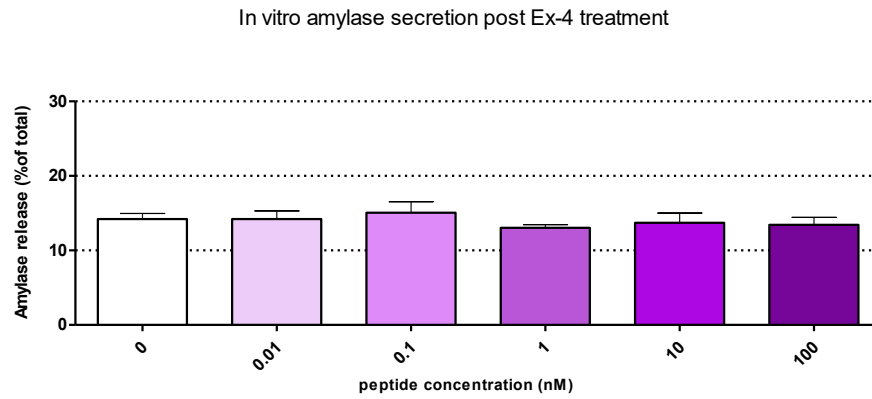


Figure 5.3. 8. Concentration response of amylase release from AR42J cells post peptide treatment at ascending concentrations for 50 min (n=5). Amylase is expressed as a percentage of the secreted amylase into the medium over the total activity of the cells. One way ANOVA with Dunnett's post-hoc test was used. Values are shown as mean $\pm$ S.E.M. * $p < 0.001$ vs. untreated cells (0).**

In vitro amylase secretion post CCK 1nM/ peptide 10nM treatment

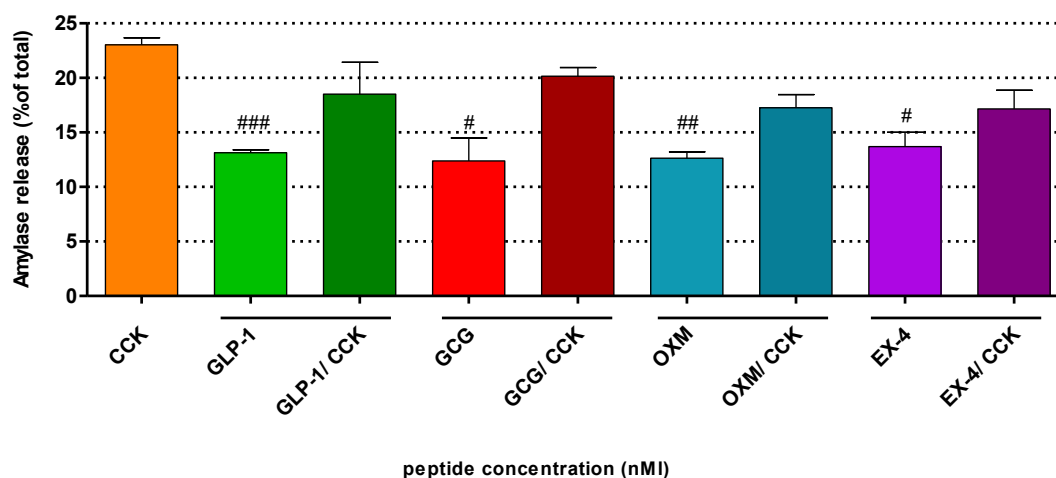


Figure 5.3. 9. Amylase release from AR42J cells post peptide (10nM)/ CCK (1nM) treatment for 50 min. Amylase is expressed as a percentage of the secreted amylase into the medium over the total activity of the cells. One way ANOVA with Dunnett's post-hoc test was used. Student t-test were used between CCK and peptides. Values are shown as mean $\pm$ S.E.M. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. CCK

5.3.5. Cell proliferation in AR42J cells and pancreatic ductal cells

To determine if the test peptides had an effect on the rate of cellular proliferation, AR42J and ductal cells were treated with 10nM of GLP-1, glucagon, oxyntomodulin and exendin-4 for 18hr. Following treatment, mRNA was extracted and the level of proliferation marker Ki-67 was analysed using quantitative PCR (Figure 5.3.10, 11). Quantitatively, compared to the untreated control cells, the test peptide did not cause a significantly higher expression of Ki-67 ($p < 0.05$).

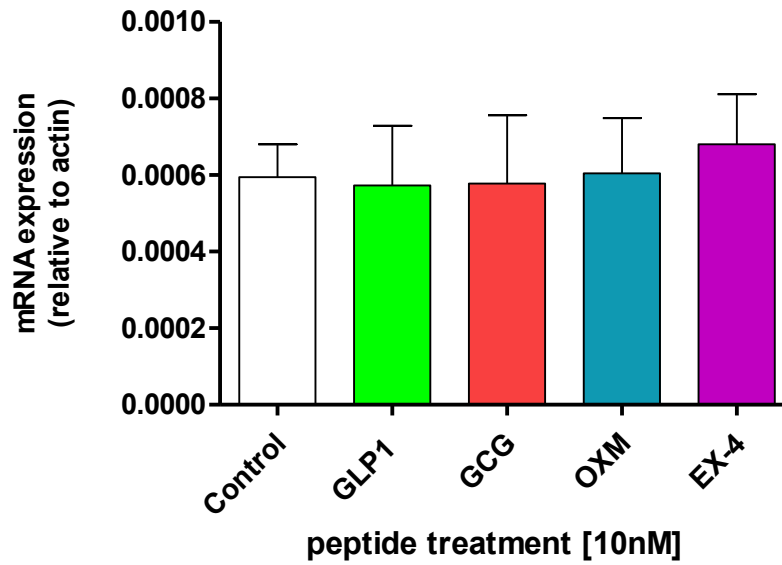


Figure 5.3. 10. Relative Ki67 mRNA expression in AR42J cells treated with GLP-1, glucagon, oxyntomodulin and exendin-4 for 18hr (n=4). Values are corrected for levels of actin and shown as mean $\pm$ S.E.M.

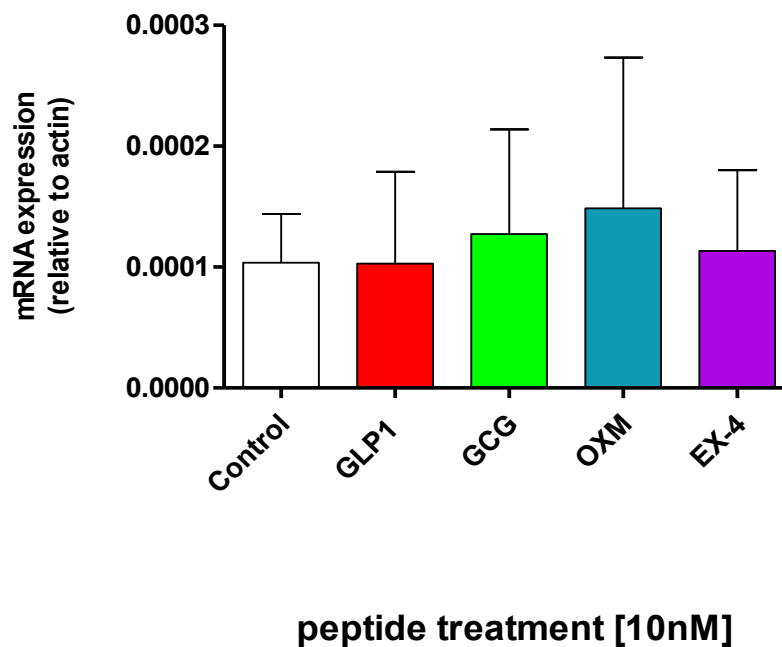


Figure 5.3. 11. Relative Ki67 mRNA expression in biliopancreatic ductal cells treated with GLP-1, glucagon, oxyntomodulin and exendin-4 for 18hr (n=3). Values are corrected for levels of actin and shown as mean $\pm$ S.E.M.

5.4. Discussion

Incretin -based glucose-lowering medications have undoubtedly been proven to be beneficial as a type 2 diabetes treatment. GLP-1 receptor agonists, such as exendin-4, have been greatly effective for both increasing insulin sensitivity and causing weight loss. However, recent reports linked them to a number of side effects, most recent of which was pancreatitis. The risk benefit analysis has shown them to be beneficial but they are not considered risk free. Co-administration of GLP-1 with glucagon has shown a promising result for weight loss as a consequence of increased energy expenditure and appetite reduction. Oxyntomodulin is a GLP-1/ glucagon receptor dual agonist and is also considered to be a possible anti-obesity treatment.

Published literature hypothesise that GLP-1 receptor agonists and DPP-IV inhibitors cause hyperplasia of pancreatic duct epithelial cells, resulting in obstruction of the duct and therefore inflammation which can lead to pancreatitis. A number of *in vivo* studies of chronic peptide administration to rodents have been completed, showing pathological changes of the pancreas and high levels of pancreatitis markers in blood, while other contradict these results. The studies completed in this chapter investigated this effect and the results showed that GLP-1 and exendin-4 do not up regulate the proliferation rate of biliopancreatic ductal cells and do not cause a significant increase of plasma amylase post IV bolus infusion, in rat. Moreover, oxyntomodulin appears to reduce plasma amylase in exogenously stimulated pancreatic secretion.

The reported pancreatitis cases were focused on GLP-1 action on the exocrine pancreas. Despite the differing outcomes of the *in vivo* studies, it is clear that there is a link between the two, the strength of which is yet to be established. For there to be a direct action, the exocrine pancreas would need to express the receptor. To confirm this, rat biliopancreatic duct cells were isolated from naive rats by a cell isolation and maintenance method optimised over a period of two months in order to achieve the appropriate cell type, number and adherence required for receptor binding studies. Whole cell receptor binding of ductal cells demonstrated a potential expression of both GLP-1 and glucagon receptors. The cells were then treated with GLP-1, exendin-4, glucagon and oxyntomodulin, and assessed

for Ki-67 proliferation marker. The outcome showed no significant increase of the proliferation rate in biliopancreatic ductal and acinar cells.

Commercially available cell line AR42J was used as a model of rat pancreatic acinar cell line. AR42J is derived initially from a transplantable tumour of a rat exocrine pancreas. This line is the only cell line currently available that, in culture, maintains many characteristics of normal pancreatic acinar cells, such as the synthesis and secretion of digestive enzymes, protein expression, growth and proliferation, and calcium signalling (253).

Acinar cells were treated with ascending doses of GLP-1, glucagon, oxyntomodulin and exendin-4, and CCK as a positive control. Both medium and cell lysates were assayed for amylase and the results were analysed and summed as total amylase activity (254, 255). None of the peptides demonstrated an increase in amylase secretion as a response to peptide treatment for 50 minutes at 37°C, when compared to untreated cells. Despite this, cells treated with CCK to demonstrate a positive control at the same concentrations showed significant increase in response to all doses.

Previous research has demonstrated that GLP-1 and glucagon did not increase amylase secretion from AR42J cells, yet GLP-1 did increase intracellular calcium and cAMP accumulation levels. Even so, none of the peptides appeared to increase tyrosine phosphorylation of cellular proteins, which is observed upon CCK treatment, providing evidence that the procedure is necessary for amylase release (255). In addition, the effect of GLP-1, glucagon and oxyntomodulin were investigated in primary isolated rat pancreatic acini, with similar results on amylase secretion (256, 257)

The effect of chronic administration of peptides *in vivo* in order to assess whether prolonged treatment increases the chances of pancreatic pathology has been investigated. Initial clinical cases claimed that GLP-1 receptor agonist treatment affect was acute. In order to investigate this, a bolus infusion of each peptide was given IV through the femoral vein and blood samples were taken over a period of 50 minutes. Results demonstrate no change in amylase secretion in response to administration of GLP-1, glucagon, exendin-4 and oxyntomodulin. Similarly, GLP-1, glucagon and exendin-4 co administration with CCK did not mitigate against the CCK- induced increase in amylase concentration. However, co-

administration of oxyntomodulin and CCK caused a significant inhibition of CCK-induced plasma amylase release *in vivo*, compared to CCK alone.

In previous studies in the anaesthetized rat, basal pancreatic secretion could not be inhibited by oxyntomodulin infusion, yet when stimulated with a CCK agonist, pancreatic secretion levels decreased. It has been suggested that a possible mechanism of this function is through the activity of gastropancreatic intrinsic nerves, or by preganglionic inhibition of excitatory vagal fibres via the central nervous system (CNS) (245, 258). This study confirmed an inhibitory function of oxyntomodulin, previously shown in overall gastric secretion.

Results from both *in vivo* and *in vitro* experiments show that there is no acute effect of GLP-1, glucagon, oxyntomodulin and exendin-4 within the first hour post administration. As the half-life of GLP-1, glucagon and oxyntomodulin is up to 6 minutes and exendin-4 is 35 minutes, it is assumed that the peak action of each peptide IV would be observed during the 50 minutes of experiment.

The results obtained in this investigation do not agree with the hypothesis stated in a number of studies linking GLP-1 to pancreatitis, a fact that can be attributed to a number of limitations. It has been shown that pancreatic ductal cell proliferation is elevated in both obese and diabetic humans, a fact that potentially predisposes them to pancreatitis (259). In addition, a study in mice pointed that a high fat diet has being a potential risk factor for pancreatic injury (260). The reasons could include the build-up of excessive fat in pancreatic tissue, resulting in β -cell apoptosis and inflammation, which leads to pancreatitis. As a result, it is possible that the outcome is also affected by high fat diet and could be further investigated in obese or diabetic *in vivo* models.

During past years, a large number of pancreatitis cases have triggered a number of critical reviews (228, 230, 261-264) that attempt to evaluate the advantages and disadvantages of GLP-1 receptor agonists and DPP-IV inhibitors. So far it seems that the benefits outweigh the potential risks, as the results on pancreatitis, but also cardiovascular effects and thyroid cancer, remain controversial while the advantages are well established. Even so, human development of pancreatic cancer can take decades and incretin-mimetic treatments have been commercially available for only 6 years. The ability of GLP-1 to inhibit glucagon secretion, which is potentially protective of the pancreas, could be an indirect mechanism of

pancreatitis development, a fact that makes GLP-1/glucagon co administration a potentially safer treatment with the same benefits and less risks. In this preliminary study, the acute effect of native peptides GLP-1, glucagon, oxyntomodulin and exendin-4 was assessed for exocrine secretions *in vivo* and *in vitro* with no significant results present. Moreover, an inhibitory effect of oxyntomodulin on the exogenously induced increase in the appearance of pancreatic enzyme in blood was found, a fact which might support that it could be useful in the treatment of diabetic obese patients, as it can have a protective effect against diabetes- induced pancreatic inflammation, in addition to weight loss induction.

5.5. Conclusion

GLP-1, glucagon, oxyntomodulin and exendin-4 don't appear to have an effect on proliferation rate of primary isolated rat epithelial pancreatic duct cells or acinar cells. Plasma amylase concentration did not increase post peptide injection *in vivo*, compared to vehicle and CCK. Of note, when oxyntomodulin was co administered with CCK, it suppressed amylase secretion compared to CCK alone. Nonetheless, *in vitro* amylase secretion post oxyntomodulin/ CCK cellular treatment was not significantly suppressed, when compared to CCK.

These results suggest that GLP-1, glucagon and exendin-4 do not have an acute effect on exocrine pancreatic function, while oxyntomodulin appears to suppress digestive enzyme secretion, yet not directly through down regulation of amylase release. Thus, it could be the safest choice for an obesity treatment, with regard to pancreatitis.

CHAPTER 6

General Discussion

Maintaining body weight in the face of fluctuations in the availability of food is an important physiological requirement of most organisms, including humans. A number of pathways ensure that even in situations of famine, food intake and energy expenditure are balanced by various adaptations in order to survive from starvation. In the past four decades, the opposite effect, an excess of available energy, is observed. Obesity, defined as BMI greater than 30 kg/ m², has become one of the biggest challenges the public health services are facing. It is the result of imbalance between energy intake and energy expenditure and it is highly related to the metabolic syndrome which is associated with increased mortality, with type 2 diabetes mellitus being the most direct outcome.

Elevated BMI is largely a result of excess adipose tissue. Within the last couple of decades the function of adipose tissue has changed from being viewed as a passive energy storage organ to releasing various highly active hormone-like molecules that participate in the regulation of metabolism and mediate immune responses. In addition, the gastrointestinal tract also produces a number of short signalling peptides and hormones which are able to control the appetite and induce insulin sensitivity. A subgroup of these is the glucagon-like peptides, the most widely studied peptide of which is GLP-1, an incretin hormone with multiple functions that can have a different function, depending on the region of its synthesis and release. GLP-1's primary action when secreted in response to food intake in the gut, is to maintain glucose homeostasis by triggering insulin secretion, while suppressing glucagon activity. Because of this, it became a target peptide for the development of pharmaceutical agents against type 2 diabetes, most important of which is exendin-4.

A number of obesity treatments became available over the past few decades, spanning from specific diets to the most recent and successful bariatric surgery. Lifestyle interventions were proven to be unsuccessful or difficult to maintain, while surgery often carries a mobility and mortality risk.

Post bariatric studies have shown that there is an increase in gut hormone levels that have an anorectic effect, pointing at an additional role to food intake control. Moreover, exendin-4 treated patients showed an inhibition of food intake and significant weight loss. The combination of observations triggered the idea that GLP-1 could pose as a potential treatment of obesity. Recently, US FDA approved a long lasting GLP-1 receptor agonist at a

high dose as an anti-obesity treatment that aspires to cause 4% body weight reduction within 16 weeks of treatment. Despite this, its common side effects include nausea, diarrhoea, constipation, vomiting and hypoglycaemia.

GLP-1 receptor agonists have also been associated with pancreatitis. The main mechanism hypothesised included GLP-1 stimulation of pancreatic ductal proliferation, thought to cause blockages and inflammation in the pancreas, as well as increase of amylase secretion. Early studies found glucagon to be protective of pancreatitis, as it appeared to reduce pancreatic secretion levels in patients. In order to assess the potential side effects of GLP-1, glucagon, oxyntomodulin and exendin-4 in the exocrine pancreas were investigated in this thesis. The two main cell types located in the exocrine pancreas, acinar and ductal cells, were investigated for increased amylase secretion on the first and proliferation rate on the latter. In addition, each peptide was administered through a bolus IV infusion in rats and plasma samples were obtained at specific time points in order to detect an increase in amylase secretion *in vivo*. Both studies demonstrated that the native peptides do not cause an acute effect on amylase secretion or cell proliferation. Of note, oxyntomodulin, a dual agonist of the GLP-1 and glucagon receptor, appeared to inhibit exogenously-stimulated amylase secretion *in vivo*, pointing to a potentially protective effect on the exocrine pancreas.

Glucagon is a pancreatic peptide that has been observed to induce weight loss either through its function to increase energy expenditure or through its possible physiological role in stimulating lipolysis, making it a potential target peptide for obesity treatment. Even so, the main problem is glucagon's glycogenolytic and gluconeogenic function through which it increases glucose concentration when low blood levels are detected.

In order to neutralize this, a co administration of GLP-1 and glucagon would potentially balance a GLP-1 peak concentration causing nausea, while counteracting the gluconeogenic effect of glucagon through the incretin effect, while making use of both peptides' ability to induce weight loss. A study in humans (132) demonstrated that co administration of native GLP-1 and glucagon increases energy expenditure and improves hyperglycaemia in overweight patients. Nonetheless, a co administration of native peptides would require very frequent injections, as the half-lives are very short.

The purpose of this thesis was to develop GLP-1 and glucagon analogues that would be equally, or more, potent as the native peptides, and have a longer half-life as to as require less frequent dosing. To achieve that, specific amino acids were substituted across the sequence of both peptides. The main focus was to make progressive changes while retain or enhance receptor binding and activation, as well as increase stability and prevent rapid degradation. Some of the analogues were successful while others were less potent than native peptides.

Initially, GLP-1 analogues were designed, with substitutions in the N-terminal, the linker region and the C-terminal. While some amino acid replacements were neutral, some cause a significant improvement in peptide efficacy or stability. Substitution in position 2 improved the half-life of the analogue, while serine and histidine in position 12 had a significant effect on peptide efficacy, confirming the literature. Interestingly, amino acid replacements in the central region demonstrated its importance in receptor binding and activation, as exenin-4 derived glutamic acid increased the efficacy of the peptide. Within the glucagon analogues, most substitutions did not increase efficacy to the glucagon receptor. However, histidine in position 20 and leucine in position 27 caused almost higher receptor activation than native glucagon. For both GLP-1 and glucagon analogues, amino acid substitution with Histidine improved the pharmacokinetic profile, as the final peptides were detectable in plasma concentration for up to 7 days. This confirmed the fact that histidine's imidazole ring and charge can offer stability when administered with positively charged zinc as it causes the formation of complexes between the peptide and zinc molecules which stabilise the molecule and prolong its half-life.

The two winning peptides (GL16 and GC17) were chosen according to their receptor activation potency, the inhibition of food intake they caused and the body weight loss they induced in mice feeding studies, and the pharmacokinetic plasma profile, as they were both present in circulation by 7 days in rats *in vivo*. Both peptides were co administered in a chronic study. Body weight and glucose tolerance were recorded. The correct and most beneficial ratio of the two peptides and therefore their affinity at the GLP-1 and glucagon receptors for safe and effective weight loss was found through different doses through the study. An excess of GLP-1 receptor action may result in similar side effects of nausea and vomiting, as seen in type 2 diabetes patients on GLP-1 treatment, while excess glucagon

receptor action could cause increased glucose concentration and hyperglycaemia. Day et al, using a single peptide co agonist, hypothesised that the optimal ratio of glucagon to GLP-1 activity is between 5:1 and 1:5 (265). It was therefore essential to investigate different ratios of the two analogues to assess the optimal dosage of both, as ligands could also cross react with different G-coupled receptors other than their own. Of note, the most effective ratio of glucagon analogue to GLP-1 analogue was 8:1 in the study described in this thesis.

In vitro experiments such as cAMP accumulation assays could explain this observation as they do provide representative data, yet they can still differ from *in vivo* effects. The cell lines used in the analogue studies overexpress the human receptors while *in vivo* studies were performed in rodents therefore the peptides aimed to bind at either mouse or rat receptors. Therefore, discrepancies may be due to the differences found between species receptor sequences or the population of receptors. As the treatment investigated is intended to be used in humans, the use of a human cell line ensures that the peptides are selected for their actions at the human receptor. Inadequacies of an overexpressing cell model are present when assessing a physiological relevance of *in vitro* data in order to predict biological effects *in vivo*. Nevertheless, *in vitro* assays provide fast, ethical, valuable and inexpensive results on a wide range of candidate molecules and can facilitate a screening process with less unknown co factors usually found *in vivo*. *In vitro* assays can therefore contribute to faster, more efficient and more targeted lead optimisation.

Nausea and vomiting cannot be assessed in rodents, as they cannot vomit, yet clinical signs such as piloerection and pica can be used to detect any signs of discomfort during a study. Moreover, the glucagon effect can be investigated through the use of metabolic cages to measure effectively a potential increase in energy expenditure. It is also crucial to research whether the weight loss observed is due to loss of adiposity or glycogen storage. Insulin levels post injection could also be measured to evaluate whether this treatment is safe for type 2 diabetes patients. Therefore, further studies, including insulin level measurement post glucose tolerance tests could be performed.

Despite the promising results, long term safety effects of the analogues should be investigated through a chronic study of frequent injections, where pancreatitis markers such as lipase and amylase levels would be regularly recorded and analysed. In addition,

histopathology of the pancreas could be performed past the end of the study. Acute effects could also be further investigated *in vivo*, by ligating the distal part of the bile duct under the liver and inserting a cannula in the proximal site through the duodenum to measure the exact amount of exocrine juice produced from the exocrine pancreas past a bolus peptide infusion.

In conclusion, the data presented in this thesis demonstrate that the GLP-1 analogue GL16 and the glucagon analogue GC17 could pose as a novel therapeutic approach for obesity. In addition, the results provide additional information on the relationship between peptide structure and function in GLP-1 and glucagon sequences. This thesis extends to assess the safety of GLP-1, glucagon, oxyntomodulin and exendin-4 in relation to the exocrine pancreas, suggesting that all four peptides do not have an acute effect on the organ.

APPENDIX

Appendix A: Amino Acids

Ala (A) Alanine

Cys (C) Cystine

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 reagents and materials other than peptides were supplied by Sigma (Poole, Dorset, UK or BDH (Poole, Dorset, UK). The principle of radioimmunoassay 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 radioimmunoassay is incubated and allowed to reach equilibrium, according to the equation:



Ag = unlabelled antigen

*Ag = radiolabelled 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 are 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 9.1, 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.

2 X: 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.

Quality Controls: includes previously aliquoted samples containing high and low levels of the antigen. These tubes allow the assays to be standardised.

Tube number	Designation
1-2	Non-specific binding
3-4	$\frac{1}{2}$ X
5-6	2 X
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
31-34	Zero
35-	Samples
Zeros	Two zeros every 50 samples
Standard curve	
Final two tubes	Excess

Table 7. 1. The general structure of the radioimmunoassay

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