The metabolic effects and therapeutic utility of novel GLP-1/Glucagon receptor co-agonists in type 2 diabetes

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

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2018

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Abstract

Type 2 Diabetes Mellitus is a metabolic disorder characterised by persistent hyperglycaemia. The global rise in obesity has fuelled an increase in the prevalence of diabetes and necessitates more effective treatments. Glucagon-like peptide-1 (GLP-1) analogues are one of the most efficacious therapeutics currently available, and simultaneously stimulate insulin secretion and increase insulin sensitivity through weight loss. Consequently, GLP-1 analogues are particularly useful for diabetic patients who are overweight or obese.

It has been hypothesised that the tolerability and therapeutic efficacy of GLP-1 analogues could be enhanced by combining GLP-1 with a second insulinotropic hormone. Based on the well-established beneficial metabolic effects of oxyntomodulin, a naturally occurring GLP-1 and glucagon receptor co-agonist, glucagon has been proposed as a co-adjunct for GLP-1. While multiple groups have demonstrated the superior weight loss efficacy of GLP-1 and glucagon receptor co-agonists versus GLP-1 analogues, the insulinotropic effects of co-agonists remain undetermined.

This project involved the development of novel GLP-1 and glucagon receptor co-agonists based on the sequence of oxyntomodulin, with targeted sequence modifications to confer longer plasma half-lives and greater potency. To determine the direct beta cell effects of co-agonists, insulin secretion assays were performed in vitro using INS-1 832/3, MIN6B1 and EndoC-βH1 beta cells. In comparison to GLP-1 analogues, my co-agonists augmented insulin release in vitro. These insulinotropic effects were preserved in vivo in a high-fat, high-sucrose fed mouse model of type 2 diabetes, where the co-agonists led to greater insulinotropic responses than GLP-1 analogues acutely. On chronic administration, co-agonists did not impair glycaemic control despite their considerable glucagon receptor activity. In addition, co-agonists induced profound weight loss chronically, which was attributed to both food intake reduction through the GLP-1 receptor and energy expenditure effects through glucagon receptor activation. Paradoxically, the weight loss did not lead to improvements in glucose tolerance.

The in vitro insulinotropic effects were associated with a signalling bias against β arrestin recruitment at the GLP-1R, and could be due to reduced receptor trafficking and internalisation. However, the in vivo characteristics show no correlation to bias, and weight loss was instead found to be positively associated with relative glucagon receptor activity of the co-agonists.

These findings highlight the potential of co-agonists as a safe therapy for weight loss in patients with diabetes, and could inform the design of more potent insulinotropic compounds for therapeutic use in type 2 diabetes.
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Declaration of contributors

I declare that this thesis has been composed solely by myself. Except where referenced otherwise explicitly, the work presented is entirely my own.

I am grateful to the following researchers for providing assistance with some experiments.

Chapter 3

Experiments were conducted with guidance from my supervisors, and without contributions from other investigators.

Chapter 4

Confocal microscopy was performed jointly with Dr Alejandra Tomas, Section of Cell Biology and Functional Genomics, Imperial College London.

Bias experiments were performed with assistance of Dr Ben Jones.

Chapters 5

Joyceline Cuenco-Shillito, Laura-Jayne Ball and Zainab Malik assisted in carrying out acute rodent studies.

Radioimmunoassays were conducted with assistance of Zainab Malik.
Acknowledgements

I am very grateful to my supervisor, Professor Steve Bloom, for giving me the opportunity to do this PhD and for his invaluable support and advice, and to James Minnion for his guidance and support throughout my time at Imperial. I would also like to thank Ben Jones and Joy Cuenco-Shillito for their assistance and advice, both scientific and otherwise.

Thank you to my parents, Mian Farooq Tahir and Naushaba Farooq, for being unfailing sources of inspiration, comfort and joy. And finally, thank you to Maham, Sana, Ahmad, and Usmaan for the laughter, camaraderie and endless entertainment.
Abbreviations

7TM 7 α helices, transmembrane domain
Aib α-amino-isobutyric acid
AUC Area under the curve
BAT Brown Adipose Tissue
BMI Body mass index
BPD Biliopancreatic diversion
BRET Bioluminescence resonance energy transfer
BSA Albumin from bovine serum fraction V
cAMP Cyclic adenosine monophosphate
CHO-K1 Chinese hamster ovary K1
DAPI 4′, 6-Diamidine-2′-phenylindole dihydrochloride
DIO Diet-induced obese
DMEM Dulbecco’s modified medium
DPP-IV Dipeptide peptidase IV
EMA European Medicines Agency
Epac Exchange protein directly activated by cAMP
FDA Food and Drug Administration
FRET Fluorescent resonance energy transfer
GDP Guanosine-diphosphate
GI gastro-intestinal
GIP Glucose-dependent insulino-tropic peptide
GLP-1 Glucagon-like peptide-1
GLP-1r GLP-1 receptor
GLP-1R/GCGR GLP-1 and glucagon receptors
GPCR G protein coupled receptor
GRK G protein receptor kinase
GTP Guanosine-5′-triphosphate
GWAS Genome wide association studies
HbA1C Haemoglobin A1c
HBSS Hanks’ Balanced Salt Solution
HEK Human embryonic kidney
HFD High-fat diet-induced
HFHS High fat, high sucrose
hGCGR Human glucagon receptor
hGLP-1R Human GLP-1 receptor
HPLC High performance liquid chromatography
HTRF Homogeneous Time-Resolved Fluorescence
i.p. Intraperitoneal
IBMX 3-isobutyl-1-methylxanthine
IPGTT Intraperitoneal glucose tolerance test
iPSCs Induced pluripotent stem cells
IRS Insulin receptor substrate
IRS-1 Insulin receptor substrate 1
LAGB Laproscopic adjustable gastric banding
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-OH kinase (PI(3)K)</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>rGCGR</td>
<td>Rat glucagon receptor</td>
</tr>
<tr>
<td>rGLP-1R</td>
<td>Rat GLP-1 receptor</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RYGB</td>
<td>Roux-en-Y gastric bypass</td>
</tr>
<tr>
<td>SG</td>
<td>Sleeve gastrectomy</td>
</tr>
<tr>
<td>SGLT2</td>
<td>Sodium–glucose cotransporter 2</td>
</tr>
<tr>
<td>SNAC</td>
<td>Sodium N-[8 (2-hydroxybenzoyl) amino] caprylate</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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1. General Introduction
1.1 Type 2 Diabetes Mellitus

Type 2 Diabetes Mellitus (T2DM) is a metabolic disorder characterised by chronic glucose dysregulation, with substantial morbidity due to resultant end organ damage.

In healthy individuals, blood glucose levels are under homeostatic regulation, and hyperglycaemia is prevented by the hormone insulin. However, insulin resistance, coupled with impaired or insufficient insulin secretion, can lead to persistently elevated blood glucose levels. This state of chronic hyperglycaemia is referred to as Type 2 Diabetes Mellitus (Stumvoll et al., 2005).

Chronic hyperglycaemia, as seen in diabetes, triggers oxidative stress, inflammation, endothelial dysfunction, and vascular complications. As a result, people with diabetes are at increased risk of chronic renal failure (diabetic nephropathy), retinal damage (diabetic retinopathy), irreversible damage to peripheral nerves (diabetic neuropathy), microvascular damage, impotency and improper wound healing. They are also two to four times more likely to suffer from cardiovascular co-morbidities as compared to non-diabetics. Approximately 5 million deaths worldwide were attributed to diabetes in 2017 (Cho et al., 2018).

1.1.1 Diabetes Prevalence and Impact

The incidence of diabetes is rising at a remarkable rate, with an increase of 200% globally from 2000 (Unwin et al., 2010). Diabetes is now considered a global epidemic, with 451 million people diagnosed worldwide, a number that is predicted to rise to 693 million within the next 30 years (Cho et al., 2018). In the UK alone, 3.8 million people suffer from diabetes, and it is estimated that this number will rise to 5.5 million by 2035 (PHE, 2016).

Diabetes and its associated co-morbidities place a significant socio-economic burden on health-care systems worldwide. The total global healthcare expenditure due to diabetes was estimated at £655 billion in 2017, and is expected to rise to £740 billion by 2045 (Cho et al., 2018).

1.1.2 Pathophysiology of Type 2 Diabetes Mellitus

Around 90% of all diabetes cases are attributed to type 2 diabetes (National Collaborating Centre for Chronic Conditions UK). Type 2 diabetes has a complicated multifactorial aetiology, and is associated with both genetic and lifestyle factors. Twin studies suggest a strong genetic component to the condition, with 100% concordance amongst monozygotic twins and 25% amongst close relatives.
However, the main modifiable risk factor for type 2 diabetes is being overweight or obese, with a body mass index (BMI) of 25 or more (NICE, 2011).

Type 2 diabetes is characterised by both insulin resistance and a reduction in insulin secretion, though the relative importance of each factor varies in individual cases (Weir, 1982).

1.1.2.1 Insulin Resistance

Insulin resistance is defined as an impairment in insulin-mediated glucose clearance, resulting from a combination of reduced glucose uptake into skeletal muscle and a failure to suppress hepatic glucose output (Cline et al., 1999, Roden, 2001). Insulin resistance is also characterised by impaired fatty acid oxidation and 'metabolic inflexibility,' namely the failure to adapt from fatty acid to glucose oxidation during an insulin stimulus (Simoneau and Kelley, 1997, Ukropcova et al., 2007).

Insulin resistance is associated with obesity and adiposity, as highlighted by genome wide association studies (GWAS) investigating beta-cell-independent risk factors for diabetes (Maes et al., 1997, Semple, 2016). In particular, insulin resistance is strongly related to visceral fat deposition (Lee et al., 2006, Pischon et al., 2008). Once adipose tissue reaches maximum expansion capacity, ectopic fat accumulation takes place in organs such as liver and muscle (Virtue S, 2008). This leads to increased free fatty acids, abnormal adipocyte behaviour such as altered secretion of adipokines, and the development of a low grade inflammatory state, all of which contribute to insulin resistance (Skurk et al., 2007, Gao et al., 2013).

Insulin resistance is also attributed to skeletal muscle mitochondrial dysfunction. Patients with T2DM are known to have a higher proportion of non-oxidative type muscle fibres, and a reduced number of skeletal muscle mitochondria (Nyholm et al., 1997, Kelley et al., 2002, Chomentowski et al., 2011). There are also reports of reduced intrinsic oxidative capacity of skeletal muscle mitochondria (Mogensen et al., 2007, Phielix et al., 2008). These mitochondrial defects lead to a reduced oxidative phosphorylation capacity that manifests as increased muscular triglyceride accumulation and defective glucose uptake, which are both key features of insulin resistance. In vivo, this leads to a reduction in skeletal muscle oxidative phosphorylation for patients with T2DM versus age and weight matched controls (Schrauwen-Hinderling et al., 2007, Phielix et al., 2008). Multiple groups have demonstrated that this defective mitochondrial function exists even in normal weight, non-diabetic subjects with insulin resistance, indicating that mitochondrial dysfunction is an early stage development in T2DM (Petersen et al., 2004, Krssak and Roden, 2004, Morino et al., 2005). Furthermore, patients with insulin resistance show an impairment in ‘mitochondrial plasticity,’ or the ability to alter mitochondrial activity and oxidation in response to insulin. These
findings hold true for both patients with overt diabetes and their first-degree relatives, suggesting impaired mitochondrial plasticity can confer genetic predisposition to insulin resistance (Petersen et al., 2005, Szendroedi et al., 2007).

Thus, due to increased lipid availability and exhaustion of lipid oxidative capacities (Bandyopadhyay et al., 2006), patients with insulin resistance frequently exhibit increased plasma and intracellular fatty acid concentrations (Reaven et al., 1988, Lara-Castro and Garvey, 2008). Elevated levels of intracellular lipids, including diacylglycerols and ceramides, can activate atypical protein kinase C isoforms. This leads to aberrant phosphorylation of cellular signalling components, impairing insulin signalling and hence resulting in insulin resistance (Dresner et al., 1999, Samuel et al., 2004, Lowell and Shulman, 2005, Holland et al., 2007).

Insulin resistance specifically is characterised by enhanced serine phosphorylation of insulin receptor substrate-1 (IRS-1). IRS-1 is a key adaptor protein, which is activated on insulin receptor activation, and recruits and serves as a scaffold for other intracellular signalling peptides. IRS-1 therefore helps propagate the downstream insulin signalling cascade. Lipid-stimulated erroneous phosphorylation of IRS-1 can attenuate the insulin signal, and even turn off the insulin response (Qiao et al., 1999, Aguirre et al., 2002).

1.1.2.2 Impaired Insulin Secretion

The role of beta cell dysfunction and impaired insulin secretion in the development of T2DM has historically been a matter of contention. The significance of beta cell dysfunction was established by a key study on Pima Indians conducted by Weyer et al. (1999). This prospective study monitored the onset of diabetes, and demonstrated that beta-cell dysfunction was present prior to any persistent hyperglycaemia in individuals who later developed diabetes. Other studies have supported these findings, suggesting that beta cell dysfunction and impaired insulin secretion is a necessary precursor to the progression of diabetes (Fukushima et al., 2004, Osei et al., 2004, Gastaldelli et al., 2004).

The role of impaired insulin secretion in the pathogenesis of diabetes has also been supported by GWAS investigating genes significantly associated with developing T2DM. A number of these genes are linked to the machinery for exocytosis and hence insulin secretion, such as ATP-sensitive potassium channel Kir6.2, and the transcription factor TCF7L2 which regulates expression of genes required for insulin granule exocytosis (Sladek et al., 2007, Chauhan et al., 2010).

Acquired impairments in beta cell function can arise due to glucotoxicity and lipotoxicity, particularly relevant for patients with obesity. Obesity results in increased circulating free fatty acids and glucose, which are toxic to beta cells (Lee et al., 1994, Robertson et al., 2004). Furthermore, obesity-
related systemic inflammation leads to circulating cytokines such as interleukin-1, -6 and -19, and tumour necrosis factor-α, which also mediate direct effects on beta cell function (Ohno et al., 1993). To explain the occurrence of fatty acid build-up in obese subjects with normal glycaemic control, the concept of ‘glucolipotoxicity’ has been proposed. The theory of ‘glucolipotoxicity’ suggests that hyperglycaemia is necessary to inhibit fatty acid oxidation, thereby facilitating excessive build-up of fatty acid metabolites (Prentki et al., 2002, Poitout and Robertson, 2002). Hence, identical levels of hyperlipidemia are toxic for beta cell function in patients with type 2 diabetes and persistent hyperglycaemia, but can be compensated by beta cell hyperfunction in normo-glycaemic patients.

Persistent hyperglycaemia brought about by beta cell dysfunction progressively impairs insulin secretion. Hyperglycaemia has profound effects on beta cell gene expression, and stimulates ‘beta cell dedifferentiation’ by changing the transcriptional pattern of the beta cells, hence altering metabolic pathways (Jonas et al., 1999, Laybutt et al., 2002). In particular, hyperglycaemia is known to reduce proinsulin transcription and hence insulin secretion (Olson et al., 1998). Over time, hyperglycaemia stimulates a non-sustainable increase in insulin secretion which worsens beta cell function, leads to a depletion of key resources and eventually inhibits further insulin secretion. This process is termed ‘beta-cell exhaustion’ (Greenwood et al., 1976, Leahy, 1996, Laedtke et al., 2000). Dysglycaemia and dyslipidaemia thus form a progressive feedback loop which ultimately leads from beta cell dysfunction to beta cell death (Deng et al., 2004).

Another pathological feature of diabetes is the deposition of amyloid fibrillary aggregates in pancreatic islets, which is associated with beta cell dysfunction and death. However, the mechanism of amyloid deposition in diabetes remains unclear as yet, and is under investigation.

1.2 Type 2 Diabetes Mellitus and Obesity

While genetic factors can influence predisposition to T2DM, environmental influence has a critical role in the development of T2DM. Obesity is the foremost modifiable risk factor for T2DM, with 90% percent of the adults presenting with T2DM worldwide being clinically overweight or obese (World Health Organization, 2005).

Obesity is classified as having a BMI of 30 or above. Over the last fifty years, increasingly sedentary lifestyles and greater availability of high-calorie, low-nutrient food has led to a dramatic rise in obesity (Swinburn et al., 2011). According to the World Health Organisation, more than 1.9 billion adults were overweight in 2016, of whom 650 million were obese. That translates to 13% of the adult population of the world being clinically obese in 2016.
Being overweight or obese increases the risk of developing debilitating conditions such as cardiovascular disease, stroke, hypertension, osteoarthritis, back pain and numerous cancers (Guh et al., 2009, Brown WV1, 2009). The strongest association of obesity is with developing type 2 diabetes. There is a threefold greater risk of developing diabetes for overweight individuals, and a seven fold greater risk for obese individuals (Abdullah et al., 2010). This close association between obesity and diabetes led to the coining of the phrase ‘diabesity’ to encompass this phenomenon (Sims et al., 1973).

The prevalence of obesity is increasing at a staggering rate, having tripled from 1975 to 2016, and is anticipated to continue rising (WHO, 2016). Given that the rise in obesity will inevitably lead to a rise in diabetes, there is great interest in developing therapies which simultaneously target both obesity and diabetes to help control this growing problem.

1.2.1 ‘Diabesity’ and available treatments

Prospective analysis suggests that around 64% of male and 74% of female cases of T2DM could have been prevented theoretically if subjects had a normal BMI (of less than 25) (Cassano et al., 1992, Chan et al., 1994, Hu et al., 2001), making a strong case for incorporating weight loss interventions in the treatment of diabetes. Furthermore, Ford et al. (1997) have reported that every additional kilogramme of weight gain can increase the risk of diabetes for an individual, suggesting that weight loss could help curtail the development of diabetes.

The efficacy of weight loss in diabetes management has been demonstrated in multiple independent studies. The Finnish Diabetes prevention study was the first to demonstrate that intensive dietary and exercise intervention, leading to an average weight loss of 4 kilograms a year, could reduce the onset of diabetes by 58% in pre-diabetic patients (Tuomilehto et al., 2001). This was corroborated by a study done the following year byKnowler et al. (2002), which showed a similar 58% reduction in the incidence of diabetes following a 3 year lifestyle intervention in patients, leading to lower basal and postprandial glucose levels. The latter study is particularly remarkable as it showed lifestyle interventions were more successful at limiting the onset of diabetes than metformin, a first line drug for diabetes. The lower rate of diabetes prevention by metformin (31% compared to 58% for the intervention group) is partly attributable to lower weight loss (2.1 compared to 5.6 kilogrammes for the intervention group). It is also of interest that while both metformin and weight loss reduce fasting plasma glucose equally, only the lifestyle intervention group showed lowered postprandial blood glucose (Knowler et al., 2002).
Recent trials have validated the impact of weight loss in diabetes, with the Look AHEAD trial showing that weight loss of 5-10% of total body weight can reduce haemoglobin A1c (HbA1c) levels, improve overall fitness and reduce the use of anti-hyperglycaemic medicines after 1 year (Look and Wing, 2010, Wing et al., 2011). A particularly promising trial published in 2018 demonstrated that intensive weight management by dietary restriction can lead to diabetes remission in patients, with 86% remission rates in patients who lost 15kg or more over the course of one year (Lean et al., 2018). Together, these findings provide robust evidence that anti-diabetic medication for obese patients should incorporate weight loss in addition to glucose lowering effects.

The most convincing argument for incorporating weight loss in diabetes management is the success of metabolic, or bariatric, surgery in treating diabetes. Bariatric surgery refers to weight loss operations that result in a functional remodelling of the intestine, through surgical diversion of the intestine and exclusion of the proximal gut, typically the duodenum and the jejunum. There are 4 key bariatric procedures: Roux-en-Y gastric bypass (RYGB), laparoscopic adjustable gastric banding (LAGB), sleeve gastrectomy (SG), and biliopancreatic diversion (BPD). Bariatric surgery is the most effective treatment of T2DM, with reports placing the likelihood of diabetes remission in patients undergoing surgery at 10 to 15 times greater than the likelihood of remission for patients receiving conventional medical therapy, irrespective of original BMI, age, severity of hyperglycaemia, or weight loss (Ribaric et al., 2014). Furthermore, diabetes remission can commence within weeks of the operation, making it the most time effective treatment for diabetes (Pories et al., 1995). While there are a number of definitions for diabetes remission, for the purposes of this discussion remission is defined as achieving the glycaemic goal of HbA1C levels <7.0 %, set out by the 2009 ADA Standards of Medical Care in Diabetes for adults. Bariatric procedures are generally safe, with a mortality rate similar to that of gall bladder surgery (Buchwald et al., 2007), and consistently mediate beneficial glycaemic effects and profound weight loss (Rubino et al., 2010). It is worth noting that intensive lifestyle programmes can achieve similar weight loss, but multiple studies have demonstrated lifestyle intervention measures lack long-term efficacy, making therapeutic intervention a necessity for sustained results (Wadden et al., 1989, Ayyad and Andersen, 2000, Foster et al., 2003, Christiansen et al., 2007, Nguyen et al., 2012).

1.3 Current treatments for Type 2 Diabetes

Current therapeutic strategies to treat hyperglycaemia involve lifestyle interventions, metformin, sulfonylureas, thiazolidinediones, exogenous insulin and incretin-based therapeutics.
Given that insulin resistance plays an important role in the development of T2DM, lifestyle interventions like exercise and dietary restriction are used to induce weight loss and hence help improve peripheral insulin sensitivity. However, the weight loss is rarely maintained post intervention, and therefore improvements are limited (Gregg et al., 2012). An alternative approach is to surgically induce weight loss, through bariatric surgery, which is known to lead to diabetes remission. The efficacy of bariatric surgery was highlighted in a recent report which recommended the surgery as safe and effective for the treatment of obesity and associated co-morbidities (Miras et al., 2018). However, due to lack of long term safety data and the expense associated with the process, bariatric surgery is not the front line treatment for diabetes, particularly for patients who have a lower BMI.

First line therapy involves metformin, an antihyperglycaemic drug that increases insulin sensitivity and reduces hepatic glucose output, but is insufficient as monotherapy in many patients. Alternatives include thiazolidinediones, which are insulin sensitizers, but lead to significant weight gain, which reduces compliance and hence their therapeutic potential. Sulphonylureas are another class of drug which enhance insulin secretion, but carry a risk of hypoglycaemic episodes due to sustained activity at low plasma glucose (Kirkman et al., 2018). Inhibitors of sodium–glucose cotransporter (SGLT) 2 are also in clinical use, are effective at lowering HbA1c levels by up to 0.9%, and have beneficial effects on cardiovascular function. However, SGLT2 inhibitors have been associated with a potential increased risk of amputation (Radholm et al., 2018, Thomas and Cherney, 2018, Verma and McMurray, 2018). In severe cases, where other drugs fail, exogenous insulin can be administered to help improve the glycaemic profile. However, the therapeutic utility of insulin is limited as it leads to weight gain, requires regular glucose monitoring, and carries a risk of cardiac failure if given with thiazolidinediones (Billings et al., 2018).

Over the last 15 years, drugs that exploit the incretin system have been developed, and are in widespread use to treat T2DM. Incretins are insulino tropic enteroendocrine hormones, including glucagon-like peptide-1 and glucose-dependent insulino tropic peptide (Elrick et al., 1964). Incretin-based therapies include inhibitors of incretin breakdown and longer-lasting incretin analogues, which will be expanded on in Section 1.5.4.

1.4 Incretins

Incretins are a group of enteroendocrine hormones which are released from the gastro-intestinal tract (GI tract) following nutrient ingestion, and potentiate insulin release from the beta cells of the
The incretin effect refers to the significant amplification of insulin secretion by hormones from the GI tract, and is quantified by comparing insulin responses to oral and intravenous glucose administration (McIntyre et al., 1964, Perley and Kipnis, 1967). In healthy subjects, oral administration causes two- to threefold larger insulin responses than the intravenous route due to engagement of incretin gut hormones. The incretin effect is believed to be one of the most important sources of insulin secretion and glucose disposal (Nauck et al., 1986b).

The two main incretins are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (Defronzo, 2009). GIP and GLP-1 are both insulinotropic hormones, produced postprandially by K and L cells in the small intestine respectively. While some studies have highlighted GIP as the more important incretin physiologically (Nauck and Meier, 2016), others have reported GLP-1 as being responsible for around 60% of the incretin effect (Kolligs et al., 1995, Defronzo, 2009).

1.4.1 Impaired Incretin Effect in Type 2 Diabetes

The incretin effect is severely reduced or abolished in diabetes, contributing to overall glucose intolerance (Nauck et al., 1986a, Knop et al., 2007). Extensive studies have shown that while GIP secretion is only mildly affected in patients with T2DM, GLP-1 secretion is profoundly impaired. In particular, the second phase of GLP-1 secretion is reduced by 50% on average in patients with T2DM, as shown in a large well controlled study (Toft-Nielsen et al., 2001a). This reduction in GLP-1 secretion manifests as an impaired incretin response (Hojberg et al., 2008, Muscelli et al., 2008), and is associated with obesity, insulin resistance, duration and severity of diabetes, and glucose intolerance (Naslund and Hellstrom, 1998, Toft-Nielsen et al., 2001b, Vilsboll et al., 2001).

In addition to reduced secretion, the insulinotropic potency of GLP-1 and GIP is reduced in T2DM, as demonstrated by Nauck et al. (1993a). Using GIP and GLP-1 infusions in healthy and diabetic subjects, this study showed that GLP-1 and GIP lose their ability to enhance insulin secretion in patients with type 2 diabetes. However, infusion of supraphysiological levels of exogenous GLP-1 restore the GLP-1 responses back to normal, suggesting that sensitivity to GLP-1 is impaired but not abolished, making it a suitable point of therapeutic intervention. In contrast, GIP infusion in patients with diabetes does not improve glycaemic control even at supraphysiological doses (Nauck et al., 1993a, Vilsboll et al., 2002).

The impairments in the incretin effect appear to be secondary to the development of diabetes (Nauck et al., 2004). In a study comparing healthy subjects, lean patients with T2DM, and patients
with chronic pancreatitis with normal or diabetic glucose tolerance it was demonstrated that the incretin effect, in particular incretin sensitivity, is lost exclusively in patients with diabetes (Knop et al., 2007). These findings, in concordance with independent twin studies, suggest that the responsiveness to incretins is lost after the development of glucose intolerance (Sladek et al., 2007, van de Bunt et al., 2015).

Given that GLP-1 retains its glucose-lowering effects even in conditions of hyperglycaemia, GLP-1 has been developed extensively as a potential therapeutic for T2DM (Kreymann et al., 1987), and is discussed in greater detail below.

1.5 GLP-1

1.5.1 GLP-1 and its physiological roles

GLP-1 is secreted postprandially from the L cells in the small intestine, and mediates a wide range of physiological effects (Orskov et al., 1994). In addition to increasing glucose-stimulated insulin secretion, GLP-1 increases biosynthesis of insulin, reduces glucagon secretion and hence hepatic gluconeogenesis, and increases insulin sensitivity even in conditions of hyperglycaemia. GLP-1 serves as a satiety signal, reducing food intake and hence stimulating weight loss (Fehmann and Habener, 1992, Drucker, 2003). GLP-1 slows down gastric emptying, a phenomenon referred to as the gastric-ileal break, which further reduces food intake and increases satiety (Wettergren et al., 1993). Finally, GLP-1 has been demonstrated to have positive effects on beta cell proliferation in animal models, increasing beta cell mass and inhibiting beta cell apoptosis (Xu et al., 1999). All of these effects are therapeutically advantageous in T2DM, making GLP-1 an ideal therapeutic candidate.

GLP-1 receptors are present on beta and delta cells of the pancreas, in the heart, kidneys, GI tract, the central nervous system and vagus nerve (Thorens, 1992, Bullock et al., 1996, Richards P, 2014, Pyke et al., 2014). In terms of insulin secretion, there is a consensus that GLP-1 mediates its effects primarily by direct action of the beta cells in islets, as demonstrated in a seminal study by Lamont et al. (2012). Their results show pancreatic GLP-1 receptors are sufficient to maintain normal glucose homeostasis, and there is a small contribution by central signalling. A recent knockout study has disputed these findings, showing that the insulin response to oral glucose is mediated by extra-islet GLP-1 receptors (Smith et al., 2014). However, both studies are in consensus that exogenous GLP-1, and GLP-1 based analogues, brings about insulinotropic effects mainly through pancreatic GLP-1 receptors.
In contrast, effects of GLP-1 on appetite and food intake are attributed to activation of the peripheral and central nervous system. Given the short half-life of GLP-1 (1 to 2 minutes) (Deacon et al., 1995b), it is unlikely that GLP-1 physically crosses the blood brain barrier to stimulate central receptors. Instead, it is postulated that GLP-1 acts locally on GLP-1 receptor-expressing vagal afferent neurons, which then transmit the signal to distant sites of action (Cabou and Burcelin, 2011, Krieger et al., 2016). This is supported by studies which demonstrated a complete inhibition of the anorectic effects of GLP-1 in vagotomised rodents and human subjects (Abbott et al., 2005, Plamboeck et al., 2013). However, this hypothesis has been disputed by a group which reported normal food intake responses to peripheral GLP-1, even in the absence of central activation, by using central administration of GLP-1 receptor antagonists (Williams et al., 2009).

1.5.2 GLP-1 receptor mediated signalling

1.5.2.1 GLP-1 receptor and G protein mediated signalling

The GLP-1 receptor belongs to the secretin family (class B) of G protein coupled receptors (GPCRs). GPCRs classically have a large N terminal extracellular domain, a transmembrane domain with 7 α helices (7TM), and a C terminal intracellular domain (Thorens, 1992). The GLP-1 receptor is thought to operate through the two-domain model, where the ligand binds to the extracellular domain, and then reorients to improve access between the ligand-bound N terminus and the extracellular face of the 7TM domain. This interaction leads to changes in the receptor conformation which then trigger intracellular signalling cascades (Al-Sabah and Donnelly, 2003, Hollenstein et al., 2014, Wootten et al., 2016).

The conformational change following ligand-binding allows GPCRs to function as guanine exchange factors for their associated intracellular G proteins. Inactive G proteins exist as heterotrimers, made of α, β and γ subunits, with the α subunit bound to guanosine-diphosphate (GDP). Following GPCR activation, the GDP is exchanged for guanosine-5'-triphosphate (GTP), and the trimer dissociates into Gα and Gβγ units (Gilman, 1987). GLP-1 is coupled to Gαs, a stimulatory G protein which activates adenylate cyclase, the enzyme responsible for synthesising secondary messenger cyclic adenosine monophosphate (cAMP) (Drucker et al., 1987).

cAMP signalling is well established as the main mediator of GLP-1-induced insulin secretion from beta cells (Pipeleers et al., 1985, Prentki and Matschinsky, 1987, Moens et al., 1996a, Montrose-Rafizadeh et al., 1997). Rise in intracellular cAMP levels activates protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac), which then mediate a range of downstream
effects, ultimately leading to an increase in intracellular Ca\textsuperscript{2+} levels (Light et al., 2002, Tsuboi et al., 2003, Kang et al., 2008). Increased cAMP levels accelerate granule mobilisation, increasing the size of the readily releasable pool of insulin vesicles, while the increase in intracellular Ca\textsuperscript{2+} levels triggers the process of insulin granule exocytosis (Lester et al., 1997, Holz, 2004, Holst and Gromada, 2004).

The GLP-1 receptor can also signal to Go and Gq subunits, but the physiological importance of these for insulin secretion has not been shown conclusively (Montrose-Rafizadeh et al., 1999).

Like other Class B GPCRs, the GLP-1 receptor can exist as a homodimer, and this interaction is important for downstream signalling. Harikumar et al. (2012) demonstrated that disrupting receptor dimerization does not affect cell surface expression, but reduces intracellular cAMP production and abolishes calcium accumulation on exposure to GLP-1. The GLP-1 receptor can also heterodimerise with the closely related GIP receptor, but this dimerization reduces the calcium response to GLP-1 and is non-conducive to insulin release (Schelshorn et al., 2012).

1.5.2.2 GLP-1 and β arrestin mediated signalling

β arrestins are a class of adaptor proteins that bind to activated 7TM receptors and are involved in downstream signalling, receptor internalisation and receptor desensitization (Lefkowitz and Shenoy, 2005, DeWire et al., 2007). Activated GPCRs are phosphorylated by G protein receptor kinases at the C terminus, and this phosphorylated site recruits β arrestins. Binding of arrestins creates steric hindrance, and hence prevents G protein interaction with the receptor. Furthermore, the arrestins serve as scaffolding proteins for recruitment of clathrin adaptor proteins, and facilitate clathrin-mediated endocytosis of the receptor. They therefore terminate the signalling cascade by reducing downstream signalling and receptor expression, a phenomenon referred to as ‘receptor desensitization’ (Goodman et al., 1996).

The GLP-1 receptor can associate with β arrestin isoforms 1 and 2 (Jorgensen et al., 2007, Sonoda et al., 2008). It is of note that while β arrestins are classically thought to mediate receptor desensitization, this has not yet been proven for the GLP-1 receptor. Recent work has instead demonstrated that arrestins can promote specific, G-protein-independent signalling cascades, through the mitogen-activated protein kinases ERK, JNK, and p38 (Lefkowitz and Shenoy, 2005). Through these non-canonical signalling pathways, arrestins can play a role in the physiological effects of GLP-1. It has been proposed that GLP-1 mediates its anti-apoptotic effects partly through β arrestin-1 (Quoyer et al., 2010). Sonoda et al. (2008) further suggested that β arrestin-1 plays a role in both cAMP dependant and independent routes of insulin secretion. Multiple studies have since supported these findings, providing strong evidence for a role for β arrestin signalling in glucose
homeostasis, insulin secretion and insulin sensitivity (Luan et al., 2009, Quoyer et al., 2010, Zhang et al., 2015, Zhu et al., 2017).

**Figure 1.5: Schematic summation of downstream signalling at the GLP-1 receptor.**

**1.5.3 GLP-1 in T2DM**

In conditions of T2DM, both the secretion and potency of GLP-1 are profoundly reduced (Toft-Nielsen et al., 2001a, Hojberg et al., 2007). However, the insulinotropic potency and glucose-lowering effects of GLP-1 can be restored by administering it at supraphysiological levels (Nauck et al., 1993a), with multiple infusion studies demonstrating that GLP-1 can preserve its effects even in patients with T2DM (Nauck et al., 1993b, Nauck et al., 1993c, Elahi et al., 1994a). These glycaemic effects are attributed to improved β-cell function and altered insulin secretory dynamics (Byrne et al., 1998, Ritzel et al., 2001, Kjems et al., 2003).

In a seminal study highlighting the therapeutic potential of GLP-1, patients with continuous subcutaneous GLP-1 infusions over 6 weeks demonstrated a 77% increase in insulin sensitivity, an improved insulinogenic index and reduced glucagon concentrations, due increased α and β-cell sensitivity, as well as reduced lipotoxicity and glucotoxicity. As a result, patients reported an average 1.3% reduction in HbA1c levels at the end of the study (Zander et al., 2002).

The main stumbling block in developing native GLP-1 as a therapeutic is its short half-life. Once in the circulation, GLP-1 undergoes rapid proteolytic degradation which, coupled with renal filtration,
gives it a half-life of 1 to 2 minutes in plasma (Orskov et al., 1993, Deacon et al., 1996, Meier et al., 2004). As expected from its plasma half-life, the beneficial glycaemic effects of GLP-1 are transient and only last 1-2 hours post administration (Gutniak et al., 1994, Ritzel et al., 1995, Nauck et al., 1996, JunttiBerggren et al., 1996). To account for the short plasma half-life of GLP-1, native GLP-1 has been trialled as thrice daily injections and continuous intravenous or subcutaneous infusions in short- and long-term studies, and proven to be highly effective in lowering blood glucose in diabetic subjects on continuous administration (Todd et al., 1997, Rachman et al., 1997, Zander et al., 2002). However, multiple daily injections and constant infusions are impractical and expensive, and hence of limited therapeutic utility.

1.5.4 GLP-1 based treatments

One of two approaches is used to therapeutically enhance GLP-1 mediated insulin secretion: giving an inhibitor to prevent breakdown of endogenous GLP-1, or giving exogenous GLP-1 receptor (GLP-1R) agonists with enhanced half-lives and improved pharmacokinetic profiles.

1.5.4.1 Inhibitors of GLP-1 breakdown

GLP-1 is broken down primarily by the enzyme dipeptide peptidase IV (DPP-IV) secreted from endothelial cells (Deacon et al., 1995a, Deacon et al., 1995b, Kieffer et al., 1995). DPP-IV acts at the N terminal dipeptide ending in Alanine or Proline, cleaving GLP-1 (7-36) to form GLP-1 (9-36), resulting in a half-life of a few minutes in circulation (Deacon et al., 1995b). The metabolite GLP-1 (9-36), although circulating at approximately 10-100 times higher concentrations than GLP-1 (7-36), is generally thought to be inactive; however some studies have suggested it might have a minor physiological role (Robinson et al., 2016, Guglielmi and Sbraccia, 2017). DPP-IV inhibitors prevent the degradation of endogenous active GLP-1, thereby improving glycaemic control. Drugs currently on the market include vidagliptin, saxagliptin and sitagliptin. Studies have shown that DPP-IV inhibitor monotherapy, or combination therapy with metformin, are safe and effective for patients with diabetes. Inhibitors are orally administered, well tolerated, and on average lead to HbA1c reductions of 0.8% without causing significant weight gain (D.S. Qi, 2008). However, more recent studies have shown that saxagliptin in particular is associated with an increased risk of heart failure (Savarese et al., 2015). Furthermore, GLP-1R agonists, exemplified by liraglutide, consistently mediate superior glycaemic control and weight loss compared to DPP-IV inhibitor sitagliptin, and have a relatively lower incidence of gastrointestinal side effects, thereby proving to be more effective therapies (Li et al., 2017).
1.5.4.2 Longer-lasting GLP-1R agonists

The current focus of GLP-1 research is to generate GLP-1R agonists which are intrinsically resistant to DPP-IV degradation and other routes of elimination. As mentioned earlier, GLP-1 receptor agonism enhances insulin secretion, beta cell function and insulin sensitivity, making GLP-1R agonists an ideal drug for diabetes.

A number of long lasting GLP-1R agonists are clinically available for the treatment of diabetes. The first licensed GLP-1 analogue was exenatide, which is based on a naturally occurring peptide called exendin-4. Exendin-4, isolated from the venom of the ‘Gila monster’ Heloderma suspectum (Eng et al., 1992), shares 53% homology with GLP-1 and is an agonist at the GLP-1 receptor (Pohl and Wank, 1998). Unlike GLP-1, it has a Glycine residue at position 2, making it resistant to DPP-IV degradation. It hence has a longer half-life and improved pharmacokinetic profile in vivo (Edwards et al., 2001a). Exendin-4 is primarily cleared by renal elimination, with a half-life of 2.4 hours in man (Bond, 2006). Synthetic exenatide reduces hyperglycaemia and enhances weight loss through its actions at the GLP-1 receptor, and is currently in wide spread clinical use for the treatment of type 2 diabetes as a twice daily injectable, called Byetta®, and a once weekly injectable, called Bydureon® (AstraZeneca). In 2013, an exenatide-based GLP-1R agonist called Lixisenatide (Lyxumia®, Sanofi-Aventis) was also licensed for daily administration, mainly as an adjunct with basal insulin.

The second GLP-1 based analogue to be licensed for therapeutic use was liraglutide (Victoza®, Novo Nordisk A/S 2012). Unlike exenatide, which is resistant to degradation by virtue of its sequence, liraglutide relies on acylation to improve its plasma half-life. Liraglutide has a covalently linked fatty acid side chain at Lysine29, which binds to plasma albumin. This reduces renal clearance and proteolytic degradation, increasing the circulatory half-life of liraglutide beyond that of GLP-1 and exenatide and making it suitable for daily administration (Knudsen et al., 2000). Liraglutide induces significant weight loss and glycaemic improvements at therapeutic doses. For example, one 56 week study reported 6.0% weight loss, and a 1.3 to 1.1% reduction in HbA1c levels, following treatment with liraglutide at 3.0 mg daily (Davies et al., 2016). Head-to-head comparison studies have shown liraglutide mediates comparable weight loss to exenatide, and superior fasting plasma glucose and HbA1c lowering effects, and exhibits a lower frequency of detrimental side effects like nausea (Buse et al., 2009, Marso et al., 2016). Furthermore, liraglutide appears to have positive cardiovascular effects, giving it an edge over exenatide as a therapeutic (Cohen and Beckey, 2016). In addition to being a licensed drug for diabetes, liraglutide has recently been approved as a stand-alone weight loss therapy, making it an ideal drug for obese diabetic patients (Davies et al., 2016, Le Roux et al., 2017).
There is extensive ongoing research into longer lasting GLP-1R agonists, as drugs with lower dosing frequencies would improve patient convenience and compliance, and potentially limit the incidence of nausea. To this end, two weekly drugs have been developed, namely albiglutide (Tanzeum®, GlaxoSmithKline) and dulaglutide (Trulicity®, Eli Lilly). Albiglutide and dulaglutide are GLP-1R agonists consisting of two GLP-1 molecules sandwiching an albumin or human immunoglobulin fragment respectively (Bush et al., 2009). The albumin and immunoglobulin fragments help reduce glomerular filtration, and lead to sustained plasma half-lives of 4 to 5 days (Dennis et al., 2002, Glaesner et al., 2010). However, albiglutide is due to be withdrawn from market by summer 2018 for financial reasons.

The latest drug to be approved for the market is semaglutide (Ozempic®, Novo Nordisk), which is a liraglutide-like molecule with a fatty acid side chain, and novel amino acid substitutions. Semaglutide was approved in 2017 for weekly subcutaneous administration, and leads to superior reductions in HbA1c and weight loss versus comparators (including exenatide, metformin, sitagliptin and insulin) in the SUSTAIN 1–5 trials. Additionally, semaglutide improves cardiovascular health in a manner similar to liraglutide (Marso et al., 2017, O’Neil et al., 2018). Semaglutide has also been optimised for daily oral administration, using a formulation containing sodium N-[8 (2-hydroxybenzoyl) amino] caprylate (SNAC) which helps absorption through the stomach. Oral administration would give semaglutide an advantage over current GLP-1R agonists in terms of ease of use, as all currently licensed drugs are injectables. Preliminary clinical trials have yielded promising results, with oral semaglutide showing comparable efficacy and tolerability to subcutaneous semaglutide (Davies et al., 2017). However, it is worth noting that initial data suggest semaglutide has a higher incidence of side effects like nausea, with 15 to 27% of subjects reporting nausea or vomiting with semaglutide versus 6 to 14% with comparators sitagliptin, exenatide and insulin (Ahren et al., 2018).
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<tr>
<th>Trade Name</th>
<th>Dosing</th>
<th>HbA1c Reduction, %</th>
<th>Potential Side Effects/ Warnings</th>
<th>Common Side Effects</th>
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<td>-1.5 to -0.5</td>
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<td>Diarrhoea, Nausea, Headaches</td>
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*Table 1.5: Summary of GLP-1R agonists available clinically in Europe and the USA. Clinical trial data from Dalsgaard et al. (2018).*
1.5.5 Side effects of GLP-1 based therapeutics

GLP-1R agonists are effective treatments for diabetes, with an average HbA1c reduction of 0.5-1.9% (Dalsgaard et al., 2018). Moreover, GLP-1R agonists have fewer complications compared to earlier drugs; they are glucose-dependant and hence self-limiting, which removes the risk of hypoglycaemic episodes, except when given concurrently with insulin or sulphonylureas. Unlike sulphonylureas and insulin therapy, GLP-1-based treatments stimulate weight loss instead of weight gain, making them a better choice for overweight patients. However, GLP-1 receptors are found in multiple tissues around the body, potentially increasing the risk of unwanted side effects. Consequently, the main limiting factor in the use of GLP-1R agonists is their side effects (Lund et al., 2014).

The most serious potential concern associated with GLP-1R agonist treatment is the risk of pancreatitis, first brought to light by an isolated case study looking a single patient (Denker and Dimarco, 2006). This effect was attributed to GLP-1-induced proliferation of pancreatic ductal cells, which could ultimately lead to auto digestion of the pancreas (Butler et al., 2010, Gier et al., 2012). However, extensive preclinical studies in non-human primates dispute this finding, and instead provide convincing evidence that GLP-1R agonists, exemplified by liraglutide, are safe and tolerable even at high doses (Nyborg et al., 2012). Large meta-analysis studies have supported this observation, and therefore GLP-1R agonists are considered safe from the risk of pancreatitis by both the FDA and the EMA (Li et al., 2014, Egan et al., 2014). Furthermore, given that T2DM itself is a risk factor for the development of pancreatitis, separating cause and association is difficult.

The most commonly reported adverse effects of GLP-1R agonists are gastrointestinal disorders, particularly nausea and diarrhoea (Sun et al., 2015). It has been observed that nausea reduces with passage of time, attributed to desensitisation as the gastric emptying effect of GLP-1 is known to undergo rapid tachyphylaxis (Nauck et al., 2011). Of note, the large albumin molecule conjugated to albiglutide is thought to impede passage across the blood brain barrier, and indeed albiglutide is associated with reduced incidence of nausea compared to liraglutide. However, the inability to cross the blood brain barrier also leads to a correspondingly lower efficacy at appetite reduction and weight loss. As a result, the overall patient satisfaction scores for albiglutide are lower than those for liraglutide despite its better side effect profile (Secher et al., 2014, Pratley et al., 2014).
Nausea as a limiting factor for GLP-1 based therapies

Nausea is the most common adverse effect of GLP-1R agonists, affecting up to 50% of all patients (Buse et al., 2009, Marre et al., 2009, Russell-Jones et al., 2009, Raskin and Mohan, 2010, Russell-Jones et al., 2012, Nauck et al., 2013, Buse et al., 2013). The high incidence of nausea due to GLP-1R agonists is particularly troubling as it leads to dose limitations, and hence limits the efficacy of the drug. If the drugs did not trigger nausea at higher doses, the therapeutic window could be widened and higher doses with greater insulinotropic effects could be prescribed (Lund et al., 2014).

The dose-dependence of insulin secretion and nausea mediated by GLP-1R agonists, specifically exenatide and liraglutide, has been shown in multiple human studies (Elbrond et al., 2002, Madsbad et al., 2004, Kadowaki et al., 2009, Henry et al., 2013). In particular the LEAD trials, which compared liraglutide at clinical doses (0.6 mg to 1.8 mg daily) to alternative therapies in long-term studies, showed a clear dose-dependence of insulinotropic effects and nausea, with the highest dose being the most efficacious but also leading to the highest incidence of vomiting and nausea (Sun et al., 2012, Zinman et al., 2012). Recent studies have corroborated these findings at even higher doses of liraglutide, as prescribed for weight loss, in both diabetic and non-diabetic obese patients (Davies et al., 2015). Therefore, it is clear that liraglutide has not yet reached its maximum therapeutic potential at the current prescribed dose of 1.8 mg daily, and is dose-limited due to emetic side effects. Weekly GLP-1R agonists show a similar parallel relationship between efficacy and incidence of adverse GI incidents (Umpierrez et al., 2016). Furthermore, the dose-finding studies for both dulaglutide and semaglutide were curtailed prematurely, with dose escalation halted prior to reaching maximal therapeutic potential, due to a high incidence of nausea and vomiting (Barrington et al., 2011, Nauck et al., 2016).

Nausea is believed to be independent of route of administration, as exenatide administered by an osmotic mini pump has a similar side effect profile to exenatide administered subcutaneously (Henry et al., 2013). Dose ramping, namely a gradual increase up to prescribed doses, is used clinically in order to reduce the incidence of nausea in patients and hence improve compliance. However, there is evidence to suggest that higher doses can lead to nausea even despite prolonged pre-acclimatisation periods (Kendall et al., 2005, Sun et al., 2012, Zinman et al., 2012).

A recent systematic review of GLP-1R agonists in Phase 3 clinical trials showed a strong correlation between the incidence of nausea and vomiting and withdrawal from clinical trials. Given that 30% of patients experienced nausea, and 10% experienced vomiting, this indicates a serious compliance issue (Bettge et al., 2017). In the ‘real-world’, rates of discontinuation are even higher, with 41% and 37% of patients prescribed exenatide or liraglutide respectively withdrawing in the U.S within 6
months (Yu et al., 2016). Similar withdrawal rates have been observed in Europe, with nausea being the most common reason (Divino et al., 2014, Lapolla et al., 2015).

In summation, GLP-1R agonists have a propensity to cause adverse gastrointestinal events, particularly nausea and vomiting, which limits their dose due to safety and tolerability concerns, and hence limits their therapeutic effect. Alternative therapies, that circumvent these side effects while maintaining beneficial effects on weight loss and glucose tolerance, are needed to provide a better treatment for type 2 diabetes. It has been shown that there is no statistical correlation between the amount of weight loss and nausea, suggesting that nausea is a side effect of GLP-1R agonism and not a mechanism of weight loss (Kendall et al., 2005). Consequently, engineering GLP-1R agonists to avoid nausea will not lower their weight loss efficacy.

### 1.5.7 Combination therapies with GLP-1 as a means of bypassing side-effects and maximising therapeutic benefits

Current treatments for diabetes, while numerous, do not provide effective glycaemic control in many cases (Keavney et al., 1995, Nathan et al., 2009). The most effective drugs on market currently are GLP-1R agonists, which are dose limited due to their emetic side effects. One possible strategy of maximising their effect would be to co-administer them with another hormone. Combination therapies are hypothesised to stimulate multiple metabolic pathways simultaneously, thereby increasing the therapeutic effect while avoiding the side effects seen at high doses of one given compound (Sadry and Drucker, 2013).

The interest in combination therapy for diabetes is inspired partly by the involvement of multiple gut peptides in the success of bariatric surgery, and partly from work done on a naturally occurring co-agonist oxyntomodulin (Wynne et al., 2005, Sjostrom et al., 2007).

### 1.6 Gastric Bypass, the most effective way of achieving diabetes remission

As mentioned earlier, gastric bypass surgery is a highly effective treatment for both obesity and diabetes. The Swedish Obese Subjects case control study in particular has provided long term, high quality data showing that bariatric surgery is threefold more effective in controlling diabetes than conventional medical therapy. Following bariatric surgery, 72% of the patients were in remission after 2 years, and 36% remained in remission even after 10 years. In contrast, patients who lost
weight through medical therapy had a remission rate of 21% at 2 years, and 12% at 10 years postsurgery (Ribaric et al., 2014).

1.6.1 Mechanism of remission following gastric bypass

Gastric bypass is the most effective therapy available for diabetes, but the aetiology of diabetes remission after surgery remains unclear (Ribaric et al., 2014). The beneficial effects of surgery are mainly attributed to the significant weight loss and profound calorie restriction post-surgery (Abbasi, 2017). Weight loss leads to a reduction in peripheral insulin resistance, and consequently the degree of weight loss post-surgery is strongly related to the improvements in glycaemic control (Sjostrom et al., 2007, Buchwald et al., 2007). For example, Dixon et al. (2008) demonstrated that the surgical groups lose significantly more weight than groups on conventional medical treatment (20% compared to 1.7% weight loss for the non-surgical group), which is associated with markedly higher remission rates in the surgical group (73% compared to 13%).

However, non-surgical patients with comparable weight loss fail to achieve comparable improvements in insulin secretion to surgical patients, suggesting the existence of weight-loss-independent effects on glycaemic control (Laferreere et al., 2008). Secondly, the positive glycaemic effects seen following surgery, including rapid improvements in insulin secretion and hyperglycaemia, an exaggerated incretin response, reduced hepatic insulin resistance and changed food preferences, all manifest prior to any significant weight change (Pournaras et al., 2010, Nannipieri et al., 2011, le Roux et al., 2011, Laferreere et al., 2011). There are a number of possible explanations for weight-loss independent glycaemic effects, such as enhanced organ insulin sensitivity, altered gut-brain axis signaling, changes in beta cell function and incretin responses, enhanced thermogenesis through brown adipose tissue, modifications in bile acid composition, and changes in gut microbiota. In particular, diabetes remission following bariatric surgery has been attributed to changes in incretins, such as GLP-1.

1.6.2 Gastric bypass induces changes in multiple hormones

Postprandial GLP-1 levels rise dramatically following surgery, and remain elevated for up to a year (Laferreere et al., 2007, Laferreere et al., 2008). These changes occur prior to significant weight loss, as early as 2 days after a procedure, and are postulated to play a role in glycaemic improvements post-surgery (le Roux et al., 2007, Laferreere B, 2007, Beckman et al., 2011). An elegant proof-of-concept in Goto-Kakizaki rats showed that improved glucose tolerance after duodenojejunal bypass is reversed
by the administration of GLP-1 receptor antagonist Exendin 9–39, providing direct evidence that improvement of glucose tolerance following a bypass-like surgery is mediated, at least in part, by enhanced GLP-1 action (Kindel et al., 2009). These findings are supported by a human study where an Exendin 9–39 infusion significantly impaired glucose tolerance and reduced insulin secretion in patients following a RYGB. Exendin 9–39 had a greater impact on surgical patients than controls, suggesting that elevated GLP-1 levels are involved in regulating insulin secretion and glucose tolerance after gastric bypass (Shah et al., 2014).

Gastric bypass leads to changes in the secretion of multiple hormones in addition to GLP-1. Despite isolated studies reporting a reduction in GIP levels (Sirinek et al., 1986, Guidone et al., 2006), overwhelming evidence suggests postprandial GIP levels increase following gastric bypass surgery, an effect that can persist for up to a year (Jorde et al., 1981, Naslund et al., 1998, LaFerrere et al., 2007, Goldfine et al., 2007, LaFerrere et al., 2008, Holter et al., 2017). The contribution of GIP to glycaemic control post-surgery has remained undefined, primarily due to the absence of a specific inhibitor for use in human studies, but recent developments in the field are promising and could help answer these questions (Gasbjerg et al., 2018).

The secretion of other products from neuroendocrine L cells, notably peptide YY (PYY) and oxyntomodulin is also enhanced after gastric bypass, which may lead to an improvement in glycaemic control (Polak et al., 1971, Ghatel et al., 1983b, Adrian et al., 1985, Kervran et al., 1987, Borg et al., 2006, le Roux et al., 2006, LaFerrere et al., 2010a). PYY, a member of the pancreatic polypeptide family (Lundberg et al., 1982), is known to have anorectic effects (Bartolome et al., 2002, Batterham et al., 2002, Roth et al., 2005). Moreover, it has been reported that the development of type 2 diabetes is associated with blunted postprandial PYY concentrations (Pittner RA, 2004, Boey et al., 2006, Viardot et al., 2008). Oxyntomodulin, a proglucagon-derived hormone, improves glucose homeostasis in humans and rodents on peripheral and central administration (Dakin et al., 2001, Cohen et al., 2003, Parlevliet et al., 2008). It has therefore been postulated that the improvements in the glycaemic profile seen post-surgery are due to simultaneous changes in the secretory profile of multiple GI tract hormones.

### 1.7 Oxyntomodulin, a naturally occurring GLP-1 and glucagon receptor co-agonist

Oxyntomodulin is a hormone co-secreted with GLP-1 from the L-cells in response to nutrient ingestion (Ghatel et al., 1983b, Le Quellec et al., 1992), which binds to and activates both GLP-1 and glucagon receptors. Glucagon is a peptide hormone secreted by α cells of the pancreas, and is
considered to be the primary gluco-regulatory hormone which opposes and counteracts insulin action (Cryer, 2014). In direct contrast to GLP-1, which lowers plasma glucose levels, glucagon enhances hepatic glucose output through gluconeogenesis and glycogenolysis, and hence raises blood glucose levels (Orskov, 1992).

The structure of oxyntomodulin consists of the 29 amino acids of glucagon with a C-terminal octapeptide tail (Bataille et al., 1981, Bataille et al., 1982, Holst, 1982). This C terminal tail enhances oxyntomodulin’s ability to bind to and activate the GLP-1 receptor, making oxyntomodulin a dual agonist for the GLP-1 and glucagon receptors, albeit with a lower potency than the native ligands (Baldissera et al., 1988, Schepp et al., 1996, Dakin et al., 2001, Jorgensen et al., 2007, Druce et al., 2009, Kerr et al., 2010, Santoprete et al., 2011, Kosinski et al., 2012).

A specific oxyntomodulin receptor has not been identified, and oxyntomodulin is instead postulated to bring about its effects through activity at both GLP-1 and glucagon receptors. Due to the postprandial rise in oxyntomodulin levels, oxyntomodulin is believed to play a physiological role in satiety like GLP-1 (Le Quellec et al., 1992).

1.7.1 Effect of oxyntomodulin on food intake, weight loss and glycaemic control

Peripheral administration of oxyntomodulin leads to a reduction in food intake in rodents and in man (Dakin et al., 2001, Dakin et al., 2002, Cohen et al., 2003, Dakin et al., 2004, Wynne et al., 2005, Wynne and Bloom, 2006, Maida et al., 2008, Kosinski et al., 2012). As these anorectic effects can be blocked by GLP-1 receptor antagonists, they are attributed to the GLP-1 receptor. Similarly, genetic ablation studies have shown that anorectic effects are preserved in glucagon receptor knockout mice, but not in GLP-1 receptor knockouts, confirming that oxyntomodulin exerts its effects on food intake specifically through the GLP-1 receptor (Dakin et al., 2001, Dakin et al., 2004, Baggio et al., 2004a). However, due to the confounding factor of compensation in germline knockouts, it is difficult to draw conclusions regarding physiological relevance from these knockout studies.

While there is a consensus that the GLP-1 receptor is involved in oxyntomodulin-mediated inhibition of food intake, the exact mechanism remains unclear. Oxyntomodulin does not reduce gastric emptying, which is partly the mechanism by which GLP-1 brings about its effects on satiety (Cohen et al., 2003, Dakin et al., 2004, Maida et al., 2008). One alternative pathway is the suppression of orexigenic hormone ghrelin by oxyntomodulin (Cohen et al., 2003, Dakin et al., 2004). However, this finding is contentious, as multiple studies have since reported unchanged ghrelin levels following
Peripheral oxyntomodulin administration in volunteers (Wynne et al., 2005, Wynne and Bloom, 2006).

Parallel to the reduction in food intake, chronic oxyntomodulin administration leads to weight loss in both rodents and humans (Dakin et al., 2001, Dakin et al., 2002, Dakin et al., 2004, Wynne et al., 2005, Wynne and Bloom, 2006). However, the magnitude of weight loss is much greater than expected for a given reduction in food intake (Dakin et al., 2002, Dakin et al., 2004, Wynne and Bloom, 2006), indicating a concomitant increase in energy expenditure. Increased energy expenditure following oxyntomodulin administration has been demonstrated in rodents (Dakin et al., 2001, Dakin et al., 2002) and in human volunteers (Wynne and Bloom, 2006). This energy expenditure effect is believed to be mediated through the glucagon receptor, as glucagon itself increases energy expenditure (Davidson et al., 1957, Davidson IWF, 1960, Tan et al., 2013), while GLP-1 is linked instead to reduced energy expenditure (Flint et al., 2000, Baggio et al., 2004a). Consistent with this hypothesis, Kosinski et al. (2012) demonstrated greater weight loss in mice subcutaneously infused with oxyntomodulin compared to mice given an equivalent GLP-1 analogue, confirming that a proportion of the weight loss following oxyntomodulin administration is due to increased energy expenditure mediated by glucagon receptor activity.

In addition to enhancing satiety and stimulating weight loss, oxyntomodulin stimulates insulin release by acting on the GLP-1 receptor, albeit to a lesser extent than native ligand GLP-1 (Du et al., 2012). These acute insulinotropic effects of oxyntomodulin have been recapitulated in humans, by a recent study showing improved glucose tolerance and enhanced insulin release on oxyntomodulin infusion (Shankar et al., 2018). It is also noteworthy that, despite its intrinsic glucagon receptor activity, oxyntomodulin does not cause hyperglycaemia following chronic administration in human volunteers (Wynne et al., 2005). Instead, chronic oxyntomodulin administration has been reported to improve glucose tolerance in insulin resistant diet-induced obese (DIO) mice, through a combination of weight loss and GLP-1 receptor mediated stimulation of insulin release (Kosinski et al., 2012).

1.7.2 Longer-lasting oxyntomodulin analogues for treatment of diabetes

Based on the beneficial metabolic effects of oxyntomodulin in vivo, there has been considerable investigation into oxyntomodulin analogues as potential treatments for obesity and diabetes. Oxyntomodulin has a short half-life in circulation, approximately 6 minutes in rodents (Kervran et al., 1990) and 12 minutes in humans (Schjoldager et al., 1988), as it is rapidly degraded by the enzymes DPP-IV and neprilysin (Zhu et al., 2003, Druce et al., 2009). Consequently, multiple groups have
developed oxyntomodulin-based, longer lasting GLP-1 and glucagon receptor (GLP-1R/GCGR) co-agonists, which stimulate significant improvements in glycaemic control and weight loss in animal models (Day et al., 2009, Druce et al., 2009, Pocai et al., 2009, Liu et al., 2010, Santoprete et al., 2011, Lynch et al., 2014b). Longer lasting oxyntomodulin analogues have superior weight loss than GLP-1 in preclinical studies, while maintaining the beneficial effects of GLP-1 on glucose homeostasis (Sadry and Drucker, 2013). However, tailoring the balance of agonism at the glucagon and GLP-1 receptor appropriately is critical to maintain beneficial glycaemic effects (Day et al., 2012).

1.8 Combination therapy involving GLP-1

As mentioned previously, there is great interest in developing combination therapies with GLP-1 (Vilsboll et al., 2002, Flatt, 2008, Knop and Taylor, 2013). Combining GLP-1 with another hormone could stimulate multiple metabolic pathways simultaneously, thereby increasing the therapeutic effect while avoiding the side effects seen at high doses (Sadry and Drucker, 2013, Lund et al., 2014).

To administer the effects of multiple hormones simultaneously, unimolecular co-agonists can be used, namely single molecules that activate multiple receptors. This is predicted to lower the incidence of side-effects seen due to hyper-activation of one given receptor or pathway, hence widening the therapeutic window and increasing the maximum tolerated dose for drugs (Finan et al., 2013, Portron et al., 2017). In comparison to giving two individual agonists, unimolecular co-agonists are simple to administer. Furthermore, regulatory aspects of development are simplified as only one molecule needs to be tested. Safety concerns regarding co-agonists include an increased risk of cross reactivity at similar receptors, leading to off target effects, as well as the possibility of preferential degradation of activity at one receptor versus the other, which would in turn change the pre-designed relative activity ratios. While there is no long-term data available to address these safety concerns for GLP-1, GIP and glucagon receptor co-agonists, preliminary results from Phase II clinical trials are promising (Ambery et al., 2018).

Results from gastric bypass studies indicate a role for enhanced GIP levels in the remission of diabetes, suggesting GLP-1 and GIP receptor co-agonism could have potential as a treatment for diabetes. The second adjunct proposed with GLP-1 is glucagon, as evidenced from the beneficial metabolic effects of oxyntomodulin.
1.9  GIP as a co-adjunct with GLP-1

Glucose-dependent insulinotropic peptide, or GIP, is a potent incretin hormone secreted postprandially from K cells of the upper small intestine (Takeda et al., 1987, Inagaki et al., 1989). GIP was originally isolated on the basis of its ability to inhibit gastric acid secretion, but later discovered to stimulate insulin secretion through direct actions on pancreatic beta cells (Brown et al., 1970, Dupre et al., 1973, Taminato et al., 1977, Adrian et al., 1978). GIP also has anti-apoptotic effects and stimulates beta cell survival proliferation (Trumper et al., 2001, Trumper et al., 2002, Ehses et al., 2003). Genetic knockdown studies have linked GIP to fat uptake and accumulation in adipose tissues, thereby associating GIP with obesity (Beck and Max, 1983, Miyawaki et al., 2002).

1.9.1  GIP in diabetes

As an incretin, GIP is the obvious candidate for co-administration with GLP-1. However, GIP loses its insulinotropic effect under hyperglycaemic conditions, and has previously been shown to cause spikes in blood glucose levels in patients with diabetes (Vilsboll et al., 2002).

In T2DM, the secretion of GIP remains unaltered but the insulinotropic effect of GIP is severely reduced (Elahi et al., 1994b, Toft-Nielsen et al., 2001a, Calanna et al., 2013). The insulinotropic effect of GIP is particularly diminished in patients with sub-optimally controlled diabetes, (Krarupe et al., 1987, Jones et al., 1987, Jones et al., 1989, Nauck et al., 1993a, Elahi et al., 1994b), with one study demonstrating that a GIP infusion worsens hyperglycaemia in patients (Chia et al., 2009). This is attributed to a lowering of GLP-1 secretion and enhancement of glucagon secretion (Chia et al., 2009). In contrast to GLP-1, administering supraphysiological levels of GIP fails to improve insulin secretion and restore the incretin effect in T2DM (Vilsboll et al., 2002, Vilsboll et al., 2003).

Given that the impaired GIP response is secondary to diabetes, it might be attributed to certain metabolic aspects of diabetes, in particular persistent hyperglycaemia. Accordingly, a small number of studies suggest that the incretin effect can be restored following the resolution of hyperglycaemia. For instance, Hojberg et al. (2007) showed that a 4 week pre-treatment with exogenous insulin lowered HbA1c levels, and consequently improved beta cell response to GIP by three to four fold. GIP efficacy is restored prior to remission from diabetes, as the glucose response itself is not normalised within 4 weeks of insulin therapy (Hojberg et al., 2009). These results were supported by a second study which showed restoration of the GIP response by treatment with a sulfonylurea prior to GIP infusion (Aaboe et al., 2009). However, these studies involved 8 and 12 patients respectively, making it difficult to draw universal conclusions.
1.9.2 GIP in the pathogenesis of obesity

In addition to reduced insulinotropic efficacy in T2DM patients, GIP has a postulated link to adiposity and obesity, explaining why GIP remained unexplored as a therapeutic for several years. A seminal study published in 2007 showed that GIP receptor knockout mice are protected from diet-induced obesity (Hansotia et al., 2007). Administration of a GIP-receptor antagonist, (Pro³)GIP, led to improved glucose tolerance and insulin sensitivity in ob/ob mice (Montgomery et al., 2010), further supporting the theory that GIP is obesogenic. Elevated GIP levels have been reported in obese patients with type 2 diabetes (Creutzfeldt et al., 1978), and a recent human study has indicated a mechanistic role for GIP in fat deposition, particularly in visceral depots (Wang et al., 2017).

However, these findings have been challenged by a study showing protective effects of GIP overexpression on diet-induced obesity in mice (Kim et al., 2012). Similarly, multiple long term studies have shown beneficial glycaemic effects of GIP analogues in rodents (Hinke et al., 2002, Widenmaier et al., 2010, McIntosh et al., 2009, Gault et al., 2011). The original knockout studies on the GIP receptor are also being questioned, and the GIP antagonist used to validate anti-obesity effects is now proposed to be a weak agonist at the GIP receptor (Faivre et al., 2012, Sparre-Ulrich et al., 2016). Research into GIP agonism for treating diabetes has therefore resumed, with multiple GLP-1/GIP receptor co-agonists in Phase I and Phase II clinical trials (Tschop and DiMarchi, 2012, Finan et al., 2013, Frias et al., 2017).

1.9.3 Co-administering GLP-1 and GIP

Synthetic GIP analogues, resistant to DPP-IV, are postulated to exhibit therapeutic potential, as described in O’Harte et al. (1999). Combining GLP-1 with GIP would be an even better therapeutic strategy: GLP-1 would restore blood glucose levels to normal, restoring GIP efficacy and hence enabling anti-diabetic actions of both incretins. This would ideally lead to an additive insulinotropic response similar to that seen physiologically, without engaging the adverse side effects seen at higher doses of GLP-1 (Nauck et al., 1993a). While GIP does stimulate glucagon secretion, even in patients with diabetes, this effect is glucose-limited and would therefore be negligible in patients with controlled glucose levels (Meier et al., 2003, Chia et al., 2009, Christensen et al., 2011, Christensen et al., 2014). Furthermore, the glucagonotropic effect of GIP could hypothetically enhance weight loss by increasing energy expenditure, as suggested by preclinical work on GIP and GLP-1 receptor co-agonism (Finan et al., 2013, Norregaard et al., 2018).
While studies in mice with targeted lesions of the both GLP-1 and GIP receptors suggest that activation of the two receptors is “additive” in terms of glycaemic effects (Hansotia and Drucker, 2005), Mentis et al. (2009) showed that co-infusing GLP-1 and GIP had no acute beneficial effects on insulin secretion beyond those of GLP-1, even though GIP independently increased insulin secretion (Mentis et al., 2009). However, recent human studies have consistently found promising results regarding the efficacy of GLP-1/GIP receptor co-agonists in vivo (Finan et al., 2016, Frias et al., 2017, Portron et al., 2017).

1.9.4 Preclinical work and clinical progress of GLP-1 and GIP receptor co-agonists

Longer lasting GLP-1/GIP receptor co-agonists have been tested in preclinical studies, and show a greater weight loss and glucose-lowering than GLP-1 receptor agonists in multiple species. In patients with T2DM, co-agonists lead to dose dependant reductions in HbA1c levels following 6 week administration. Furthermore, direct comparison studies in healthy volunteers found that co-agonists are more effective at improving insulin sensitivity and glucose tolerance then market leader liraglutide (Finan et al., 2013). A recent phase II clinical trial investigated a distinct balanced, acylated GLP-1/GIP receptor co-agonist, in patients with inadequately controlled T2DM, and demonstrated that this dual agonist was safe, well tolerated, and improved glycaemic control and reduced body weight to a similar extent as clinical doses of liraglutide (Frias et al., 2017). Similar glycaemic improvements have been reported for a third co-agonist developed by Novo Nordisk, which is currently in Phase II clinical trials (Portron et al., 2017, Schmitt et al., 2017).

1.10 Glucagon as a co-adjunct with GLP-1

Glucagon has been proposed as an alternative co-adjunct with GLP-1. GLP-1, oxyntomodulin and glucagon are all products of the pre-pro-glucagon gene and show substantial sequence homology. As mentioned above, glucagon is secreted by α cells of pancreatic islets and is traditionally considered to be the hormone that opposes the action of insulin in peripheral tissues, particularly the liver. The relative ratio of insulin to glucagon determines the rate of gluconeogenesis (de novo synthesis of glucose) and glycogenolysis (breakdown of glycogen reserves in the liver), and hence regulates plasma glucose levels (Cryer, 2014).

Glucagon release is stimulated by hypoglycaemia, and inhibited by hyperglycaemia, insulin, and GLP-1. In direct contrast to GLP-1, glucagon increases glycogenolysis and hepatic glucose output, leading to a rise in blood glucose levels (Orskov, 1992). Glucagon is therefore expected to exacerbate
chronic hyperglycaemia in people with diabetes, and glucagon antagonists have been proposed as potential drugs for diabetes (Scheen et al., 2017). However, co-administering GLP-1 and glucagon has been shown to bypass the hyperglycaemic effects of glucagon, while retaining glucagon’s positive metabolic effects (Tan et al., 2013, Cegla et al., 2014). As GLP-1 and glucagon receptor co-agonism can have beneficial glycaemic effects, glucagon is considered a suitable adjunct for diabetes treatment.

In addition to its glycaemic effects, glucagon stimulates a range of beneficial metabolic outcomes. Acute glucagon administration reduces food intake in animals and humans (Davidson IWF, 1960, Goodridge and Ball, 1965, Heckemeyer et al., 1983, Woods et al., 2006), while chronic exposure leads to enhanced lipolysis and weight loss (Salter, Chan et al., 1984, Geary, 1990, Geary et al., 1993). Glucagon also enhances energy expenditure, as shown using pair feeding studies in rodents and indirect calorimetry in humans (Salter et al., 1957, Tan et al., 2013, Cegla et al., 2014, Salem et al., 2016). One human study showed that, while there was no significant change in resting energy expenditure during a GLP-1 infusion, energy expenditure increased significantly during glucagon infusion by an average of 146.99 kcal/day. This energy expenditure effect was retained on co-infusing GLP-1 and glucagon (Tan et al., 2013). As glucagon can induce weight loss through a mechanism distinct from the anorectic effects of GLP-1, coupling the two together could lead to synergistic weight loss, which would be of particular therapeutic value in obese diabetic patients.

1.10.1 Co-administering GLP-1 and glucagon

The rationale behind GLP-1 and glucagon combination therapy is to combine the energy expenditure and lipolytic effects of glucagon with anorectic and insulinotropic effects of GLP-1, yielding a drug that improves insulin secretion and has superior weight loss. Over time, the enhanced weight loss would improve insulin sensitivity, hence further improving the glycaemic profile.

The interest in GLP-1 and glucagon receptor (GLP-1R/GCGR) co-agonists stems from the promising metabolic profile of oxyntomodulin, as detailed above in Section 1.7. Oxyntomodulin has beneficial glycaemic effects in vivo (Wynne et al., 2005, Kosinski et al., 2012), which have since been replicated and improved upon in a number of studies involving synthetic GLP-1R/GCGR co-agonists.

1.10.2 Preclinical work into GLP-1 and glucagon receptor co-agonists

Given the sequence homology between GLP-1 and glucagon, particularly at the N terminal region, and the structural similarity between their receptors, it is possible to create a single molecule that
activates both the GLP-1 and glucagon receptors. Multiple research groups have developed GLP-1R/GCGR co-agonists, and proved that it is practically possible to make dual agonists.

The seminal paper in this field was by Pocai et al. (2009), who developed a DPP-IV-resistant GLP-1R/GCGR co-agonist using oxyntomodulin as their foundation. To improve pharmacokinetics, a cholesterol moiety and mini polyethylene glycol (PEG) spacer were incorporated, which enhanced binding to plasma proteins and hence increased half-life in circulation. The study reported superior weight loss in diet-induced obese (DIO) mice following chronic treatment with the co-agonist in comparison to a GLP-1R-specific agonist. Furthermore, the co-agonist improved glucose tolerance, and induced more pronounced increases in plasma insulin levels than a GLP-1R agonist.

This study was followed shortly by a paper by Day et al. (2009), showing similar results for a different oxyntomodulin derivative. Day et al. (2009) developed a glucagon-based peptide, with enhanced activity at the GLP-1 receptor. An α-amino-isobutyric acid (Aib) substitution was made at the DPP-IV cleavage site to reduce proteolytic degradation of the peptide, and a 40 KDa linear PEG moiety was added to enhance plasma half-life. In preclinical studies, this compound showed significant weight loss, increased energy expenditure, and improved hepatic steatosis when compared with a selective GLP-1R agonist in DIO mice.

Recently, a new GLP-1R/GCGR co-agonist has been developed with site-specific substitutions to reduce degradation, and a fatty acid side chain to facilitate binding to albumin for an increased half-life. The co-agonist has superior weight loss and comparable glucose lowering to current market leader liraglutide in DIO mice. This study is particularly significant as the superior weight loss seen in rodents translated to healthy non-human primates, although no changes in fasting glucose or insulin levels were observed in the latter. This might reflect on study design rather than drug potency, as they were testing on healthy cynomolgus monkeys who are expected to have robust glycaemic control (Henderson et al., 2016). This co-agonist has since been tested in Phase II clinical trials, where it is effective at enhancing glucose tolerance and inducing weight loss (Ambery et al., 2018).

These independent investigations provide proof of concept that GLP-1R/GCGR co-agonists have significantly greater weight loss than pure GLP-1R agonists, and have beneficial or neutral glycaemic effects (Maida et al., 2008, Day et al., 2009, Pocai et al., 2009, Henderson et al., 2016, Ambery et al., 2018). Multiple pharmaceutical companies have therefore developed GLP-1R/GCGR co-agonists for therapeutic use, and are currently in Phase 1 or Phase 2 clinical trials (Ambery et al., 2018, Tillner et al., 2018, Brandt et al., 2018). However, research to date focuses primarily on the weight loss effects of these co-agonists, and the mechanism behind their glycaemic effects remains unexplored.
1.10.3 Mechanism for GLP-1R/GCGR co-agonists mediated insulin release

The mechanism behind the insulinotrop effect of GLP-1R/GCGR co-agonists has not been determined. Understanding their mechanism of action, and physiological relevance, can lead to the development of novel anti-diabetes treatments.

Using glucagon to reduce hyperglycaemia may seem counter-intuitive, but a variety of mechanisms have been proposed to explain this. One explanation is that sustained glucagon receptor activity could potentiate GLP-1-mediated insulin release. As GLP-1’s insulinotropic effects depend on the blood glucose levels, they tend to be self-limiting (Samols et al., 1966). The hyperglycaemia brought about by glucagon could therefore help prolong GLP-1’s insulinotropic effect (Kreymann et al., 1987, Tan et al., 2013). Furthermore, glucagon itself has been shown to stimulate insulin secretion during intravenous infusion in human volunteers (Samols et al., 1965). This may be an indirect effect, by increasing glucose availability and hence glucose stimulated insulin secretion, or a direct effect on beta cells (Samols et al., 1966, Christophe, 1996, Huypens et al., 2000). However, the physiological relevance of this remains unclear.

It is also unclear whether the direct action of glucagon on beta cells is mediated by the glucagon or the GLP-1 receptor. It has been postulated that the insulinotropic effects of glucagon are mediated by the glucagon receptor itself (Kawai et al., 1995, Huypens et al., 2000). However, while glucagon receptors are expressed on beta cells, the potency of glucagon at stimulating insulin secretion in purified rat islet cells is several orders of magnitude lower than the potency of GIP and GLP-1 (cAMP half-maximal concentrations of 1nM compared to 50 and 10 pM respectively) (Moens et al., 1996a).

1.10.4 Human trials on GLP-1 and glucagon receptor co-administration

While there is a dearth of studies looking at insulinotrop effects of GLP-1R/GCGR co-agonists, an isolated human study has reported that co-infusion of native GLP-1 and glucagon leads to beneficial metabolic effects in vivo. This key study by Tan et al. (2013) investigated the effects GLP-1 and glucagon co-infusion in comparison to individual administration, in overweight or obese volunteers without diabetes. As could be expected, GLP-1 reduced plasma glucose levels beyond placebo, and induced a rise in plasma insulin levels, while glucagon led to a rise in blood glucose levels and a correspondent increase in plasma insulin. Co-infusing GLP-1 with glucagon partially blunted the hyperglycaemic effect of glucagon, and led to a synergistic insulin response which was 2.4 and 6 fold higher than that of glucagon and GLP-1 respectively. However, any conclusions about mechanism are limited by the confounding factor of hepatic glucose output in vivo. The relative insulin levels in
the different treatment groups mirror the relative blood glucose levels, suggesting that the increased insulin release seen on co-infusion of the two peptides could merely be in response to the increased blood glucose levels resulting from glucagon action on the liver. In order to ascertain the mechanism whereby GLP-1 and glucagon work synergistically, a reductive approach is required, looking at the direct action of the two hormones on beta cells.

**Aims:** This project aims to establish the effect of GLP-1R/GCGR co-agonists on insulin secretion compared to GLP-1R agonists, to investigate the underlying mechanism, and to investigate the effect of co-agonists on appetite regulation and glucose tolerance *in vivo*.
2. Materials and Methods
2.1 Peptides

GLP-1 (7-36) NH₂, glucagon, oxyntomodulin, and exendin-4 were purchased from Bachem, Ltd. (Merseyside, UK). GLP-1 (7-36) NH₂ will henceforth be referred to as GLP-1. Liraglutide was purchased from Imperial College Healthcare NHS Trust.

GLP-1R/GCGR co-agonists were synthesised by and purchased from Insight Biotechnology Limited (Middlesex, UK). Peptides were synthesised using solid phase peptide synthesis, with each amino acid sequentially added from the C to the N terminus. Peptides were then purified using reverse phase preparative high performance liquid chromatography (HPLC), followed by lyophilisation. Peptide purity was determined by reverse-phase HPLC and by matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS). All peptides supplied had >95% purity. Peptide sequences are detailed in Appendix A.

Lyophilised aliquots were reconstituted for each experiment.

2.2 In Vitro Assays

2.2.1 Cell culture and maintenance

Chinese Hamster Ovary (CHO) K1 cells, over expressing the human GLP-1 receptor or the rat glucagon receptor, were produced and validated by previous lab members. Human embryonic kidney (HEK) cells expressing the rat GLP-1 receptor were a gift from Professor Bernard Thorens (University of Lausanne, Switzerland). CHO-K1 cells stably over expressing the human glucagon receptor were purchased from Invitrogen Life Technologies (Paisley, UK). INS-1 823/3 cells were a gift from Professor Christopher Newgard (Duke University, USA). MIN6B1 cells were a gift from Professor Philippe Halban (University of Geneva, Switzerland). The human pancreatic cell line, EndoC-βH1, was a gift from Professor Guy Rutter (Imperial College London). PathHunter CHO-GLP-1R β arrestin-1 and β arrestin-2 reporter cell lines were purchased from DiscoveRx. MIN6B1 cells stably expressing N-terminal snap-tagged human GLP-1 receptor were a gift from Dr Alejandra Tomas (Imperial College London, UK).

All cell lines were maintained at 37°C, with 5% CO2. The constitution of the media is as follows. INS-1 823/3, MIN6B1 and EndoC-βH1 cell lines were cultured as per Groot Nibbelink et al. (2016) and
Ravassard et al. (2011). All cell culture reagents were purchased from Invitrogen Life Technologies (Paisley, UK), unless specified otherwise.

**CHO-K1 cells over-expressing the human glucagon receptor or rat glucagon receptor**

- Dulbecco’s modified medium
- 4.5 g/l glucose
- 1% antibiotic (100 U/ml Penicillin and 100 μg/ml Streptomycin)
- 10% dialyzed foetal bovine serum
- 0.1 mM non-essential amino acids (Sigma-Aldrich)
- 25 mM HEPES (pH 7.3) (Sigma-Aldrich)

**CHO-K1 cells over-expressing the human GLP-1 receptor**

- Dulbecco’s modified medium
- 4.5 g/l glucose
- 1% antibiotic (100U/ml Penicillin and 100ug/ml Streptomycin)
- 10% dialyzed foetal bovine serum
- 0.1 mM non-essential amino acids (Sigma-Aldrich)
- 25 mM HEPES (pH 7.3)
- 200 μg/ml Genetecin

**HEK cells over-expressing the rat GLP-1 receptor**

- Dulbecco’s modified medium
- 4.5 g/l glucose
- 1% antibiotic (100U/ml Penicillin and 100ug/ml Streptomycin)
- 10% dialyzed foetal bovine serum

**INS-1 823/3 cells**

- RPMI-1640 11mM D-glucose
- 1% antibiotic (100U/ml Penicillin and 100ug/ml Streptomycin)
- 10% dialyzed foetal bovine serum
- 2 mM L-glutamine
- 10 mM HEPES
- 1 mM sodium pyruvate
• 0.05 mM 2-mercaptoethanol

**MIN6B1 cells**

- Dulbecco’s modified medium
- 4.5 g/l glucose
- 1% antibiotic (100U/ml Penicillin and 100ug/ml Streptomycin)
- 15% dialyzed foetal bovine serum
- 0.05 mM 2-mercaptoethanol
- 2 mM L-glutamine

**EndoC-βH1 cells**

- Dulbecco’s modified medium
- 1 g/L glucose
- 1% antibiotic (100U/ml Penicillin and 100ug/ml Streptomycin)
- 2% Albumin from bovine serum fraction V (BSA)
- 0.05 mM 2-mercaptoethanol
- 10 mM nicotinamide
- 5.5 μg/ml transferrin
- 6.7 ng/ml sodium selenite

**PathHunter CHO-GLP-1R and CHO-GCGR cells expressing β arrestin-1 and -2**

- Complete Culture medium 6 (DiscoveRx)

**MIN6B1 cells stably expressing N-terminal snap-tagged human GLP-1 receptor**

- Dulbecco’s modified medium
- 4.5 g/l glucose
- 1% antibiotic (100U/ml Penicillin and 100ug/ml Streptomycin)
- 15% dialyzed foetal bovine serum
- 0.05 mM 2-mercaptoethanol
- 1 mg/ml Geneticin G418
2.2.2  cAMP accumulation bioactivity assay

cAMP levels were measured using a HTRF® (Homogeneous Time-Resolved Fluorescence) based cAMP assay (cAMP Dynamic 2 kit, Cisbio Bioassays (Codolet, France)), which uses a competitive immunoassay to measure intracellular cAMP levels. A SpectraMax i3x plate reader (Molecular Devices) installed with an HTRF cartridge was used to read the assays. Protocols used were as per kit instructions, and an eight point standard curve was included in each experiment.

2.2.2.1  Determination of cAMP EC$_{50}$ values of co-agonists at glucagon and GLP-1 receptors

The following method was used to measure cAMP synthesis in all receptor overexpressing cell lines. Cells were plated at a density of 10,000 cells/well in serum free Dulbecco’s modified medium (DMEM), in white half area 96 well plates. The indicated concentration range of peptide was prepared using serum-free DMEM containing 100 μM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX).

Cells were incubated with 25 μL of test peptide in duplicate for 30 minutes at 37°C, after which the assay was terminated by addition of HTRF detection reagents reconstituted in the manufacturer’s cAMP lysis buffer. After 60 minutes incubation with the detection reagents at room temperature, the plates were read in homogenous format.

Data were analysed by 4-parameter fitting using Graphpad Prism 7.0. Results presented as means with SEM.

2.2.2.2  16 hour concentration response experiments with INS-1 823/3 cells

Cells were plated overnight, at a density of 20,000 cells/well in complete media, in white half area 96 well plates. On the day of the assay, the cells were serum starved for 60 minutes in serum-free RPMI with 3 mM of glucose. Test peptides were prepared in reduced serum RPMI (3% FBS, 11 mM glucose).

Cells were incubated in a volume of 20 μL of test peptides, in duplicate for 16 hours at 37°C. After 16 hours, 5 μL of IBMX (final concentration 0.25 mM) was added to each well, prepared in reduced serum RPMI (3% FBS, 11 mM glucose) at 5 times the indicated final concentration. Following a further 10 minute incubation, the assay was terminated by addition of HTRF detection reagents, reconstituted in the manufacturer’s cAMP lysis buffer. The plates were read in homogenous format after 60 minutes incubation with the detection reagents at room temperature.
Data were analysed by 4-parameter fitting using Graphpad Prism 7.0. Results are expressed normalised to the cAMP response to basal media (11 mM glucose), and expressed as means with SEM.

### 2.2.3 Insulin Secretion Assays

Insulin secretion assays were performed as described below on INS-1 823/3, MIN6B1 and EndoC-βH1 cells. Insulin levels were quantified using a HTRF® based insulin assay (Cisbio Bioassays (Codolet, France)). The sandwich immunoassay measures fluorescent resonance energy transfer (FRET) between two fluorescent monoclonal antibodies on binding on insulin. The FRET signal intensity is proportional to insulin concentrations, and is used to quantify insulin levels in the sample. A SpectraMax i3x plate reader (Molecular Devices) installed with an HTRF cartridge was used to read the assays, and a standard curve was run in each experiment as per the standardised protocol.

#### 2.2.3.1 Concentration response experiments with INS-1 823/3 and MIN6B1 cells

Cells were cultured in T75 flasks in their respective media. Prior to the assay, cells were washed and incubated in low glucose (3 mM) RPMI medium overnight. On the day of the experiment, cells were plated at a density of 30,000 cells/well in complete RPMI media (11 mM glucose), in 96 well plates. Test peptides were prepared in RPMI medium (11 mM glucose), and added to duplicate wells, to achieve a final concentration of 1 μM to 1 pM.

After a 16 hour incubation, the plates were removed, and gently agitated on a plate shaker for 5 minutes to ensure homogenous distribution of insulin secreted into the supernatant. 10 μL aliquots of supernatant were removed from each well, and diluted 1:10 in Hank’s balanced salt solution (HBSS) supplemented with 0.1% BSA. Cells were lysed by addition of 10 μL of 10% TritonX-100 to the cells plates, and lysate samples diluted as appropriate in HBSS supplemented with 0.1% BSA.

Insulin release into the supernatant and total well insulin content was measured using the Insulin High Range Assay Kit (Cisbio Bioassays (Codolet, France)), as per kit instructions. Percentage insulin secretion was calculated per well, and then normalised to the average response to 11 mM glucose alone for each experiment. This resulting ratio is referred to as the ‘insulin simulation index’ or ‘fold insulin release’.

Data were analysed by 4-parameter fitting using Graphpad Prism 7.0, and expressed as means with SEM.
2.2.3.2 Concentration response experiments with EndoC-βH1 cells

Cells were plated at a density of 70,000 cells/well in complete media, in white half area 96 well plates two days prior to the assay. The night before the assay, the cells were washed and incubated overnight with low glucose media (EndoC-βH1 growth medium as detailed above, but with 2.8mM glucose). On the day of the assay, the cells were serum starved for 60 minutes in Krebs-Ringer solution (115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂·2H₂O, 10 mM HEPES and 0.2% BSA) supplemented with 0.5 mM glucose.

Test peptides were prepared in Krebs-Ringer solution, supplemented with 15 mM glucose and 0.25 mM IBMX. Following the 60 minute pre-incubation, the cells were washed and incubated in a volume of 100 μL of test peptides, in duplicate for 16 hours at 37°C. After 16 hours, 50 μL aliquots of supernatant were removed from each well. The samples were centrifuged at 3000 rpm for 5 minutes at 4°C to remove any cellular debris, and the supernatant was recovered. Samples were diluted as appropriate in Krebs-Ringer solution. Insulin release into the supernatant was measured using the Insulin Ultra-Sensitive Assay Kit (Cisbio Bioassays (Codolet, France)).

The cells were lysed by adding 50 μL of cell lysis buffer (Tris pH 8.0, 0.02M, 0.1% Triton X-100, 0.137 M NaCl, 0.002M EGTA), containing antiprotease, to each well in the cell plate. The samples were agitated on a plate shaker for 5 minutes to lyse the cells, and then centrifuged at 3000 rpm for 5 minutes at 4°C. The supernatant (lysate) was recovered to measure total insulin content. Lysate samples were diluted as appropriate in kit diluent, and insulin content measured using the Insulin High Range Assay Kit (Cisbio Bioassays (Codolet, France)), as per kit instructions.

Percentage insulin secretion was calculated per well, and then normalised to the average response to 11 mM glucose alone for each experiment. This resulting ratio is referred to as the ‘insulin simulation index’ or ‘fold insulin release’. Data were analysed by 4-parameter fitting using Graphpad Prism 7.0, and expressed as means with SEM.

Statistical Analyses

Intra-experimental replicates were averaged to give a single result per experiment, and the n number represents the total individual experiments. Randomised block ANOVA were used to calculate significance as the experimental conditions were matched. Post hoc tests are indicated in figure legends.
2.2.4 Bias Measurements

2.2.4.1 β Arrestin Recruitment in PathHunter reporter cell lines

PathHunter CHO-GLP-1R and CHO-GCGR β arrestin-1 and β arrestin-2 cells were plated overnight at a density of 10,000 cells/well, in CP2 medium (DiscoverX), in 96 well half area white plates. On the day of the assay, medium was removed, and test peptides (2 x 10^{-5} to 10^{-10}M) prepared in CP2 added to the cell plates, in a volume of 20 μL. Each condition was tested in duplicate. Following a 30 minute incubation at 37°C, the assay was terminated by adding 10 μL of PathHunter detection reagents. After a 60 minutes incubation with detection reagents at room temperature, plates were read using a SpectraMax i3x plate reader (500 ms integration time). Data were analysed by 4-parameter fitting using Graphpad Prism 7.0. Results were normalised to the maximal arrestin response to the native ligand (GLP-1 or glucagon) for each test system, and expressed as means with SEM.

2.2.4.2 cAMP accumulation assays in PathHunter reporter cell lines

Cells were plated at a density of 20,000 cells/well in CP2 (DiscoverX), in white half area 96 well plates. Cells were incubated with test peptide (2 x 10^{-8} to 10^{-13}M) prepared in CP2 (DiscoverX), for 30 minutes at 37°C. Each condition was tested in duplicate. Following a 30 minute incubation, the assay was terminated by addition of HTRF detection reagents reconstituted in the manufacturer’s cAMP lysis buffer (cAMP Dynamic 2 kit, Cisbio Bioassays (Codolet, France)). After a 60 minutes incubation with detection reagents at room temperature, the plates were read in homogenous format. Data were analysed by 4-parameter fitting using Graphpad Prism 7.0. Results were normalised to the maximal cAMP response to the native ligand (GLP-1 or glucagon) for each test system, and expressed as means with SEM.

2.2.4.3 Quantification of Bias

To quantify the degree of bias between cAMP, β arrestin-1 and β arrestin-2 pathways, the Black and Leff Operation model was used (Kenakin and Christopoulos, 2013, van der Westhuizen et al., 2014). Concentration response data from the cAMP and β arrestin assays was fitted to the following equation, as per the aforementioned model:

\[
Response = \text{basal} + \frac{(E_{\text{max}} - \text{basal}) \cdot \frac{[A]}{K_A}^n \cdot [A]^n}{[A]^n \cdot \frac{1}{K_A}^n + (1 + \frac{[A]}{K_A}^n)^n}
\]
Basal is the basal response of the system and \( E_{\text{max}} \) is the maximal response. \([A]\) is molar agonist concentration, \( K_A \) is the dissociation constant for that specific pathway, and \( n \) is the transducer slope which links occupancy to response. \( \tau \) is the efficacy of the agonist as defined in the model.

\( E_{\text{max}} \) was defined as the maximal response to control agonist (GLP-1 or glucagon) for each experiment. As per the operational model, \( E_{\text{max}}, n, \) and basal are system-specific parameters, and were hence fit globally.

Concentration response data obtained from the experiments were used to quantify a value for \( \log(\tau/K_A) \), the ‘transduction coefficient.’ This is a measure of the efficacy of an agonist at activating a specific pathway. \( \log(\tau/K_A) \) values for each agonist at each given pathway were normalised to the benchmark peptide by subtracting, to give \( \Delta \log(\tau/K_A) \). To determine the bias between two pathways for each agonist, \( \Delta \log(\tau/K_A) \) of one pathway were subtracted from the other, giving the bias factor \( \Delta \Delta \log(\tau/K_A) \).

Where possible, cAMP and β arrestin assays were performed in parallel to minimise variation. As bias can be time dependant, the assays were all conducted for 30 minutes. Each assay was analysed independently to calculate the bias factor.

**Statistical Analyses**

Intra-experimental replicates were averaged to give a single result per experiment, and the \( n \) number represents the total individual experiments. Randomised block ANOVA or paired t-tests were used to calculate significance as the experimental conditions were matched. Post hoc tests are indicated in figure legends.

### 2.2.5 Microscopy experiments with SNAP-GLP-1R cells

MIN6B1 cells, stably expressing N-terminal-SNAP-tagged GLP-1R (pSNAP-GLP-1R, Cisbio Bioassays (Codolet, France)) were used for confocal microscopy.

Cells were seeded overnight, at a density of 100,000 cells per well, onto glass coverslips in 12 well plates in complete growth medium. Following overnight incubation, cells were covalently labelled using 1 \( \mu \)M of probe, SNAP-Surface-488 (New England Biolabs), for 1 hour in complete medium at 37°C. The probe specifically labels SNAP-GLP-1Rs present on the cell surface.

After a 1 hour incubation, cells were washed thrice with phosphate buffered saline (PBS), and then incubated with 100 nM of test peptides for 15 minutes at 37°C. Medium was then removed, cells washed once with PBS, and fixed using a 20 minute incubation with 4% paraformaldehyde (in PBS) at
4°C. Coverslips were mounted for imaging in Vectashield Hardset with 4′, 6-Diamidine-2′-phenylindole dihydrochloride (DAPI). Images were acquired with a Zeiss LSM-780 inverted confocal laser-scanning microscope with a 63x/1.4 numerical aperture oil-immersion objective and analysed using Image J software.

2.3 In Vivo Experiments

All animal procedures were approved by the British Home Office Animal (Scientific Procedures) Act 1986.

Project license: 70/8068 (rat studies) and P0A6474AE (mouse studies).

2.3.1 Acute Mouse Studies

Regular Chow-fed Mice: Male C57BL/6J mice (8-10 weeks) were purchased from Charles River, Margate, and group housed (5 per cage). Mice were kept at controlled temperature (21–23°C), with a 12:12 hour light to dark schedule (lights on at 7 a.m.), and handled regularly to minimise stress. The animals were provided ad libitum access to water and kept on a standard RM1 chow diet (Special Diet Services, Witham, UK). Mice were acclimatised for at least one week prior to experimental onset, and had a minimum of a one week wash out period between experiments.

High Fat Diet Fed Mice: Male C57BL/6J mice (4-6 weeks) were purchased from Charles River, Margate, and group housed (3 per cage). Mice were kept at controlled temperature (21–23°C), with a 12:12 hour light to dark schedule (lights on at 7 a.m.), and handled regularly to minimise stress. The animals were provided ad libitum access to water and kept on a high fat, high sucrose (HFHS) diabetogenic diet (AIN-76A, TestDiet) for 8-12 weeks prior to experiments. Mice were single-housed before commencing the feeding study. Mice were acclimatised for at least one week prior to experimental onset, and had a minimum of a one week washout period between experiments. This mouse model is referred to as ‘DIO mice’ in the thesis.

2.3.1.1 Assessment of effect of peptide on blood glucose levels

Mice were weighed and fasted overnight (12 hours) prior to the procedure, and randomised to treatment groups to ensure that average body weight was consistent across all groups. Peptides were prepared at the indicated concentrations in saline (0.9% NaCl). Mice were injected intra-peritoneally (i.p.) with 20 μL of saline or test peptide at the indicated dose. Lateral tail vein
venesection was carried out, and tail vein bleeds performed just before peptide injection, and at 60 and 120 minutes after injections. Blood glucose levels were monitored using a Freestyle Optium Neo glucose meter (Abbott Diabetes Care, Alameda, CA), and 20 μL blood samples were taken for insulin measurement. Samples were collected into lithium heparin-coated microvette tubes, centrifuged for 8 minutes at 10,000 g, and plasma separated and analysed for insulin levels (Mouse Serum Insulin Kit, Cisbio Bioassays (Codolet, France)).

The procedure was repeated after a week’s washout period, with an identical protocol except that the animals were fed instead of fasted.

2.3.1.2 Intra-Peritoneal Glucose Tolerance Tests

Mice were weighed and fasted overnight (12 hours) prior to the procedure, and randomised to treatment groups to ensure that average bodyweight was consistent across all groups.

Peptides were prepared at the indicated concentrations in saline (0.9% NaCl). Mice were injected i.p. with 20 μL of saline or test peptide at the indicated dose. Mice were then injected i.p. with a weight-adjusted dose of 2 g/kg of D-glucose, as a 20% dextrose solution, either immediately after the peptide or after a 4 hour delay (maximum injection volume of 10 mL/kg). Tail vein bleeds were performed just before peptide and glucose injections, and at the indicated time points after injections. Blood glucose levels were monitored using a Freestyle Optium Neo glucose meter (Abbott Diabetes Care, Alameda, CA).

At indicated time points, 20 μL blood samples were also taken for insulin measurement. Samples were collected into lithium heparin-coated microvette tubes, centrifuged for 8 minutes at 10,000 g, and plasma separated and analysed for insulin levels (Mouse Serum Insulin Kit, Cisbio Bioassays (Codolet, France)).

For procedures with high fat diet fed mice, the protocol remained identical but the glucose load was lowered to 1.5 g/kg of body weight.

2.3.1.3 Acute Feeding Studies

Animals were weighed and fasted at 7 a.m. on the morning of the study, and randomised to treatment groups, ensuring that average bodyweight was consistent across all groups. The study started at 7 p.m., following 12 hours starvation, to coincide with the beginning of the dark phase.

Peptides were reconstituted to the indicated concentrations using saline (0.9% NaCl). Mice received a single subcutaneous injection of 20 μL of either peptide or saline, using a 0.5 mL insulin syringe and
29 gauge needle. After the injection, mice were returned to their cage and given access to a pre-weighted amount of food. The food remaining in the hopper (including any pieces of food found on the cage floor) was measured for up to 24 hours post injection, at regular intervals as indicated, using a balance accurate to 0.01 g. Mice were given continual access to water throughout the studies. Any external disturbances, such as bedding changes, were avoided during the study.

2.3.2 Chronic Mouse Studies

Animals were weighed at 7 a.m. on the morning of the study, and randomised into treatment groups to ensure that that average bodyweight stayed consistent across all groups. Animals were injected at 7 p.m. daily, to coincide with the beginning of the dark phase.

Peptides were prepared to the indicated concentrations using saline (0.9% NaCl). Animals were given 20 μL i.p injections of either peptide or saline daily, using a 0.5 mL insulin syringe and 29 gauge needle. On the first day of the study, mice were given a pre-weighted amount of food, which was not topped up through the course of the study. Mice were allowed to feed ad libitum throughout the study, and food and bodyweight were measured daily at the time of injection. Peptide doses were increased with the passage of time based on results, as indicated. Animals were allowed continual access to water throughout the studies.

Following 12 days daily administration, a glucose tolerance test was conducted, as detailed above in Section 2.3.1.2. In brief, mice were given a final i.p. injection of saline or peptide at 7 p.m. the night prior to the study, and then fasted at 6 a.m. the morning after. After a 6 hour fast, the mice had a pre-dose bleed at 12 p.m., followed by an i.p. glucose injection of 1.5 g/kg of body weight (maximum injection volume of 10ml/kg). Further blood samples were obtained for glucose and insulin measurements at time= 30, 60, 90 and 120 minutes following i.p. glucose injection.

Following the intraperitoneal glucose tolerance test (IPGTT), mice were sacrificed using cervical dislocation and body fat composition was analysed using an EchoMRI™-500.

2.3.3 Acute Rat Studies

Adult male Wistar rats weighing 300-500 g (Charles River, Margate, UK) were individually housed under controlled conditions, at a temperature of 21–23°C, with a 12:12 hour light to dark schedule (lights on at 7 a.m.), and handled regularly to minimise stress. Animals were allowed ad libitum access to water and RM1 diet (Special Diet Services, Witham, UK).
2.3.3.1 Acute Feeding Studies

Rats were weighed and fasted overnight (12 hours) before the study started, and randomised to treatment groups, to ensure that average bodyweight was consistent across all experimental groups. Peptides were reconstituted in ZnCl$_2$ slow release diluent.

On the morning of the study, rats were injected subcutaneously with 20 μL of either saline or peptide, using a 0.5 ml insulin syringe and 29 gauge needle. Rats were returned to their cage and given access to a pre-weighed amount of food. Food and body weight were measured at regular intervals, up to 72 hours following injections, using a balance accurate to 0.1 g. Animals were allowed continual access to water. Bedding changes were avoided during the study.

2.3.3.2 Pharmacokinetic Studies

Adult male Wistar rats weighing 300-350 g were purchased from Charles River, Margate, UK. Rats were administered test peptides at 1 mg in 10 μL of saline through subcutaneous injection. 100 μL blood samples were obtained through tail vein venesection on the day prior to injection, and at 30 minutes, 3, 24 and 48 hours post injection. Samples were collected into lithium heparin-coated microvette tubes, centrifuged for 8 minutes at 10,000 g, and the plasma separated.

Peptide levels in rat plasma at the different time points were quantified using a sensitive in-house radioimmunoassay. A representative purified N terminal (residues 1-18) sequence of the co-agonists was used to raise antiserum (RA553) in rabbits. RA553 was then coupled to bovine serum albumin (BSA) by glutaraldehyde, and used at a final dilution of 1:3000. This antibody specifically cross-reacts with the N terminal sequence common to the co-agonists, including the Aib residue at position 2, and hence the radioimmunoassay measures co-agonist N terminal reactivity. Radiolabelled (125I) N terminal co-agonist fragment was synthesised by direct iodination and purified by reverse phase HPLC.

Exendin-4 was used as a control. Purified exendin-4 was used to raise antiserum (R4) in rabbits. R4 was then coupled to BSA by glutaraldehyde, and used at a final dilution of 1:3500. The antibody specifically cross-reacts with exendin-4. Radiolabelled (125I) analogue exendin-4(1-30)-Tyr$^{31}$ was synthesised by direct iodination and purified by reverse phase HPLC.

The assay was performed in a volume of 0.7 mL of 0.06 M phosphate EDTA buffer (0.05 M Na$_2$HPO$_4$.2H$_2$O, 0.0006 M KH$_2$PO$_4$, 0.01 M disodium-EDTA.2H$_2$O, 0.008M NaN$_3$), containing 0.3% BSA, in a final PH of 7.4. All samples were assayed in duplicate. Standard curves were prepared using purified peptide in assay buffer at a concentration of 2 pmol/mL, and added in duplicate at volumes
of 1, 3, 5, 10, 20, 50 and 100 μL. Following a 3 day incubation at 4°C, the samples were separated into free and antibody bound label using charcoal. Both pellet and supernatant were counted for 180 seconds in a γ-counter (model NE1600, NE Technology Ltd, Reading, UK), and the ratio of free to bound label was calculated. Plasma peptide concentrations were then calculated in terms of the individual peptide standard curves using two phase exponential decay (Graphpad Prism 7.0). For each rat, individual standard curves were prepared to account for any background differences, and accordingly results are expressed normalised to individual baselines.

2.3.4 Chronic Rat Studies

Rats were weighed at 9 a.m. on the morning of the study, and randomised into treatment groups to ensure that that average bodyweight remained consistent across all groups. Rats were injected at 2 p.m. daily, which is late in the light phase.

Peptides were prepared to the indicated concentrations in ZnCl₂ slow release diluent. Rats were given 20 μL subcutaneous injections of either vehicle or peptide daily, using a 0.5 mL insulin syringe and 29 gauge needle. Peptide doses were increased with the passage of time based on results, as indicated.

On the first day of the study, rats were given a pre-weighed amount of food, which was not topped up through the course of the study. They were allowed to feed ad libitum throughout the study, and food and body weight were measured daily at the time of injection. Animals were allowed continual access to water throughout the studies.

Following 9 days daily administration, a glucose tolerance test was conducted. At 6 a.m. on the morning of the study, rats were given their final subcutaneous injection of peptide or vehicle, and fasted. At 12 p.m., the animals had a pre-dose bleed to measure blood glucose levels. Following the bleed, rats were injected i.p. with 2 g/kg of body weight glucose. Blood glucose levels were measured at 30, 60 and 120 minutes after glucose administration.

Pair-fed groups within the study were administered vehicle injections daily as above. The rats were given a restricted, weighed amount of food daily which was equivalent to the mean food intake by their corresponding peptide group over the previous 24 hours. Food hoppers and cage floors were examined to ensure all the food was consumed daily by the pair-fed group.

Statistical Analyses

Data was analysed using Graphpad Prism 7.0.
All results are presented as means with SEM. Food intake and bodyweight data were analysed using one-way or two-way ANOVAs, with appropriate post hoc tests as indicated in the figure legends.
3. Development of GLP-1 and glucagon receptor co-agonists, and investigation into their insulinotropic properties
3.1 Introduction

3.1.1 Oxyntomodulin, a naturally occurring GLP-1R/GCGR co-agonist

GLP-1, glucagon and oxyntomodulin are all products of the pre-pro-glucagon gene, which is expressed in alpha cells in the pancreas and L-cells in the intestine, as well as in a group of neurons in the caudal nucleus of the solitary tract (NTS) (Orskov et al., 1986, Mojsov et al., 1987, Larsen et al., 1997). Tissue-specific post-translational processing of the pre-pro-glucagon precursor peptide generates different hormonal products in different tissues. In intestinal L-cells, processing by enzyme prohormone convertase 1/3 produces GLP-1, GLP-2 and oxyntomodulin, while processing by prohormone convertase 2 in pancreatic alpha cells predominately produces glucagon (Rouille et al., 1994, Tucker et al., 1996, Rouille et al., 1997, Habib et al., 2012).

As it is derived from the same precursor peptide, oxyntomodulin shares sequence homology with both GLP-1 and glucagon, and consequently is a naturally occurring co-agonist of the GLP-1 and glucagon receptors. It binds to and activates both receptors, albeit with a 10 fold lower potency than glucagon and a 100 fold lower potency than GLP-1 at their respective receptors (Bataille et al., 1982, Gros et al., 1993, Schepp et al., 1996, Dakin et al., 2001, Jorgensen et al., 2007).

A specific oxyntomodulin receptor has not been identified yet, making it difficult to ascertain the physiological role of oxyntomodulin. Oxyntomodulin is co-secreted from the L-cells postprandially, along with GLP-1, in proportion to calorie intake, and is therefore postulated to have a role in satiety (Ghatei et al., 1983a, Le Quellec et al., 1992). Oxyntomodulin has also been identified in the caudal NTS in the brain, suggesting a potential role as a neurotransmitter (Larsen et al., 1997).

3.1.2 The effect of oxyntomodulin on food intake and body weight

Oxyntomodulin reduces food intake and induces weight loss in vivo. Central administration of oxyntomodulin acutely reduces food intake in fed and fasted rats (Dakin et al., 2001). Accordingly, chronic treatment with oxyntomodulin, through either intracerebroventricular or intraperitoneal administration, leads to a reduction in both food intake and body weight gain in rats (Dakin et al., 2002, Dakin et al., 2004).

A seminal study by Cohen et al. (2003) demonstrated that these beneficial metabolic effects can translate to humans. In this study, a 90 minute intravenous oxyntomodulin infusion reduced ad libitum food intake and 12 hour cumulative food intake by 19.3% and 11.3% respectively in healthy
human volunteers. Such a reduction in food intake would bring about significant weight reduction over time. However, it is worth noting that 24 hour cumulative food intake was not significantly different compared to control, suggesting that the effects of native oxyntomodulin are transient and cannot be sustained post infusion.

Evidence for the therapeutic utility of oxyntomodulin first came from a study by Wynne et al. (2005), which showed that the anorectic effects of oxyntomodulin are preserved in obese patients. In the 4 week study, patients given thrice daily injections of oxyntomodulin lost 2.3 kilogrammes on average, and reported a 25% to 35% reduction in food intake over the course of the study. As oxyntomodulin mediates profound weight loss in overweight and obese patients, it therefore has potential as a therapeutic for obesity.

3.1.3 Glucagon and GLP-1 receptor co-agonism mediates synergistic weight loss

The anorectic effects of oxyntomodulin are attributed to GLP-1 receptor activation, as these effects can be blocked using GLP-1 receptor antagonists, and are abolished specifically in GLP-1 receptor knockout mice (Dakin et al., 2001, Dakin et al., 2004, Baggio et al., 2004a, Baggio et al., 2004b).

However, pair-feeding studies have established that weight loss mediated by oxyntomodulin cannot be explained completely by the reduction in food intake, suggestive of alternative independent mechanisms of weight loss (Dakin et al., 2002, Dakin et al., 2004). A study from Kosinski et al. (2012) supported these findings, showing oxyntomodulin induces greater weight loss compared to an equivalent GLP-1 analogue in mice, suggesting a GLP-1-independent mechanism of weight loss.

The superior weight loss mediated by oxyntomodulin is ascribed to increased energy expenditure due to glucagon receptor activation (Davidson et al., 1957, Davidson IWF, 1960, Tan et al., 2013). Oxyntomodulin increases energy expenditure in both rodents and man, creating an energy deficit and hence stimulating further weight loss (Dakin et al., 2001, Dakin et al., 2002, Wynne et al., 2006).

The enhanced weight loss in response to oxyntomodulin is thus ascribed to concomitant activation of the GLP-1 and glucagon receptors, which leads to increased energy expenditure in addition to a reduction in food intake.

3.1.4 Glucagon and GLP-1 receptor co-agonism in diabetes

As demonstrated by oxyntomodulin, coupling GLP-1 and glucagon receptor activity can enhance weight loss beyond the effects of GLP-1 alone, making it an ideal therapeutic strategy for obesity.
However, the safety of GLP-1R/GCGR co-agonists for diabetic patients remains under question, as the intrinsic glucagon receptor activity of oxyntomodulin could increase hepatic glucose output, and could hence worsen hyperglycaemia in diabetes (Dinneen et al., 1995). These concerns were allayed by results from a 4 week chronic administration study, where oxyntomodulin did not lead to hyperglycaemia in human volunteers despite prolonged administration (Wynne et al., 2005).

Furthermore, oxyntomodulin has actually been shown to improve glucose metabolism in rodents both acutely and chronically. Chronic oxyntomodulin administration lowers plasma glucose levels in insulin resistant DIO mice, which is postulated to be through a combination of weight loss and GLP-1R mediated stimulation of insulin release (Maida et al., 2008, Parlevliet et al., 2008, Kosinski et al., 2012). Similar glycaemic improvements have recently been demonstrated in humans by Shankar et al. (2018). In a series of randomized, placebo-controlled, crossover trials in obese and overweight subjects, with or without diabetes, an intravenous infusion of oxyntomodulin led to improved glucose tolerance and enhanced insulin release compared to placebo prior to any significant weight loss. In T2DM patients, the glucose lowering effects of oxyntomodulin were comparable to the effects of the clinical dose of liraglutide (0.6 mg). These findings not only show that there is no deterioration of glycaemic control due to the glucagon component of oxyntomodulin, but actually suggest a potential therapeutic role for dual agonists in type 2 diabetes.

This potential acute insulinotropic effect, coupled with superior weight loss on chronic administration, suggests that oxyntomodulin-based co-agonists could be effective drugs for diabetes as well as obesity.

3.1.5 Optimising oxyntomodulin for clinical use

In order to optimise oxyntomodulin for clinical use, both ease of administration and safety must be considered. As oxyntomodulin has a short half-life in plasma, human studies till date have relied on infusions or thrice daily injections to maintain oxyntomodulin levels in vivo. For clinical purposes, it would be better to have a longer lasting oxyntomodulin derivative, with less frequent dosing schedules.

Secondly, the glucagon activity of oxyntomodulin could lead to harmful side effects in patients with diabetes. Hence it is critical to optimise the ratio of GLP-1 and glucagon receptor activation to ensure that the drug does not lead to hyperglycaemia in vivo.
3.1.5.1 Increasing the half-life of oxyntomodulin

Oxyntomodulin has a short circulating half-life, of approximately 12 minutes in humans (Schjoldager et al., 1988) due to renal clearance and rapid proteolytic degradation by enzymes including DPP-IV (Zhu et al., 2003, Druce et al., 2009). In order to develop oxyntomodulin as a potential therapeutic, modifications are needed to enhance its half-life.

One strategy to increase plasma half-life is to add a side chain onto the active compound. Side chains include cholesterol moieties (Pocai et al., 2009, Santoprete et al., 2011), fatty acids (Druce et al., 2009, Kerr et al., 2010), or polyethylene glycol (PEG) side chains (Day et al., 2009), all of which bind to plasma proteins to form a larger complex molecule. This helps to keep the peptide sequence intact, while reducing glomerular filtration due to an increased hydrodynamic radius, hence increasing circulating half-life (Harris and Chess, 2003). Furthermore, as exemplified by liraglutide, addition of a fatty acid side chain stimulates binding to plasma albumin, which reduces proteolytic degradation in addition to reducing filtration (Larsen PJ, 2001). While these modifications increase the circulating half-life, they often concurrently reduce receptor binding and activity. For example, PEGylation at the C terminus can lead to a reduction in receptor activation of 42 and 134 fold respectively at the GLP-1 and glucagon receptors (Bianchi et al., 2013). C-terminal derivatization of oxyntomodulin can specifically reduce activity at the GLP-1 receptor by 1.5 to 38 fold (Santoprete et al., 2011). Furthermore, addition of larger side-chains can restrict access to the brain, which is postulated to be a site of action for oxyntomodulin, and can hence limit the satiety effects of analogues (Dakin et al., 2004).

An alternative strategy to prolong plasma half-life is to circumvent proteolytic degradation. One of the main enzymes responsible for breaking down oxyntomodulin *in vivo* is DPP-IV, which acts at the N terminal dipeptide His^1^-Ser^2^ (Druce et al., 2009). Replacing this Serine residue can reduce degradation by DPP-IV, however, in most reported cases, the extended life-life comes at the cost of efficacy at the glucagon receptor (Druce et al., 2009, Pocai et al., 2009, Kerr et al., 2010, Lynch et al., 2014a).

While each modification has its limitations, using a combination of multiple strategies can successfully increase the half-life and preserve activity of oxyntomodulin-based analogues, as demonstrated by a number of groups. Pocai et al. (2009), coupled a D-Serine substitution at position 2 with a cholesterol side chain, extending the half-life of their active analogue to 1.7 hours. The addition of the cholesterol side-chain was found to increase receptor activity, potentially by increasing affinity to lipid rafts in the membrane and hence increasing proximity to the target receptors (Santoprete et al., 2011). The resulting peptide had a pharmacokinetic profile that allowed...
dosing every two days, and led to greater weight loss and improved glucose tolerance in DIO mice than pure GLP-1R agonists. Furthermore, chronic administration of the peptide in non-human primates led to enhanced weight loss and improved glycaemic control, at a lower dose than a pure GLP-1R agonist (Lao J, 2013). Similarly, Henderson et al. (2016) used derivatization with a fatty acid side-chain and conservative amino acid substitutions to generate a GLP-1R/GCGR co-agonist with a plasma half-life of 5 hours, which had superior weight loss and comparable glucose lowering to liraglutide in DIO mice.

Together, these studies indicate that it is possible to create potent oxyntomodulin-based, GLP-1R/GCGR co-agonists which have longer half-lives in vivo.

3.1.5.2 Optimising GLP-1 versus glucagon receptor activity

Achieving the right balance of GLP-1 versus glucagon receptor activity is a key feature of analogue design. Drug development studies to date have demonstrated that in rodents and non-human primates, GLP-1R/GCGR co-agonists are more efficacious at inducing weight loss than GLP-1R-specific agonists (Day et al., 2009, Pocai et al., 2009, Lao J, 2013, Henderson et al., 2016). However, the optimal ratio of GLP-1 versus glucagon receptor activity remains undetermined.

As discussed earlier, high levels of glucagon receptor activity can lead to hyperglycaemia due to increased hepatic glucose output and gluconeogenesis, and hence raise safety concerns for diabetic patients. However, insufficient glucagon receptor activity would create a GLP-1R-biased agonist, and engineer out the beneficial energy expenditure and weight loss effects of glucagon. Tailoring the GLP-1R/GCGR activity ratio appropriately is critical to maintain significant weight loss while avoiding detrimental effects on glucose tolerance.

Native oxyntomodulin is biased to the glucagon receptor versus the GLP-1 receptor, with an average reported bias of 5:1, although the exact ratio remains contentious (Jorgensen et al., 2007, Kerr et al., 2010, Kosinski et al., 2012). While some groups use oxyntomodulin as a basis for developing GCGR-biased co-agonists (Pocai et al., 2009), others have successfully developed GLP-1R-biased co-agonists as potential therapeutic agents (Day et al., 2009).

Day et al. (2009) investigated the effects of altering receptor activity ratios on metabolic parameters and found, as expected, that increasing glucagon receptor activity enhances weight loss but leads to a deterioration of glycaemic control in DIO mice. For example, a 10-fold GCGR-biased analogue mediates the most significant weight loss, for minimal reduction in food intake, and improves glucose tolerance chronically by improving insulin sensitivity. However, this co-agonist could lead to hyperglycaemia on acute administration. Following a single injection, as plasma co-agonist levels
drop, a heavily GCGR-biased co-agonist will continue to activate the GCGR but no longer be able to activate the GLP-1R, leading to hyperglycaemia. In contrast, a balanced GLP-1R/GCGR co-agonist, with a 1:1 receptor activity ratio, concomitantly activates both receptors and hence retains its beneficial glycaemic effects over time (Day et al., 2009, Day et al., 2012). Further investigation by the group indicated that a ratio lying between 1:1 and 10:1 ratio in the favour of glucagon is optimal for weight loss, but a 5:1 ratio in the favour of glucagon is a cut-off point for glycaemic control, beyond which glucose tolerance is significantly impaired (Day et al., 2009).

In keeping with these findings, Henderson et al. (2016) recently developed a GLP-1R-biased analogue, with a 5:1 ratio of GLP-1 versus glucagon receptor activity. This compound does not lead to measurable hyperglycaemia acutely, and instead mediates superior glucose lowering acutely and superior weight loss and glucose lowering chronically in DIO mice in comparison to GLP-1R agonist liraglutide. Interestingly, the compound did not lead to improvements in basal plasma glucose and insulin levels following chronic administration in non-human primates, despite causing significant weight loss. This is potentially due to the animal model used: the study relied on healthy, normal weight primates, hence the maintenance of fasting insulin levels is not entirely unexpected. It would have been more relevant to use obese or insulin resistant models, and conduct a glucose tolerance test.

While the literature mainly focuses on GLP-1R-biased co-agonists, there are some reports of successful GCGR-biased co-agonists (Pocai et al., 2009, Kerr et al., 2010). Pocai et al. (2009) developed a GCGR-biased co-agonist, with a 1:10 ratio of GLP-1 versus glucagon receptor activity, and reported a profound reduction of body weight, and improvements in glycaemic control, following chronic administration in DIO mice. Furthermore, the weight loss and improvements in metabolic parameters were more significant than those stimulated by a pure GLP-1R agonist. However, they did not report acute changes in plasma glucose or insulin levels on administration, making it difficult to determine whether the enhanced glucagon activity leads to hyperglycaemia acutely in vivo.

In conclusion, a balanced or moderately GCGR-biased co-agonist is optimal for significant weight loss with minimal food intake reduction, but sufficient GLP-1R activity must be preserved in order to maintain glycaemic control. It is of note that most optimisation work has been done in mice, and further studies are needed to investigate the optimal activity ratios across species.
3.1.6 Designing a GLP-1R/GCGR co-agonist

As part of a drug design and development project, I developed a range of GLP-1R/GCGR co-agonists, making modest substitutions to enhance plasma half-life and optimise relative receptor activities (Koth et al., 2012). The aim of this project was to develop a co-agonist which would be suitable as a therapeutic to stimulate enhanced insulin secretion and significant weight loss in patients with type 2 diabetes.

Oxyntomodulin, as a naturally occurring co-agonist, served as the blueprint for peptide design. As oxyntomodulin has a significantly lower potency at the GLP-1R and GCGR compared to native ligands, targeted sequence modifications were made to enhance receptor activity (Kerr et al., 2010, Santoprete et al., 2011, Kosinski et al., 2012, Koth et al., 2012).

Oxyntomodulin is essentially the 29 amino acid residues of glucagon followed by a C terminal octapeptide tail. As native glucagon has a very low affinity for and efficacy at the GLP-1 receptor, this suggests that the C terminal tail is capable of conferring affinity for the GLP-1 receptor. In keeping with this hypothesis, addition of the final 4 residues of GLP-1 to glucagon increases GLP-1 receptor binding 169 fold (Hjorth et al., 1994), and addition of a C terminal amide to glucagon results in a 6 fold increase in GLP-1R activity (Day et al. 2009). Therefore, the optimal strategy for generating co-agonists is to add the C terminus of GLP-1 to the N terminus of glucagon, generating significant binding and activity at both receptors (Hjorth et al., 1994, Runge et al., 2003).

Additional substitutions were made to enhance potency at both the GLP-1 and glucagon receptors. Oxyntomodulin, GLP-1 and glucagon are all derived from the pre-pro-glucagon gene and hence exhibit a high degree of sequence homology, particularly at the N terminus. Of the first 14 N terminal residues, 10 are conserved across glucagon, GLP-1 and oxyntomodulin, as well as in other family B GPCR ligands, suggesting that they might be essential for receptor activity. Furthermore, modifications of these N terminal residues reduces GCGR activity, without effecting GLP-1R activity (Pocai et al., 2009, Du et al., 2012, Kosinski et al., 2012). Accordingly, to preserve GCGR activity, these residues were not substituted in the process of compound development. With regards to GLP-1R activity, substitutions of the non-conserved N-terminal residues in GLP-1 has minimal impact on GLP-1 receptor affinity and potency, suggesting that the N-terminal residues of GLP-1 are not responsible for selective recognition or binding by the GLP-1 receptor (Hjorth et al. 1994; Runge et al. 2003b).

In contrast to the N terminus, the C terminus is more divergent, with only 4 conserved residues across GLP-1 and glucagon. Swapping the divergent C-terminal residues of GLP-1 leads to a 475 fold
reduction in GLP-1 receptor affinity, demonstrating that the C terminus is critical for GLP-1 receptor activity (Hjorth et al. 1994). Consequently, the C terminus of my peptides was based on that of native GLP-1.

The co-agonists investigated in this project had a glucagon-based N terminus, and a C terminal tail of 6-7 residues to confer GLP-1R activity. Conservative substitutions were also made between residues 12-29 to help increase GLP-1 receptor activity. To improve pharmacokinetics, modifications were made to increase the circulating half-life and stability of the peptides. For this purpose, substitutions were made at positions 2 and 3, which are the site of action for DPP-IV. All the peptides tested in this project had an α-amino-isobutyric acid (Aib) in position 2 instead of Serine, conferring resistance to DPP-IV degradation (Deacon et al., 1998, Santoprete et al., 2011). As mentioned above, the addition of side-chains, like PEG or cholesterol, is postulated to prevent access through the blood-brain barrier. This could block any potential central effects of these compounds, and reduce their potency in vivo (Dakin et al., 2004). Therefore, I have not investigated any derivatized peptides.
Figure 3.1.6: Amino acid sequences of pre-pro-glucagon family peptides and analogues.

See Appendix A for the complete sequences of Analogues 1, 2 and 3. See Appendix B for amino acid abbreviations.
3.1.7 Investigating insulinotropic effects of co-agonists in vitro

There are multiple examples GLP-1R/GCGR co-agonists which bring about beneficial glycaemic effects on chronic treatment in pre-clinical species (Pocai et al., 2009, Day et al., 2009, Du et al., 2012, Kosinski et al., 2012, Henderson et al., 2016), although delineating the contribution of weight loss to the observed metabolic improvements is difficult. The improvements on chronic treatment are secondary to weight loss, and are hence attributed to reduced insulin resistance as opposed to improved insulin secretion.

There is a paucity of studies looking at the acute insulinotropic actions of co-agonists, prior to any significant weight loss. Tan et al. (2013) demonstrated that co-infusion of glucagon and GLP-1 leads to an acute synergistic insulinotropic effect in healthy volunteers, partially blunting the hyperglycaemic effect of glucagon. These findings have recently been replicated for native oxyntomodulin, which significantly increased insulin secretion and enhanced glucose lowering following infusion in obese or diabetic human volunteers (Shankar et al., 2018). However, the increased insulin release seen in vivo on could be secondary to increased blood glucose levels, brought about by glucagon action on the liver. Hence, interpreting mechanism from these two studies is impossible due to the confounding factor of hepatic glucose output in vivo. In order to investigate the mechanism whereby GLP-1R/GCGR co-agonists work, a reductive in vitro approach is needed.

There are a number of in vitro and ex vivo models that can be used to investigate the insulinotropic effects of peptides.

3.1.7.1 Immortalised Cell Lines

Beta cell research employs a range of insulinoma-derived, immortalised cell lines. As cell lines can be propagated rapidly, they are convenient for high throughput assays. However, as with any insulinoma cell line, they become less homogenous with time, and can lose their beta cell characteristics at later passages (Cheng et al., 2012). Another concern is these cells can themselves secrete glucagon-derived peptides, which alters background insulin levels and makes it harder to ascertain the specific impact of a peptide (Masur et al., 2005). However, as similar hormonal feedback is seen physiologically between alpha and beta cells, cell lines can be argued to be physiologically relevant.

Of the immortalised cell lines, rat INS-1 832/3 (Asfari et al., 1992) and mouse MIN6B1 (Miyazaki et al., 1990) cells in particular have a high cell insulin content and give robust glucose-stimulated insulin
secretion. Human derived beta cells have recently become available as well, including the 1.1B4 cells and EndoC-βH1 cells (McCluskey et al., 2011, Ravassard et al., 2011). The former have low insulin secretion levels, making it hard to determine any differences between peptides, but the latter are useful for screening.

### 3.1.7.2 Induced Stem Cell Lines

Using specific growth factors, human islet cells can be developed from human embryonic stem cells (Rezania et al., 2014), but these are rare and their use is restricted due to ethical concerns. An alternative is to use induced pluripotent stem cells (iPSCs), which are differentiated human cells that can be de-differentiated to form progenitor cells and then reprogrammed into islet cells (Pagliuca et al., 2014). This circumvents the ethical limitations of embryonic tissue. Furthermore, as iPSCs can be precisely programmed to recapitulate different variations of type 2 diabetes, this facilitates research into specific forms of diabetes. However, inducing stem cells is an expensive process, with low yield (ranging from 2% to 25%) of mature insulin-positive cells (Jiang et al., 2007, Zhang et al., 2009, Rezania et al., 2014). Of the insulin-positive, glucose-responsive cells, a majority are poly-hormonal, co-expressing glucagon and/or somatostatin with insulin (Rezania et al., 2011, Kelly et al., 2011).

### 3.1.7.3 Rodent and Human Islets

Intact islets can be recovered from mice and rats following collagenase digestion of the pancreas (Neuman et al., 2014). Islets are a more physiological model than isolated beta cells, as they capture the complex paracrine signalling seen within the beta cell microenvironment, which includes alpha cells which secrete glucagon, delta cells which secrete somatostatin, and PP cells which secrete pancreatic polypeptide. Furthermore, the intact three-dimensional structure of islets is known to influence their responsiveness in terms of the speed and amount of insulin release (Johnston et al., 2016).

Primary human islets can be obtained from cadaveric donors. Due to species-specific differences in the islet micro-architecture, human islets are a more relevant model than mouse islets (Cabrera et al., 2006). Human islets have a more homogenous distribution of alpha and beta cells, and a different local blood flow than mouse islets, which implies differences in paracrine intra-islet signalling (Nyman et al., 2008). However, human islets are hard to source, often get damaged and necrotic while being transferred to the laboratory, and are substantially more variable than rodent islets (Lyon et al., 2016).
For this project, INS-1 832/3 cells were used for high-throughput screening due to their robust insulin responses (Hohmeier et al., 2000). Key peptides were tested on MIN6B1 and EndoC-βH1 cells as well, to confirm that the observations held true across species.

3.2 Aims

1. Develop co-agonists of the GLP-1 and glucagon receptors
2. Characterise the potency and receptor activity ratios of co-agonists \textit{in vitro}
3. Investigate insulinotropic effects of co-agonists on beta cell lines relative to GLP-1R specific agonists

3.3 Results

3.3.1 Effect of co-administering native GLP-1 and glucagon on insulin secretion

To determine the effect of co-administering GLP-1 and glucagon on insulin secretion, sustained 16 hour insulin secretion assays were performed, using the immortalised rat beta cell line INS-1 823/3 cells. Cells were incubated with the indicated range of peptide concentrations for 16 hours. Resulting insulin concentrations in cell supernatants were determined using the HTRF Insulin assay kit (Cisbio International, France) as per manufacturer’s instructions. Insulin release was normalised to basal insulin release in response to 11 mM of glucose, and this normalised response is henceforth referred to as ‘fold insulin release.’

Results showed that co-administration of native GLP-1 and glucagon did not affect either the EC$_{50}$ or the maximal fold insulin release compared to GLP-1 alone, suggesting that the insulinotropic effect seen is primarily due to GLP-1.
Figure 3.3.1: Insulin secretion from INS-1 823/3 cells on co-administering GLP-1 and glucagon.

Insulin release from INS-1 823/3 cells (30,000 cells/well, passage 23-43) was measured after a 16 hour incubation with GLP-1, glucagon, or an equimolar combination of GLP-1 and glucagon. Insulin secretion was normalised to the response seen to 11 mM glucose, giving fold insulin release. Each peptide concentration was tested in duplicate in each experiment. Data shown are the mean of 4 separate experiments ± SEM, with a 4 parameter logistic fit of averaged data.

3.3.2 Effect of co-agonists on human GLP-1 receptor mediated cAMP accumulation

Dual functionality of synthetic GLP-1R/GCGR co-agonists was validated using CHO-K1 cell lines stably expressing human GLP-1R or GCGR.

The efficacy of co-agonists was compared to native GLP-1 by determining their EC$_{50}$ values of cAMP production in vitro, using a cell line overexpressing the human GLP-1 receptor (hGLP-1R) (Figure 3.3.2 and Table 3.3.3).

At the hGLP-1R, Analogue 3 was the most potent co-agonist, with an EC$_{50}$ value 0.67 times lower than GLP-1. Analogue 2 was equipotent to GLP-1, while Analogue 1 had 4 times lower potency. All three co-agonists were significantly more potent than oxyntomodulin (p<0.01, analysed using a one-way ANOVA with Tukey’s post hoc corrections).
Figure 3.3.2: Efficacy of co-agonists in CHO-K1 cells over expressing hGLP-1R.

cAMP accumulation was measured after a 30 minute incubation with endogenous ligand GLP-1, oxyntomodulin or co-agonists. Each peptide concentration was tested in duplicate in each experiment. cAMP accumulation was quantified using a Cisbio HTRF assay. Data shown are the mean of at least five separate experiments ± SEM, with a 4 parameter logistic fit of averaged data.

3.3.3 Effect of co-agonists on human glucagon receptor mediated cAMP accumulation

The efficacy of the co-agonists at the glucagon receptor was investigated by determining their efficacy at stimulating cAMP production \textit{in vitro}, using a CHO-K1 cell line overexpressing the human glucagon receptor (hGCGR). The native agonist, glucagon was used as a control (Figure 3.3.3 and Table 3.3.3).

At the hGCGR, Analogue 2 and Analogue 3 had similar efficacies as glucagon, while Analogue 1 has an efficacy 1.40 fold lower than the native ligand. All three co-agonists were significantly more potent than oxyntomodulin (p<0.001, analysed using a one-way ANOVA with Tukey’s post hoc corrections).
**Figure 3.3.3: Efficacy of co-agonists in CHO-K1 cells over expressing hGCGR.**

cAMP accumulation was measured after a 30 minute incubation with endogenous ligand glucagon, oxyntomodulin or co-agonists. Each peptide concentration was tested in duplicate in each experiment. cAMP accumulation was quantified using a Cisbio HTRF assay. Data shown are the mean of at least five separate experiments ± SEM, with a 4 parameter logistic fit of averaged data.

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<td>Glucagon</td>
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<td>Oxyntomodulin</td>
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</tr>
<tr>
<td>Analogue 1</td>
<td>45.9 ± 16.6</td>
<td>3.97</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>12.3 ± 3.40</td>
<td>1.06</td>
</tr>
<tr>
<td>Analogue 3</td>
<td>7.77 ± 1.40</td>
<td>0.67</td>
</tr>
</tbody>
</table>

**Table 3.3.3: Summary of the mean EC<sub>50</sub> values for the native peptides and analogues at the human GLP-1 and glucagon receptor.**

CHO-K1 cells over-expressing the human GLP-1 or glucagon receptor were incubated for 30 minutes with a range of peptide concentrations. Intracellular cAMP levels were quantified by a Cisbio HTRF assay. EC<sub>50</sub> values are the mean of at least 5 separate experiments ± SEM. The ratio was calculated by dividing the analogue EC<sub>50</sub> by control peptide EC<sub>50</sub>. 

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3.3.4 Effect of co-agonists on rat GLP-1 receptor mediated cAMP accumulation

In order to corroborate effects in rodent beta cell lines and in planned rodent studies (Chapter 5), the co-agonists were also investigated for their activities at the rat GLP-1 receptor (rGLP-1R), using a HEK cell line overexpressing the rGLP-1R (Figure 3.3.4 and Table 3.3.5-1). At the rGLP-1R, the relative potencies of the peptides were similar to those seen at the human receptor. Analogue 3 was the most potent, with a 3 fold lower efficacy than GLP-1. Analogue 1 and 2 had a 7 and 4 fold lower efficacy than GLP-1 respectively. All the analogues remained more potent than oxyntomodulin (p<0.001, analysed using a one-way ANOVA with Tukey’s post hoc corrections).

![Graph showing efficacy of co-agonists in HEK cells over expressing rGLP-1R.](image)

*Figure 3.3.4: Efficacy of co-agonists in HEK cells over expressing rGLP-1R.*

Cells were incubated with endogenous ligand GLP-1, oxyntomodulin or co-agonists for 30 minutes. Each peptide concentration was tested in duplicate in each experiment. cAMP accumulation was quantified using a Cisbio HTRF assay. Data shown are the mean of at least three separate experiments ± SEM, with a 4 parameter logistic fit of averaged data.

3.3.5 Effect of co-agonists on rat glucagon receptor mediated cAMP accumulation

*In vitro* efficacy of the co-agonists was further investigated at the rat glucagon receptor (rGCGR), using cAMP assays in CHO-K1 cells overexpressing the rGCGR (Figure 3.3.5 and Table 3.3.5-1). Analogue 2 appeared more potent than glucagon itself, while Analogue 3 appeared equipotent. Analogue 1 had the lowest efficacy, at 1.4 fold the efficacy of the native ligand. In contrast to the hGCGR, the analogues appeared equipotent to oxyntomodulin at the rGCGR (p>0.05, analysed using a one-way ANOVA with Tukey’s post hoc corrections).
Figure 3.3.5: Efficacy of dual agonists in CHO-K1 cells over expressing rGCGR.

Cells were incubated with endogenous ligand glucagon, oxyntomodulin or co-agonists for 30 minutes. Each peptide concentration was tested in duplicate in each experiment. cAMP accumulation was quantified using a Cisbio HTRF assay. Data shown are the mean of at least five separate experiments ± SEM, with a 4 parameter logistic fit of averaged data.

### Table 3.3.5-1: Summary of the mean EC\textsubscript{50} values for the native peptides and analogues at the rat GLP-1 and glucagon receptor.

<table>
<thead>
<tr>
<th></th>
<th>rGLP-1R</th>
<th></th>
<th>rGCGR</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC\textsubscript{50} (nM) ± SEM</td>
<td>Ratio of EC\textsubscript{50} to GLP-1</td>
<td>EC\textsubscript{50} (nM) ± SEM</td>
<td>Ratio of EC\textsubscript{50} to GCG</td>
</tr>
<tr>
<td>GLP-1</td>
<td>6.29 ± 1.03</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glucagon</td>
<td>ND</td>
<td>ND</td>
<td>0.28 ± 0.063</td>
<td>1</td>
</tr>
<tr>
<td>Oxyntomodulin</td>
<td>176 ± 70.0</td>
<td>28.0</td>
<td>0.40 ± 0.14</td>
<td>1.41</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>44.2 ± 15.3</td>
<td>7.06</td>
<td>0.40 ± 0.12</td>
<td>1.40</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>27.6 ± 6.94</td>
<td>4.39</td>
<td>0.15 ± 0.02</td>
<td>0.53</td>
</tr>
<tr>
<td>Analogue 3</td>
<td>20.9 ± 7.63</td>
<td>3.32</td>
<td>0.29 ± 0.09</td>
<td>1.01</td>
</tr>
</tbody>
</table>

HEK and CHO-K1 cells over-expressing the rat GLP-1 and glucagon receptor respectively were incubated for 30 minutes with a range of peptide concentrations. Intracellular cAMP levels were quantified by a Cisbio HTRF assay. EC\textsubscript{50} values are the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the analogue EC\textsubscript{50} by control peptide EC\textsubscript{50}.

Relative activity at the GCGR versus the GLP-1R are quantified in Table 3.3.5-2. At the human receptors, Analogue 1 appears GCGR-biased, Analogue 2 appears equipotent at both the GLP-1R and
GCGR, and Analogue 3 appears GLP-1R-biased. In contrast, all analogues appear GCGR-biased in rat receptor systems, with Analogue 2 being the most biased.

<table>
<thead>
<tr>
<th></th>
<th>Human Receptor</th>
<th>Rat Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLP-1R activity$^a$</td>
<td>GCGR activity$^b$</td>
</tr>
<tr>
<td>Oxyntomodulin</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>0.25</td>
<td>0.72</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>0.94</td>
<td>0.99</td>
</tr>
<tr>
<td>Analogue 3</td>
<td>1.51</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Table 3.3.5-2: Summary of the relative activity values for oxyntomodulin and analogues at human and rat GLP-1 and glucagon receptors.

Values are calculated from geometric mean EC$^{50}$ ± SEM from at least four independent experiments.

GLP-1R activity relative to GLP-1 = Geometric mean EC$^{50}$ GLP-1/Geometric mean EC$^{50}$ Analogue

GCGR activity relative to glucagon = Geometric mean EC$^{50}$ glucagon/geometric mean EC$^{50}$ Analogue

GCGR/GLP-1R ratio = GCGR activity relative to glucagon/GLP-1R activity relative to GLP-1

GCGR/GLP-1R ratio greater than 1 suggests glucagon bias

# GLP-1R activity relative to GLP-1; $GCGR activity relative to glucagon

3.3.6 Effect of co-agonists on insulin secretion in INS-1 832/3 cells

To investigate the effect of the synthetic co-agonists on insulin secretion, rat INS-1 832/3 cells were used. Cells were incubated with the indicated range of peptide concentrations for 16 hours, and insulin concentrations in cell supernatants determined using the HTRF insulin assay kit (Cisbio International, France) as per manufacturer’s instructions. Insulin release was normalised to basal insulin release in response to 11 mM of glucose, and expressed as ‘fold insulin release.’ Results were compared to control peptides, GLP-1R agonists GLP-1 and exendin-4.

Analogue 1, 2 and 3 stimulated dose-dependent insulin secretion from INS-1 832/3 cells, with maximum fold insulin release of 4.54 ± 0.40, 4.49 ± 0.49 and 4.16 ± 0.23 respectively. In contrast, GLP-1R agonists GLP-1 and exendin-4 have a lower maximum fold insulin release of 3.48 ± 0.47 and 2.35 ± 0.16 respectively.

Analogue 1, 2 and 3 stimulated a significantly greater maximal insulin release compared to both GLP-1 (p=0.003, 0.005, and 0.013 respectively) and exendin-4 (p=0.0001, 0.0001 and 0.0001
respectively). On average, the analogues had a maximum fold insulin release which was 1.3 fold higher than that of GLP-1.

A)  

**Graph:**

- **Analogue 1**
- **GLP-1**
- **Liraglutide**
- **Exendin-4**

**Y-axis:** Fold Insulin Release

**X-axis:** Log [agonist] (M)
Figure 3.3.6: Effect of co-agonists on insulin secretion from in INS-1 823/3 cells.

Insulin release into the supernatant was measured after a 16 hour incubation with GLP-1, exendin-4, liraglutide and (A) Analogue 1, (B) Analogue 2 or (C) Analogue 3. Separate graphs are shown for clarity but represent the same dataset. Insulin secretion was normalised to the response seen to 11 mM glucose, giving fold insulin release. Each peptide concentration was tested in duplicate in each experiment. Data shown are the mean ± SEM of five individual experiments performed at passages 25-43, with a 4 parameter logistic fit of averaged data. Results analysed using randomised block ANOVA with Dunnett’s post hoc test.


<table>
<thead>
<tr>
<th>Peptide</th>
<th>EC$_{50}$ (nM) ± SEM</th>
<th>Maximum Fold Insulin Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>7.23 ± 1.21</td>
<td>3.48 ± 0.47</td>
</tr>
<tr>
<td>Exendin-4</td>
<td>1.33 ± 0.68</td>
<td>2.35 ± 0.16</td>
</tr>
<tr>
<td>Liraglutide</td>
<td>1.55 ± 0.40</td>
<td>2.71 ± 0.28</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>16.2 ± 4.44</td>
<td>4.54 ± 0.40</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>4.35 ± 0.51</td>
<td>4.49 ± 0.49</td>
</tr>
<tr>
<td>Analogue 3</td>
<td>3.34 ± 1.40</td>
<td>4.16 ± 0.23</td>
</tr>
</tbody>
</table>

Table 3.3.6: Insulinotropic effects of co-agonists and control peptides in INS-1 823/3 cells.

INS-1 823/3 cells were incubated for 16 hours with a range of peptide concentrations in the presence of 11 mM glucose. Insulin release into the supernatant was quantified by an immunoassay, and normalised to insulin release from cells treated with 11 mM glucose alone to yield fold insulin release. Mean values from duplicate wells were used to calculate EC$_{50}$ and maximum fold insulin release in individual experiments, and values presented are a mean of at least 4 separate experiments ± SEM.

3.3.7 Effect of co-agonists on insulin secretion in MIN6B1 cells

The effect of the co-agonists on insulin secretion from the mouse pancreatic beta cell line MIN6B1 cells was also investigated using the same protocol as above. Results were compared to control peptides, GLP-1R agonists exendin-4 and liraglutide.

All analogues stimulated dose-dependent insulin secretion from MIN6B1 cells. Analogues 1, 2 and 3 gave a significantly greater maximal insulin release (Analogue 1= 1.79 ± 0.06, Analogue 2= 2.05 ± 0.06, Analogue 3= 1.84 ± 0.09, Exendin-4=1.51 ± 0.09) compared to exendin-4 (p = 0.0432, 0.0002, and 0.0147 respectively). Analogue 2 gave the highest maximum fold insulin release.

These results demonstrated that co-agonists have more potent insulinotropic responses in vitro in comparison to GLP-1R-specific agonists.
**Figure 3.3.7: Effect of co-agonists on insulin secretion from MIN6B1 cells.**

Insulin release into the supernatant was measured after a 16 hour incubation with exendin-4, liraglutide or the analogues. Insulin secretion was normalised to the response seen to 11 mM glucose, giving fold insulin release. Each peptide concentration was tested in duplicate in each experiment. Data shown are the mean ± SEM of five individual experiments performed at passages 25-43, with a 4 parameter logistic fit of averaged data. Results analysed using randomised block ANOVA with Dunnett’s post hoc test.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EC_{50} (nM)± SEM</th>
<th>Maximum Fold Insulin Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>10.4 ± 4.49</td>
<td>1.83 ± 0.12</td>
</tr>
<tr>
<td>Exendin-4</td>
<td>1.40 ± 1.14</td>
<td>1.51 ± 0.09</td>
</tr>
<tr>
<td>Liraglutide</td>
<td>1.29 ± 0.44</td>
<td>1.62 ± 0.08</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>18.6 ± 8.67</td>
<td>1.79 ± 0.06</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>7.91 ± 5.70</td>
<td>2.05± 0.06</td>
</tr>
<tr>
<td>Analogue 3</td>
<td>2.86 ± 1.37</td>
<td>1.84 ± 0.09</td>
</tr>
</tbody>
</table>

**Table 3.3.7: Insulinotropic effects of co-agonists and control peptides in MIN6B1 cells.**

MIN6B1 cells were incubated for 16 hours with a range of peptide concentrations in the presence of 11 mM glucose. Insulin release into the supernatant was quantified by an immunoassay, and normalised to insulin release to 11 mM of glucose alone to yield fold insulin release. Each individual experiment was done in duplicate. EC_{50} values are the mean of at least 4 separate experiments ± SEM.
3.3.8 Effect of co-agonists on insulin secretion in EndoC-βH1 cells

Insulin secretion assays were repeated on a human pancreatic beta cell line EndoC-βH1. Results were compared to control peptides liraglutide and exendin-4.

Analogue 1, 2 and 3 stimulated dose-dependent insulin secretion from EndoC-βH1 cells, with maximum fold insulin release of 2.08 ± 0.16, 1.90 ± 0.17 and 1.96 ± 0.14 respectively. In contrast, GLP-1R agonists exendin-4 and liraglutide have a lower maximum fold insulin release of 1.50 ± 0.06 and 1.57 ± 0.16 respectively.

Analogue 1, 2 and 3 stimulated a significantly greater maximal insulin release compared to both exendin-4 (p = 0.0004, 0.009 and 0.003 respectively) and liraglutide (p= 0.01, 0.04, and 0.01 respectively). Analogue 1 gave the highest maximum fold insulin release.

These results confirm that co-agonists have potent insulinotropic responses in vitro in comparison to GLP-1R-specific agonists in human, rat and mouse cell lines.

![Figure 3.3.8](image)

**Figure 3.3.8: Insulinotropic effects of co-agonists and control peptides in EndoC-βH1 cells.**

Insulin release into the supernatant was measured after a 16 hour incubation with exendin-4, liraglutide or the analogues. Insulin secretion was normalised to the response seen to 11 mM glucose, giving fold insulin release. Each peptide concentration was tested in duplicate in each experiment. Data shown are the mean ± SEM of five individual experiments performed at passages 93-106, with a 4 parameter logistic fit of averaged data. Results analysed using randomised block ANOVA with Dunnett’s post hoc test.
### Table 3.3.8: Summary of the mean EC₅₀ values for the native peptides and analogues for insulin secretion from EndoC-BH1 cells.

EndoC-BH1 cells were incubated for 16 hours with a range of peptide concentrations in the presence of 11 mM glucose. Insulin release into the supernatant was quantified by an immunoassay, and normalised to insulin release to 11 mM of glucose alone to yield fold insulin release. Each individual experiment was done in duplicate. EC₅₀ values are the mean of 5 separate experiments ± SEM. Results analysed using randomised block ANOVA with Dunnett’s post hoc test.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EC₅₀ (nM) ± SEM</th>
<th>Maximum Fold Insulin Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exendin-4</td>
<td>0.65 ± 0.20</td>
<td>1.50 ± 0.06</td>
</tr>
<tr>
<td>Liraglutide</td>
<td>8.01 ± 1.34</td>
<td>1.57 ± 0.16</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>3.32 ± 8.67</td>
<td>2.08 ± 0.16</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>3.13 ± 1.66</td>
<td>1.90 ± 0.17</td>
</tr>
<tr>
<td>Analogue 3</td>
<td>3.07 ± 0.92</td>
<td>1.96 ± 0.14</td>
</tr>
</tbody>
</table>

#### 3.4 Discussion

The aim of the work presented in this chapter was to develop and validate GLP-1R/GCGR co-agonists. *In vitro* test systems were used to characterise co-agonists in terms of their potency at GLP-1 and glucagon receptors, and immortalised beta cell lines were used to investigate their insulinotropic properties.

GLP-1R/GCGR co-agonists were designed based on the amino acid sequence and secondary structure of oxyntomodulin. As native oxyntomodulin has a short circulating half-life and relatively low potency at both GLP-1 and glucagon receptors, sequence modifications were made to improve these properties (Bataille et al., 1982, Gros et al., 1993, Schepp et al., 1996, Dakin et al., 2001, Jorgensen et al., 2007). The impact of these substitutions was assessed using intracellular assays, namely cAMP synthesis assays. Both GLP-1 and glucagon receptors are G protein coupled receptors which can couple to Gαs on activation, and stimulate synthesis of cAMP as a secondary messenger (Sutherland and Rall, 1958, Drucker et al., 1987). Thus, by quantifying the concentration of peptide needed to stimulate half-maximal cAMP production, referred to as the EC₅₀ value, the potency of each co-agonist at a given receptor can be determined.

Accordingly, CHO-K1 cells expressing human glucagon or GLP-1 receptors were used for intracellular cAMP assays. At the human glucagon receptor, Analogues 2 and 3 were equipotent to the native ligand glucagon, while Analogue 1 was two times weaker than glucagon. At the human GLP-1
receptor, Analogues 2 and 3 were again equipotent to native GLP-1, while Analogue 1 was four times weaker than GLP-1. The co-agonists were significantly more potent than oxyntomodulin at both human GLP-1 and glucagon receptors, demonstrating that the substitutions were effective at increasing receptor activity.

To assess inter species variation, and to facilitate interpretation of in vivo animal studies, cAMP assays were repeated in HEK and CHO-K1 cells expressing rat receptors. To account for intrinsic differences in the different receptor systems, agonist potency was normalised to the native ligand for each receptor. The co-agonists remain consistently more potent compared to oxyntomodulin in rat receptor systems, demonstrating that the substitutions made to the co-agonists successfully increased GLP-1 and glucagon receptor activity beyond that of oxyntomodulin. On average, the co-agonists are 20 and 18 times more potent than oxyntomodulin at the human GLP-1 and glucagon receptors respectively. In contrast, the co-agonists are only 6 and 2 times more potent than oxyntomodulin at the rat GLP-1 and glucagon receptors respectively (Table 3.3.5-2). This reduction in relative potency at rat receptors is primarily because oxyntomodulin itself is significantly more potent at the rat receptors than human receptors. However, there are no published studies that can reaffirm these findings. The activity ratios I obtained for oxyntomodulin at the human receptors are in keeping with receptor activity reported previously by Henderson et al. (2016) and Kerr et al. (2010), but neither group tested oxyntomodulin in rat receptor systems.

In addition to being a marker for receptor binding and activation, intracellular cAMP assays also indicate the insulin secretory potential of peptides. While there are some suggestions that cAMP-independent, G\textalpha_q-mediated pathways may be involved in physiological insulin secretion, there is a consensus that pharmacological insulin release as discussed here is primarily through cAMP signalling (Shigeto et al., 2015). Both GLP-1 and glucagon activate cAMP signalling cascades which contribute to insulin release from beta cells (Moens et al., 1996, Pipeleers et al., 1985, Schuit and Pipeleers, 1985). A threshold level of cAMP is necessary to stimulate insulin secretion, and increases in cAMP levels above this threshold can modulate the amplitude of the insulin response (Pipeleers et al., 1985, Prentki and Matschinsky, 1987). The increased potency of co-agonists in cAMP assays relative to oxyntomodulin is therefore suggestive of enhanced potency for insulin secretion.

While the overall trend for enhanced potency at cAMP signalling, and the relative order of potency of co-agonists, is preserved across human and rat glucagon and GLP-1 receptors, there are certain species-specific differences. For instance, Analogue 2 appeared twice as potent as glucagon at the rat receptor, while it was equipotent to glucagon at the human receptor (Table 3.3.5-2). Greater interspecies variation was seen at the GLP-1 receptor where, on average, co-agonists were three
times weaker at the rat GLP-1 receptor than the human receptor in comparison to native GLP-1. The relative potencies of the analogues also varied. For example, Analogue 1 appeared four times weaker than Analogue 2 and 3 at the human GLP-1 receptor, but this difference was reduced to two fold at the rat GLP-1 receptor. This observed inter-species variation at the GLP-1R might be due to a difference in receptor structure, as human and rat GLP-1 receptor share 88% homology compared to a 97% homology between the rat and mouse receptors (Moon et al., 2012). In particular, the sequence divergences across the human and rat receptor isoforms involve incorporation of charged residues in the rat receptor versus uncharged residues in the human receptor. These changes in charged residues in particular are predicted to effect receptor conformation and coupling (Knudsen et al., 2012). In contrast, the glucagon receptor is more conserved across rats and humans, with 90% sequence homology between rat and human in comparison to a 75.2% homology between mouse and human receptors (Lok et al., 1994, Menzel et al., 1994, Burcelin et al., 1995). Furthermore, human, rat and mouse glucagon receptors share a common structural motif, with four N-linked glycosylation sites and a RLAR sequence in the third intracellular loop (Jelinek et al., 1993, Svoboda et al., 1993, Lok et al., 1994, Macneil et al., 1994, Burcelin et al., 1995). Given that these molecular differences could manifest as differences in receptor affinity and activity in vivo, it is important to continually consider human receptor activity to generate an effective drug.

GLP-1R/GCGR activity ratios were generated using EC50 values obtained from cAMP assays, as per earlier studies (Henderson et al., 2016). It has been demonstrated that co-agonists mediate superior weight loss through dual activation of the GLP-1 and glucagon receptors, while positive glycaemic effects are mediated mainly by the GLP-1 receptor in vivo (Day et al., 2009, Henderson et al., 2016). The optimal GLP-1R/GCGR activity ratio is critical to achieve maximal weight loss while minimising the hyperglycaemic tendencies of glucagon. My analogues are based on oxyntomodulin, and are therefore expected to show bias towards GCGR activation, which in turn is predicted to lead to greater weight loss. Other dual GLP-1R/GCGR agonists in development are predominately GLP-1R agonists, with a fraction of the glucagon activity that oxyntomodulin has at the human receptors. For example, Henderson et al. (2016) have developed a peptide with a hGCGR versus hGLP-1R activity ratio of 0.19, and Day et al. (2009) have developed a peptide with a ratio of 0.25. Therefore, Analogues 1, 2 and 3, with more oxyntomodulin-like hGCGR versus hGLP-1R activity ratios of 2.86, 1.05 and 0.58 respectively, are expected to produce greater weight loss. Interestingly, in rat receptor systems, my co-agonists appear even more biased towards GCGR activation. This glucagon receptor bias is created mainly by a selective reduction in GLP-1 receptor activity at the rat receptor as compared to the human receptor, suggesting that the sequence modifications specifically affect rat GLP-1R activation. This is surprising, considering that the two receptor sub forms share 88% amino
acid homology, but a similar hGLP-1R preferring analogue has been reported previously by Tibaduiza et al. (2001). However, as the latter study involved a non-peptide ligand, I cannot extrapolate which sequence changes stimulated preferential activity at the human receptor based on earlier data.

To assess potency of co-agonists at potentiating glucose stimulated insulin secretion from beta cells, *in vitro* insulin secretion assays were utilised. Typically, *in vitro* insulin secretion assays have incubation times of 20 minutes to 1 hour, in order to replicate physiological conditions with short-acting stimuli. As the purpose of this project was to develop a new therapeutic for daily or weekly use, using a short term assay would be unsuitable. Instead a sustained incubation period of 16 hours was used to model an *in vivo* pharmacological set up. However, the model is limited in that it cannot replicate *in vivo* drug elimination (de Witte et al., 2016). In addition to GLP-1, exendin-4 and liraglutide were used as controls. Due to their increased plasma half-lives of 1.5 hours and 13 hours respectively, exendin-4 and liraglutide are more relevant comparators for the modified co-agonists (Elbrond et al., 2002, Linnebjerg et al., 2007). The insulin secretion protocol was adapted from Hohmeier et al. (2000). The washing steps were eliminated by seeding the cells along with peptide, thereby reducing variation due to cell loss and yielding more reliable results.

Three different immortalised pancreatic cell lines, rat INS-1 823/3, mouse MIN6B1, and human EndoC-βH1 cells were used, based on their robust, quantifiable insulin responses. I also carried out initial studies on rat Rin-mf5 cells and human 1.1B4 cells as additional pancreatic cell models, but was unable to obtain conclusive results due to low levels of insulin secretion which could not be measured using available assays. In the rat, mouse and human cell lines tested, co-agonists stimulate a significantly higher maximal insulin release as compared to GLP-1, exendin-4 and liraglutide, demonstrating that the co-agonists are more effective at stimulating insulin secretion than selective GLP-1R agonists across species. As these insulinotropic effects are observed *in vitro*, they are independent of any interplay from hepatic glucose output, and suggest a direct effect of co-agonists on insulin secretion from beta cells.

The order of insulinotropic potency varied across species, with Analogue 1 giving the highest fold insulin secretion in rat and human cell lines, while Analogue 2 had the highest insulin secretion in mouse cells. In the absence of mouse cell cAMP data, it is difficult to correlate these inter-species differences to cAMP potencies. It is of note that across human and rat receptors, where cAMP data is available, the order of potency (EC50) for cAMP and for insulin secretion is identical, and is conserved across species. However, one of the key features of these co-agonists is enhanced maximal insulin release, which does not appear to correlate to cAMP potency. For instance,
Analogue 1 has the highest maximum fold insulin release at both human and rat receptors despite having the lowest potency for GLP-1R and GCGR cAMP.

The relative contribution of GCGR activity to the observed insulinotropic responses was also investigated. While the physiological relevance of glucagon-mediated insulin secretion remains contentious (Kawai et al., 1995, Moens et al., 2002), it is well established that glucagon can potentiate insulin release through direct action on the beta cell, via the GCGR at low doses, and the GLP-1 receptor at supraphysiological concentrations exceeding 10 nM (Moens et al., 1996a, Moens et al., 1998). Furthermore, in vivo studies have previously demonstrated that co-administering glucagon with GLP-1 brings about a synergistic increase in insulin secretion, suggesting a potential insulinotropic effect for GLP-1R/GCGR co-agonists (Tan et al., 2013). However, in the absence of in vitro investigations, the mechanism behind this synergy remains undetermined, as the observed synergistic insulin response could be a secondary effect of elevated blood glucose brought about by glucagon. Therefore, to investigate the effect of GLP-1 and glucagon co-administration on insulin secretion, and to explore the mechanism behind synergistic insulin release seen in vivo, I carried out in vitro insulin secretion assays using rat pancreatic cell line, INS-1 823/3. By removing the confounding factor of hepatic glucose output, this in vitro test system specifically looks at the effects of glucagon and GLP-1 on beta cell stimulation. In keeping with earlier findings, my results show that glucagon only stimulates measurable insulin secretion at concentrations exceeding 100 nM. Furthermore, an equimolar combination of GLP-1 and glucagon has no beneficial effects on insulin secretion beyond those of GLP-1 alone. This is expected at lower concentrations, where GLP-1 has the dominant effect on insulin secretion due to its lower EC$_{50}$ value. However, at higher concentrations where glucagon can stimulate substantial insulin secretion, we would expect a greater insulin response from a combination of glucagon and GLP-1 than from GLP-1 alone. The lack of any additive or synergistic response from glucagon might be due to receptor saturation by GLP-1 at the high concentrations indicated. As co-administering glucagon with native GLP-1 had no beneficial effect on insulin secretion, it is postulated that the improved insulinotropic profile of the co-agonists is independent of glucagon receptor activity. These findings are limited by the absence of a potent, specific GCGR antagonist, which would allow me to define the relative contribution of the GCGR to insulin secretion mediated by co-agonists. Extensive mechanistic studies were conducted, as detailed in Chapter 4, to further explore the mechanism behind enhanced insulinotropic effects of co-agonists.

This chapter describes the in vitro profile of GLP-1R/GCGR co-agonists with respect to their insulinotropic potential. Most work in this field to date relies on in vivo assessments of insulin secretion to estimate therapeutic viability, and limited data is available regarding in vitro potency of
previously developed co-agonists. Henderson et al. (2016) have developed a co-agonist, MEDI0382, which is more potent at improving glucose tolerance in vivo than liraglutide. In vitro, MEDI0382 has low potency at cAMP synthesis, with an $EC_{50}$ 3 and 10 fold higher than GLP-1 and glucagon respectively. MEDI0382 also has lower maximal fold insulin release than GLP-1 and liraglutide in INS-1 823/3 cells. In comparison to MEDI0382, Analogue 2 and 3 are more potent at cAMP, while Analogue 1 is equipotent to MEDI0382. All three analogues are also more effective than GLP-1 and liraglutide in vitro with respect to the magnitude of insulin response. This suggests that my compounds, which have a better in vitro profile in terms of both cAMP responses and insulin secretion, would have good therapeutic prospects in vivo.

Work by Pocai et al. (2009) and Kerr et al. (2010) similarly shows that weak co-agonists can mediate dramatic weight loss and improvements in glucose tolerance in vivo. Pocai et al. (2009) developed a dual agonist which was 40 times weaker than GLP-1 and 3 times weaker than glucagon for cAMP at the murine receptors, but led to enhanced weight loss and more pronounced improvements in glucose tolerance compared to a matched GLP-1R specific agonist. Kerr et al. (2010) developed a longer lasting oxyntomodulin analogue, with similar in vitro efficacy to oxyntomodulin at cAMP (10 to 100 times reduced relative to glucagon and GLP-1 respectively). Despite reduced potency at cAMP, the analogue lowered plasma glucose by 35-43% and raised plasma insulin by 66-82% following an acute glucose challenge in mice. It is hard to assess therapeutic viability relative to current GLP-1 treatments as these studies did not include a relevant comparator. However, given the in vitro efficacy is much lower than that of my co-agonists, these studies suggest that my compounds would have dramatic beneficial effects in vivo.

In summary, a range of in vitro assays, using both secondary messenger and functional endpoints, consistently validated the analogues as GLP-1/GCGR co-agonists, capable of stimulating a greater maximal insulin release in vitro than pure GLP-1 agonists. As this effect cannot be replicated by simply giving GLP-1 and glucagon together, the mechanism behind the observed insulin effect remains unclear, and shall be explored in the next chapter.
4. Investigation into the mechanism behind insulinotropic effects of GLP-1R/GCGR co-agonists
4.1 Introduction

4.1.1 Mechanism of GLP-1 induced insulin secretion

GLP-1 induces insulin secretion from beta cells primarily through G protein-mediated cAMP signalling (Moens et al., 1996b, MontroseRafizadeh et al., 1997, Kang et al., 2001, Hashiguchi et al., 2006, Kelley et al., 2009, Chepurny et al., 2009). Rise in intracellular cAMP levels activates effectors Protein Kinase A (PKA) and exchange protein directly activated by cAMP (Epac2), which then mediate a range of downstream effects such as enhancing proinsulin transcription and insulin biosynthesis, and accelerating granule mobilisation to increase the size of the readily releasable pool of insulin vesicles (Lester et al., 1997, Holz, 2004, Holst and Gromada, 2004). Ultimately, cAMP signalling leads to an increase in intracellular Ca\(^{2+}\) levels through both PKA and Epac2-dependant mechanisms (Light et al., 2002, Tsuboi et al., 2003, Kang et al., 2008). Increased Ca\(^{2+}\) levels trigger insulin granule exocytosis, leading to insulin secretion.

4.1.2 β arrestin mediated signalling

In addition to the classical G protein signalling pathway, the GLP-1 receptor can couple to β arrestins, a class of adaptor proteins which partly mediate the physiological effects of GLP-1.

Activated GPCRs are phosphorylated by G protein receptor kinases (GRKs) at the C terminus, and this phosphorylated site recruits β arrestins. Binding of arrestins creates steric hindrance, preventing G protein interaction with the receptor. Furthermore, arrestins serve as scaffolding proteins for recruitment of clathrin adaptor proteins such as AP2, and facilitate clathrin-mediated endocytosis of the receptor (Premont and Gainetdinov, 2007, DeWire et al., 2007). They therefore terminate the G protein signalling cascade by reducing downstream signalling and surface receptor availability, a phenomenon referred to as ‘receptor desensitization’ (Goodman et al., 1996). However, recent work has demonstrated that G protein signalling in fact continues post receptor internalisation, mediated by arrestins (Tsvetanova and von Zastrow, 2014).

A large number of studies over the past 20 years report that β arrestins can also promote G-protein-independent signalling cascades by recruiting and scaffolding a number of signalling proteins including mitogen-activated protein kinases ERK, Akt, JNK, and p38 (Luttrell et al., 1999a, Luttrell et al., 1999b, DeFea et al., 2000a, DeFea et al., 2000b, Lefkowitz and Shenoy, 2005, DeWire et al., 2007). Interestingly, this dogma has been recently challenged by a study in which β arrestin and/or G
proteins were deleted by CRISPR-Cas9 or pharmacologically inactivated. In this study, ERK phosphorylation was preserved in cells lacking both β arrestins, but completely lost in the absence of G proteins (Grundmann et al., 2018).

### 4.1.3 β arrestin isoforms

Two sub-types of β arrestins have been identified: β arrestin-1 and β arrestin-2, which share 78% sequence homology at the amino acid level (Lefkowitz and Shenoy, 2005). There is a degree of redundancy between the isoforms, as evidenced by single isoform knockouts. However, certain physiological roles of β arrestins are isoform specific and cannot be compensated for (Conner et al., 1997, Bohn et al., 1999, DeWire et al., 2007, Schmid and Bohn, 2009). In particular, loss or dysfunction of β arrestin-2 has been linked to a reduction in insulin signalling in vivo, contributing to the development of insulin resistance (Luan et al., 2009).

### 4.1.4 GLP-1 receptor and β arrestin signalling

On activation, the GLP-1 receptor can associate with both β arrestin isoforms (Jorgensen et al., 2007, Sonoda et al., 2008). GLP-1, glucagon and oxyntomodulin can all recruit β arrestins to the GLP-1R, with a relative order of potency that is identical to the relative cAMP potencies of the agonists (Jorgensen et al., 2005).

It is of note that, while β arrestins are classically thought to mediate receptor desensitization, this has not yet been conclusively proven for the GLP-1 receptor. Quoyer et al. (2010) demonstrated that, in addition to the classic GPCR-mediated, PKA-dependent signalling cascade, GLP-1 partly mediates anti-apoptotic effects through a PKA-independent, β arrestin-1-mediated route. An elegant study conducted by Sonoda et al. (2008) supported this claim, and further suggested that β arrestin-1 plays a role in both cAMP-dependent and -independent routes of insulin secretion. Following on from this seminal paper, a number of studies have confirmed a physiological role for both β arrestin-1 and -2 in glucose homeostasis and insulin secretion (Usui et al., 2004, Luan et al., 2009, Zhang et al., 2013, Zhu et al., 2017).

### 4.1.5 β arrestins in GLP-1-mediated glucose homeostasis

A role for β arrestins in insulin secretion was first proposed by Sonoda et al. (2008), who demonstrated physical association between β arrestin-1 and the GLP-1 receptor in rat beta cell
models. Following RNAi mediated β arrestin-knockdown, there is a 35% reduction in GLP-1-mediated cAMP signalling and a 60% impairment in GLP-1 potentiation of glucose-stimulated insulin secretion, demonstrating the physiological relevance of β arrestin signalling. The reduced cAMP responses can be rescued by phosphodiesterase inhibitor IBMX and adenylate cyclase stimulator forskolin, suggesting that β arrestin-1 works through the Go\_s-cAMP pathway, upstream of adenylate cyclase. In the absence of any changes in basal receptor expression, GLP-1-induced internalisation or total intracellular insulin content, it was hypothesised that the impaired insulin secretion was independent of receptor recycling and due to a secretory defect. The study was unable to investigate β arrestin-2, as an effective knockdown could not be generated.

β arrestin-2 has since been shown to play a role in GLP-1 mediated insulin secretion and insulin sensitivity (Luan et al., 2009). There is a significant reduction in β arrestin-2, and to a lesser extent β-arrestin-1, at both protein and messenger RNA levels in liver and muscle of db/db mice and high-fat diet-induced (HFD) insulin-resistant mice. β arrestin-2 levels are also significantly reduced in islets from these mouse models, which are known to have impaired beta cell function. A similar reduction in β arrestin levels has been seen in clinical samples from a small diabetic patient cohort, suggesting a role for β arrestin-2 in particular in the pathogenesis of beta cell dysfunction, and the development of diabetes and insulin resistance (Zhang et al., 2013).

The physiological relevance of β arrestin in vivo was illustrated by studies done on whole body β arrestin-2 knockout mice. The knockouts have reduced glucose tolerance, increased endogenous glucose production and impaired insulin sensitivity. Hyperglycaemic clamp studies revealed these knockouts also had reduced acute and late phase insulin secretion, while ex vivo studies on cultured islets showed a 50% reduction in glucose stimulated insulin secretion (Luan et al., 2009, Zhang et al., 2013). β arrestin-2 is therefore postulated to regulate both insulin sensitivity and insulin secretion. Based on these findings, db/db mice and HFD-fed whole body β arrestin-2 knockouts were administered exogenous β arrestin-2, using adenoviral vectors, to improve their glucose homeostasis. A threefold rise in β arrestin-2 protein levels led to a reduction in postprandial blood glucose levels, and improvements in glucose tolerance and insulin sensitivity. Similarly, mice overexpressing β arrestin-2 had lower post prandial insulin and glucose levels, and augmented insulin sensitivity (Luan et al., 2009). β arrestin-2 could therefore have therapeutic potential in the treatment of diabetes, although the relative improvement compared to wild type mice was not addressed in these studies.

While these studies support a role for β arrestin-2 in glucose homeostasis, they rely on whole body, germ-line knockouts, making it impossible to rule out confounding developmental and compensatory
changes. Furthermore, these findings have been disputed by a study which reports no effect of β arrestin knockdown on glucose homeostasis (Ravier et al., 2014). Definitive evidence for a physiological role of β arrestins in islet function was provided by Zhu et al. (2017), who developed a conditional, beta-cell-specific knockout of β arrestin-2 in adult mice. In vivo, the knockout had a profound impairment in insulin secretion and reduced glucose tolerance, in keeping with findings from Luan et al. (2009) and Zhang et al. (2013). However, these observed impairments were conditional on a high fat diet. Ex vivo analysis on islets from knockout mice showed constitutively impaired glucose-stimulated insulin secretion. The incongruity between in vitro and in vivo results could be due to compensatory in vivo mechanisms, such as hormonal stimulation of beta cells, or involvement of non-beta-cell pathways, which help maintain normal glucose homoeostasis in knockouts in the absence of an environmental trigger.

Studies on immortalised beta cell lines support a role for β arrestins in glucose homeostasis, as glucose stimulated insulin secretion is completely inhibited in human beta cells following β arrestin-2 knockdown (Zhu et al., 2017). A recent study by Jones et al. (2018) contradicted these earlier findings, and reported an increase in insulin secretion following β arrestin-1 and -2 knockdown in rat INS-1 832/3, mouse MIN6B1 and human EndoC-βH1 cell lines. However, the latter study used prolonged (16 hour) insulin secretion assays, and did not investigate acute insulin secretion. They also noted that the differences in receptor internalisation and cAMP signalling following β-arrestin knockdown were strongly time-dependent, suggesting that the prolonged nature of their insulin assay may explain the discrepancy between their findings and other published data.

4.1.6 Mechanism of action of β arrestins in glucose homeostasis

The mechanism whereby arrestins enhance insulin secretion and improve glucose tolerance in vivo remains unclear.

As β arrestins are classically associated with receptor trafficking, it has been postulated that arrestins could act directly at the GLP-1 receptor, bringing about changes in receptor internalisation and cell surface expression which in turn lead to changes in insulin secretion (Jorgensen et al., 2005). However, there are contradicting results from different groups, with some failing to note any effect of β arrestin knockdown on GLP-1 receptor trafficking (Sonoda et al., 2008, Thompson and Kanamarlapudi, 2015).
An alternative possibility is that β arrestins act downstream of the insulin receptor, suggested by multiple studies which show that β arrestins can regulate insulin sensitivity. Investigations into downstream insulin signalling pathways have consequently identified multiple sites of action whereby β arrestins could act to impact insulin sensitivity.

On activation, the insulin receptor phosphorylates insulin receptor substrate (IRS) proteins. IRS proteins have a key role in the physiological regulation of glucose-stimulated insulin secretion (Withers et al., 1998, Jhala et al., 2003, Usui et al., 2004, Rhodes, 2005, Park et al., 2006, Weir and Bonner-Weir, 2007, Cantley et al., 2007, Terauchi et al., 2007, Danial et al., 2008). IRS proteins stimulate the phosphatidylinositol-3-OH kinase (PI(3)K)–Akt pathway (Sun et al., 1991), which activates Akt. Akt is a serine/threonine kinase which phosphorylates downstream kinases and transcription factors, serving as a key effector molecule to mediate the metabolic effects of insulin (Burgering and Coffer, 1995, Franke et al., 1995, Biddinger and Kahn, 2006).

It has been proposed that β arrestins effect glucose homeostasis by regulating insulin receptor substrates, IRS-1 and IRS-2. β arrestin-1 can modulate gene expression and degradation of IRS-2 (Jhala et al., 2003, Kang et al., 2006), and altered regulation of IRS-2 was identified as the mechanism behind reduced GLP-1-mediated insulin secretion in β arrestin-1 knockdowns by Sonoda et al. (2008). Similarly, Usui et al. (2004) showed that reduced β arrestin-1 levels led to enhanced IRS-1 degradation and increased cellular insulin resistance, while overexpression of β-arrestin-1 protected against insulin-induced degradation of IRS-1.

There is also some evidence that β arrestins work by regulating Akt, the downstream effector of insulin. Akt phosphorylation and activation is regulated by the kinase Src (Datta et al., 1996, Wong et al., 1999, Craxton et al., 1999, Chen et al., 2001, Jiang and Qiu, 2003). Luan et al. (2009) showed that insulin triggers a concomitant increase in Akt/β arrestin-2 association and Akt/Src interaction, suggesting that β arrestins can serve as scaffolding proteins for Akt and Src. In β arrestin-2 knockout mice, the co-association of Src and Akt reduces drastically, and manifests as a 40% reduction in Akt activation. Conversely, β arrestin-2-overexpressing mice show an increased Akt/Src association. It has therefore been hypothesised that that β arrestin-2 influences glucose homeostasis by regulating the Src-Akt interaction and hence downstream insulin signalling.

Lastly, Zhu et al. (2017) proposed a role for Ca\(^{2+}\) signalling in β arrestin-2 mediated glucose homeostasis. Their study used a conditional, beta cell knockout to demonstrate β arrestin-2 is necessary for normal Ca\(^{2+}\) entry into beta cells, which in turn regulated exocytosis. This hypothesis ties in with earlier observations from Sonoda et al. (2008) and Zhang et al. (2013), which reported insulin secretory defects following β arrestin knockdown.
In spite of considerable evidence supporting a role for β arrestins in mediating glucose and insulin homeostasis through the GLP-1R, the precise mechanism underlying it remains undetermined. ‘Ligand-induced bias’ is a recent concept in the field which could help answer these questions.

### 4.1.7 Ligand Induced Bias

Different ligands, with different structures, will stabilise distinct receptor active-state conformations, leading to recruitment of different second messengers. As each ligand activates only a selective subset of the entire signalling repertoire available, different ligands can have specific, distinct signalling profiles at the same receptor. This concept is referred to as ‘functional selectivity’ or ‘ligand induced bias’ (Urban et al., 2007, Costa-Neto et al., 2016).

Bias is particularly relevant when discussing the GLP-1 receptor. Variations in the amino acid sequences of GLP-1R agonists lead to differential abilities of these agonists to recruit intracellular effector proteins and stimulate downstream signalling. Bias in GLP-1R agonists can be quantified as selective activation of G protein signalling versus β arrestin recruitment. Exendin-4, glucagon and dual agonist oxyntomodulin are all intrinsically biased towards G protein signalling as opposed to β arrestin recruitment, relative to native ligand GLP-1 (Jorgensen et al., 2007). As this study utilised bioluminescence resonance energy transfer (BRET) assays to directly measure β arrestin recruitment, as opposed to relying on secondary messenger outputs, it is reliable. In contrast, earlier studies which failed to demonstrate bias may have been limited by study design, as spare receptors and the use of signal amplification in secondary messenger assays can mask subtle differences in receptor activation (Wootten et al., 2016).

### 4.1.8 The impact of bias on insulin secretion

There are number of examples in the literature regarding biased GLP-1R agonists, engineered by incorporating β amino acids (Hager et al., 2016, Hager et al., 2017) or targeting peptide helicity (Plisson et al., 2017). However, most studies do not look specifically at bias in the context of insulin responses. Two independent studies to date have investigated the impact of bias on insulin secretion, and reported that the G protein biased analogues exhibit better glucose regulation and are potential candidates for drug development.

Zhang et al. (2015) developed a GLP-1R agonist termed “P5”, which exhibits bias towards G protein activation over β arrestin-1 and -2 recruitment compared to GLP-1 and exendin-4. In vivo, P5 acutely lowers blood glucose levels in lean, DIO and ob/ob mice, with superior glucose lowering effects to
exendin-4 in the former two mouse models. Chronic treatment with P5 leads to superior improvements in hyperglycaemia in ob/ob and DIO mice in comparison to exendin-4. Interestingly, in all mouse models tested, the insulinotropic effect of P5 was lower than that of exendin-4, which was interpreted as demonstrating that the superior glucose regulation was due to increased insulin sensitivity, as opposed to insulin secretion.

In contrast, a recently published study from Jones et al. (2018) shows G protein biased agonists can enhance insulin secretion in comparison to exendin-4. The study investigated G protein biased GLP-1R agonists, generated through single amino acid substitutions at N terminus. These agonists showed a marked reduction in β arrestin-1 and -2 recruitment, which in turn led to reduced endocytosis of surface receptors. An interesting finding from the study was that these biased co-agonists had a shorter residency time at the receptor and exhibited faster recycling once internalised. Coupled together, these trafficking alterations led to increased receptor surface expression and reduced desensitization in vitro, which led to enhanced insulin secretion compared to exendin-4. However, in order for the trafficking effects to translate into differences in insulin secretion from human islets, prolonged studies were needed, with 16 hour incubations employed to mimic pharmacological drug exposure. In keeping with the in vitro results, superior in vivo results were also time-dependant, with the glucose lowering and insulinotropic effects of the analogues exceeding exendin-4 only after a time delay of 4 hours. While previous studies have demonstrated a positive role for β arrestins in insulin secretion through non-canonical downstream signalling pathways, these longer incubation times and the study design highlighted the trafficking and desensitization effects of β-arrestin recruitment, potentially superseding any acute benefits of β arrestin mediated signalling (Sonoda et al., 2008, Quoyer et al., 2010). Altered receptor trafficking and reduced receptor desensitization of the G protein biased analogues could therefore lead to an enhanced insulinotropic profile over time.

4.2 Aims

1. Determine whether the co-agonists display bias between β arrestin recruitment and cAMP signalling
2. Investigate the effect of bias on in vitro insulin secretion
3. Determine the effect of GLP-1R bias on receptor internalisation
4.3 Results

4.3.1 cAMP responses in INS-1 823/3 cells

In order to determine the contribution of prolonged cAMP signalling to the observed insulin secretion effects of the analogues, 16 hour cAMP secretion assays were performed on rat INS-1 823/3 cells (as described in Methods, section 2.2.2). GLP-1, and GLP-1 analogues liraglutide and exendin-4, had similar maximal cAMP responses (1.85 ± 0.069, 1.81 ± 0.066, and 1.62 ± 0.057 respectively), with the order of efficacy in keeping with their insulin responses.

Analogues 2 and 3 had higher maximal fold cAMP responses than GLP-1 (2.71 ± 0.098 and 2.78 ± 0.146 respectively versus 1.85 ± 0.069), which is in keeping with their enhanced insulinotropic potential. However, Analogue 1 had a maximal cAMP response of 1.94 ± 0.171, which is significantly lower than the other co-agonists.

![Figure 4.3.1: Efficacy of dual agonists at stimulating G protein signalling in INS-1 823/3 cells.](image)

Cells were incubated with indicated peptides for 16 hours. cAMP accumulation was quantified using a Cisbio HTRF assay. Each peptide concentration was tested in duplicate in each experiment. Data shown are the mean of at least three separate experiments ± SEM, with a 4 parameter logisitic fit of averaged data.
4.3.2 Functional pharmacology of co-agonists at the GLP-1 receptor

To determine relative agonist activity at G protein and β arrestin signalling pathways, G protein dependent cAMP synthesis and β arrestin-2 recruitment were measured.

The experiments were performed in parallel in PathHunter CHO-K1 cells expressing the human GLP-1 receptor. Dose response curves were generated using four-parameter curve fitting, as seen in Figure 4.3.2.

Figure 4.3.2: Effect of co-agonists on (A) cAMP synthesis and (B) β arrestin-2 (βarr2) recruitment in PathHunter CHO-GLP-1R cells.

Cells were incubated with co-agonists for 30 minutes. Data represented as mean ± SEM, after normalisation to maximum response to GLP-1. n=5, with a 4-parameter logistic fit of averaged data.
Table 4.3.2: Potency and efficacy of co-agonists at cAMP and β arrestin signalling in PathHunter CHO GLP-1R cells.

$E_{\text{max}}$ normalised to GLP-1 $E_{\text{max}}$. Data represented as mean ± SEM, n=5. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs GLP-1, analysed using randomised block ANOVA with Dunnett’s post hoc test.

All three co-agonists show a significant reduction in β arrestin recruitment relative to GLP-1, through reductions in either potency (pEC$_{50}$) or efficacy ($E_{\text{max}}$), or both. The co-agonists also show reductions in the cAMP signalling pathways relative to GLP-1.

These alterations in signalling are suggestive of pathway bias, which was then calculated and confirmed.

4.3.3 Quantifying bias at the GLP-1 receptor

To quantify the bias between the cAMP and β arrestin signalling pathways, a modified version of the Black and Leff operational model of agonism was used (Kenakin et al., 2012, van der Westhuizen et al., 2014). For each peptide, the dose response data was then fit to the following equation, as described in detail in Methods Section 2.2.4.
This equation calculates the ‘transduction ratio’ or ‘τ/Kₐ’ for each given peptide, which is a measure of the efficacy of an agonist at both activating a specific pathway and generating a measurable readout (Table 4.3.2).

Kₐ is the affinity constant, which is dependent on the affinity of the agonist for both the receptor and the specific downstream pathway. τ measures the efficacy of the agonist at triggering a specific downstream response, and depends on the agonist and the receptor system. Therefore, τ/Kₐ is a more complete measure of efficacy than EC₅₀ values, as it accounts for both potency and maximal response in a given receptor system. As τ/Kₐ is dependent on cell type, it is expressed relative to a reference agonist, which here is the native ligand GLP-1. Thus, the log (τ/Kₐ) values for each agonist were normalised to GLP-1, yielding Δ log (τ/Kₐ), a measure of agonist activity relative to reference peptide for a given pathway. This value is considered to be independent of receptor density (Kenakin and Christopoulos, 2013). To determine the intrinsic bias of a peptide between G protein and arrestin signalling pathways, Δ log (τ/Kₐ) of one pathway were subtracted from the other, giving the bias factor ΔΔ log (τ/Kₐ). The anti-logarithm of this value is considered to numerically describe the degree of bias. To simplify statistical analysis, calculations were performed on the logarithms.

**Figure 4.3.3: Quantification of biased agonism at the GLP-1 receptor.**
Data indicates mean ± 95% confidence intervals of bias factor $\Delta \Delta \log \left( \frac{1}{K_A} \right)$ values, with reference agonist GLP-1 assigned a bias factor of 0.

4.3.4 Correlation between bias and insulin secretion

Relative bias at the GLP-1 receptor ($\Delta \Delta \log \left( \frac{1}{K_A} \right)$) was plotted against maximal insulin secretion from INS-1 823/3 cells for a range of co-agonists, in order to investigate the relationship between bias and insulin secretion. In addition to Analogues 1, 2 and 3, other novel GLP-1R/GCGR co-agonists were included in order to determine a trend. A positive correlation is seen, such that increased bias towards G protein signalling leads to enhanced insulin secretion.

Of note, exendin-4 appeared to deviate somewhat from this trend, and its bias towards G protein signalling is not translated into enhanced insulin release. This is potentially due to the high degree of sequence divergence between exendin-4 and GLP-1, in contrast to the sequence similarity between GLP-1 and all other tested compounds. Previously published studies looking into bias and insulin secretion consistently use reference compounds with comparable sequences for bias calculations and correlations (Zhang et al., 2015, Jones et al., 2018). Consequently, the correlation between bias and insulin secretion was calculated after excluding Exendin-4 from the data set.
Figure 4.3.4: Correlation of bias factors at the GLP-1 receptor (ΔΔlog(τ/K_A)) and maximal insulin secretion (E_{max}). (A) Including exendin-4, (B) Excluding exendin-4, quantified with a linear regression. Further co-agonists are included in addition to Analogues 1, 2 and 3.
4.3.5 Functional pharmacology of co-agonists at the glucagon receptor

To determine bias at the glucagon receptor, G protein dependant cAMP synthesis and β arrestin-1 recruitment assays were repeated in PathHunter CHO-K1 cells expressing the glucagon receptor. Earlier experiments in our laboratory indicated that GLP-1R and GCGR β arrestin-1 and β arrestin-2 responses tend to be well matched for peptides, meaning that measuring both provides little additional information. Dose response curves were generated using four-parameter curve fitting, as seen in Figure 4.3.5.

Figure 4.3.5: Effect of co-agonists on (A) cAMP synthesis and (B) β arrestin-1 (βarr1) recruitment in PathHunter CHO-GCGR cells.

Cells were incubated with co-agonists for 30 minutes. Data represented as mean ± SEM, after normalisation to maximum response to glucagon. n=5, with a 4 parameter logistic fit of averaged data.
The dose responses were used to calculate bias factors for each analogue, relative to glucagon. As both pathways were measured in parallel, bias was calculated on a per-assay basis to minimise error. The bias factors are shown in Figure 4.3.6, with log (τ/Kₐ) values given above in Table 4.3.5. None of the co-agonists show ligand induced bias relative to glucagon.
Figure 4.3.6: Quantification of biased agonism at the glucagon receptor.

Data indicates mean ± 95% confidence intervals of bias factor $\Delta \Delta \log(\tau/K_A)$ values, with reference agonist glucagon assigned a bias factor of 0.

4.3.7 GLP-1R internalisation in response to co-agonists

To investigate whether the differential activation of $\beta$ arrestin pathways manifested as changes in receptor trafficking and surface GLP-1R density, confocal microscopy was used.

MIN6B1 cells, stably expressing the N-terminally SNAP-tagged GLP-1R, were incubated with the test agonists for 30 minutes. Receptor internalisation was visualised under a confocal microscope. Representative images from this experiment are shown below. Incubation with GLP-1 induced profound receptor internalisation in comparison to vehicle. However, treatment with Analogues 1 and 3 helped retain receptor surface expression, with Analogue 1 appearing most potent at reducing internalisation.
MIN6B1-SNAP-GLP-1R cells were labelled with SNAP-Surface 488 (green) and treated with (A) vehicle, (B) GLP-1, (C) Analogue 1, (D) Analogue 2, and (E) Analogue 3 for 30 minutes (all peptides were at 100 nM). Cells were then thoroughly washed and fixed at 4°C to arrest further trafficking.
4.4 Discussion

The three GLP-1R/GCGR co-agonists show enhanced maximal insulin secretion compared to GLP-1 and GLP-1R agonists in vitro. The aim of the work presented in this chapter was to determine the mechanism behind these observed changes.

GLP-1 mediated potentiation of insulin secretion is attributed mainly to G protein signalling leading to cAMP synthesis (Drucker, 2006, Holst, 2007, Nauck, 2009). Therefore, cAMP synthesis in response to each co-agonist was measured. To facilitate relevant comparisons between cAMP responses and insulin secretion, rat beta cell line INS-1 823/3 was used, as insulin responses in this cell line had been established previously. Similar to the insulin assays, cAMP assays were conducted with a 16 hour stimulation period, as an approximation of sustained in vivo drug exposure and to allow for manifestation of receptor desensitization and down regulation effects. In keeping with their enhanced maximal insulin secretion in INS-1 823/3 cells, Analogues 2 and 3 show enhanced maximal cAMP stimulation in comparison to GLP-1, exendin-4 and liraglutide. Analogue 1 is an exception, as it has similar maximal cAMP stimulation to GLP-1 despite having significantly higher insulin secretion.

The enhanced sustained insulin and cAMP responses in vitro for my co-agonists could relate to differential receptor trafficking and desensitisation over time, which is associated with β arrestin signalling (Goodman et al., 1996, Premont and Gainetdinov, 2007, DeWire et al., 2007). Accordingly, β arrestin recruitment in response to the co-agonists was investigated. Specifically the relative activation of β arrestin and G protein signalling cascades was measured to quantify ‘ligand induced bias’, namely the differential activation of downstream signalling cascades. Bias was investigated at the level of receptor activation and engagement of the G protein and β arrestin pathways, by measuring G protein-dependent cAMP generation and β arrestin recruitment. The assays were conducted in parallel in PathHunter CHOK-1 cells. As these signalling responses are time-dependant, all assays were conducted for a matched length of time. The relative bias between signalling pathways was calculated using a well-established operational model of agonism (Kenakin et al., 2012, Kenakin, 2015).

At the GLP-1R, all three co-agonists showed a significant reduction in both cAMP signalling and β arrestin signalling relative to GLP-1, through reductions in either or both potency and efficacy. The impairment in β arrestin signalling was more profound than that in cAMP signalling, creating a significant bias towards G protein signalling relative to native ligand, GLP-1. At the glucagon receptor the co-agonists showed no significant bias compared to native ligand, glucagon.
The bias observed at the GLP-1R could be due to sequence modifications made while developing the co-agonists. The co-agonists are derived from oxyntomodulin, and have sequence similarities with glucagon, both of which are intrinsically biased towards G protein signalling relative to GLP-1 (Jorgensen et al., 2007). Furthermore, all three co-agonists tested here have key modifications at the N terminus. Recent insights into the structure of the GLP-1 receptor, based on crystallography, cryo-electron microscopy and mutagenesis studies, have highlighted the N terminal region of GLP-1R agonists as a key site for receptor interaction and ligand-induced bias (Wootten et al., 2016, Jazayeri et al., 2017, Zhang et al., 2017). In particular, the co-agonists have α-aminoisobutyric acid at position 2. This specific substitution has previously been shown to reduce β arrestin recruitment to a point where it is non-quantifiable, thereby creating a strong bias away from arrestin signalling and towards G protein activation (Hager et al., 2016).

To investigate whether bias against β arrestin recruitment was associated with altered receptor internalisation, confocal microscopy was employed. Murine beta cell line MIN6B1, expressing SNAP-GLP-1R, was surface-labelled and imaged following a 30 minute incubation with either GLP-1 or the co-agonists. In comparison to native GLP-1, the co-agonists induced markedly less internalisation of the GLP-1R and maintained cell surface receptor expression. Analogue 1 had the lowest transduction ratio for the β arrestin pathway, and accordingly induced the least internalisation. However, these findings are limited as the receptor internalisation was not quantified formally. To conclusively show a role of arrestins in differential insulin responses, it would have been ideal to use knockdown of either or both β arrestin isoforms here, and monitor changes in receptor trafficking and insulin secretion. A recent publication from my laboratory showed that even with both β arrestins deleted by CRISPR-Cas9, GLP-1R internalisation was only modestly slowed (Jones et al. 2018). An important caveat is that signalling profiles of the agonists tested here are likely to differ in additional ways beyond cAMP generation and β arrestin recruitment, which could have a role in downstream responses such receptor trafficking and insulin release.

In summary, these findings suggest that biased co-agonists with reduced β arrestin recruitment help maintain receptor surface expression during prolonged stimulation, and hence stimulate enhanced insulin secretion. This contrasts with earlier studies, which have highlighted a positive role of β arrestin-mediated signalling in insulin secretion (Usui et al., 2004, Jorgensen et al., 2007, Sonoda et al., 2008, Luan et al., 2009, Quoyer et al., 2010, Zhang et al., 2013, Zhu et al., 2017), but is important to note that these earlier studies relied on acute in vitro insulin secretion assays as opposed to the pharmacological incubation times employed here. My findings are corroborated by a recent study by Jones et al. (2018), who used similar prolonged, pharmacological incubations and found that G protein biased analogues induce less receptor internalisation and enhance insulin secretion in vitro.
This apparent link between reduced receptor internalisation and enhanced insulin secretion suggests a new approach for insulinotropic drug design. One limitation of this approach is that agonists with the least internalisation propensity tend to exhibit lower receptor affinity, which ultimately supersedes any potential advantage in maintaining high levels of surface receptor (Jones et al., 2018).

The degree of G protein bias at the GLP-1R is positively correlated to maximal insulin secretion for the co-agonists, with an R squared value of 0.73, however I cannot deduce causation from the current data. Only three other published studies till date have investigated the relationship between G protein bias and insulin secretion (Zhang et al., 2015, Plisson et al., 2017, Jones et al., 2018). Of these studies, Zhang et al. (2015) did not investigate in vitro insulinotropic effects, and failed to show enhanced insulin secretion in vivo in response to their G protein biased analogue. As this study was acute and in vivo, it is difficult to draw comparisons. In keeping with our findings, Plisson et al. (2017) found that insulin secretion is enhanced in G protein biased analogues. However, they proposed that increased potency of insulin secretion was due to increased potency (reflected as a lower EC50) of cAMP signalling. This appears to contradict my results, which focus on efficacy rather than potency, as my co-agonists mediate greater maximal insulin secretion in INS-1 823/3 cells despite significantly lower cAMP potencies. It is of note that Plisson et al. (2017) did not formally quantify bias, making it impossible to confirm the relationship between bias and insulin secretion, and used acute insulin secretion assays. My findings are supported by Jones et al. (2018), where prolonged in vitro assays found a strong positive correlation between G protein bias and insulin secretion.

While co-agonists can potentially stimulate insulin secretion by direct action at the GCGR (Moens et al., 1996a, Moens et al., 1998), earlier experiments involving GLP-1 and glucagon co-administration suggest that the enhanced insulinotropic activity of co-agonists is not mediated by GCGR activity (see Figure 3.3.1). However, these conclusions are limited by the absence of an effective, specific GCGR antagonist. No significant bias is seen at the GCGR for co-agonists relative to reference compound glucagon, reiterating that activity at the GLP-1R is the key factor determining differential insulin responses.

GLP-1R/GCGR co-agonists tested here exhibit preferential bias towards G protein activation as opposed to β arrestin recruitment, which manifests in vitro as enhanced maximal insulin secretion. This is attributed at least in part to reduced receptor internalisation following agonist treatment. By preserving receptor sensitivity and surface expression over the course of prolonged incubation times, biased ligands can generate a greater insulinotropic response. Biased agonists, which activate specific downstream signalling cascades, are of particular interest in drug development, as they
could specifically activate therapeutic pathways while minimizing unwanted side effects (Whalen et al., 2011, Kenakin and Christopoulos, 2013, Appleton and Luttrell, 2013). My results further show that biased GLP-1R/GCGR co-agonists have enhanced insulin secretion \textit{in vitro}, suggesting that they could be more effective as well as being safer.
5. Metabolic effects of GLP-1R/GCGR co-agonists \textit{in vivo}
5.1 Introduction

5.1.1 Beneficial weight loss effects of GLP-1R/GCGR co-agonists in vivo

Oxyntomodulin, and synthetic GLP-1R/GCGR co-agonists based on oxyntomodulin, mediate profound weight loss in vivo (Dakin et al., 2001, Dakin et al., 2002, Dakin et al., 2004, Wynne et al., 2005, Wynne et al., 2006, Day et al., 2009, Pocai et al., 2009, Kerr et al., 2010, Kosinski et al., 2012, Henderson et al., 2016). Furthermore, GLP-1R/GCGR co-agonists can bring about greater weight loss than selective GLP-1R agonists (Dakin et al., 2001, Dakin et al., 2002, Baggio et al., 2004a, Wynne et al., 2006, Day et al., 2009, Kosinski et al., 2012, Henderson et al., 2016). GLP-1 receptor activation restricts food intake and enhances satiety, while glucagon receptor activation mediates an increases in energy expenditure (Davidson et al., 1957, Davidson IWF, 1960, Tan et al., 2013). The effect on energy expenditure mediated by the glucagon receptor were first highlighted in a rat pair-feeding study where glucagon administration resulted in a greater reduction of body weight as compared to pair-fed animals (Salter, 1960).

As co-agonists bring about weight loss by simultaneously activating two distinct pathways, as opposed to just activating the GLP-1 receptor, it is postulated that they would cause less nausea for a given amount of weight loss. Early human studies on native oxyntomodulin support this hypothesis (Wynne et al., 2005, Wynne et al., 2006), while preliminary data on synthetic GLP-1R/GCGR co-agonists confirms that they are well tolerated even at doses mediating profound weight loss (Ambery et al., 2018). However, studies involving a direct comparison between GLP-1R-specific analogues and co-agonists are needed in order to draw definitive conclusions.

5.1.2 Glycaemic effects of oxyntomodulin

In addition to mediating weight loss, oxyntomodulin shows beneficial glycaemic effects in mouse models and in human volunteers (Kosinski et al., 2012, Du et al., 2012, Shankar et al., 2018).

In lean and diet-induced insulin-resistant mice, oxyntomodulin acutely improves glucose tolerance and increases insulin secretion. However, higher doses of oxyntomodulin are required to achieve the same glucose lowering as a GLP-1R-specific agonist matched for potency at the GLP-1R (Maida et al., 2008, Parlevliet et al., 2008, Du et al., 2012). This indicates that while oxyntomodulin stimulates insulin secretion, the glucagon component might be overall detrimental to glycaemic control. Chronic oxyntomodulin administration in rodents similarly leads to improved glycaemic control, and
chronic treatment in DIO mice has been reported to normalise basal glucose levels (Pocai et al., 2009). These chronic effects reflect the profound weight loss mediated by co-agonists, and are correspondingly associated with improvements in insulin sensitivity as opposed to insulin secretion. However, despite superior weight loss compared to matched GLP-1R agonists, oxyntomodulin brings about comparable lowering of plasma glucose to GLP-1R agonists. This is surprising, as we would expect the superior weight loss to translate into superior insulin sensitivity and glycaemic control. Chronic studies therefore also suggest that the glucagon activity of oxyntomodulin might be detrimental to glycaemic control (Kosinski et al., 2012, Pocai et al., 2009).

The acute insulinotropic effects of oxyntomodulin translate to humans, and were first highlighted by a human study that reported increased plasma insulin levels on oxyntomodulin administration (Wynne et al., 2006). A recent study has similarly shown that oxyntomodulin infusion leads to significant insulin secretion in diabetic and non-diabetic patients. Remarkably, in patients with diabetes, the acute glucose lowering effect of an intravenous infusion of oxyntomodulin (3.0 pmol/kg/min – translating to 1.24 mg over 24 hours in a 70 kg human) was comparable to the glycaemic effects of a prescribed daily dose of liraglutide (0.6 mg) (Shankar et al., 2018), although direct dose comparisons are difficult due to the different clearance rates of both molecules, as well as the impact of albumin binding of liraglutide on the concentration of “free” agonist available for GLP-1R binding.

It is difficult to draw conclusions regarding the chronic effects of oxyntomodulin administration on glycaemic control in humans, due to a lack of suitable studies looking specifically at changes in glucose tolerance. However, it has been shown previously that 4 week chronic oxyntomodulin administration in humans does not lead to hyperglycaemia, although the response to a glucose load was not investigated in this study (Wynne et al., 2005).

5.1.3 Glycaemic effects of GLP-1R/GCGR co-agonists

As opposed to their well-established beneficial effects on weight loss, the insulinotropic effect of synthetic co-agonists remains relatively unexplored. As discussed in earlier chapters, the hyperglycaemic effects of glucagon receptor activation can be detrimental for maintaining glycaemic control, raising a safety concern in terms of developing co-agonists for use in T2DM (Day et al., 2012). Most GLP-1R/GCGR co-agonists are therefore designed to preferentially activate the GLP-1R. The rationale behind this is to incorporate dual GLP-1R/GCGR activation to enhance weight loss, while retaining prominent GLP-1R activity to overcome the hyperglycaemic tendencies of glucagon.
Published data to date shows co-agonists improve glucose tolerance and lower blood glucose levels \textit{in vivo} in DIO mouse models (Day et al., 2009, Pocai et al., 2009, Kerr et al., 2010, Henderson et al., 2016). To minimise the hyperglycaemic tendencies of co-agonists, both Henderson et al. (2016) and Day et al. (2009) developed GLP-1R- preferring co-agonists, with a hGCGR \textit{versus} hGLP-1R activity ratio of 0.19 and 0.25 respectively (which signifies a signalling potency favouring hGLP-1R activation by a factor of 5 and 4 respectively). In contrast, Pocai et al. (2009) developed a GCGR- preferring analogue with a murine GCGR \textit{versus} GLP-1R activity ratio of 2.27. Despite the varied receptor preference, all three co-agonists were as potent at glucose lowering as GLP-1R-selective agonists (Day et al., 2009, Pocai et al., 2009, Henderson et al., 2016). However, most studies rely on chronic assessments of glucose tolerance, post weight loss, making it difficult to define the relative contributions of enhanced insulin sensitivity versus direct insulinotropic effects of the co-agonists. A further complicating factor relates to the impact of sustained improvements in glycaemia on beta cell function, namely the alleviation of glucotoxicity, which could improve the insulin secretory capacity in a manner independent of direct insulinotropic effects of receptor activation.

The first study that specifically investigated glycaemic effects of synthetic co-agonists was by Day et al. (2009), who developed a GLP-1R-preferring co-agonist. The co-agonist induced greater weight loss than GLP-1 (25% of body weight as compared to 9% respectively). Following a month long chronic treatment, the co-agonist significantly improved glucose tolerance, and enhanced insulin sensitivity, as demonstrated by reduced basal glucose and insulin levels. Pocai et al. (2009) similarly reported chronic treatment with a co-agonist led to twice as much weight loss as a GLP-1 analogue (25% as compared to 12% body weight). The weight loss was reflected in enhanced insulin sensitivity, as the co-agonist lowered plasma insulin twice as much as the GLP-1 control. At the end of chronic treatment, both the co-agonist and GLP-1R selective agonist normalised plasma glucose levels. However, despite superior weight loss, the co-agonist mediated no further improvements in acute glucose tolerance beyond the effects of the GLP-1R selective agonist.

One of the latest co-agonists being developed for clinical administration is MEDI0382. Following one-month chronic administration, both liraglutide and MEDI0382 led to a normalisation of fasting plasma glucose levels in DIO mice. However, MEDI0382 led to significantly greater weight loss than liraglutide (30% as opposed to 21% of body weight respectively), and consequently lowered basal plasma glucose and insulin levels to a greater extent. In spite of the greater weight loss and insulin sensitivity, the co-agonist mediated similar improvements in glucose tolerance as liraglutide following the chronic study.
As these results were obtained in *in vivo* studies, it is difficult to ascertain mechanism linking co-agonist treatment to changes in glycaemia. In all three studies cited here, basal insulin levels were significantly lowered following chronic treatment, suggesting that the improvements in glycaemic control are due to increased insulin sensitivity, which is secondary to weight loss (Day et al., 2009, Pocai et al., 2009, Henderson et al., 2016). None of these studies measured insulin responses during glucose tolerance tests, so conclusions regarding insulin secretion cannot be drawn.

Contrary to these studies, Kerr et al. (2010) found an increase in basal insulin levels following chronic administration in mice of their oxyntomodulin-like co-agonist, along with lowered plasma glucose levels. An intraperitoneal glucose tolerance test following the study showed enhanced glucose tolerance accompanied by an enhanced insulinotropic response (57% increase in insulin area under the curve (AUC)). They also reported moderate improvements in insulin sensitivity, suggesting both insulinotropic and insulin sensitising actions were at play.

A better idea of mechanism can be drawn from acute *in vivo* studies, which measure the impact of co-agonists prior to any significant weight loss. Only two studies have reported results of acute IPGTTs, performed with single dose agonist administration, with both showing consistently lower glucose excursions on administering a co-agonist, comparable to the glucose lowering effects of liraglutide (Kerr et al., 2010, Henderson et al., 2016). Kerr et al. (2010) found that this glucose lowering effect was associated with an enhanced insulin response, measured as a 58-66% increase in the insulin AUC. These findings are supported by a clinical trial where co-infusing glucagon with GLP-1 led to a synergistic insulinotropic effect in healthy volunteers (Tan et al., 2013). Contrary to these findings, Henderson et al. (2016) found no correlation between the insulin response and glucose lowering effect of their peptide, but this might be due to the apparent high degree of intra-group variability in their results.

Henderson et al. (2016) and Kerr et al. (2010) also investigated insulin secretion *in vitro*, to get an estimate of the direct insulinotropic action of their peptides on beta cells. Neither group found enhanced insulin secretion in response to their co-agonists versus specific GLP-1R agonists. These compounds still went on to have salutary effects on glycaemic control *in vivo*, which is promising in context of my co-agonists.

In conclusion, a substantial pre-existing body of work indicates that *in vivo* administration of GLP-1R/GCGR co-agonists has no harmful hyperglycaemic effects, and instead enhances insulin secretion acutely, and enhances weight loss and insulin sensitivity chronically. While the chronic weight loss effects outweigh those of GLP-1R-specific agonists, the glycaemic benefits of co-agonists are paradoxically comparable to those of GLP-1R-specific agonists. However, as earlier studies have
mainly focused on GLP-1R-preferring analogues, it would be interesting to explore the differences in in vivo effects of my GCGR-preferring agonists. Secondly, as my co-agonists have greater insulinotropic effects than GLP-1R agonists in vitro, they could potentially improve upon the glycaemic effects mediated by earlier published co-agonists in vivo.

5.1.4 In vivo metabolic profiles of G protein biased agonists

In addition to being GLP-1R/GCGR co-agonists, the analogues tested here are also G protein biased agonists of the GLP-1 receptor. As biased agonists show enhanced, sustained insulin secretion in vitro, due to reduced receptor internalisation and desensitisation, it is postulated that they could mediate superior insulin responses in vivo. This hypothesis is in contradiction to a finding by Baggio et al. (2000), which showed exendin-4-induced desensitization to be an exclusively in vitro phenomenon that does not affect in vivo glycaemic control in mouse models.

To date, only two studies have investigated glycaemic effects of GLP-1R biased analogues, with contradictory results. One biased agonist developed by Zhang et al. (2015) stimulated enhanced insulin secretion in vitro, but failed to stimulate similar insulin responses in vivo. The agonist does have superior glucose lowering effects to exendin-4 acutely and chronically, in both lean and insulin resistant mouse models, but these effects are attributed to increased insulin sensitivity as opposed to insulin secretion.

In direct contrast, a recent study from my laboratory (Jones et al., 2018) found that in vitro insulinotropic effects could translate in vivo. The group employed a different study design to show that their G protein biased analogue had superior insulinotropic effects in vivo, but this effect was time dependant and only manifested 4 hours post injection. In the absence of pharmacokinetic differences, the enhanced insulin response was ascribed to reduced desensitisation and sustained signalling in vivo by biased analogues. The biased analogue continued to improve glucose tolerance beyond exendin-4 even in a chronic setting. Although insulin responses were not quantified chronically, there was no significant weight difference between the groups suggesting that the observed differences were due to insulin secretion as opposed to sensitivity.

5.2 Aims

1. Investigate the acute effects of co-agonists on glucose tolerance and weight loss in vivo, in comparison to exendin-4
2. Determine the therapeutic profile and anti-diabetic efficacy of co-agonists compared to exendin-4 in chronic studies

5.3 Results

5.3.1 Pilot dose-finding study into the effects of co-agonists on glucose homeostasis in lean mice

To determine appropriate doses for glucose tolerance tests, an initial dose finding study was conducted in lean C57BL/6J mice. GLP-1R agonist exendin-4 was used as a comparator, and vehicle (0.9% NaCl) was used as a negative control. Analogue 2 was used as an exemplar for all three co-agonists. Weight adjusted doses of exendin-4 and Analogue 2 of 1 nmol/kg, 3 nmol/kg and 10 nmol/kg were administered as single intraperitoneal (i.p.) injections following an overnight fast. 30 minutes after the injection, mice were given an i.p. injection of glucose at 2 g/kg of body weight. Blood glucose levels were monitored as indicated using tail vein venesection.

Results are presented in Fig 5.3.1. Analogue 2 stimulated a dose-dependent increase in glucose tolerance. Following an i.p. glucose challenge, peak blood glucose concentration was lower than vehicle for all three groups given Analogue 2, and the glucose AUC was significantly reduced by a dose 10 nmol/kg compared to vehicle (p=0.008).

The AUC for groups administered 10 nmol/kg of Analogue 2 and 1 nmol/kg of exendin-4 was comparable. Based on these findings, exendin-4 was administered at a dose of 1 nmol/kg and co-agonists were administered at a dose of 10 nmol/kg in all further mouse studies, unless specified otherwise.
Figure 5.3.1: Effect of acute co-agonist administration on blood glucose homeostasis in mice.

Overnight fasted male C57BL/6J mice (n=4) received an i.p injection of vehicle or indicated doses of peptide, followed 30 minutes later by a glucose challenge of 2 g/kg i.p. glucose at t=0. (A) Blood glucose concentrations (B) The area under the curve for blood glucose, both expressed as mean ± SEM. Statistical analysis carried out using one-way ANOVA and post hoc tests with Sidak correction *p <0.05; **p <0.01, ***p<0.001, versus vehicle.

5.3.2 Effect of acute co-agonist administration on blood glucose homeostasis in lean mice

To investigate the effect of co-agonists on glucose tolerance in vivo in comparison to exendin-4, acute IPGTTs were performed on lean mice following an overnight fast of 12 hours.

Basal blood glucose levels remained comparable across all groups following peptide administration, as assessed by the 0 minute time point. After an i.p. glucose challenge, glucose excursion was lowered significantly by all three co-agonists, and glucose lowering effects were comparable to those of exendin-4 (p > 0.1).
Analogues 1, 2 and 3 lowered the glucose AUC significantly, with AUC values of 1041 ± 129.5, 866.8 ± 71.88, and 1050 ± 153.5 mmol/L*min, in comparison to 1562 ± 118.5 mmol/L*min for vehicle (all p<0.01). The AUC values for the co-agonists were comparable to exendin-4 (all p > 0.1); hence, the co-agonists bring about significant glucose lowering in vivo on acute administration.

![Graph A]

**Figure 5.3.2: Effect of acute co-agonist administration on glucose tolerance in mice.**

Overnight fasted male C57BL/6J mice (n=6) received an i.p injection of vehicle, 1 nmol/kg of exendin-4, or 10 nmol/kg of co-agonists, followed 30 minutes later by a 2 g/kg i.p. glucose challenge. (A) Blood glucose concentrations (B) The area under the curve for blood glucose, both expressed as mean ± SEM. Statistical
5.3.3 Delayed effects of co-agonist administration on blood glucose homeostasis in mice

IPGTTs were also conducted with a time delay after administering peptide, to investigate whether the desensitisation effects seen in vitro translate to glycaemic differences in vivo. For this purpose, fasted mice were given a glucose challenge 4 hours after peptide administration. It is hypothesised that this time delay will allow for the agonist-related GLP-1R trafficking differences to manifest as differences in insulin secretion and glucose tolerance in vivo (Jones et al., 2018). To normalise experimental conditions and facilitate comparison with the earlier IPGTT, overnight food restriction was adjusted such that mice had fasted for 12 hours at the time of glucose administration.

Results are presented in Fig 5.3.3. Four hours post-administration, Analogues 1, 2 and 3 significantly lowered basal plasma glucose levels in comparison to vehicle (p= 0.0004, 0.0001 and 0.002 respectively). Numerically, Analogues 1, 2 and 3 lowered basal glucose levels to a greater extent than exendin-4, with mean plasma glucose values of 4.20 ± 0.29, 3.95 ± 0.26 and 4.63 ± 0.42 mmol/L respectively, as compared to 5.33 ± 0.52mmol/L for exendin-4. However, these results failed to achieve significance.

Following an i.p. glucose challenge, peak glucose excursion was significantly lower for all three co-agonists in comparison to vehicle (all p<0.01). The glucose lowering effect was comparable to that of exendin-4. Analogue 2 in particular lowered peak glucose levels more than exendin-4, with mean plasma glucose levels of 8.15 ± 1.19 mmol/L compared to 10.08 ± 0.92 mmol/L for exendin-4; this difference was however statistically non-significant.

Analogues 1, 2 and 3 lowered the glucose AUC significantly, with AUC values of 732.6 ± 57.11, 819.3 ± 92.23, and 868.6 ± 147.2 mmol/L*min, in comparison to 1489 ± 116.1 mmol/L*min for vehicle (all p<0.01). The AUC values for the co-agonists were numerically lower than that for exendin-4, but the results failed to reach significance (all p > 0.1). This indicates that co-agonists bring about significant glucose lowering in vivo on acute administration, with an effect at least as pronounced as that of Exendin-4 following a time delay of 4 hours.
Figure 5.3.3: Effect of acute co-agonist administration on glucose tolerance after a 4-hour delay in mice.

Overnight fasted male C57BL/6J mice (n=5-6) received an i.p injection of vehicle, 1 nmol/kg of exendin-4, or 10 nmol/kg of co-agonists, followed 4 hours later by a 2 g/kg i.p. glucose challenge. (A) Blood glucose concentrations (B) The area under the curve for blood glucose, all expressed as mean ± SEM. Statistical analysis carried out using one-way ANOVA and post hoc tests with Holm-Sidak correction. *p <0.05; **p <0.01, ***p<0.001, versus vehicle controls.

5.3.4 Effect of acute co-agonist administration on basal glucose levels in DIO mice

C57BL/6J mice were kept on a high fat, high sucrose diet for 4 months to induce obesity and glucose intolerance, hence developing a diet-induced model of diabetes (DIO mice) (Omar et al., 2012). To
investigate the effect of co-agonists on basal blood glucose levels, and determine whether there is a measurable hyperglycaemic effect, co-agonists were administered to fed DIO mice, in the absence of a glucose challenge. Blood glucose and insulin levels were measured at regular intervals, as indicated below.

Analogues 1, 2 and 3 significantly lowered basal plasma glucose levels in fed mice, with glucose AUC values of 979.5 ± 29.7, 968 ± 22.9, and 1016 ± 35.0 mmol/L*min respectively, in comparison to 1349 ± 54.7 mmol/L*min for vehicle (all p<0.0001). The glucose lowering effects of the co-agonists were comparable to those of exendin-4, which had an AUC of 994 ± 42.9 mmol/L*min (all p>0.1).

Analogue 2 was the only peptide to mediate a significant acute rise in plasma insulin levels from basal at t=60 minutes (p= 0.03, analysed by a two-way ANOVA corrected using Tukey’s multiple comparison test). Of note, exendin-4 failed to stimulate an increase in plasma insulin levels in ad libitum fed mice, while maintaining its glucose lowering effects. This lack of an insulinotropic response following an oral glucose stimulus has been shown previously for exendin-4, and the glucose lowering effects are instead attributed to delayed gastric emptying (Edwards et al., 2001b, Kolterman et al., 2003, Ionut et al., 2008).
Figure 5.3.4-1: Effect of acute co-agonist administration on blood glucose in fed DIO mice.

Male DIO mice (n=6) received an i.p injection of vehicle, 1 nmol/kg of exendin-4, or 10nmol/kg of co-agonists. (A) Blood glucose concentrations, (B) The area under the curve for the change in blood glucose, (C) Plasma insulin levels.
insulin concentrations, all expressed as mean ± SEM. Statistical analysis for AUC\textsubscript{glucose} carried out using one-way ANOVA and post hoc tests with Tukey’s correction. *p <0.05; **p <0.01, ***p<0.001, ****p<0.0001 versus vehicle. Statistical analysis for plasma insulin levels carried out using two-way ANOVA and post hoc tests with Tukey’s correction, # p <0.05 versus basal insulin levels.

To eliminate the effect of agonist-specific gastric emptying delays impacting delivery of dietary glucose to the circulation, the study was repeated following an overnight fast (Figure 5.3.4-2).

Analogues 1, 2 and 3 all significantly lowered blood glucose levels in fasted mice, with glucose AUC values of 619.5 ± 24.1, 652 ± 23.1, and 663.5 ± 49.7 mmol/L*min respectively, in comparison to 843 ± 48.9 mmol/L*min for vehicle (p=0.008, 0.025, and 0.037 respectively). The glucose lowering effects of the co-agonists were comparable to those of exendin-4, which had an AUC of 647 ± 63.9 mmol/L*min (all p>0.1). analogue 1 numerically had the greatest glucose lowering effect. Despite the reduction in glucose excursion, neither the co-agonists nor exendin-4 brought about a significant rise in plasma insulin levels compared to vehicle in fasted mice (all p>0.5).

The results confirm that the co-agonists do not worsen hyperglycaemia in diabetic rodent models, but rather lower basal glucose levels in both fed and fasted mice.
**Figure 5.3.4-2: Effect of acute co-agonist administration on basal blood glucose in fasted DIO mice.**

Overnight fasted male DIO mice (n=6) received an i.p injection of vehicle, 1 nmol/kg of exendin-4, or 10 nmol/kg of co-agonists. (A) Blood glucose concentrations (B) The area under the curve for blood glucose (C) Plasma insulin concentrations, all expressed as mean ± SEM. Statistical analysis for AUC_glucose carried out using one-way
Statistical analysis for plasma insulin levels carried out using two-way ANOVA and post hoc tests with Tukey’s correction. *p < 0.05; **p < 0.01, ***p<0.001 ****p<0.0001 versus vehicle.

5.3.5 Effect of acute co-agonist administration on glucose tolerance in DIO mice

Acute glucose tolerance tests were carried out on DIO mice, 30 minutes after an injection of vehicle, 1 nmol/kg of exendin-4 or 10 nmol/kg of co-agonists.

Basal blood glucose levels remained comparable across all groups following peptide administration, as assessed by the 0 minute time point. Following an i.p. glucose challenge, peak glucose excursion was lowered by all three co-agonists, and glucose lowering effects were comparable to those of exendin-4 (all p > 0.1).

Analogues 1, 2 and 3 lowered the glucose excursion significantly, with glucose AUC values of 1485 ± 183.0, 1311 ± 55.7, and 1247 ± 104.5 mmol/L*min respectively, in comparison to 2562 ± 148.6 mmol/L*min for vehicle (all p < 0.0001). The AUC values for the co-agonists were comparable to exendin-4, which had an AUC of 1405 ± 98.6 (all p > 0.1).

Analogues 1 and 2 brought about a significant increase in plasma insulin levels, with insulin AUC values of 232.0 ± 39.0 and 240.5 ± 31.3 ng/mL*min respectively, as compared to 101.8 ± 23.4 ng/mL*min for vehicle (p < 0.03). For Analogue 2, these findings are in keeping with earlier studies on fed DIO mice (Figure 5.3.4-1). Exendin-4 and Analogue 3 did not bring about a significant rise in plasma insulin levels compared to vehicle. There is no direct association between glucose tolerance and insulin secretion, as co-agonists and exendin-4 all have comparable glucose excursions despite markedly different insulin responses.
Figure 5.3.5-1: Effect of acute co-agonist administration on glucose tolerance in DIO mice.

Overnight fasted male DIO mice (n=6) received an i.p injection of vehicle, 1nmol/kg of exendin-4, or 10nmol/kg of co-agonists, followed 30 minutes later by a 1.5 g/kg i.p. glucose load. (A) Blood glucose concentrations (B) The area under the curve for the change in blood glucose (C) Plasma insulin concentrations (D) The area under the curve for insulin concentrations, all expressed as mean ± SEM. Statistical analysis carried out using one-way ANOVA and post hoc tests with Tukey’s correction. *p <0.05; **p <0.01, ***p<0.001 ****p<0.0001 versus vehicle.

Delayed IPGTTs were also conducted to investigate sustained effects of the peptides in DIO mice (Figure 5.3.5-2). In contrast to earlier findings from lean mice, neither exendin-4 nor the co-agonists improved glucose tolerance or enhanced insulin secretion after a 4 hour delay.
Figure 5.3.5-2: Effect of delayed co-agonist administration on glucose tolerance in DIO mice.

Overnight fasted male DIO mice (n=6) received an i.p injection of vehicle, 1 nmol/kg of exendin-4, or 10 nmol/kg of co-agonists, followed 4 hours later by a 1.5 g/kg i.p. glucose load. (A) Blood glucose concentrations (B) The area under the curve for the change in blood glucose (C) Plasma insulin concentrations (D) The area under the curve for insulin concentrations, all expressed as mean ± SEM. Statistical analysis carried out using one-way ANOVA and post hoc tests with Tukey’s correction. *p <0.05; **p <0.01, ***p<0.001, ****p<0.0001 versus vehicle.

5.3.6 Effect of acute co-agonist administration on food intake in DIO mice

The acute effect of co-agonists on food intake was assessed using DIO mice. DIO mice were fasted for 12 hours prior to a single i.p. injection of vehicle, 2.5 nmol/kg of exendin-4, or 25 nmol/kg of co-agonists, given at the onset of the dark phase. The mice were given access to HFHS diet during the course of the study and food intake was monitored at regular intervals.

A single injection of 25 nmol/kg of co-agonists did not significantly change acute food intake in *ad libitum* fed mice. In contrast, exendin-4 at a dose of 2.5 nmol/kg significantly decreased food intake compared to vehicle controls, albeit only during 4-12 hours post injection (p=0.009).
Figure 5.3.6: Effect of acute co-agonist administration on food intake in DIO mice.

Acute food intake in DIO mice \((n=5-6, \text{ average body weight } 41.3g)\) was monitored following a single i.p. injection of vehicle, 2.5 nmol/kg of exendin-4, or 25 nmol/kg of co-agonists. Peptide was administered 30 minutes after the onset of the dark phase. (A) Cumulative food intake (B) Interval food intake during 8-12 hours post injection. Data represented as mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Dunnett correction. *\(p <0.05\); **\(p <0.01\), ***\(p<0.001\), ****\(p <0.0001\) versus vehicle controls.

5.3.7 Effect of chronic co-agonist administration on food intake and body weight in DIO mice

To investigate the chronic effect of co-agonists, DIO mice were injected daily with peptide at the onset of the dark phase, and food intake and body weight was monitored. The doses of all treatments were up-titrated as the study proceeded to achieve an exendin-4 dose that curtailed weight gain. For exendin-4, the starting dose was 2.5 nmol/kg, which was doubled twice to achieve a final dose of 10 nmol/kg, which was administered daily for the final 8 days of the study. Co-agonists had a starting dose of 25 nmol/kg, and were given at a final dose of 100 nmol/kg for the last 8 days of the study.
Daily injections of the co-agonists did not reduce food intake significantly, with cumulative food intake of 32.4 ± 1.04 g, 37.0 ± 2.29 g and 43.0 ± 3.98 g for Analogues 1, 2 and 3, respectively, compared to 39.6 ±2.32 g for vehicle (all p>0.01). The co-agonists had comparable food intake to exendin-4, which had cumulative food intake of 39.1 ± 1.13 g (all p>0.01).

Despite no significant difference in food intake, animals given co-agonists on average weighed 4.71 g less than vehicle treated animals after 12 days. Analogues 1 and 2 in particular significantly reduced body weight, with a mean 12 day weight loss of 7.07 ± 0.79 g and 3.54 ± 0.52 g respectively, as compared to a weight gain of 0.57 ± 0.37 g for vehicle (p= 0.0001 and 0.004 respectively). Analogue 3 also stimulated weight loss of 1.8 ± 1.03 g, but the results failed to reach significance.

All three co-agonists numerically brought about greater weight loss than exendin-4, which had a mean weight loss of 0.18 ± 0.44 g. Furthermore, Analogues 1 and 2 mediated significantly greater weight loss than exendin-4 in spite of comparable food intake (p= 0.0001 and 0.006 respectively).
**Figure 5.3.7: Effect of chronic co-agonist administration on food intake and body weight in DIO mice.**

DIO mice (n=5-6, initial body weight 41.6g) received daily i.p injections of vehicle, exendin-4 or the co-agonists. Doses were doubled over the course of the study, as indicated on the graphs, to reach a final concentration of 10 nmol/kg for exendin-4 and 100 nmol/kg for co-agonists. Daily injections, and food and body weight measurements were carried out at 7.30pm, following the onset of the dark phase. (A) Cumulative food intake and (B) body weight changes during the 12 day study. Results are shown as mean ± SEM. Statistical analysis was carried out using one-way ANOVA and post hoc tests with Tukey's correction.

**5.3.8 Effect of chronic co-agonist administration on glucose homeostasis and glucose tolerance in DIO mice**

Following the 12 day chronic study, an acute glucose tolerance test was carried out to investigate the effect of chronic administration on glucose homeostasis. On the morning of the IPGTT, mice were
fasted for 6 hours, injected i.p. with 1.5 g/kg glucose, and blood glucose levels were monitored at indicated time points. In order to distinguish from chronic effects from acute effects of the peptides, mice were not injected with peptide on the morning of the IPGTT.

Basal blood glucose and insulin measurements were taken to look at the impact of chronic peptide treatment on glucose homeostasis. Basal blood glucose levels remained comparable across all groups following chronic peptide administration, as assessed by the 0 minute time point. To investigate the impact of chronic treatment on insulin homeostasis, homeostatic model assessment (HOMA) indices were calculated using the HOMA2 calculator (Table 5.3.8). The indices consist of three components which serve as surrogate markers of insulin resistance (HOMA-IR), beta cell function (HOMA2 %B) and insulin sensitivity (HOMA2 %S) (Levy et al., 1998). Chronic treatment with co-agonists tends to improve the insulin resistance seen in DIO mice, as indicated by lower HOMA-IR values. Treatment with Analogues 1 and 2 in particular lowered HOMA-IR significantly (p=0.005 and 0.012 respectively). Concurrently with improvements in insulin resistance, Analogue 1 and 2 mediate improvements in insulin sensitivity, as indicated by higher HOMA2 %S values (p=0.04 and 0.02 respectively). The improvement in insulin sensitivity parallels improvements in weight loss (Figure 5.3.7). However, none of the co-agonists impact insulin secretion or beta cell function on chronic administration, demonstrated by the lack of significant changes in HOMA2 %B.

<table>
<thead>
<tr>
<th></th>
<th>HOMA-IR</th>
<th>HOMA2 %B</th>
<th>HOMA2 %S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4.80 ± 1.12</td>
<td>80.8 ± 25.7</td>
<td>25.6 ± 7.22</td>
</tr>
<tr>
<td>Exendin-4</td>
<td>4.35 ± 0.86</td>
<td>87.0 ± 18.8</td>
<td>24.6 ± 4.04</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>0.78 ± 0.04 **</td>
<td>31.1 ± 9.33</td>
<td>126 ± 6.70*</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>1.49 ± 0.53 *</td>
<td>46.2 ± 17.2</td>
<td>132 ± 39.1*</td>
</tr>
<tr>
<td>Analogue 3</td>
<td>3.22 ± 0.68</td>
<td>44.5 ± 6.65</td>
<td>45.4 ± 15.7</td>
</tr>
</tbody>
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Table 5.3.8: HOMA indices following chronic treatment with co-agonists.

Results calculated using the HOMA2 calculator (https://www.dtu.ox.ac.uk/homacalculator/). Data represented as mean ± SEM, n=5-6. *p<0.05, **p<0.01 vs vehicle, analysed using one-way ANOVA with Dunnett’s post hoc correction.

Following an i.p. glucose challenge, only exendin-4 significantly reduced peak glucose excursion, with peak blood glucose levels of 13.88 mmol/L compared to 24.53 mmol/L for vehicle (p= 0.009). A trend towards improved glucose tolerance was seen with Analogues 1, 2 and 3, with glucose AUC values of 2365 ± 280.5, 2105 ± 307.5, and 2007 ± 366.5 mmol/L*min respectively, in comparison to 2653 ±
115.8 mmol/L*min for vehicle; however none of these results reached statistical significance. In contrast, exendin-4 significantly lowered the glucose AUC compared to vehicle, with an AUC value of 1641 ± 106.4 mmol/L*min (p= 0.04).

Despite the reduction in glucose excursion, neither the co-agonists nor exendin-4 brought about a significant rise in plasma insulin levels compared to vehicle (all p>0.5). Exendin-4 and Analogue 3 showed a numerically greater insulinotropic response compared to vehicle, with insulin AUC values of 83.3 ± 15.9 and 102.1 ± 29.0 ng/mL*min respectively, compared to 70.9 ± 4.00 ng/mL*min for vehicle. As these two treatment groups also had the greatest glucose lowering, this suggests an association between the insulinotropic response and glucose tolerance. Analogue 1, which mediated the greatest weight loss, had the lowest insulin response, with an insulin AUC of 32.7 ± 3.74 ng/mL*min. This may reflect on enhanced insulin sensitivity, consistent with the greater weight reduction.

**Figure 5.3.8: Effect of chronic co-agonist administration on blood glucose homeostasis in DIO mice.**

DIO mice (n=5-6) were fasted for 6 hours, and injected i.p. with a 1.5 g/kg glucose load. (A) Blood glucose concentrations (B) The area under the curve for the change in blood glucose (C) Plasma insulin concentrations
The area under the curve for insulin concentrations, all expressed as mean ± SEM. Statistical analysis carried out using one-way ANOVA and post hoc tests with Tukey’s correction. *p <0.05; **p <0.01, ***p<0.001, ****p<0.0001 versus vehicle.

5.3.9 Pharmacokinetic profiles of co-agonists in slow release diluent

The ability of Zinc to stabilise peptides and protect them from proteolytic degradation in vivo is well established, and is used commercially to stabilise preparations of insulin, recombinant human growth hormone and α-interferon (Bartus et al., 1998, Dunn, 2005, Li, 2014, Havelund et al., 2015). Previous research from our group has developed a slow-release peptide formulation using zinc chloride (ZnCl₂) which helps maintain sustained plasma peptide levels in rodents. This enables reliable, longer-term studies into food intake and body weight following a single peptide injection. Furthermore, as this experimental design eliminates the need for multiple injections, it minimises interference from environmental factors.

Histidine is a classical zinc chelating ligand, and work from Phillips et al. (2010) has demonstrated that incorporating Histidine residues in insulin hexamers facilitates Zn²⁺ chelation and insulin depot formation (Phillips et al., 2010). Analogues 1, 2 and 3 all have a Histidine residue at position 3, and a five residue Histidine tail at the C terminus. This increased number of Histidine residues is predicted to enhance peptide-zinc binding and hence depot formation (Laity et al., 2001, Blindauer, 2008). Additionally, the co-agonists have Glutamate residues which are negatively charged at physiological pH, and further facilitate chelation with zinc (Campbell M. K., 2012). Control peptide exendin-4 also has a number of glutamate residues, which would facilitate chelation, but exendin-4 is expected to chelate less well with Zn²⁺ due to the relative lack of Histidines.

In the rat experiments described below, all peptides were prepared in a solution of ZnCl₂, with a final ZnCl₂ to peptide ratio of 0.7:1. This peptide-zinc solution has a pH range of 4-5, and at these low pH values Histidine is protonated and positively charged, which in turn increases peptide solubility. Post administration, Histidine residues are deprotonated at a physiological pH of 7 and lose their positive charge. Accordingly, the peptide becomes insoluble in vivo, and forms a subcutaneous depot that releases peptide into circulation over time (Campbell M. K., 2012).

To investigate pharmacokinetics in vivo, naïve male Wistar rats were injected subcutaneously with 700 nmol/kg (1mg) of peptide, in an injection volume of 10μL, with a ZnCl₂ to peptide ratio of 0.7:1. Blood samples were obtained at regular intervals as indicated, and plasma peptide levels were measured by radioimmunoassay (detailed in Methods, section 2.3.3.2).
All three co-agonists and exendin-4 reached maximum plasma concentrations from $t=0$ to 3 hours. The AUC values for Analogues 1, 2 and 3 were comparable ($p>0.01$), indicating similar peptide exposure over the course of 72 hours. All three co-agonists remained detectable even after 72 hours, with a final plasma concentration that did not fall below half maximal. This finding facilitates direct comparison of the three co-agonists in acute feeding studies.

In contrast to the co-agonists, exendin-4 levels fall rapidly, and reach half-maximal concentrations by 72 hours. As a much lower dose of agonist was planned for chronic administration studies than used for pharmacokinetic analysis, the apparent faster net disappearance of exendin-4 could result in periods of zero agonist exposure with an infrequent dosing schedule. To avoid this issue, and normalise animal handling across groups, in chronic studies including exendin-4, all peptides were administered daily.

Figure 5.3.9: Pharmacokinetic profiles of the co-agonists in Wistar rats.

Naïve rats (n=4) received subcutaneous injections of 1 mg of co-agonist or exendin-4, formulated in ZnCl$_2$ slow release diluent (0.7:1, zinc to peptide). Plasma peptide levels were measured at indicated time points using an RIA, and the results for (A) Analogue 1, (B) Analogue 2, (C) Analogue 3 and (D) Exendin-4 are shown as means ± SEM.

5.3.10 Effect of acute co-agonist administration on food intake and body weight in rats

Analogue were formulated in ZnCl$_2$ slow release diluent (0.7:1 ratio of zinc to peptide) and administered by subcutaneous injection to fasted rats, at a weight-adjusted dose of 10 nmol/kg.
Following a single injection, food intake and body weight were monitored at regular intervals as indicated.

A single dose of Analogues 1, 2 and 3 resulted in a significant reduction in food intake during the 8-24 hour time period (p <0.05, p <0.001 and p <0.01 respectively). Analogues 2 and 3 continued to inhibit food intake during the 24-48 hour time period (p <0.0001 and p <0.05 respectively), but no significant differences were seen in food intake during 48-144 hours.

Analogues 1, 2 and 3 reduced body weight significantly 48 hours post injection compared to vehicle (p= 0.007, 0.0001 and 0.0001). Analogues 2 and 3 reduced cumulative food intake up to 48 hours post injection (P <0.001), and stimulated the most significant weight loss in keeping with this superior food intake reduction. Interestingly, all co-agonists continued to significantly reduce weight gain from 48 hours to 6 day time period, in the absence of any significant changes in food intake. Animals treated with Analogues 1, 2 and 3 had an average 6 day weight gain of 18.0 ± 1.54 g, 6.75 ± 4.48 g and 10.2 ± 3.79 g compared to 33.8 ± 2.25 g for vehicle (p= 0.0009, 0.0001 and 0.0001 respectively).

In summation, all analogue treated groups had significantly lower body weight 6 days post injection compared to vehicle. Despite no significant reduction in 6 day cumulative food intake, Analogues 1, 2 and 3 significantly reduced 6 day cumulative weight gain, by an average of 46%, 80% and 69% compared to vehicle. The relative potency of weight loss corresponds positively with the extent of GCGR activity noted *in vitro*. 

Figure 5.3.10: Effect of acute administration of co-agonists on food intake and bodyweight in rats.

(A) Interval food intake (B) Cumulative food intake and (C) Cumulative bodyweight change in overnight fasted adult male Wistar rats (n=5, initial body weight 410g) after a single subcutaneous injection of vehicle or 10 nmol/kg of co-agonists in a slow release formulation (ZnCl₂ to peptide ratio 0.7:1). Data shown as the mean ± SEM. Statistical analysis carried out using one way ANOVA and post hoc tests with Dunnett’s correction, *p <0.05; **p <0.01, ***p<0.001, ****p<0.0001 compared to vehicle.
5.3.11 Effect of chronic co-agonist administration on food intake and body weight in rats

To investigate the effect of chronic co-agonist administration on body weight and food intake in rats, adult male Wistar rats were injected daily with vehicle, exendin-4 or Analogue 2 at the indicated doses. Analogue 2 was chosen as an exemplar as it mediated the most profound weight loss in acute studies. Both Analogue 2 and comparator exendin-4 were formulated in ZnCl₂ slow release diluent (0.7:1 ratio of zinc to peptide). As shown by pharmacokinetic analysis, the peptides have comparable 24 hours exposure when administered using this protocol (see Figure 5.3.9). It is noted that the co-agonists have longer exposure times than exendin-4, and could therefore be accumulated over time following daily injections; however to avoid periods of zero agonist exposure with exendin-4 at the lower doses used in this chronic study (compared to for pharmacokinetic measurements), and to minimise differences in animal handling and stress across the groups, both exendin-4 and Analogue 2 were injected daily.

Body weight and food intake were measured daily. To determine whether weight loss involved food-intake-independent mechanisms, stimulated by glucagon receptor activity, pair-fed groups were included. The pair-fed groups were given a restricted amount of food each day, matched to the food intake for the corresponding experimental group over the last 24 hours.

Initially, both co-agonist and exendin-4 were trialled at doses of 12.5 and 37.5 nmol/kg. Analogue 2 caused profound weight loss even at the lower dose, and was hence administered at 12.5 nmol/kg. Analogue 2 was later reduced to 8 nmol/kg on day 3 due to significant weight loss. Exendin-4 was trialled at 12.5 nmol/kg and 37.5 nmol/kg, and the lower dose of exendin-4 (12.5 nmol/kg) was discontinued on day 3 as it appeared ineffective at reducing food intake and body weight. Exendin-4 at 37.5 nmol/kg was chosen as a better comparator for Analogue 2. As the weight loss effects appeared to plateau on day 3, the dose was increased to 50 nmol/kg, but further dose escalation was prevented by excessive food intake reduction and signs of illness in the treated group.

Analogue 2 significantly lowered body weight during the 8 day chronic study, with a cumulative weight loss of 79.8 ± 4.25 g compared to a mean weight gain of 30.5 ± 2.93 g for vehicle (p<0.0001). Furthermore, Analogue 2 significantly lowered body weight compared to its pair-fed group, which had a mean weight gain of 0.86 ± 1.96 g (p <0.0001), confirming that the weight loss is not purely due to food intake reduction, and suggesting an energy expenditure effect. In contrast, exendin-4 treated animals and their corresponding pair-fed group showed similar weight loss effects (p= 0.99), confirming that the weight loss due to GLP-1R specific agonists is mediated primarily by reduced energy intake.
Analogue 2 brings about 8 times greater weight loss than exendin-4, which has a mean cumulative weight loss of 10.6 ± 3.23 g (p <0.0001), while maintaining a numerically higher cumulative food intake of 190.4 ± 7.26 g compared to 165.6 ± 8.07 g for exendin-4 (p= 0.05). Furthermore, up until Day 7 of the study, Analogue 2 had significantly higher food intake than exendin-4 (p<0.01) while maintaining significantly higher weight loss (p<0.0001). In summary, the co-agonist mediates greater weight loss than exendin-4, a GLP-1R agonist, with less reduction in food intake, while being administered at a lower dose.
Figure 5.3.11: Effect of chronic administration of co-agonists on food intake and body weight in rats.

(A) Cumulative food intake and (B) cumulative body weight change in adult male Wistar rats (n=6-7, initial mean body weight 498g) after daily injections of vehicle, 37.5nmol/kg of exendin-4, or 12.5nmol/kg of Analogue 2 (unless indicated otherwise) in a slow release formulation (ZnCl$_2$ to peptide ratio 0.7:1). Data shown as the mean ± SEM. Statistical analysis carried out using one way ANOVA and post hoc tests with Tukey’s correction, *p <0.05; **p <0.01, ***p<0.001, ****p <0.0001 for exendin-4 and Analogue 2 treatment groups compared to vehicle, *p<0.05, **p <0.01, ***p <0.001, ****p <0.0001 for exendin-4 compared to Analogue 2.
5.3.12 Effect of chronic co-agonist administration on glucose tolerance in rats

After the 8 day chronic study, an acute glucose tolerance test was carried out. Following a 6 hour fast, rats were injected subcutaneously with peptide, followed 30 minutes later by an i.p. injection of 2 g/kg glucose. Blood glucose levels were monitored regularly, as indicated.

Analogue 2 significantly lowered basal blood glucose levels following chronic administration, to 4.33 ± 0.24 mmol/L compared to 5.60 ± 0.20 mmol/L for vehicle (p=0.0007). In contrast, chronic treatment with exendin-4 did not affect basal blood glucose levels relative to vehicle (p<0.5). Analogue 2 also lowered basal blood glucose levels significantly in comparison to exendin-4, which had basal blood glucose levels of 5.89 ± 0.20 mmol/L (p<0.0001).

Despite the enhanced weight loss and improved basal glucose, Analogue 2 did not have a measurable effect on glucose tolerance, with a glucose AUC value of 1156 ± 30.1 mmol/L*min, compared to 1158 ± 51.3 mmol/L*min for vehicle. Paradoxically, exendin-4 significantly impaired glucose tolerance relative to vehicle (glucose AUC value of 1493 ± 95.0 mmol/L*min, p=0.008).
Figure 5.3.12: Effect of chronic co-agonist administration on blood glucose homeostasis in rats.

Following the chronic study, adult male Wistar rats (n=5-6) were fasted for 6 hours. Rats were injected subcutaneously with vehicle, 50 nmol/kg of exendin-4, or 8nmol/kg of Analogue 2, followed 30 minutes later by a 2 g/kg i.p. glucose challenge. (A) Basal blood glucose concentrations (B) Blood glucose concentrations during the GTT (C) The area under the curve for the change in blood glucose, all expressed as mean ± SEM. Statistical analysis carried out using one-way ANOVA and post hoc tests with Tukey’s correction. *p <0.05; **p <0.01, ***p<0.001, versus vehicle.
5.4 Discussion

The work presented here describes the in vivo effects of GLP-1R/GCGR co-agonists in rodents, in comparison to GLP-1R agonist exendin-4. The aim was to determine whether the superior insulinotropic effects observed in vitro translate to meaningful physiological outcomes in vivo.

In vitro models provide a reliable, reproducible set up for high throughput screening. In the context of this project, in vitro models were particularly useful as a reductive approach to look at the impact of co-agonists on insulin secretion independent of liver glucose production and weight loss effects. However, in vitro models cannot recapitulate the milieu of factors influencing in vivo responses, such as pharmacokinetics and pharmacodynamics, inter-organ communication, and neural innervation. Therefore, in vivo studies are necessary to assess the physiological relevance and therapeutic utility of the co-agonists.

It is important to study these co-agonists in diabetic models to determine whether the hypothesised glycaemic effects are preserved in disease conditions. Various diabetic rodent models are available, including models of: monogenic diabetes, polygenic diabetes, chemically-induced diabetes and diet induced diabetes (King, 2012). I chose to use diet induced obese mice, as this model encapsulates the environmental effect in the pathogenesis of diabetes, and is hence the most relevant comparison available to human T2DM progression. While polygenic models also have an aetiology similar to the disease causation in man (Fuchsberger et al., 2016), most polygenic models carry additional, genotype-specific physiological defects that can confound research (Nakamura and Yamada, 1967). In contrast, monogenic and streptozotocin-induced mouse models are aetiologically remarkably different from T2DM, bringing into question their physiological relevance (Srinivasan et al., 2005, King, 2012).

Consequently, I used a diet induced obese mouse model for in vivo studies. Lean C57BL/6J mice were kept on a high fat, high sugar diet for 4 months prior to experiments to induce obesity, insulin resistance, and glucose intolerance (Surwit et al., 1988, Fisher-Wellman et al., 2016). The main drawback of the model is that there is less impairment of glucose tolerance as compared to monogenic models, making it difficult to measure subtle effects of treatments, which might explain the relatively high doses of peptide needed to achieve a measurable response. Secondly, the combination of low hourly intake of HFHS diet (as low as 0.15 g in the dark phase) and the suboptimal physical integrity of HFHS pellets reduced the precision of food intake measurements across the groups. To account for this, pair feeding studies were conducted on Wistar rats instead, where minimum hourly food intake was 0.50 g, using standard chow.
The long-term therapeutic profile of GLP-1R agonists results from a combination of insulinotropic effects, improvements in beta cell function, and increased insulin sensitivity due to weight loss, making it difficult to delineate the relative contribution of each component. It is therefore useful to conduct acute, single dose studies, prior to any significant weight loss, to specifically investigate the insulin secretion effects of the co-agonists. In the acute studies conducted in this project, co-agonists enhanced insulin release in vivo, in keeping with the insulinotropic effects seen in vitro. Co-agonists acutely improved glucose tolerance, and lowered peak glucose excursion in both lean and DIO mice. Acute insulinotropic actions by GLP-1R/GCGR co-agonists have only been demonstrated once before, by Kerr et al. (2010). As the latter study did not include a GLP-1 comparator, this is the first study to show superior insulin secretion in vivo in response to acute administration of a GLP-1R/GCGR co-agonist versus a GLP-1R specific agonist (Figure 5.3.5-1). Furthermore, the relative potency of co-agonists at stimulating insulin secretion in vitro from murine beta cell lines is similar to their insulinotropic efficacy in vivo, with Analogue 2 stimulating the greatest insulin response in both settings. This suggests that the enhanced insulin response seen in vivo could be due to enhanced stimulation of insulin release from beta cells. There is a consensus that synthetic GLP-1R and GLP-1/GCGR co-agonists can mediate direct effects on islets, which supports this hypothesis (Du et al., 2012, Smith et al., 2014). However, no correlation was seen between the insulinotropic response and the extent of glucose lowering by the co-agonists, suggesting both insulin sensitivity and insulin secretion are involved in bringing about the overall observed changes in glucose homeostasis. This work was limited by the absence of pharmacokinetic data in mice, as different pharmacokinetic profiles could also explain the differential insulin responses. Furthermore, my in vitro results question the contribution of GCGR agonism to the insulinotropic response with Analogue 1, 2 and 3 and suggest that the effect could relate instead to differential GLP-1R pharmacology and signalling bias.

A primary safety concern with GLP-1R/GCGR co-agonists is that the GCGR activity will increase endogenous glucose production and hence worsen hyperglycaemia in diabetic patients. Contrary to this assumption, all three co-agonists significantly lowered plasma glucose levels in both fed and fasted DIO mice, with the glucose lowering effects comparable to those of exendin-4, showing that the glucagon component in these co-agonists does not stimulate persistent hyperglycaemia. In the fed mice exclusively, glucose lowering was accompanied by an enhanced insulin response. This might reflect on the glucose-dependence of insulin secretion mediated by the peptides (fasted mice have basal plasma glucose levels of 6.53 mmol/L compared to 9.60 mmol/L for fed mice), consistent with parent compound GLP-1 (Goke et al., 1993, Qualmann et al., 1995, Nauck et al., 1998). The ability of the co-agonists to modulate glucose tolerance in fasted mice even in the absence of insulinotropic
responses implies the involvement of insulin-independent mechanisms, although preliminary HOMA-IR data suggests that there are no significant changes in beta cell function or in insulin sensitivity following acute peptide administration in fasted mice (data not shown). From the available information, I cannot confirm the contribution of impaired insulin secretion and sensitivity to the observed metabolic profiles, but it would be interesting to carry out insulin tolerance tests and hyperglycaemic and hyperinsulinemic-euglycemic clamps to help answer these questions.

To determine the long-term profile and therapeutic utility of the co-agonists, chronic studies were performed in DIO mice. Surprisingly, in spite of profound weight loss and acute insulin secretion, none of the co-agonists improved glucose tolerance following 12 day chronic administration. In comparison, exendin-4, which mediated the least weight loss, improved glucose tolerance significantly. The relative potency of the co-agonists also varied dramatically from the acute studies: while Analogues 1 and 2 stimulated the highest insulin secretion acutely, they had the lowest insulin secretion and glucose tolerance following chronic administration. The connection between insulin responses and glucose tolerance following chronic co-agonist administration was tenuous: of the co-agonists, only Analogue 3 had a numerically greater insulinotropic response compared to vehicle, and correspondingly had the greatest glucose lowering effect of the three co-agonists. While this is in keeping with earlier studies (Kerr et al., 2010) and suggestive of an association between the insulinotropic response and glucose tolerance, the differences in glucose tolerance achieved in this project were minimal and did not reach significance. In general, my co-agonists showed a trend towards reduced insulin responses on chronic administration, and a negative correlation was seen between the insulin AUC and weight loss. Analogue 1 in particular is interesting as it has a lower insulin response than vehicle or the other co-agonists, but mediates comparable glucose lowering. This is potentially due to improvements in insulin sensitivity secondary to weight loss. This hypothesis is supported by the HOMA indices, which demonstrate that co-agonists lead to improvements in insulin resistance in parallel with inducing weight loss. For Analogue 1 and 2, which mediate the greatest weight loss, the order of potency for weight loss is identical to the order of potency of improving insulin resistance (HOMA-IR). Furthermore, the observed metabolic changes are associated specifically with improvements in insulin sensitivity and are independent of improvements in beta cell function.

In accordance with mouse studies, chronic administration of co-agonists in rats led to significant weight loss and lowered basal plasma glucose, but failed to improve glucose tolerance relative to vehicle. Exendin-4 had a paradoxical hyperglycaemic response during the IPGTT, but this probably reflects more on experimental design than peptide pharmacology. Such hyperglycaemic responses have been noted previously by Perez-Tilve et al. (2010), and are thought to be specific to acute
exendin-4 administration in lean, non-diabetic animals (Young et al., 1999, Fehse et al., 2005). It would be interesting to repeat the chronic study without exendin-4 injections prior to the IPGTT. Furthermore, future work would include repeating the study in diabetic rat models, to determine the impact of co-agonists in disease conditions in rats. The second key parameter monitored in chronic studies was weight loss. All co-agonists induced profound weight loss in DIO mice, with Analogues 1, 2 and 3 causing a 17.0%, 9.0% and 4.4% reduction in body weight respectively, in comparison to a weight gain of 2.0% for vehicle treated animals. The peptides were more efficacious than exendin-4, which was ineffective at causing weight loss (overall weight loss of only 0.4% of body weight). Of the co-agonists tested, Analogue 1 mediated the most profound weight loss. This weight loss effect correlates to the receptor activity for Analogue 1: of the three analogues, Analogue 1 has the lowest GLP-1 receptor activity in rat and human cell lines, and has a significant glucagon receptor preference. Particularly at the human receptor system, which shares greater homology with murine receptors (Knudsen et al., 2012), Analogue 1 is the most GCGR-preferring co-agonist. This preference for glucagon versus GLP-1 receptor activation could explain both the superior weight loss and the limited insulinotropic potential. However, in the absence of murine receptor data, I cannot draw definitive conclusions. These results support the hypothesis that the intrinsic glucagon receptor activity of co-agonists enhances weight loss beyond that mediated by GLP-1R selective agonists. Of particular significance is the finding that co-agonists had comparable food intake to exendin-4 despite markedly greater weight loss, confirming the involvement of GLP-1R-independent mechanisms. It is worth noting that the peptides were given at a 10 times higher dose than exendin-4, which is consistent with the higher EC50 value seen for co-agonists in in vitro studies. This calls into question the canonical way of ranking potencies based on EC50 values, as these analogues mediate superior physiological effects despite lower cAMP potencies; comparisons are however complicated by the absence of pharmacokinetic data of each agonist injected without zinc. In line with mouse studies, co-agonists co-injected with zinc brought about significantly greater weight loss in rats, while maintaining significantly higher food intake than the exendin-4 comparator group. Pair feeding studies in rats confirmed that the weight loss due to co-agonists cannot be explained entirely by food intake reduction through GLP-1R activation. Instead, the superior weight loss is postulated to be due to increased energy expenditure brought about by glucagon receptor activity (Day et al., 2009, Kosinski et al., 2012, Tan et al., 2013, Cegla et al., 2014, Henderson et al., 2016). It has previously been shown that increasing glucagon receptor activity increases weight loss brought about by GLP-1R/GCGR co-agonists (Pocai et al., 2009, Kosinski et al., 2012). My findings
support this hypothesis, as the degree of GCGR-preference seen at rat receptors in vitro co-relates strongly to the extent of weight loss seen on acute co-agonist administration in rats. To determine the contribution of energy expenditure to the observed weight loss, future studies would include direct measurements by indirect calorimetry of animals housed in metabolic cages.

In both mouse and rat feeding studies, the exendin-4 dose was increased with the course of the study to achieve measurable reductions in food intake and body weight. This dose-dependency of metabolic effects of exendin-4 has been reported previously (Young et al., 1999, Yang et al., 2014, Hou et al., 2015). Furthermore, the latter studies also noted a similar plateauing of weight loss effects shortly after the start of chronic studies. The initial extensive weight loss has been attributed to dehydration due to diuresis and adipsia, but further investigation is needed to confirm this. It is surprising that, despite similar pharmacokinetic profiles in vivo in rats, significantly different doses of exendin-4 and Analogue 2 are needed in order to attain measurable effects on food intake and body weight. This may reflect on differential pharmacodynamics and receptor activity: exendin-4 was previously noted to have a significantly lower EC\textsubscript{50} value for activation of beta cells insulin responses compared to the co-agonists (Table 3.3.6). It is also worth mentioning that the exendin-4 appears more potent than co-agonists in DIO mice, but is less potent relative to co-agonists in Wistar rats. This could be due to species-specific dose-dependence of food intake effects in vivo, which has been reported previously for exendin-4 (Mack et al., 2006). Alternatively, it could be due to differential sensitivity to exendin-4 in diabetic rodents (DIO mice) versus non-diabetic rodents (Wistar rats).

In comparison to earlier studies, my co-agonists mediate superior weight loss in DIO mice. The increased potency of the co-agonists, as measured in receptor cAMP assays compared to Pocai et al. (2009) and Kerr et al. (2010), could be responsible for the enhanced potency of weight loss in vivo. Secondly, as my co-agonists have higher preference for glucagon receptor activation (relative activity ratios of 2.86, 1.05 and 0.58 compared to 0.19 at the human receptor for Henderson et al. (2016) and 0.25 for Day et al. (2009)), the effect of the glucagon component would be more noticeable in my peptides, which might explain this superior weight loss. However, the GLP-1R versus GCGR activity ratio must be tailored appropriately in order to avoid hyperglycaemia in diabetic patients, although endogenous insulin is predicted to effectively outweigh hyperglycaemic tendencies of glucagon in patients with early-stage Type 2 diabetes (El Youssef et al., 2014).

The ability of co-agonists to mediate superior weight loss without a concomitant reduction of food intake is particularly promising, as it suggests that co-agonists would lead to less nausea than GLP-1R agonists. As mentioned earlier, GLP-1R agonists are associated with nausea at higher doses, which limits their dose and hence efficacy (Kolterman et al., 2005, Ratner et al., 2010, Barrington et al.,
As many as 50% of the patients prescribed GLP-1R agonists suffer from nausea, which is closely linked to treatment discontinuation (Shyangdan et al., 2011, Bettge et al., 2017). In the absence of an emetic reflex, acute food intake reduction is used as a measure of nausea in rodents (Horn, 2014, Kenward et al., 2015). In mice, exendin-4 reduces food intake significantly at earlier time points, namely 4 to 12 hours post administration, whereas co-agonists administered at 10 times the dose have no measurable effect, suggesting that co-agonists would lead to less nausea in humans than exendin-4, and could accordingly be given at higher doses to achieve greater therapeutic effect. The usual caveats about unmeasured pharmacokinetic differences apply.

In addition to being GLP-1R/GCGR co-agonists, these analogues are also G protein-biased GLP-1R agonists. Recent work by Jones et al. (2018) has reported that desensitisation effects of biased agonists noted in vitro can take up to 4 hours to manifest in vivo. Consequently, delayed IPGTTs were conducted to investigate the effect of biased analogues in vivo. Consistent with Jones et al. (2018), the co-agonists show a time-dependant glucose lowering effect, which exceeds that of exendin-4 only after 4 hours. However, this phenomenon appears specific to lean mice for my co-agonists, as a similar delayed study in DIO mice showed no lowering of plasma glucose at 4 hours. Furthermore, in contrast to Jones et al. (2018), daily administration of my biased analogues did not improve glucose tolerance chronically. The discrepancy in results might be due to different pharmacokinetics of my co-agonists.

In keeping with another earlier study, co-agonists with greater G protein bias mediated greater glucose lowering in vivo acutely (Zhang et al., 2015). However, the positive chronic effect on glucose tolerance, secondary to enhanced insulin sensitivity, seen by both groups (Zhang et al., 2015, Jones et al., 2018) was not recapitulated here. There is a suggestion of increased insulin sensitivity for Analogues 1 and 2, which have lower HOMA-IR values following chronic administration, and exhibit lower insulin excursions for comparable glucose tolerance as vehicle. Hence, it would have been useful to investigate insulin sensitivity markers. The inconsistency between my findings and the earlier studies in terms of chronic effects could be due to the glucagon component of my co-agonists, which alters their metabolic profile and leads to profound weight loss chronically without changes in glycaemic control.

In summation, the co-agonists bring about an acute enhancement of insulin release in comparison to exendin-4, as predicted by the in vitro data. The superior acute insulinotropic response compared to exendin-4 is a novel finding for the co-agonists. However, in the studies presented, this does not translate into glycaemic improvements chronically and further investigations are required to establish the clinical utility of co-agonists for a diabetes treatment. At a minimum, my findings...
provide reassurance that the co-agonists described, despite their GCGR activity, do not result in deteriorations in glucose tolerance. This is also the first study to report significant body weight reduction, in the absence of changes in food intake, in response to synthetic GLP-1R/GCGR co-agonists in DIO mice (Pocai et al., 2009, Day et al., 2009, Kerr et al., 2010, Henderson et al., 2016). These results suggest a therapeutic role for co-agonists as weight loss agents, which are safe to use in patients with type 2 diabetes.
6. General Discussion
Type 2 diabetes mellitus is a metabolic disorder fast approaching the status of a global epidemic, with over 451 million patients diagnosed worldwide (Cho et al., 2018). Type 2 diabetes results from a combination of insulin resistance and impaired insulin secretion, and manifests as persistently elevated blood glucose levels. This chronic glucose dysregulation is associated with a number of co-morbidities, increasing the risk of the death in patients with type 2 diabetes by a reported 28.4% (2017). In 2017, 5 million deaths were attributed to diabetes, highlighting the impact of this condition and the dire need for better management (Cho et al., 2018).

There are a number of treatments available for type 2 diabetes, including metformin, sulphonylureas, exogenous insulin, DPP-IV inhibitors and GLP-1 analogues. The latter are particularly useful; studies have consistently found liraglutide and semaglutide to be the most effective drugs on the market (Madsbad, 2009, Ahren et al., 2018). However, despite advances in drug development, only 40.8% of patients in England achieved the NICE recommended treatment targets in 2016-17 (NHS, 2018), indicating an urgent need for more effective treatments for diabetes.

While GLP-1 analogues are in widespread clinical use, dose-escalation studies suggest that these drugs have yet to reach their maximal efficacy, and are dose-limited prematurely due to a propensity for causing undesirable side effects such as nausea at higher doses (Kolterman et al., 2005, Ratner et al., 2010, Barrington et al., 2011, Nauck et al., 2016). One possible way of minimising deleterious side effects while maximising glycaemic control is to combine GLP-1 with a second insulinotropic peptide. This approach is predicted to theoretically lower receptor occupancy of any one receptor subtype, hence lowering drug exposure and minimising the propensity for GLP-1 related side effects. There is a lack of evidence for this as co-agonists are yet to be trialled extensively in humans, but initial work on GLP-1R/GIPR co-agonists provides proof of concept, showing that co-agonists can lower blood glucose without inducing nausea (Finan et al., 2013, Portron et al., 2017). The only prolonged studies published to date on GLP-1R/GIPR and GLP-1R/GCGR co-agonists demonstrated that co-agonists were well tolerated, but did not compare the incidence of nausea for a dual agonist versus GLP-1R agonist liraglutide (Frias et al., 2017, Ambery et al. 2018). In addition to reducing side effects, coupling GLP-1 with another insulinotropic peptide could also enhance insulin secretion and glucose tolerance (Sadry and Drucker, 2013, Lund et al., 2014).

The interest in combination therapies with GLP-1 stems from the success of bariatric surgery. Bariatric surgery is currently the most effective treatment for type 2 diabetes, with one meta-analysis quantifying the remission rate for surgery at 63.5% versus 15.6% for conventional medical therapy (Ribaric et al., 2014). It is postulated that surgery brings about beneficial effects on glycaemic tolerance at least partly by simultaneously altering levels of multiple hormones (Pournaras
et al., 2010, Nannipieri et al., 2011, le Roux et al., 2011, LaFerrere et al., 2011). In particular, post-prandial GLP-1, GIP and oxyntomodulin levels are elevated post-surgery, which has fuelled investigation into GLP-1 combination therapy with either GIP or glucagon (Jorde et al., 1981, Naslund et al., 1998, LaFerrere et al., 2007, Goldfine et al., 2007, LaFerrere et al., 2008, LaFerrere et al., 2010b, Holter et al., 2017). With regards to glucagon, the impact of surgery remains contentious, with some studies reporting a reduction in glucagon levels (Korner et al., 2006, Umeda et al., 2011). However, overwhelming evidence suggests that there is an increase in post-prandial glucagon levels post-bypass, from 1 month to 5 years following the operation, although the physiological relevance of this change remains undetermined (Falken et al., 2011, Jorgensen et al., 2012, Jorgensen et al., 2013, Jimenez et al., 2013, Salehi et al., 2014).

This project in particular investigates the therapeutic utility of combining GLP-1 with glucagon in order to maximise the insulinotropic effects of GLP-1. While this approach seems counter-intuitive given the hyperglycaemic tendencies of glucagon, there is substantial empirical evidence to support it gleaned from studies done on oxyntomodulin. Persistently elevated oxyntomodulin levels have been proposed to play a role in the improved glycaemic control and weight loss seen following a gastric bypass (LaFerrere et al., 2010a, Falken et al., 2011). In human volunteers, both native oxyntomodulin and a co-infusion of GLP-1 and glucagon have previously been shown to mediate beneficial glycaemic effects (Wynne et al., 2005, Tan et al., 2013, Shankar et al., 2018).

Additionally, oxyntomodulin stimulates greater weight loss than GLP-1R-specific agonists by mediating a concomitant reduction in food intake through GLP-1 receptor activation and increase in energy expenditure through glucagon receptor activation (Dakin et al., 2002, Wynne et al., 2006, Kosinski et al., 2012). The enhanced weight loss mediated by oxyntomodulin leads to improvements in insulin sensitivity, and hence further improves glucose tolerance. As 90% of the patients suffering from diabetes are obese or overweight, an oxyntomodulin-like drug that targets obesity and diabetes concurrently would be an ideal strategy (NHS, 2011).

**Design and development of a unimolecular GLP-1R/GCGR co-agonist**

The aim of the work presented in this thesis was to couple GLP-1 and glucagon receptor activity to stimulate enhanced insulin secretion and superior weight loss than GLP-1R-specific agonists. This can be achieved by either co-administering GLP-1 and glucagon simultaneously, or by developing co-agonists of the two receptors. I chose to develop unimolecular co-agonists as, in comparison to giving two individual agonists, co-agonists have a simplified dosing regimen and are therefore safer and easier to administer. Of particular relevance for my co-agonists, the intrinsic structure of the co-agonists also led to ligand-induced bias, which improved the insulinotropic profile of co-agonists in
However, co-agonists are restricted in terms of their relative GLP-1 and glucagon receptor activity ratio: co-administration of GLP-1 and glucagon can accommodate for individualised receptor activity ratios, whereas my co-agonists were limited to a specific pre-engineered activity ratio. Furthermore, as co-agonists are designed to activate multiple receptors, they carry an increased risk of cross reactivity at similar receptors which can lead to off target effects. Investigating the side-effect profile of my co-agonists was beyond the scope of this project, but initial safety data from GLP-1R/GCGR co-agonists in Phase II clinical trials is promising, with one 41 day trial on a co-agonist finding no significant increase in the occurrence of treatment-emergent adverse events (Ambery et al., 2018). In the absence of long-term safety trials in humans, it remains unclear whether co-agonists will have a greater risk of off target effects and hence reduced clinical safety. However, as multiple co-agonists are currently in Phase I or Phase II clinical trials, these questions will be answered shortly.

I developed GLP-1R/GCGR co-agonists based on the sequence of oxyntomodulin, with modifications to enhance their potency and plasma half-life. The three co-agonists presented had a glucagon-based backbone, with a C terminal tail to confer GLP-1R activity, and showed significantly greater potency than oxyntomodulin at both GLP-1 and glucagon receptors. Unlike previously published co-agonists, which tend to be GLP-1R-preferring, my co-agonists were more oxyntomodulin-like and had substantial GCGR activity. Furthermore, the co-agonists had a potency within 10 fold of the native ligand at each receptor, which is a significant improvement on previously published co-agonists (Pocai et al., 2009, Kerr et al., 2010, Kosinski et al., 2012). To confer resistance to DPP-IV mediated degradation, an Aib substitution was made at the DPP-IV site of action (Deacon et al., 1998, Santoprete et al., 2011). Consequently, the co-agonists were stable over 16 hour incubations with immortalised beta cell lines (as assessed by secondary cAMP assays), and had a sustained 72-hour pharmacokinetic profile in vivo in slow release ZnCl₂ diluent.

**Effects of co-agonists on glycaemic control**

This project primarily focused on the insulinotropic properties of GLP-1R/GCGR co-agonists.

While a synergistic insulinotropic response has been seen previously on co-administering GLP-1 and glucagon in vivo, it remains unclear whether this insulin response was secondary to increased glucose excursions mediated by glucagon (Tan et al., 2013). To determine the insulinotropic actions of co-agonists independent of hepatic glucose output, in vitro assays were conducted on beta cell lines. My co-agonists stimulated greater insulin responses, specifically greater maximal insulin release, compared to GLP-1 and GLP-1 analogues exendin-4 and liraglutide in vitro. In rat beta cell line INS-1 823/3, my co-agonists on average stimulated a 62% greater maximal insulin response...
compared to liraglutide. The superior insulinotropic effect was preserved across cell lines from three different species, confirming that it is not a species-specific phenomenon. No published study to date has reported similar in vitro insulinotropic profiles for GLP-1R/GCGR co-agonists, demonstrating a clear difference between my co-agonists and other drugs currently in development, and is suggestive of an advantage with regards to their insulinotropic profile.

These results demonstrate a direct effect of the co-agonists on insulin secretion through beta cells, suggesting that intrinsic glucagon receptor activity could have an insulinotropic effect. However, in the absence of specific glucagon receptor antagonists and effective siRNA knockdown in beta cell lines, the exact contribution of glucagon receptor activity could not be elucidated. Instead, an indirect assessment was completed by co-administering native glucagon and GLP-1. Co-administration of glucagon with GLP-1 surprisingly failed to improve on the insulinotropic potential of GLP-1 alone. This suggests that the enhanced in vitro insulinotropic potential of the co-agonists is not entirely due to additional glucagon receptor activity, and might involve other pathways. One alternative explanation is enhanced stability and activity of my co-agonists versus GLP-1R-specific agonists during the prolonged in vitro assays. A direct comparison of relative peptide stabilities during prolonged in vitro incubations could not be conducted, as high performance liquid chromatography (Agilent 1100 Series, Agilent Technologies, Germany) could not detect peptides at the concentrations used (maximum peptide concentrations used were 0.004 mg/L, while the detection limit of the equipment was 0.1 mg/ml). Instead, secondary cAMP assays were employed to estimate peptide stability by confirming the in vitro potency, and found no difference between stability of co-agonists and the GLP-1R agonist controls (results not shown).

The enhanced insulin secretion seen in vitro was instead associated with bias at the GLP-1R for G protein signalling versus β arrestin mediated signalling. A strong positive correlation was seen between maximal in vitro insulin secretion for the co-agonists and the degree of G protein bias at the GLP-1R. During prolonged stimulation, this bias helped maintain receptor surface expression, and hence stimulated enhanced insulin secretion. This study was limited in that bias was only investigated between Gαs and β arrestin signalling, even though GLP-1R agonists can also signal through Gαq and ERK1/2 pathways (Arnette et al., 2003, Shigeto et al., 2015). Nonetheless, the concept of biased GLP-1R signalling is exciting as it raises the possibility of engineering drugs that specifically activate therapeutic pathways while avoiding undesirable side effects (Rajagopal et al., 2010, Koole et al., 2013). Several β arrestin-biased (Violin et al., 2010, Shonberg et al., 2013) and G protein-biased (Gesty-Palmer et al., 2006, DeWire et al., 2013, Manglik et al., 2016) peptides have shown beneficial effects in preclinical studies, but limited success has been seen as yet at the clinical stage (Viscusi et al., 2016). In addition to developing biased analogues, allosteric ligands can also be
used to direct downstream signalling and intracellular protein recruitment (Koole et al., 2010, Wootten et al., 2013), which could modify the therapeutic profile of drugs and hence minimise their side effects (Appleton and Luttrell, 2013).

For therapeutic purposes, it is critical that the in vitro observations translate in vivo. In vitro models cannot recapitulate the complexities of in vivo systems, including the pharmacokinetic profiles of drugs. Secondly, receptor overexpressing cell lines are a simplistic model of beta cells, and lack the repertoire of intracellular accessory proteins that modulate GPCR trafficking, localisation and activity (Magalhaes et al. 2012). For this project, β arrestins were particularly critical in terms of their association with the GLP-1R. Other accessory proteins include RAMPs, which modulate agonist activity at the glucagon receptor (Jorgensen et al. 2007, Appleton & Luttrell 2013, Weston et al. 2015). Therefore, in vivo studies are necessary to determine the therapeutic utility of co-agonists by confirming the effects on insulin secretion are retained in vivo.

Results from my in vivo studies consistently demonstrated co-agonists lowered basal glucose and enhanced glucose tolerance on acute administration to DIO mouse models. The improved glucose tolerance was accompanied by an increase in insulin secretion in vivo, validating the earlier in vitro studies. The GLP-1R: GCGR activity ratio for my co-agonists is significantly higher than previously published peptides from other research groups and therefore these results are the first to demonstrate that enhanced glucagon receptor activity, similar to that of oxyntomodulin, does not deteriorate basal glycaemic control or glucose tolerance, but instead leads to significant improvements in glycaemic control acutely. Chronic administration of co-agonists surprisingly failed to have an effect on glucose tolerance. Given the profound weight loss brought about by co-agonists, it is counter-intuitive that they do not improve glucose tolerance. However, Analogues 1 and 2, which mediate the greatest weight loss, did show a trend for improved insulin sensitivity following chronic administration. In contrast, exendin-4 significantly improved glucose tolerance despite mediating markedly lower weight loss.

Interestingly, the in vivo data from rats shows a hyperglycaemic response on exendin-4 administration. This has been previously documented (Perez-Tilve et al., 2010), and could be circumvented by using diabetic rat models instead in future studies (Young et al., 1999). Future preclinical studies would also investigate oral glucose challenges. In order to look specifically at beta cell effects, and to avoid engaging the incretin effect, glucose was administered intraperitoneally in the project as opposed to orally. To determine whether there is any additional insulinotropic impact upon varying route of administration, it would be valuable to conduct oral gavage studies. It would
also be worthwhile to repeat studies with current market leaders liraglutide and semaglutide as GLP-1 comparators instead of exendin-4.

While the insulinotropic properties of co-agonists were primarily associated with G protein bias in vitro, no similar correlation was observed in vivo. In contrast, previously published studies have shown superior improvements in glycaemic control following chronic administration with biased analogues compared to exendin-4, but it is worth noting that these studies focused on GLP-1R-specific biased agonists (Zhang et al., 2015, Jones et al., 2018). Therefore, the in vivo profile of my co-agonists is not directly comparable to existing literature. The most relevant comparator is the study done by Jones et al. (2018), which found a similar association between G protein bias at the GLP-1R and enhanced maximal insulin secretion in vitro. In contrast to work by Jones et al. (2018), my biased co-agonists did not show a time-dependant glucose lowering effect in DIO mice, nor did they improve glucose tolerance chronically. The discrepancy in acute results might be due to different pharmacokinetics of my co-agonists, while the difference in chronic results may be due the more dominant glucagon effect in my co-agonists. Additionally, Jones et al. (2018) did not note weight loss in response to their co-agonists in vivo, which may reflect on the lower doses used in the study (0.24 nmol/kg compared to 100 nmol/kg for my co-agonists). It would therefore be interesting to investigate the effects of my co-agonists at sub-threshold doses for weight loss in order to decipher the impact of bias in vivo. The results obtained in vivo from this project indicate that the degree of glucagon receptor activity has a more dominant impact on metabolic parameters than the extent of bias.

As mentioned previously, the main safety concern of GLP-1R/GCGR co-agonists is the risk of hyperglycaemia mediated by glucagon, as hyperglycaemia can overstimulate already hyperactive beta cells in diabetes and lead to beta cell exhaustion chronically (Meier and Bonadonna, 2013). In fact, the bi-hormonal hypothesis of diabetes holds an excess of glucagon as culpable for diabetes pathogenesis as lack of insulin (Unger and Orci, 1975). However, this hypothesis has not gained a real foothold in the scientific community, as it is rooted in studies on patients with type 1 diabetes who lack counter-regulation by insulin (Gerich et al., 1974a, Gerich et al., 1974b). The hypothesis is also based on reports of resistance to diet-induced diabetes in GCGR knockout mice, which lack credence as knockouts have multiple compensatory mechanisms which mask their phenotypes (Gelling et al., 2003, Conarello et al., 2007, Lee et al., 2011, Lee et al., 2012). Recent studies have demonstrated that, in the absence of insulin signalling, blocking glucagon receptor activity fails to improve glycaemic control, confirming that insulin is the key hormone in diabetes pathogenesis (Neumann et al., 2016, Damond et al., 2016, Holst et al., 2017). In addition, co-administration studies have shown that combining GLP-1 with glucagon blunts the hyperglycaemic tendencies of
glucagon (Tan et al., 2013). In patients with early-stage type 2 Diabetes, endogenous insulin is predicted to have the dominant effect and hence there is minimal risk of my co-agonists stimulating hyperglycaemia and ketoacidosis (El Youssef et al., 2014). The results I obtained in DIO mice support this hypothesis, as both acute and chronic co-agonist administration did not lead to a deterioration in glycaemic control or in glucose tolerance. However, giving an oxyntomodulin-like co-agonist would be impossible in conditions of type 1 diabetes, where the intrinsic glucagon activity would lead to diabetic ketoacidosis in the absence of endogenous insulin.

Overall, the in vivo data suggests that co-agonists have negligible hyperglycaemic tendencies and are safe for use in patients with diabetes, but have limited therapeutic utility as a standalone treatment for diabetes as shown by the chronic studies.

Effects of co-agonists on weight loss

In addition to exploring potential insulinotropic benefits, the aim of incorporating glucagon receptor activity was to harness the superior weight loss seen in response to dual GLP-1R/GCGR agonists. To determine the weight loss effects of co-agonists in comparison to GLP-1R-specific agonists in preclinical species, DIO mice and Wistar rats were used. In keeping with earlier findings (Pocai et al., 2009, Kosinski et al., 2012, Henderson et al., 2016), my co-agonists stimulated significantly greater weight loss than GLP-1R agonist exendin-4 in both rodent models. The superior weight loss is ascribed to glucagon receptor activity, as increasing glucagon receptor activity has been shown to increase the potency of weight loss in mice versus native oxyntomodulin (Pocai et al., 2009, Kosinski et al., 2012). Accordingly, the rGCGR: rGLP-1R activity ratio for my co-agonists co-relates strongly to the extent of weight loss in rats, with GCGR-prefering co-agonists mediating greater weight loss. This work is limited by lack of murine cell data: to correlate the observed metabolic effects in DIO mice to glucagon receptor activity for my co-agonists, cAMP assays will be conducted in cell lines expressing mouse GLP-1 and glucagon receptors as part of my future work. However, work done in DIO mice by Pocai et al. (2009) provides supporting evidence for this hypothesis.

Pair-feeding studies in rats suggest that my co-agonists increase energy expenditure, as the group administered active peptide showed an average weight loss of 79.8 g, while the pair-fed group showed an average weight gain of 0.86 g over the same time period. This demonstrates that my co-agonists mediated weight loss through food-intake-independent mechanisms. To confirm the contribution of energy expenditure to the observed weight loss, it would be useful to measure energy expenditure directly in metabolic CLAMS cages.
Increased energy expenditure in response to co-agonists is canonically associated with glucagon receptor activity (Du et al., 2012, Kosinski et al., 2012). GLP-1 does not increase energy expenditure in humans, with one study actually reporting a reduction in energy expenditure following GLP-1 infusion (Flint et al., 2000, Tan et al., 2013, Cegla et al., 2014, Schmidt et al., 2014). Indirect evidence for a role for glucagon comes from co-infusion studies of glucagon and GLP-1, where glucagon enhances thermogenesis (Tan et al., 2013, Cegla et al., 2014). Similarly, an elegant study conducted by Kosinski et al. (2012) used targeted sequence substitutions to generate a GLP-1R-specific oxyntomodulin derivative that mediated less weight loss than native oxyntomodulin despite mediating similar food intake. This is suggestive of enhanced energy expenditure in response to oxyntomodulin-like dual agonists. Finally, a study published in 2018 using selective antagonists confirmed that the glucagon receptor, not the GLP-1 receptor, mediates the energy expenditure effects of oxyntomodulin (Scott et al., 2018).

The precise mechanism behind the energy expenditure effects of glucagon remains unclear and has been attributed to brown adipose tissue (BAT) activation and/or stimulation of futile cycles by glucagon (Billington et al., 1991, Samoilov et al., 2005). Glucagon-induced BAT activation in particular seems to be the likely explanation, as glucagon receptors are expressed in BAT (Morales et al., 1998), and multiple studies have provided evidence for the role of BAT activation in GCGR-mediated thermogenesis (Joel, 1966, Cockburn et al., 1967, Kuroshima and Yahata, 1979, Yahata and Kuroshima, 1982, Billington et al., 1987, Billington et al., 1991). However, all these studies relied on indirect methods to measure BAT activation, and did not demonstrate changes in levels of the key molecular marker of BAT activation, namely Uncoupling Protein 1 (UCP-1). Furthermore, a recent study using thermal imaging has reported that glucagon infusion enhances energy expenditure independent of changes in BAT activation (Salem et al., 2016). The role of BAT activation in glucagon-induced thermogenesis therefore remains undetermined. It would be interesting to measure changes in UCP-1 expression in BAT following chronic administration of my co-agonists in rodent models to determine their effects on energy expenditure and BAT activation.

In comparison to previously published GLP-1R/GCGR co-agonists, my co-agonists bring about greater weight loss in DIO mice. As mentioned above, this superior weight loss could be due to increased glucagon receptor activity: my co-agonists tend to be GCGR-preferring while co-agonists developed by other groups have been GLP-1R-preffering instead (Day et al., 2009, Henderson et al., 2016). There are very few examples of GCGR-preferring co-agonists in the literature, with only one group investigating the weight loss effects of GCGR-preferring co-agonists in DIO mice (Pocai et al., 2009, Kosinski et al., 2012). My co-agonists exhibit superior weight loss efficacy in comparison to these
earlier GCGR-preferring co-agonists, which is potentially due to the increased potency of my co-agonists at GLP-1 and glucagon receptors as measured in cAMP assays.

Of the studies cited above, two reported that the enhanced weight loss in rodent models was reflected in enhanced food restriction for GLP-1R/GCGR co-agonists compared to GLP-1R-specific agonists (Pocai et al., 2009, Kosinski et al., 2012). In contrast, Henderson et al. (2016) developed a co-agonist that led to greater weight loss than GLP-1R agonist liraglutide while maintaining a higher food intake than lirgalutide. The latter study involved co-agonists with higher individual potencies at the GLP-1 and glucagon receptors, and is hence more comparable to my co-agonists. However, even in this study, the co-agonist did lead to a significant reduction in food intake compared to vehicle. In direct contrast to the earlier studies, my co-agonists bring about a significant reduction in body weight in DIO mice without significantly reducing food intake. This is ascribed to the increased glucagon receptor activity of my co-agonists, which enables weight loss independent of food restriction.

The ability of my co-agonists to stimulate profound weight loss without restricting food intake is novel and implies a reduced tendency for nausea and vomiting. As mentioned previously, GLP-1R agonists are dose-limited due to emetic side effects, and hence limited in terms of efficacy (Kolterman et al., 2005, Ratner et al., 2010, Barrington et al., 2011). In this project, co-agonists were administered at 10 times higher doses than the GLP-1 comparator in DIO mice. Despite the higher dose, co-agonists did not induce an acute reduction in food intake, which is indicative of a lower propensity for causing nausea (Horn, 2014, Kenward et al., 2015). The superior weight loss in DIO mice could therefore be due to the higher doses used for co-agonists as opposed to increased intrinsic potency, suggesting that GLP-1R/GCGR co-agonists have increased efficacy due to an increased dosing window.

In contrast to dosing in DIO mice, rats were administered co-agonists at a lower dose than exendin-4, but still exhibited greater weight loss. No formal assessments of nausea were made, such as conditioned taste aversion studies or experimental measurements of pica. However, rats injected with exendin-4 displayed signs of aversion, such as hunching, reduced locomotion and periods of little food intake. Consequently, the experiment was curtailed early for animal welfare reasons. The observation that exendin-4 leads to nausea-like symptoms in rats at sub-threshold doses, while my co-agonists mediate 8 times greater weight loss without causing observable behavioural changes, is particularly promising and supports the hypothesis that co-agonists have lower tendencies to cause nausea. However, it would be useful to repeat these studies with experimental assessments to investigate the effect of co-agonists on nausea further. Conclusive evidence of the impact of co-
agonists on nausea can only be obtained from human trials, as any rodent studies are only estimates of aversion and are not directly translatable to nausea in man.

In summation, my co-agonists produce superior weight loss for minimal food intake restriction in DIO mice, which is novel for GLP-1R/GCGR co-agonists and potentially reflects on the greater glucagon receptor activity. The co-agonists therefore appear to be ideal therapeutic candidates for an anti-obesity treatment, particularly for patients with diabetes.

**Potential benefits and detrimental side-effects of GLP-1R/GCGR co-agonists**

T2DM and the metabolic syndrome are closely associated with non-alcoholic fatty liver disease (NAFLD). NAFLD is characterised by accumulation of fat in the liver, and is fast becoming the primary cause of liver disease across the world (Ray, 2013). NAFLD increases hepatic insulin resistance, aiding the progression of T2DM, and is a robust predictor of T2DM (Choi et al., 2013, Park et al., 2013). Glucagon stimulates lipolysis, reduces lipogenesis and lowers serum triglyceride and cholesterol levels (Parmley et al., 1968, Unger, 1985, More et al., 2017). Accordingly, glucagon has been proposed as a therapy for hepatic steatosis and NAFLD (Liang et al., 2004, Charbonneau et al., 2005, Conarello et al., 2007, Junker et al., 2016). The glucagon receptor activity of my co-agonists could therefore have beneficial effects on plasma cholesterol levels and hepatic lipid deposition, and could serve as a prophylactic treatment for hepatic steatosis and NAFLD.

GLP-1R/GCGR co-agonists could lead to muscle wasting, which is particularly relevant for my GCGR-prefering co-agonists. Glucagon facilitates hepatic gluconeogenesis by stimulating amino acid uptake into hepatocytes and enhancing protein catabolism (Fitzpatrick et al., 1977, Schade et al., 1979, Fehlmann et al., 1979, Boden et al., 1996). Correspondingly, elevated glucagon levels, such as those seen in glucagonoma patients, are associated with increased risk of muscle wasting (Nair et al., 1987, Barazzoni et al., 1999). However, wasting in response to co-agonists has never been demonstrated conclusively in pre-clinical species (Pocai et al., 2009, Day et al., 2012). Results from this project similarly demonstrated that there is no significant loss of lean mass following chronic co-agonist administration in DIO mice (results not shown). Furthermore, these co-agonists are being developed for patients with T2DM who retain the capacity to secrete insulin, which would help counter the catabolic actions of glucagon. Finally, as seen in glucagonoma patients, the enhanced protein breakdown rate can be successfully compensated by giving a high protein diet where necessary.
Future Therapeutic Strategies

In addition to further preclinical work, translation into humans poses several hurdles. The increased efficacy of my co-agonists in mice could be attributed to dose escalation versus exendin-4, which cannot be replicated in human trials where there are greater limitations on doses that can be administered. Long-term safety data is also needed to establish the safety profile of the drugs, particularly with respect to off target receptor cross reactivity by co-agonists. Finally, the ratios for GCGR versus GLP-1R activation will require further optimisation to account for species-specific differences that might arise in vivo.

While my co-agonists mediated profound weight loss, they failed to enhance insulin secretion and glycaemic control on chronic administration. In order to increase their insulinotropic effect, the co-agonists can be combined with a third insulinotropic peptide, such as GIP, to develop triple agonists for the treatment of obesity and diabetes (Finan et al., 2015, Jall et al., 2017). Such unimolecular tri-agonists are predicted to harness the insulinotropic effects of GLP-1 and GIP, and mediate weight loss through complementary GLP-1 and glucagon activity. In a sophisticated study, Finan et al. (2015) demonstrated that adding glucagon receptor activity to a GLP-1/GIP co-agonist dramatically enhances weight loss without causing a further reduction in food intake in obese and diabetic mouse models, and hence helps to improve insulin sensitivity. Through a series of experiments involving whole-body receptor knockout mice, the weight loss effects were ascribed to glucagon and GLP-1 receptor activation, while basal glucose levels were linked primarily to GLP-1 with a small but significant contribution from the GIP receptor. In keeping with my findings, both GLP-1 and glucagon receptor activity appeared to be involved in enhancing acute glucose tolerance. The study allayed concerns of hyperglycaemic tendencies of glucagon, as it found that incorporating GLP-1R/GIPR activity at as low as a tenth of the activity at the GCGR is sufficient to buffer against the hyperglycaemic effects of glucagon. Surprisingly, this is in contrast to earlier studies by the same group, which found that comparable glucagon and GLP-1 activity was the optimal strategy to maintain glucose regulation (Day et al., 2012). However, the earlier study investigated co-agonists containing a single incretin, GLP-1, while the new tri-agonist combines two incretins, GLP-1 and GIP. This suggests that tri-agonists can buffer hyperglycaemic tendencies of glucagon more effectively, enabling a higher safe dose of glucagon and hence facilitating greater weight loss. Other groups have investigated tri-agonists, confirming their beneficial glycaemic effects and demonstrating positive insulinotropic effects both acutely and chronically in diabetic mouse models (Gault et al., 2013, Bhat et al., 2013). Notably, the latter studies had considerably lower success in in terms of body weight loss compared to Finan et al. (2013). This failure is probably reflective of the potency of the tri-agonists employed, as Gault et al. (2013) developed tri-agonists with 100-fold lower potency than
native ligands and a 1000-fold lower potency than the tri-agonists developed by Finan et al. (2013). In summation, combining all three peptides could widen the therapeutic window, enabling a better therapeutic profile and better clinical outcomes in terms of glycaemic control and weight loss. GIP/GLP-1 co-agonists have recently been shown to mediate neuroprotective effects in rat and mouse models, suggesting a role in treatments for Alzheimer’s and Parkinson’s diseases, and are therefore of great interest therapeutically (Tamargo et al., 2017, Jalewa et al., 2017, Shi et al., 2017).

In conclusion, this project validates novel GLP-1R/GCGR co-agonists as highly effective anti-obesity drugs, which are safe for use in pre-clinical diabetic models. The co-agonists demonstrate enhanced insulinotropic potential in vitro, and on acute administration in vivo, which is linked to G protein signalling bias at a molecular level. The acute insulinotropic effects fail to translate into chronic improvements in glycaemic control in vivo, and merit further investigation.
## Appendix A

### Peptide Sequences

|     | 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 |
|-----|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 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 |   |   |   |   |   |   |   |   |   |
| Analogue 1 | H | A | B | H | G | T | F | T | S | D | Y | S | R | Y | L | D | Q | R | R | A | Q | E | F | I | E | W | L | L | H | T | H | H | H | H | H | P | S | N | H | 2 |   |   |   |   |   |   |   |   |   |
| Analogue 2 | H | A | B | H | G | T | F | T | S | D | Y | S | R | Y | L | D | E | R | R | A | Q | E | F | I | E | W | L | L | H | G | H | H | H | H | H | G | L | S | N | H | 2 |   |   |   |   |   |   |   |   |   |
| Analogue 3 | H | A | B | H | G | T | F | T | S | D | Y | S | K | Y | L | D | E | K | R | A | H | E | F | I | E | W | L | L | H | G | H | H | H | H | H | S | W | N | H | 2 |   |   |   |   |   |   |   |   |   |

*A fatty acid side-chain [N-(1-oxohexadecyl)-L-γ-glutaMyl] is attached at residue 20 of liraglutide*

- **Yellow**: Conserved residues in GLP-1, glucagon and oxyntomodulin
- **Red**: Conserved residues in glucagon and oxyntomodulin
- **Blue**: Conserved residues in GLP-1
- **Green**: Conserved residues in oxyntomodulin
- **Pink**: AIB, α-amino-isobutyric acid
Appendix B

Amino Acid Abbreviations

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


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