Glucagon-like peptide-1 receptor biased agonism and its functional consequences in type 2 diabetes

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

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Abstract

Glucagon-like peptide-1 (GLP-1) is released by the intestine in response to food ingestion, and plays a key role in postprandial metabolism. Furthermore, pharmacological GLP-1 receptor agonists are an effective treatment for type 2 diabetes, as they stimulate pancreatic beta cells to secrete insulin, and increase insulin sensitivity via weight loss.

GLP-1R activation is coupled to insulin secretion via cyclic AMP generation. However, recent evidence suggests an important additional role of non-canonical pathways such as recruitment of β-arrestins, which can act as protein scaffolds to coordinate activation of other signalling proteins. Paradoxically, arrestin recruitment may negatively modulate signalling by inducing receptor desensitisation and sequestration from the plasma membrane, although this has not yet been demonstrated for the GLP-1R.

Biased agonism is a new concept in pharmacology, defined by differential activation of divergent signalling pathways from the same receptor by different agonists. In this project, peptide agonists based on GLP-1 and its homologue exendin-4, but with a variety of substitutions close to the peptide N-terminal region, were designed and tested for biased signalling between cAMP generation and β-arrestin recruitment. To determine the downstream consequences of bias, selected agonists were tested for insulin secretion in vitro using INS-1 832/3 cells and human islets. Despite an important role for β-arrestin recruitment in insulin secretion suggested by the literature, agonists with minimal β-arrestin recruitment were considerably more insulinotropic than those with robust arrestin responses. This was due to reduced GLP-1R desensitisation.

When tested in high-fat, high-sucrose fed mice, a rodent model of type 2 diabetes, agonists with minimal arrestin recruitment were more effective than exendin-4 at preventing hyperglycaemia. Surprisingly, appetite suppression with biased peptides was unaltered, suggesting that these compounds might achieve glycaemic improvements without concomitant increases in nausea. As such, biased agonists targeting the GLP-1R may represent a therapeutic advance in type 2 diabetes treatment.
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Declaration of contributors

I confirm all work presented in this thesis is my own except where referenced. Additionally, I am grateful to the following researchers who assisted with some experiments:

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

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### Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>7TM</td>
<td>7-transmembrane</td>
</tr>
<tr>
<td>AM</td>
<td>Allosteric modulator</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin 2 type 1 receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>B2-AR</td>
<td>β2-adrenergic receptor</td>
</tr>
<tr>
<td>BETP</td>
<td>4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine</td>
</tr>
<tr>
<td>BG</td>
<td>Benzyl guanine</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5'-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca$^{2+}$-induced Ca$^{2+}$ release</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DERET</td>
<td>Diffusion-enhanced resonance energy transfer</td>
</tr>
<tr>
<td>ECD</td>
<td>Extra-cellular domain</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Administration</td>
</tr>
<tr>
<td>Epac2</td>
<td>Exchange protein activated by cAMP-2</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>eWAT</td>
<td>Epidydimal white adipose tissue</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drugs Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FRET</td>
<td>Forster resonance energy transfer</td>
</tr>
<tr>
<td>G protein</td>
<td>Guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GCGR</td>
<td>Glucagon receptor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinoitropic polypeptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLP-1</td>
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</tr>
<tr>
<td>GLP-1R</td>
<td>GLP-1 receptor</td>
</tr>
<tr>
<td>GLP-2</td>
<td>Glucagon-like peptide-2</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein receptor kinase</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Haemoglobin A1c</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
</tr>
<tr>
<td>HFHS</td>
<td>High fat, high sucrose</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HTRF</td>
<td>Homogenous time-resolved fluorescence</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5 triphosphate</td>
</tr>
<tr>
<td>IPGTT</td>
<td>Intra-peritoneal glucose tolerance test</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>KATP</td>
<td>Adenosine triphosphate-sensitive potassium channel</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTC</td>
<td>Medullary thyroid carcinoma</td>
</tr>
<tr>
<td>NAM</td>
<td>Negative allosteric modulator</td>
</tr>
<tr>
<td>NAS</td>
<td>Non-alcoholic score</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>OXM</td>
<td>oxyntomodulin</td>
</tr>
<tr>
<td>PAC1R</td>
<td>Pituitary adenylate cyclase 1 receptor</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase activating polypeptide</td>
</tr>
<tr>
<td>PAM</td>
<td>Positive allosteric modulator</td>
</tr>
<tr>
<td>PC2</td>
<td>prohormone convertase 2</td>
</tr>
<tr>
<td>PC3</td>
<td>prohormone convertase 3</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide tyrosine-tyrosine</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RAMP</td>
<td>Receptor activity modifying protein</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum Ca²⁺-ATPase</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TMDD</td>
<td>Target-mediated drug disposition</td>
</tr>
<tr>
<td>TMR</td>
<td>Transmembrane region</td>
</tr>
<tr>
<td>TR-FRET</td>
<td>Time-resolved FRET</td>
</tr>
<tr>
<td>TRF</td>
<td>Time-resolved fluorescence</td>
</tr>
<tr>
<td>V2R</td>
<td>Vasopressin type 2 receptor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
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1 General introduction
1.1 Type 2 diabetes and the need for new treatments

Type 2 diabetes mellitus (T2DM) is an increasingly common disorder in which chronic dysregulation of multiple metabolic processes leads to serious end organ damage, including cardiovascular disease, blindness, kidney disease, and limb amputation. It is estimated that 382 million adults have diabetes, and that this will increase to 592 million by 2035 (1). In the UK, T2DM currently affects 3.5 million people, a figure expected to rise to 5.5 million by 2035, with a total cost to the national economy of £21.8 billion (2).

“Classical” treatments for T2DM include lifestyle interventions, metformin, sulphonylureas, and insulin replacement. Lifestyle changes to promote weight loss are often tried, but improvements are limited and weight regain common when the intervention ends (3). Metformin is usually the first line pharmacological treatment and is safe, but many patients do not achieve adequate glycaemic control on metformin monotherapy. Sulphonylureas reduce hyperglycaemia but increase the risk of hypoglycaemia, weight gain and death (4). Insulin replacement therapy is sometimes required in later stages of T2DM when pancreatic β cell function cannot be rescued by other drugs, but is unpopular with patients due to requirement for injections, more frequent blood glucose monitoring, impositions on lifestyle (e.g. driving). Insulin also causes weight gain and hypoglycaemic events.

In spite of these and other treatments, data from the UK Quality Outcomes Framework indicated that in 2012/13, 36% of patients with T2DM in England failed to achieve a target HbA1c of <7.5% (5). It is apparent therefore that, in addition to preventative measures, new treatments are needed. Recently it has been recognised that bariatric surgery is a very effective treatment for T2DM. Roux-en-Y gastric bypass, a type of bariatric surgery, causes substantial weight loss and leads to long-term remission of diabetes in a large proportion of patients, as well as reductions in microvascular and macrovascular complications (6). However, the risk of several complications associated with this procedure, including perioperative death (7), long-term micronutrient deficiencies (8), postprandial hypoglycaemia (9) and autonomic dysfunction leading to orthostatic hypotension (10), means many patients wish to explore other options.

The therapeutic effect of Roux-en-Y bypass and related surgical procedures is dependent partly on the exaggerated release of gastrointestinal hormones, including glucagon-like peptide-1 (GLP-1) (11). GLP-1 normally functions to facilitate postprandial homeostasis by promoting satiety and insulin secretion. Replicating these beneficial effects by exogenously administered GLP-1 receptor (GLP-1R) agonists, or agents which promote the actions of endogenous GLP-1, is now an established treatment modality for T2DM, and the refinement of this treatment is the subject of this thesis.
1.2 Normal glucose homeostasis

Diabetes mellitus is characterised by many metabolic abnormalities, but it is diagnostically defined in relation to abnormal glucose homeostasis. According to the current diagnostic guidelines, diabetes can be diagnosed if any of the following criteria are met (12):

1. HbA1c ≥6.5%
2. Fasting plasma glucose ≥7.0 mmol/L
3. Plasma glucose ≥11.1 mmol/L 2 hours after a 75g oral glucose challenge
4. Plasma glucose ≥11.1 mmol/L with classical symptoms of hyperglycaemia

In order to understand the pathophysiology and treatment of T2DM, it is necessary to first review normal glucose physiology. In healthy individuals blood glucose is tightly regulated between 4 and 7 mmol/L in spite of intermittent and unpredictable provision of glucose from ingested food. Glucose is a critical fuel and metabolic precursor in all body tissues, so ensuring availability of circulating glucose, and its entry into cells, is of paramount importance. The primary glucoregulatory hormone is insulin, produced by pancreatic β cells in the islets of Langerhans. Insulin acts to promote tissue uptake of absorbed glucose following meal ingestion.

1.2.1 Insulin production

Insulin is a peptide hormone which coordinates the response to the nutrient load absorbed following a normal meal. Systemically available insulin is produced exclusively in pancreatic beta cells (13). Secretion of insulin is tightly regulated in response to changes in nutrient availability, both via direct β cell nutrient sensing, and indirectly via hormonal and neurally-mediated signals. In healthy individuals, circulating insulin concentrations rapidly increase 5 – 10-fold under experimentally induced hyperglycaemia (14).

1.2.2 Insulin action

Insulin receptors are expressed in many mammalian tissues (15). In the post-prandial state, insulin drives entry of absorbed glucose into peripheral tissues, in particular adipose tissue and muscle, via upregulation of the facultative glucose transporter GLUT4 (16). Additionally, insulin acts on the liver to promote storage of excess glucose as glycogen.
1.2.3 Glucagon

A second hormone, glucagon, produced by pancreatic islet alpha cells, plays a permissive role in sustaining endogenous glucose production. Once nutrient absorption is complete, the body must shift to a catabolic state in which peripheral tissues are partly or completely supplied by endogenously produced glucose. The primary source of endogenous glucose production is the liver, with additional contributions from kidney and intestinal tissue. Liver-derived glucose is generated from glycogen breakdown and gluconeogenesis, a process whereby glucose is \textit{de novo} synthesised from non-carbohydrate precursors including amino acids, glycerol and lactate. These processes are stimulated by glucagon, the secretion of which is increased in the fasting state due to lack of insulin and alpha cell detection of declining blood glucose, to maintain hepatic glucose output. These same processes are also to varying degrees inhibited by the hepatic action of insulin, and consequently only active when beta cell insulin secretion ceases in the post-absorptive state.

1.3 Pathophysiology of T2DM

T2DM is primarily a disorder of insulin, and is characterised by relative insulin insufficiency due to a combination of reduced insulin secretory capacity and tissue insulin resistance, resulting in hyperglycaemia. Both genetic and environmental factors play important roles in T2DM development. Twin studies reveal 72% of T2DM risk is heritable (17), although only a small proportion of genetic determinants underpinning this effect have been identified, even with large studies involving whole genome sequencing of many thousands of people (18). The explosion in global T2DM prevalence over the past 30 years clearly implicates environmental changes as the permissive factor in allowing the disease to emerge in genetically predisposed individuals.

1.3.1 Beta cell failure

Genome-wide association studies (GWAS) have identified a number of loci associated with T2DM with an apparent role in pancreatic development and insulin secretion (19-21). The best studied polymorphism, which is mapped to the gene encoding the transcription factor TCF7L2, is associated with an odds ratio of 1.7 of developing T2DM, and appears to act by regulating expression of a number of genes required for insulin granule exocytosis (22).

Systemic insults resulting from excess calorie ingestion, obesity and the metabolic syndrome lead to biochemical abnormalities which are directly toxic to beta cells, in particular increases in circulating glucose and free fatty acids (23). Beta cell damage occurs additionally due to obesity-related low grade systemic inflammation characterised by increases in circulating cytokines such as interleukin-1, -6 and
tumour necrosis factor-α (24). Furthermore, increased insulin demand results in endoplasmic reticulum stress due to incorrect processing of an unsustainably increased flux of newly synthesised insulin precursors; the resulting unfolded protein response leads to global translational downregulation, ultimately reducing insulin secretion (25). As dysglycaemia and dyslipidaemia are both cause and consequence of beta cell failure, a progressive feedback loop is established which leads, over many years, from subclinical insulin insufficiency, to overt T2DM, and ultimately to total beta cell failure in some patients, for whom exogenous insulin therapy is then required.

The relative importance of beta cell dysfunction versus beta cell death in human T2DM is currently unresolved. It is known from autopsy studies that beta cell mass in patients with T2DM is substantially reduced (26). On the other hand, it has been demonstrated that beta cell function can to a significant extent be restored even in some patients with longstanding T2DM (27). It is likely that reversible beta cell dysfunction is of primary importance early in the disease, with reduced beta cell mass increasingly important in later stages. Additionally, part of the apparent reduction in beta cell mass observed in T2DM is in fact likely not due to cell death, but rather de-differentiation to a progenitor islet cell with reduced beta cell “identity” (28).

### 1.3.2 Insulin resistance

Tissue resistance to the action of insulin prevents uptake of glucose and other metabolites by adipose tissue and skeletal muscle, and failure to suppress glucagon secretion and hepatic glucose production, leading to hyperglycaemia. A number of rare monogenic disorders causing severe insulin resistance have been identified which affect components of the insulin receptor signalling pathway, with mutations in the insulin receptor (29) and AKT2 (30) being well characterised examples.

Outwith rare Mendelian disease, non-beta cell GWAS hits for T2DM are predominantly associated with excess body weight, which itself is highly implicated in the development of insulin resistance (31). The best known are polymorphisms in FTO, the function of which is incompletely understood but likely involves long-range enhancer activity on another gene, IRX3 (32), and is associated with an average 3kg increase in body weight (33). As for T2DM per se, weight is highly heritable (34) but only a fraction of the observed heritability has been uncovered despite large scale efforts (35).

Insulin resistance is well correlated with body mass index (BMI) in population studies (36), although this masks a more complex aetiology in which adipose tissue failure and depot-specific fat accumulation are more important than overall adiposity. The metabolic sequelae of obesity emerge once the expansion capacity of an individual’s adipose tissue is reached, at which point fat can no longer be stored appropriately and accumulates in other organs such as liver and muscle where
lipotoxicity leads to insulin resistance (37). Furthermore, once buffering capacity is reached, abnormal adipocyte behaviour leads to altered secretion of adipokines and development of a low grade inflammatory state, both of which could contribute to development of insulin resistance (38,39). The stronger epidemiological relationship between metabolic dysfunction and abdominal girth, a marker of visceral adiposity, than for BMI (40), is consistent with the consensus view that visceral fat is more metabolically active than subcutaneous fat, and plays a more important role in T2DM pathogenesis (41).

1.4 Regulation of insulin secretion by incretin hormones

Circulating nutrients directly stimulate insulin release from beta cells. However, insulin secretion is also modulated by hormonal cues which reflect food ingestion and energy status. The most apparent example of this is termed the “incretin effect”, defined as the increased insulin response after oral ingestion of glucose, compared to that when the same glucose load is administered parenterally. The incretin effect is estimated to account for 50-70% of insulin secreted in response to oral glucose (42). Importantly, incretin-stimulated insulin secretion only occurs in the presence of a permissive blood glucose concentration, and does not cause hypoglycaemia.

1.4.1 Discovery of intestinal factors responsible for the incretin effect

Stimulation of pancreatic exocrine secretion by a factor isolated from intestinal mucosa was first described in 1901 (43). Glucose-lowering properties of the same extract when administered to patients with diabetes was subsequently noted (44) in a report that predates the first use of pancreatic extracts for the same purpose (45) and subsequent identification of insulin (46). The term “incretin” was first proposed in 1932 (47) but a conclusive demonstration of enhanced insulinotropism by oral vs. intravenous glucose was not reported until the 1960s (48), when the invention of radioimmunoassay made measurement of plasma insulin-like immunoreactivity possible. Advances in protein purification led to the eventual identification of gastric-inhibitory polypeptide (GIP) as a physiological incretin in man, released by the intestine in response to glucose ingestion (49) and capable of stimulating secretion of insulin (50). Following this discovery, GIP has been routinely referred to as “glucose-dependent insulinotropic polypeptide”, which conveniently shares the same acronym.

The second major incretin to be identified was glucagon-like peptide-1 (GLP-1). Glucagon-like immunoreactivity in intestinal extracts was reported in 1968 to stimulate insulin secretion without the usual hyperglycaemic effect of glucagon (51). The prediction of the existence of GLP-1 from the sequence of the preproglucagon gene (52) predates the specific identification of the bioactive peptide
itself. Detection of GLP-1 in intestine by immunohistochemistry was first reported in 1985 (53), and its release from isolated intestine (54) and increased plasma concentrations after a meal noted soon after (55). The incretin action of GLP-1 was subsequently demonstrated (56) by infusion into humans at physiological levels, and notably its insulinotropic effect was found to be substantially greater than that of GIP.

1.4.2 GLP-1 production

GLP-1 is encoded within the preproglucagon gene, a protein precursor for a family of peptides including glucagon itself, GLP-1, GLP-2, the dual GLP-1 / glucagon receptor agonist oxyntomodulin (OXM), glicentin, and glicentin-related pancreatic polypeptide. The translated gene product is processed by prohormone convertase (PC) enzymes in a tissue-specific manner to generate the different peptides; in pancreatic alpha cells, the major convertase isoform is PC2 which produces glucagon, whereas in intestinal enteroendocrine cells known as "L-cells", PC3 dominates to produce the full length GLP-1 peptide, GLP-1(1-37) (57). This peptide undergoes further proteolytic cleavage and amidation to produce GLP-1(7-36)NH₂, which is the primary biologically active form of the molecule.

L-cells are found throughout the intestinal mucosa but particularly in the distal jejunum and ileum (58). They possess nutrient-sensing receptors and transporters which allow them to respond to glucose (e.g. via sodium-glucose co-transporters (59)), amino acids (e.g. via the calcium sensing receptor (60)), and fatty acids (e.g. via free fatty receptors (61)), coupling meal ingestion to GLP-1 release.

1.4.3 Physiological actions of GLP-1

GLP-1 receptors are distributed in multiple tissues including pancreatic islets (beta and delta cells), heart, vascular smooth muscle, vagus nerve and specific areas in the central nervous system (62,63). In addition to stimulating insulin secretion, GLP-1 exerts multiple other metabolic actions including promotion of satiety and reduction in food intake (64), slowing of gastric emptying (65), suppression of glucagon release (66) and protection of beta cells from apoptosis (67).

1.4.4 Endocrine or paracrine action of GLP-1?

Whether GLP-1 acts as an endocrine hormone, stimulating relevant tissues via the systemic circulation, is a matter of debate. The circulatory half life of GLP-1(7-36)NH₂ is short (1-2 minutes) due
to rapid and extensive degradation by the serine exopeptidase dipeptidyl dipeptidase-4 (DPP-4), generating GLP-1(9-36)NH$_2$, which traditionally has been considered an inactive metabolite. Therefore, the amount of GLP-1 that might reach receptors in the central nervous system (CNS) is likely to be small. Instead, GLP-1 might act on more local GLP-1 receptor (GLP-1R)-expressing vagal afferent neurons close to the site of its release, with physiological effects at distant tissues being mediated by neural signals (68). Contrasting evidence exists to support a primary role of beta cell GLP-1Rs (69) and extra-islet GLP-1Rs (70) in physiological GLP-1-induced insulin secretion, although both of these studies conclude that pharmacological GLP-1R agonism involves the beta cell directly.

1.4.5 GLP-1R signal transduction in beta cells

The GLP-1R couples the binding of GLP-1 to a variety of downstream beta cell responses. Historically, the GLP-1R, like other related receptors, was believed to signal in a linear manner exclusively via guanine nucleotide binding proteins (G proteins). It is now recognised that divergent signalling events resulting from recruitment of other protein interactors, such as β-arrestins, are an important part of the GLP-1 response. As arrestins also play a role in receptor desensitisation, a complex balance of signal initiation and termination is struck, which can be ligand-specific.

1.4.6 The GLP-1 receptor

The GLP-1R is a secretin family (class B) G protein coupled receptor (GPCR). This class of receptors is characterised by a large extracellular N-terminal domain (ECD), a transmembrane domain consisting of 7 α-helices (7TM), and an intracellular C-terminus (71). The mechanism of ligand-GLP-1R binding and activation is typically described according to the "two domain model", in which the ligand first binds to the receptor ECD and is thereby orientated to facilitate interaction between ligand N-terminal and the surface of the receptor 7TM region, with this subsequent interaction responsible for inducing receptor conformation changes required to trigger intracellular signalling cascades (72-74). Recent evidence suggests that in fact the GLP-1R ECD may in fact play a role in signal transduction rather than simply serving as an affinity trap (75).

1.4.7 G protein signalling by the GLP-1R

G proteins in their inactive state exist as heterotrimeric composed of α, β and γ subunits. Agonist binding to the GPCR leads to a conformational change in the receptor which allows it to induce guanine nucleotide exchange in the G protein. Gα and Gβγ subunits then dissociate and initiate signalling events (76).
Similar to the closely related glucagon receptor, the GLP-1R is coupled to generation of 3',5'-cyclic adenosine monophosphate (cAMP) (77) via activation of the stimulatory G protein subunit Gaα, which in turn activates adenylate cyclase. In beta cells the primary transmembrane adenylate cyclase responsible for GLP-1R signal transduction is isoform 8 (ADCY8) (78,79). Immediately downstream of cAMP are protein kinase A (PKA) and “exchange protein activated by cAMP” (Epac2), both of which are coupled to insulin secretion (80,81) via multiple mechanisms including activation of ATP-sensitive potassium (KATP) channels (82,83) and amplification of calcium-induced calcium release by activation of ryanodine receptors and inositol 1,4,5 triphosphate (IP3) receptors (B4).

Other G proteins recruited by the GLP-1R include Gaq and Gai/o (85). In contrast to the body of evidence linking cAMP signalling to insulin secretion, the physiological importance of these additional pathways is not clear. However, a recent report highlighted a potential role for Gaq in GLP-1-induced insulin release (86).

1.4.8 Role of β-arrestins in GLP-1R signalling

Following G protein activation, GPCRs are phosphorylated at the C-terminus by G protein receptor kinases (GRKs). GRK-phosphorylated receptors can then recruit β-arrestins, which act as multifunctional scaffold proteins, orchestrating both signal generation and signal termination (87).

Arrestins were originally identified as negative regulators of photoactivated visual rhodopsin signalling in retinal cells (88), and subsequently found to play a similar role for the β2-adrenergic receptor (β2-AR) in non-retinal tissue (89), hence "β"-arrestins. Arrestin binding causes receptor desensitisation (i.e. resistance to continuous or repeat activation after an initial stimulus) by sterically hindering interaction of the receptor with further G proteins, and also by promoting receptor endocytosis through recruitment of clathrin adaptor proteins such as AP2 (90), preventing further exposure to extracellular ligand. In contrast, arrestins are also known to initiate signalling via mitogen-activated protein kinase (MAPK) family proteins, for example by recruiting the non-receptor tyrosine kinase c-Src, which activates extracellular signal-regulated kinases 1 and 2 (ERK1/2) (91).

Two isoforms of β-arrestin exist (1 and 2), which exhibit 78% sequence identity (92); both are ubiquitously expressed and are to a certain extent thought to be able to functionally substitute for each other. Mice lacking either β-arrestin1 (93) or β-arrestin2 (94) exhibit a grossly normal phenotype, whereas dual knockout is embryonically lethal (95). However, numerous examples of functional specificity of each isoform have now been demonstrated (96).
The GLP-1R associates with both β-arrestin isoforms (97,98). Interestingly, this interaction is transient, and does not persist after GLP-1Rs are internalised (99). This observation qualifies the GLP-1R as "class A with regard to arrestin recruitment"; in contrast, class B (with regard to arrestin recruitment) receptors, such as the angiotensin 1A receptor (100), co-internalise with arrestin bound. GLP-1R recruitment of β-arrestin1 leads to ERK1/2 phosphorylation, which is coupled to anti-apoptotic effect in beta cells via phosphorylation and inactivation of the pro-apoptotic protein Bcl-2-associated death promoter (BAD) (101). In this study, β-arrestin1-mediated ERK1/2 phosphorylation was sustained and spatially constrained to the cytoplasm, and distinct from a transient G protein-mediated nuclear phospho-ERK signal. β-arrestin1 signalling is also implicated in GLP-1-induced insulin secretion (98), and in this study, both ERK phosphorylation and cAMP responses were attenuated by β-arrestin1 silencing, at odds with a purely G protein-mediated mechanism for adenylate cyclase activation. GLP-1R recruitment of β-arrestin2 has also been detected in HEK293 and COS-7 cells (97,102) but as yet no physiological role for this interaction has been demonstrated. Notably, clear evidence of a role for β-arrestins in GLP-1R desensitisation has not been reported, although has been hinted at by the generation of GLP-1R-β-arrestin2 fusion proteins, or co-overexpression of β-arrestin2 with GRK5 in GLP-1R HEK293 cells (97).

GLP-1R signalling pathways are concisely summarised in figure 1.1.

![Figure 1.1. Schematic summarising G protein and arrestin signalling by the GLP-1R.](image-url)
1.4.9 Interplay of GLP-1R trafficking and signalling

After ligand binding, many GPCRs are sequestered from the plasma membrane and internalised into the cell. The GLP-1R exhibits a strong propensity to internalise, with a half time \( t_{1/2} \) of 2-3 minutes at saturating agonist concentrations \((103,104)\). Multiple mechanisms may be involved in GLP-1R internalisation. Clathrin-mediated endocytosis was reported as the main mode of internalisation in insulinoma and fibroblast cell lines \((103)\). On the other hand, a dominant negative clathrin mutant \((105)\) and pharmacological inhibition of clathrin \((106)\) failed to block GLP-1R internalisation in HEK293 and BRIN-BD11 insulinoma cells, respectively. Whether β-arrestin recruitment is required, as it is for other receptors \((90)\), is disputed, as RNA-mediated silencing \((98)\) and dominant negative overexpression \((105)\) of β-arrestin1 did not affect GLP-1R internalisation, although a fusion protein of GLP-1R and β-arrestin2 increased agonist-induced surface receptor loss, as did overexpression of GRK5 which is believed to promote β-arrestin2-GLP-1R interaction \((97)\). Caveolin, thought to play a role in non-clathrin-mediated endocytosis has also been implicated in GLP-1R internalisation \((105,107)\). Dynamin, involved in both clathrin-dependent and clathrin-independent endocytosis, appears to play an important role, as indicated by reduced internalisation when it is ablated genetically \((108)\) or pharmacologically \((106)\).

Agonist-stimulated receptor endocytosis has traditionally been believed to represent a signal-terminating event, intended to protect the cell from overstimulation. This dogma has been challenged recently with several reports of persistent generation of cAMP from internalised receptors. This phenomenon was initially reported for the parathyroid hormone (PTH) receptor, as demonstrated by prolonged signal from a Forster resonance energy transfer (FRET) probe that detects cAMP production after washout of surface bound agonist \((109)\). Similar effects were observed with the pituitary adenylate cyclase 1 receptor (PAC1R) \((110)\) and the vasopressin type 2 receptor (V2R) \((111)\). Further evidence that GPCRs can remain active in endosomal compartments was provided using nanobodies which specifically recognise the active conformation of the β2-AR, which localised to early endosomes as well as the plasma membrane \((112)\). Spatially constrained cAMP signalling may have functional consequences; for example cAMP-response element (CRE)-associated transcription results predominantly from endosomal rather than plasma membrane originating cAMP, as determined using specific ligands and photoactivatable compartment-targeting adenylate cyclase isoforms \((113)\).

A possible additional mechanism for an apparent requirement of receptor internalisation for sustained signalling is the role played by endocytosis and subsequent recycling back to the plasma membrane in receptor resensitisation. Receptors phosphorylated by GRKs following agonist stimulation become desensitised, and to restore cellular responsiveness, a mechanism is required for dephosphorylation. This process is typified by the β2-AR, which undergoes dephosphorylation by protein phosphatase 2A (PP2A) in acid conditions in early endosomes, prior to recycling back to the plasma membrane \((114)\).
As this process requires an initial internalisation event, preventing endocytosis might abrogate signalling responses through accumulation of desensitised receptors at the cell membrane.

One study has examined the role of internalised GLP-1Rs in coupling to downstream response (106). Pharmacological inhibition of GLP-1 internalisation blunted signalling in BRIN-BD11 insulinoma cells as measured by a CRE-dependent luminescent transcriptional reporter, and was claimed in this report lead to a reduction in insulin secretion (although alternative interpretations of the data are possible).

### 1.5 Pharmacological incretins in the treatment of T2DM

GLP-1R agonism enhances both β cell function and insulin sensitivity (via weight loss), making it an attractive pharmacological target for T2DM treatment. Furthermore, the glucose-dependency of the insulinotropic effect leads to a low risk of hypoglycaemia in comparison to other insulin secretagogues such as sulphonylureas. However, the short plasma half life of GLP-1 itself limits its therapeutic application (115,116). Three approaches have been trialled to circumvent this limitation:

1. Reducing degradation of endogenous GLP-1 by inhibiting the enzyme DPP-4
2. Development of peptide GLP-1R agonists with reduced susceptibility to degradation and clearance
3. Non-peptidic GLP-1R agonists

#### 1.5.1 DPP-4 inhibition

DPP-4, also known as CD26, targets peptides with an alanine or proline as the penultimate residue from their N-terminus (117), including GLP-1 and GIP. Consequently, inhibition of DPP-4 increases circulating levels of active GLP-1(7-36)NH₂, promoting GLP-1 actions and improving glycaemia (118). Several DPP-4 inhibitors are currently approved for the treatment of T2DM, including (in the UK) sitagliptin, saxagliptin, alogliptin, vildagliptin and linagliptin. On average, this class of agent leads to HbA1c reductions of ~0.8% without causing weight gain (or weight loss) (119). DPP-4 agents are orally administered and well tolerated, but a signal of increased risk of congestive heart failure was noted in a large cardiovascular outcome trial of saxagliptin (120), the aetiology of which is unclear.

#### 1.5.2 Peptide GLP-1R agonists

DPP-4 degradation is the primary determinant of GLP-1 clearance. Therefore, substitution of alternative amino acids in place of the usual Ala² in GLP-1 increases plasma stability and biological activity (121). In fact, these mutant peptides display only modestly prolonged pharmacokinetics in
comparison to native GLP-1 as degradation by the neutral endopeptidase NEP-24.11 becomes a limiting factor for in vivo stability once DPP-4 is inhibited (122). NEP-24.11 targets multiple sites within the GLP-1 peptide (123) and therefore rational introduction of sequence substitutions to GLP-1 to reduce NEP-24.11 sensitivity without major losses in biological potency is challenging.

However, the lizard peptide exendin-4, originally isolated from the venom of the Gila monster Heloderma suspectum (124) is a GLP-1 homologue which is resistant to both DPP-4 and NEP-24.11, thereby increasing its plasma half life approximately 10-fold in comparison to GLP-1 after intravenous injection (125). Exendin-4 is primarily cleared via the renal route at a rate determined by glomerular filtration (126). Synthetic exendin-4 (Exenatide) was found to be effective in improving both glycaemia and weight in T2DM (127,128), and is now in routine clinical usage for this indication, administered twice daily by subcutaneous injection. The more potent effects of this and other GLP-1R agonists compared to DPP-4 inhibitors likely reflect the substantially greater circulating agonist concentration achieved by the former strategy versus the modest increase in active GLP-1 when DPP-4 is inhibited.

The second-to-market GLP-1R agonist, liraglutide, utilises a different strategy to prolong biological half-life. This peptide is palmitoylated at Lys\textsuperscript{20} which facilitates non-covalent bonding to albumin in plasma, reducing both proteolytic degradation and renal clearance (129). Consequently, liraglutide exhibits a prolonged pharmacokinetic profile in comparison to exenatide, allowing once-daily dosing. Glucose- and weight-lowering properties of liraglutide are similar to exenatide, but liraglutide currently enjoys two additional advantages; firstly, as well as treatment of T2DM, it is also approved (at a higher dose) for weight loss in people who do not have diabetes (130), and also a recent large trial demonstrated it leads to long-term improvements in cardiovascular outcomes in T2DM patients (131).

Reducing dose frequency may be desirable to reduce nausea associated with plasma drug level fluctuations, for patient convenience and to improve adherence. Therefore GLP-1R agonists have now been developed which allow weekly or less dosing. Albiglutide (132) and dulaglutide (133) consist of, respectively, human albumin or a human IgG4 Fc fragment, coupled to two “optimised” GLP-1 molecules. Both exhibit half-lives of several days and are administered once weekly. Semaglutide, a next generation liraglutide-like molecule containing further refinements to the peptide sequence and acyl chain, is also given weekly. Semaglutide shares liraglutide’s proven cardiovascular benefits (134), and interestingly, is under investigation for possible oral administration, achieved by formulation with novel incipients to prevent intestinal degradation and enhance absorption. Additionally, novel delivery methods of exenatide have been developed, including by slowly absorbed microspheres (135) and via year-long osmotic minipump (136).
1.5.3 Small molecule GLP-1R agonists and allosteric modulators

Peptide agonists, with few exceptions, must be given by injection to avoid degradation in the digestive tract. Therefore, small molecule agonists which can be taken orally are an appealing alternative.

Novo Nordisk identified a series of quinoxalines, the most effective of which is known as “compound 2”, which allosterically enhances GLP-1R ligand affinity and signal transduction, and demonstrated short term in vivo efficacy (137). A structurally distinct pyrimidine compound with similar pharmacological properties, known as compound B or “BETP”, was later reported (138). Whilst these compounds display weak partial agonism in isolation, they are powerful positive allosteric modulators (PAMs) of GLP-1R activity, in particular for less potent endogenous GLP-1R ligands such as GLP-1(9-36)NH₂. It is proposed that the high circulating concentrations of this metabolite, which is otherwise probably inactive, could become biologically useful in the presence of GLP-1R PAMs (139). Interestingly, compound 2 and BETP have been found to share an identical mode of action; both are reactive electrophiles which form stable adducts to Cys³⁴⁷ of the GLP-1R, enhancing orthosteric agonist affinity and efficacy (140). Unfortunately this reactivity is not restricted to the GLP-1R, resulting in rapid inactivation in plasma via covalent binding to plasma proteins, precluding further development for clinical use. Recently, a GLP-1R PAM with improved stability was described (141), which both potentiates insulin secretion and penetrates the CNS (which might therefore allow appetite suppression).

Non-peptidic orthosteric GLP-1R agonists have also been described. Boc5, a substituted cyclobutane, is a GLP-1R partial agonist with anti-diabetic activity in mice (142). TT15 (143) behaves similarly. Neither of these compounds have been taken forward into clinical development.

1.5.4 Side effects of GLP-1R therapy

The most common adverse effect of treatment with GLP-1R agonists is nausea. This most likely represents overstimulation of the anorectic brain centres responsible for these agents’ weight lowering effects. Around 30-50% of patients experience nausea at treatment initiation (144), despite careful dose titration. GLP-1R agonists may differ somewhat in their propensity to induce nausea, with an apparent correlation with duration of action; for example, 40% vs 50% of patients experience nausea with weekly vs twice daily exenatide (145). This could result from greater GLP-1R desensitisation with prolonged continuous exposure compared to twice daily dosing, as the gastric emptying effect of GLP-1 is known to undergo rapid tachyphylaxis (146). Additionally, other long acting agents which are conjugated to large plasma proteins (eg albiglutide) may exhibit slower penetration to brain regions responsible for nausea (147).
The wide distribution of GLP-1Rs raises the possibility of non-therapeutic effects in other tissues. Exocrine pancreas pathology has attracted the greatest attention. A possible association of GLP-1R agonist therapy with pancreatitis was first raised in a single-patient case report (148). A seemingly plausible mechanism for how this might occur involves incretin-induced proliferation of GLP-1R-expressing pancreatic ductal cells, leading to duct stenosis and thus pancreatic autodigestion by trapped digestive enzymes (149). Rodents harbouring a mutation associated with high risk of pancreatic neoplasia responded to exenatide treatment with development of focal proliferation of exocrine pancreas tissue (150). However, preclinical studies in genetically normal animals have in general not supported an increased rate of pancreatitis; for example, in rodents and non-human primates administered extremely high dose liraglutide for up to two years there was no evidence of pancreatic pathology (151). Whilst some post-marketing studies reported an apparent increased risk of pancreatitis in incretin-treated patients (152,153), such studies can suffer from ascertainment bias and insufficient controlling for confounding (for example, the risk of pancreatitis is increased in T2DM). A large meta-analysis failed to identify any signal of pancreatic pathology from pooled data of several incretin trials (154), and the current view of the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) is that there is insufficient evidence to attribute increased pancreatitis risk to treatment with GLP-1-based agents (155).

During preclinical development liraglutide was found to stimulate hyperplasia of thyroid C-cells (156). Indeed, the GLP-1R was reported to be expressed in rodent medullary thyroid carcinoma (MTC, for which C-cell hyperplasia is a precursor) in the 1990s (157). This observation raises concerns as to whether incretin treatment could increase the risk of MTC. As MTC is extremely rare, randomised trials are unlikely to be large enough to detect changes in incidence, so monitoring of calcitonin, a biomarker of MTC, is used as a surrogate. Thus far, no evidence of increased calcitonin release has been found in response to GLP-1R agonism in humans (158,159). Of note, human thyroid contains a much lower proportion of C-cells than in rodent, and no evidence of GLP-1R expression in human or non-human primate thyroid was found using an extensively validated GLP-1R antibody (63). The FDA currently takes the view that the current evidence does not support an increased risk of thyroid pathology in humans (160).

1.6 Biased agonism

A two-state model of receptor activation dominated pharmacological thinking for many years, in which, once bound, a ligand may induce the receptor to adopt an “active” conformation, which is coupled to downstream response, for example by recruitment of G proteins (161). The inherent ability of the ligand to preferentially stabilise the active conformation determines its behaviour as a full
agonist, partial agonist, or antagonist (162). In this model, the downstream response is generally considered to be unidimensional, either coupled to a single pathway, or to more than one pathway but with equivalent activation of each. In fact, for many years data has been available which implies differential activation of separate pathways by different ligands, for example at serotonin (163) and glucagon (164) receptors. Another example describes a reversed potency ranking for cAMP and inositol phosphate generation by two pituitary adenylate cyclase activating polypeptide (PACAP) isoforms at the PACAP1 receptor (165).

Increasing awareness of this phenomenon has now crystallised as a concept known variously as “biased agonism”, “ligand bias” or “functional selectivity”. The single active receptor conformation underpinning previous models has evolved to a theory of multiple active conformations, each facilitating coupling to a particular pathway, with differential stabilisation of each conformation by different ligands. Structural evidence for pathway-specific receptor conformations has now been provided by 19F-NMR (166).

GPCR activation often induces several downstream responses at the cellular level, some desirable and some less desirable, which may individually result from engagement of specific signalling pathways. Biased agonism therefore provides a framework to selectively promote therapeutic actions whilst minimising adverse effects. For example, standard angiotensin 2 receptor type 1 (AT1R) blockade reduces blood pressure (desirable) but also impairs cardiac performance (undesirable) in rats. However, TRV12002, a biased ligand for the AT1R which prevents G protein signalling by the endogenous agonist angiotensin 2 whilst allowing β-arrestin recruitment, maintains the anti-hypertensive effect whilst paradoxically increasing myocardial contractility (167). Carvedilol (168) and nebivolol (169) are β-blockers used to treat cardiac failure with hitherto unexplained survival benefits in comparison to other agents of this class; accumulating evidence suggests that the fact that they allow persistent β-arrestin signalling at the β2-AR despite G protein antagonism underpins their apparent clinical utility (170). Several further examples of novel and pre-existing agonists exhibiting biased signalling properties have been identified, including for µ-opioid (171), serotonin (172), cannabinoid (173), PTH (174) and adenosine (175) receptors.

In addition to their potential therapeutic utility, biased agonists may be useful as research tools to understand specific cellular responses. By selectively stabilising specific receptor conformations, structural insights into G protein- or arrestin- associated receptor states may be obtained (176). "Knocking out” specific pathway engagements using biased ligands may facilitate the study of determinants of receptor trafficking (177,178), post-translational modifications (179), cell growth (180), death (181), and transcriptional responses (182).
Interestingly, exploration of signalling responses to endogenous GLP-1R ligands has revealed differences in pathway engagement between GLP-1(7-36)NH₂, glucagon and OXM, with the latter two revealed to be biased towards cAMP signalling. Furthermore, small molecule allosteric modulators (discussed in 1.6.3) of the GLP-1R have also been demonstrated to induce additional signal bias when these endogenous agonist are used as orthosteric probes (143). Due to the instability of this and related compounds, the physiological consequences of this effect has not been demonstrated.

Recently, a peptide-based agonist of the GLP-1R was identified which exhibited agonist bias, favouring G protein signalling over β-arrestin recruitment (183). In this study, a peptide library was generated based on exendin-4 but with the N-terminal 8 amino acids removed and replaced with scrambled sequences of 7 amino acids. Via lentiviral expression these peptides were expressed tethered to a membrane protein with an interspaced linker sequence, which allowed autocrine activation of HEK293-GLP-1R cells. Flow cytometry was used to identify the most efficacious sequence on the basis of receptor activity measured by a bioluminescent reporter. The favoured compound identified, termed "P5", exhibited little similarity to exendin-4 in the novel N-terminal sequence; the distal 31 amino acids were however identical. P5 was characterised as a biased GLP-1R ligand, retaining full agonist activity for cAMP generation but only partial agonist activity for β-arrestin1 recruitment. When tested for anti-diabetic activity in mice acutely via intra-peritoneal glucose tolerance tests (IPGTTs), the compound was found to exhibit better glucose-lowering action than exendin-4, despite reduced insulinotropic effect and no change to insulin sensitivity. This paradoxical finding was unexplained, although improvements in glycaemic control after chronic administration of P5 vs exendin-4 appeared to be associated with changes to adipogenesis and some markers of insulin sensitivity.

The structural basis of GLP-1R bias has been extensively investigated by the group led by Wootten and Sexton at Monash University. Disruption by mutagenesis of hydrogen bond networks existing between transmembrane helices selectively altered GLP-1R signalling profiles in a ligand-dependent manner, implying an important role for these polar interactions in mediating pathway-specific active receptor conformations (184). An extensive alanine scan of extracellular regions of the GLP-1R and testing of mutants for altered affinity, cAMP generation, calcium response and ERK phosphorylation, using GLP-1, exendin-4 and oxyntomodulin as orthosteric probes, revealed pathway- and ligand-specific receptor regions responsible for biased signalling (74). These findings may provide a useful framework for structure-based design of compounds to selectively target particular downstream responses.
1.7 Conclusion

GLP-1R agonism stimulates insulin secretion and inhibits appetite, and is thus an attractive pharmacological target for treatment of T2DM. Much effort has gone into development of incretin-based drugs. Thus far, peptide-based GLP-1R agonists have proven the most effective, with DPP-4 inhibition also now in routine clinical usage. Current evidence supports a good safety profile for GLP-1-based drugs, although an increased pancreatitis risk has not been conclusively excluded. The main drawback of incretin therapy is a high rate of nausea particularly during treatment initiation.

The past several years has witnessed substantial progress in understanding of intracellular signalling pathways engaged by GPCRs. Several receptors couple to multidimensional signalling networks involving multiple G proteins and β-arrestins, with distinct downstream consequences. The recognition that different pathways can be selectively targeted by biased ligands provides exciting new opportunities for drug discovery, potentially allowing identification of novel agents with greater efficacy or fewer adverse effects.

Evidence for biased GLP-1R signalling (by OXM) was first reported in 2007, and whilst a number of biased allosteric molecules developed since have proved useful as research tools, none are sufficiently stable for therapeutic use, and only recently was a peptide-based biased GLP-1R agonist described and demonstrated to outperform the non-biased comparator compound in a chronic administration study in vivo.

An in depth exploration of ligand-based structural determinants of biased GLP-1R signalling, and the downstream consequences of this in cellular and animal models, is the subject of this thesis.
2 Methods
2.1 Study approvals

All studies involving human tissue were approved by the National Research Ethics Committee (NRES) London (Fulham) “Signal transduction in isolated human islets: regulation by glucose and other stimuli” (REC 07/H0711/114). Islets were isolated under the approval of NRES Oxfordshire (REC 09/H0605/2) (Oxford, United Kingdom), Comitato di Bioetica Azienda Ospedaliero-Universitaria Pisana (34058) (Pisa, Italy), Comitato Etico Istituto Scientifico San Raffaele (Milan, Italy), and the Central Institutional Review Board on Clinical Research of Geneva University Hospitals (CER 05-028 [05-065]) (Geneva, Switzerland). Where required, consent from next of kin was obtained before use of tissue for scientific research. All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project Licence 70/7596).

2.2 Peptides

Exendin-4, exendin(9-39) and GLP-1 (7-36)NH₂ were purchased from Bachem. Custom peptides were obtained from Insight Biotechnology. Sequences of peptides used are given in appendix A. Experiments were performed with peptide freshly prepared from lyophilized aliquots.

2.3 Cell culture and generation of stable cell lines

PathHunter CHO-GLP-1R β-arrestin1 and 2 reporter cell lines (DiscoverX) were maintained in complete Culture Medium 6 (DiscoverX). INS-1 832/3 cells (a kind gift from Prof Christopher Newgard, Duke University, USA) were maintained in RPMI-1640 supplemented with 10% FBS, 11 mM D-glucose, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and 1% penicillin/streptomycin. Stable CHO-SNAP-GLP-1R cells were generated using wildtype CHO-K1 (ECACC) transfected using Lipofectamine 2000 with N-terminal-SNAP-tagged GLP-1R (pSNAP-GLP-1R, Cisbio) and selected with G418 (1 mg/ml); a monoclonal population was then generated by amplification of single cells obtained from fluorescence-assisted cell sorting (FACS) of cells labelled with SNAP-surface 488 (New England Biolabs). CHO-SNAP-GLP-1R cells were maintained in DMEM supplemented with 10% FBS, 20 mM HEPES, 2% non-essential amino acids, 1% penicillin/streptomycin, and 0.5 mg/ml G418.
2.4 Human islet isolation & culture

Our laboratory regularly receives human islets isolated from normoglycaemic cadaveric donors at centers in Oxford, Geneva, Milan, Pisa and Milan. These are viable for several days when cultured in RPMI-1640 supplemented with 5.5 mM D-glucose, 10% FBS, 1% penicillin/streptomycin, and 0.25 μg/μl fungizone. Donor characteristics are indicated in appendix B.

2.5 Cyclic AMP assays

Cyclic AMP accumulation was determined by HTRF (cAMP Dynamic 2 kit, Cisbio) and read using a SpectraMax i3x plate reader (Molecular Devices) installed with an HTRF cartridge. A standard curve was run in each assay according to the manufacturer’s instructions.

Concentration response experiments using CHO cells: Cells were seeded at a density of 10,000 cells/well in complete CHO medium in white half area 96-well plates and cultured overnight. On the day of the assay, growth medium was aspirated and 25 μL agonist prepared in serum-free DMEM was applied in duplicate in the absence of any phosphodiesterase inhibitors, and incubated for the indicated time period at 37°C. The assay was terminated by addition of HTRF detection reagents reconstituted in the manufacturer’s cAMP lysis buffer to the plate and read in homogenous format after 60 minutes incubation at room temperature. Data were analysed by 4-parameter fitting using Graphpad Prism 6.0.

Pulse-chase experiment (figure 3.16): CHO-SNAP-GLP-1R cells, seeded overnight at a density of 10,000 cells/well in a 96-well plate, were exposed to peptides at a concentration of 10 nM in quadruplicate without any phosphodiesterase inhibitors for 10 minutes at 37°C. After washing three times with 100 μL cold HBSS, 50 μL serum free DMEM supplemented with 10 μM exendin(9-39) was applied and the plate returned to 37°C for the remainder of the experiment. At the indicated timepoints, 50 μL of the cAMP lysis buffer was added to the plate, and once lysis was complete, 25 μL was transferred to a fresh 96-well half area plate and stored at 4°C until the end of the assay, at which point detection reagents were added in parallel to all samples.

INS-1 832/3 cAMP timecourse: 30,000 cells/well were seeded overnight in white half area 96-well plates (1 per timepoint) in complete INS-1 medium. The day of the assay, cells were carefully washed once with Hank’s buffered salt solution (HBSS), and 20 μL serum free RPMI with 3 mM glucose was added to each well. After 60 minutes, 5 μL of peptide were then added at 5X concentration, supplemented with IBMX at a final concentration of 25 μM, in duplicate, and returned to 37°C. At
indicated timepoints, the assay was terminated by addition of detection reagents reconstituted in cAMP lysis buffer.

INS-1 832/3 homologous desensitisation: 30,000 cells/well were seeded at a density in white, half area 96-well plates and cultured in complete INS-1 medium. After 24 hours, agonist at a target concentration of 100 nM, or vehicle (growth medium), was added to each well and incubated overnight. After 16 hours, medium was aspirated, wells carefully washed twice with HBSS, and replaced with 20 µL serum free RPMI + 3 mM glucose for 60 minutes at 37°C. 5 µL GLP-1 (target concentration 100 nM) + IBMX (target concentration 100 µM), prepared in serum free RPMI + 3 mM glucose, was then added to each well to stimulate cAMP generation and incubated for a further 10 minutes at 37°C before the reaction was terminated and analysed as above.

Human islet homologous desensitisation: 150 islets per condition were incubated overnight in 2ml complete RPMI, 11 mM glucose, ± agonist at 100 mM, in 6 well untreated plates. After 16 hours, islets were washed three times in HBSS + 0.1% BSA, and resuspended in HBSS + 0.1% BSA containing 100 nM GLP-1 and 500 µM IBMX for 30 minutes, in triplicate. 100 nM HTRF lysis buffer was then added, islets disrupted by sonication, centrifuged, and an aliquot of supernatant transferred to a white 96-well half area plate, and cAMP determined by HTRF as above.

2.6 Measurement of β-arrestin recruitment

PathHunter CHO-GLP-1R β-arrestin1 and 2 reporter cell lines were seeded overnight in 96-well half area white plates at a density of 10,000 cells/well in CP2 medium (DiscoverX). The day of the assay, medium was aspirated and replaced with peptides prepared in CP2 at the indicated concentration, in duplicate, in a volume of 20 µL. After a 90 minute incubation at 37°C, 10 µL PathHunter detection reagents were added and incubated for 60 minutes at room temperature before reading the luminescent signal from each well (500 ms integration time) with a SpectraMax i3x plate reader. Data were analysed by 4-parameter fitting.

2.7 Quantitation of biased signalling

Bias between cAMP, β-arrestin1 and β-arrestin2 responses was determined using modifications to the operational model of agonism, as previously described (185,186). Because bias is time-dependent (187), a 90 minute incubation period was used for all pathways. Concentration response data were fit to the following equation, which represents the aforementioned model, to derive transduction ratios (\(\tau/K_a\)) values for each agonist in each pathway:
Response = basal + \frac{(E_{max} - basal) \left( \frac{\tau}{K_A} \right)^n [A]^n}{[A]^n \left( \frac{\tau}{K_A} \right)^n + \left( 1 + \frac{[A]}{K_A} \right)^n}

In this equation, basal is the basal level of response of the system, \( E_{max} \) is the maximum response of the system, \( \tau \) is a measure of agonist efficacy as defined in the operational model, \( K_A \) is the pathway-specific affinity constant, \([A]\) is the molar agonist concentration, and \( n \) is the transducer slope which links receptor occupancy to response. Basal, \( E_{max} \) and \( n \) were fit globally as they are system-specific parameters which are not dependent on the agonist. The maximum response in \( \beta \)-arrestin recruitment assays was established by including GLP-1 in each experiment, as arrestin responses for exendin-4-derived agonists were submaximal.

Log(\( \tau/K_A \)) values for each agonist were then normalised by subtracting the log(\( \tau/K_A \)) for the reference agonist, exendin-4, in each pathway, giving \( \Delta \log(\tau/K_A) \), and to determine bias between two pathways, \( \Delta \Delta \log(\tau/K_A) \) values from one pathway were subtracted from the other pathway, yielding \( \Delta \Delta \log(\tau/K_A) \), or log bias factor. When possible, \( \beta \)-arrestin and cAMP assays were performed in parallel using agonists doses prepared from the same aliquot to minimise variability, with bias calculated on a per-assay basis.

2.8 Cell surface GLP-1R ELISA

PathHunter CHO-GLP-1R cells were seeded overnight at a density of 50,000 cells/well in complete CHO medium without selection antibiotics. The day of the assay, medium was aspirated and replaced with serum-free DMEM supplemented with freshly prepared peptide at the indicated concentration in triplicate, and incubated at 37°C for 90 minutes. The plate was then placed on ice to arrest further receptor trafficking, and washed three times with 100 µL cold HBSS. Cells were fixed in 2% PFA, pH 7.4 in PBS, for 30 minutes at 4°C. A cell surface ELISA was then used to detect remaining GLP-1R at the cell surface using an extensively validated monoclonal anti-human GLP-1R antibody (63) (Mab 3FS2, Developmental Studies Hybridoma Bank, University of Iowa). Non-specific binding sites were first blocked using 200 µL PBS + 2% BSA for 60 minutes at room temperature. 40 µL anti-GLP-1R antibody (1:100 in PBS + 2% BSA) was then applied and incubated overnight at 4°C. Cells were then washed 5 times in 100 µL PBS + 0.1% BSA, followed by application of 40 µL HRP-conjugated rabbit anti-mouse secondary antibody (ab6728, Abcam, Cambridge, UK, 1:2000 in PBS + 2% BSA) for 60 minutes at room temperature. After a further 5 washes with 100 µL PBS + 0.1% BSA, 50 µL TMB substrate (Thermo Fisher Scientific) was added and incubated for 10 minutes at room temperature. The reaction was terminated with addition of 50 µL 1M HCl, and absorbance read using a SpectraMax i3x plate reader at
450 nm. Surface expression was determined as absorbance from peptide-treated wells relative to that obtained from control-treated wells.

### 2.9 DERET assay to measure GLP-1R internalisation

CHO-SNAP-GLP-1R cells were seeded overnight at a density of 20,000 cells/well in complete CHO medium without G418 in 96-well half area white plates. On the day of the assay, surface SNAP-GLP-1Rs were covalently labelled with 40 nM Lumi4-Tb (Cisbio) prepared in HBSS + 1% BSA, 20 µL per well, for 60 minutes at 37°C. Where metabolic inhibitors were added to prevent internalisation (103) (figure 3.19), this was done 20 minutes before the end of labelling by adding 2 µL of inhibitors at 11X target concentration (10 mM NaN₃, 20 mM 2-deoxyglucose); these were then present throughout the experiment. After washing three times with 100 µL HBSS, 45 µL of 24 µM fluorescein prepared in warm HBSS was added. The plate was transferred to a Flexstation 3 plate reader (Molecular Devices) pre-heated to 37°C, and serial baseline readings were taken over 10 minutes by reading in time resolved fluorescence mode with the following parameters: excitation 340 nm, emission 520 nm (cutoff 495 nm) and 620 nm (cutoff 570 nm), delay time 400 us, integration time 1500 us. Peptides were added in triplicate at 10X target concentration in a volume of 5 µL and TR-FRET serially monitored thereafter. To determine cell surface receptor loss, the signal obtained at 620 nm was divided by that obtained at 520 nm at each timepoint after first subtracting signal from “blank” wells containing fluorescein but no labelled cells. The signal was then normalised as a fold change to the average baseline.

### 2.10 SNAP-tag endpoint internalisation and recycling assay

CHO-SNAP-GLP-1R cells were seeded overnight at a density of 20,000 cells/well in complete CHO medium without G418 in 96-well half area white plates. Separate plates were used for internalisation and recycling. Cells were washed once in HBSS and compounds were added in serum free medium + 0.1% BSA for 60 minutes, before washing three times with cold HBSS to arrest trafficking. At this point, the “internalisation” plate was placed in the cold room, and to the “recycling” plate was added 100 µL warm serum free medium + 0.1% BSA supplemented with 10 µM exendin(9-39). Recycling was allowed to proceed for 60 minutes. Both recycling and internalisation plates were washed one further time and then cells were labelled in the plate with 40 µM Lumi4-Tb in HBSS + 1% BSA for 60 minutes at 4°C. Cells were then washed a further three times, and surface SNAP-GLP-1R was then detected as the signal at 620 nm measured by TR-FRET as above, after first subtracting signal from blank wells.
2.11 TR-FRET binding assays

When N-terminal SNAP-GLP-1R is covalently labelled using suitable SNAP-probes, the close proximity of the probe to peptide FITC moiety allows agonist binding to be directly monitored by FRET. The long-lived fluorescence of terbium cryptate makes this a suitable FRET donor for use in high throughput plate reader assays as fluorescence recordings can be time-gated to eliminate short-lived autofluorescence from cells and plate walls (188). CHO-SNAP-GLP-1R cells, seeded overnight at 20,000 cells/well in complete CHO medium without G418 in white 96-well half area plates, were labelled for 60 minutes at 37°C with 40 µM Lumi4-Tb in 20 µL HBSS + 1% BSA with addition of FITC-conjugated agonists ± non-FITC agonists, depending on the assay. In each case agonist binding was monitored at 37°C by TR-FRET using a Flexstation 3 plate reader set up with the following parameters: excitation 340 nm, emission 520 nm (cutoff 495 nm) and 620 nm (cutoff 570 nm), delay time 50 µs, integration time 300 µs. Binding was quantified as the signal obtained at 520 nm divided by that obtained at 620 nm. Non-specific binding for each agonist was measured in the presence of an excess (10 µM) of unlabelled agonist, and the 520/620 ratio derived at each timepoint from this subtracted from total binding to obtain specific binding. Assay specific protocol aspects are as follows:

Association then dissociation assay (figure 3.18): After labelling, FITC-conjugated agonists (10 nM) were added for 10 minutes (association phase) during which binding was monitored, followed by washing with HBSS and monitoring of agonist dissociation in the presence of 10 µM exendin(9-39) to prevent rebinding. Binding data was fit using Graphpad Prism using the "association then dissociation" model of kinetic binding to calculate $k_{on}$ and $k_{off}$.

Surface dissociation assay (figure 3.20): Metabolic inhibitors (10 mM NaN$_3$, 20 mM 2-deoxyglucose) were added 45 minutes before the end of the labelling period to prevent endocytosis, and then were present for the remainder of the assay. 30 minutes before the end of the labelling period FITC-agonist was added and prebound to surface receptors before washing and immediate measurement of dissociation in the presence of 10 µM exendin(9-39) to prevent rebinding. Binding data was fit using Graphpad Prism using the "dissociation kinetics" model to calculate $k_{off}$.

Competitive kinetic binding assay (figure 3.22): Metabolic inhibitors (10 mM NaN$_3$, 20 mM 2-deoxyglucose) were added 15 minutes before the end of the labelling period to prevent endocytosis, and then were present for the remainder of the assay. After labelling, cells were washed, and exendin-4-FITC ± unlabelled agonist at the indicated concentration were added with immediate measurement of binding at 60 second intervals by TR-FRET. Binding data from a dose-response curve of exendin-4-FITC were analysed using the “two or more concentrations of hot” kinetic binding model in Graphpad Prism to determine the $k_{on}$ and $k_{off}$ for the fluorescence ligand on a per-experiment basis. These data were then included in the analysis of binding data for unlabelled compounds (added in competition...
with exendin-4-FITC) to enable calculation of $k_{on}$ and $k_{off}$ of unlabelled exendin-4, ex-phe1 and ex-asp3 using the "kinetics of competitive binding" model in Graphpad Prism (189).

### 2.12 Endosomal binding

A two-step process was used to label SNAP-GLP-1Rs with BG-SS-biotin (a kind gift from New England Biolabs), a cleavable SNAP probe incorporating a reducible disulfide bond between O6-benzylguanine and biotin moieties, and streptavidin-terbium cryptate (Cisbio), to allow selective stripping of probe from surface receptors without de-labelling internalised receptors. CHO-SNAP-GLP-1R cells were seeded at a density of 20,000 cells/well in 96-well half area white plates and cultured overnight in complete CHO medium. The day of the assay, growth medium was aspirated and cells were labelled with 5 µM BG-SS-biotin in 20 µL HBSS + 1% BSA for 60 minutes at 37°C. Cells were then washed three times with 100 µL HBSS, after which streptavidin-terbium cryptate was added at a concentration of 0.24 µg/ml active streptavidin moiety in 20 µL HBSS + 1% BSA, and incubated for 60 minutes at 37°C. Cells were then washed once with HBSS, and 50 µL FITC-agonist was added in triplicate at a concentration of 100 nM in warm HBSS and incubated for 30 minutes at 37°C to induce internalisation. At the end of this incubation, the plate was moved to the cold room, agonist was removed and washed once with cold HBSS to arrest further endocytosis, and cells were then exposed for 5 minutes to cold sodium 2-sulfanylethanesulfonate (MesNa) prepared at 500 mM in alkaline TNE buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.6), a membrane impermeant reducing agent which cleaves the BG-SS-biotin disulfide bond to release the probe from cell surface receptors. Cells were then washed twice with HBSS, after which 50 µL exendin(9-39) prepared in warm HBSS was added to each well to block further binding to surface receptors. Thus, FRET can only occur between FITC-agonist and labelled SNAP-GLP-1R which was internalised before MesNa washing. The plate was immediately transferred to a Flexstation 3 plate reader at 37°C to serially monitor TR-FRET using the same parameters as for the dissociation kinetics experiments. Endosomal binding was calculated the ratio of signal obtained at 520 nm to that obtained at 620 nm, having first subtracted readings from blank wells (not labelled with BG-SS-biotin) at each wavelength.

### 2.13 Calcium flux assay in CHO-GLP-1R cells

PathHunter CHO-GLP-1R cells were seeded at a density of 50,000 cells per well in black, clear bottom, 96-well plates and cultured overnight in complete CHO media without selection antibiotics. The day of the assay, culture media was removed and replaced with calcium dye solution (Calcium 6 [Molecular Devices] prepared in HBSS + 20 mM HEPES, supplemented with probenecid 2.5 mM) for two hours. Where inhibitors were used (figure 3.23), these were added 15 minutes before the end of the dye-loading phase. The plate was then transferred to a Flexstation 3 plate reader at 37°C set up to read
with the following parameters: excitation 485 nm, emission 525 nm (cutoff 515 nm). Fluorescence was monitored every 2 seconds for a 16 second baseline, followed by robotic addition of the indicated concentration of GLP-1 prepared in HBSS at 3X target concentration, in duplicate, and serial monitoring of fluorescence for a further 90 seconds. Peak fluorescence after agonist addition was expressed relative to average baseline signal to establish a fold change, and GLP-1 concentration response data was analysed by 4-parameter fitting.

Where the calcium flux assay was used to determine desensitisation (figure 3.24), the protocol was adapted to include exposure to agonist during dye-loading. Agonist at the indicated concentration was added to the dye solution for the first 90 minutes at 37°C, followed by washing four times with HBSS. Fresh dye solution (60 µL) without agonist was then added to each well for a further 30 minutes before transferring the plate to the Flexstation 3 plate reader, with response to GLP-1 measured as before. To exclude agonist-induced depletion of intracellular calcium stores, ATP 10 µM was included as a control in each experiment.

### 2.14 Labelling and visualisation of surface SNAP-GLP-1Rs

CHO-SNAP-GLP-1R cells, 100,000 cells per well, were seeded onto glass coverslips in 12 well plates in complete CHO medium. After overnight incubation, cells were labelled with 1 µM SNAP-Surface-488 (New England Biolabs) in complete medium for 1 hour at 37°C. This probe only labels surface SNAP-GLP-1Rs. After washing 3 times with HBSS, cells were treated ± 100 nM exendin-4 for 30 minutes at 37°C, washed, and fixed with 2% PFA for 30 minutes at 4°C. Coverslips were mounted in Vectashield Hardset with DAPI and images acquired with a Zeiss LSM-780 inverted confocal laser-scanning microscope with a 63x/1.4 numerical aperture oil-immersion objective and analyzed in ImageJ.

### 2.15 Imaging of human islet GLP-1Rs

For examining agonist-induced loss of surface GLP-1R in human islets, 100 human islets per condition were incubated overnight in 2 ml complete RPMI, 11 mM glucose, ± unlabelled agonist at 100 mM, in 6 well untreated plates. 16 hours post agonist addition, islets were placed on ice to arrest further trafficking, washed three times by centrifugation at 350G for 3 minutes in cold HBSS + 0.1% BSA, and then exposed to exendin-4-FITC at 10 µM in HBSS + 1% BSA for 60 minutes, at 4°C under gentle agitation, to label surface GLP-1Rs. Islets were then washed in cold HBSS + 0.1% BSA and fixed with 4% PFA in PBS for 30 minutes at 4°C, washed again, and transferred to 70% ethanol before suspension in 4% agarose in order to form small agarose plugs. Plugs were left to cool at room temperature, placed in processing cassettes and dehydrated through serial ethanol gradient (70, 90, and 100%) and Histochoice clearing agent (Sigma) before embedding in paraffin blocks using the Histoembedder.
embedding station (Leica). 1 µm sections were cut with a Leica Jung RM2035 microtome with low-profile disposable blades and placed on poly-lysine-coated microscope slides before de-waxing in Histochoice and rehydration in ethanol and water. Sections were stained by immunofluorescence for FITC with a rabbit polyclonal anti-FITC antibody (Thermo Fisher Scientific) plus a secondary Alexa Fluor 488 antibody (Life Technologies) and a guinea pig anti-human insulin antibody (Dako) plus secondary Alexa Fluor 546 antibody (Life Technologies) prior to mounting and imaging by confocal microscopy as above.

2.16 Insulin secretion experiments

Overnight secretions in INS-1 832/3 cells: The night before the experiment, cells in T75 flasks were washed twice and placed in fresh low-glucose (3 mM) medium. The day of the experiment, 30,000 cells/well were seeded into 96 well plates in complete medium with 11 mM glucose. Agonist or vehicle (complete medium) was then immediately added at 10X target concentration, and cells were incubated overnight at 37°C. After 16 hours, the plate was removed and gently agitated for 5 minutes on a plate shaker to ensure homogenous distribution of secreted insulin within the culture medium without dislodging cells from the plate base. A 10 µL sample of medium was withdrawn from each well and diluted directly 1:10 in HBSS + 0.1% BSA. Cells were then lysed without removing remaining culture medium by addition of 10 µL HBSS + Triton-X100 at a final concentration of 1%. Appropriate sample dilutions were prepared in HBSS + 0.1% BSA, and analysed by HTRF (Cisbio) in white 384 well low volume plates. Percentage secreted insulin was determined for each well, and then expressed relative to the average glucose-only response from each experiment as “insulin stimulation index” (ISI).

Acute secretions in INS-1 832/3 cells: 20,000 cells/well were seeded into 96 well plates and cultured for 24 hours in complete medium with 11 mM glucose. The night before the secretion experiment, medium was replaced with low glucose medium. On the day of the experiment, cells were carefully washed twice with HBSS and compounds added to wells in HBSS + 0.1% BSA, and incubated for the indicated time period. Insulin samples were then taken and processed as above.

Overnight secretions in human islets: Human islets were washed twice in RPMI, and then incubated overnight with 1 ml complete RPMI, 11 mM glucose, +/- 100 nM agonist, in 12 well plates (30 size-matched islets per well, each condition in triplicate). After 16 hours, medium and islets were transferred to microtubes and centrifuged at 350G for 3 minutes. A sample of supernatant was removed, and the islet pellet was washed twice in RPMI, after which acid ethanol (70% ethanol in 0.015 M HCl, 0.1% Triton-X100) was added and sonication performed. Supernatant and islet insulin
content were measured by HTRF after appropriate dilution, percentage secreted insulin determined, and expressed relative to glucose-only response from each experiment as ISI.

**Acute secretions in human islets:** Acute secretions were performed as for overnight, except the stimulation was done in modified Kreb's Ringer bicarbonate-HEPES buffer (130 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgSO₄, 0.5 mM KH₂PO₄, 2.0 mM NaHCO₃ and 10 mM HEPES) saturated with 95% O₂/5% CO₂, adjusted to pH 7.4, and containing 11 mM glucose.

**2.17 Islet calcium imaging to measure homologous desensitisation**

Ca²⁺ imaging were performed as previously described (190). Briefly, islets were incubated for 1 hour with Fluo-2 (10 µM) in a HEPES-bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 24 mM NaHCO₃, 0.5 mM Na₂HPO₄, 5 mM HEPES, 2.5 mM CaCl₂, and 1.2 mM MgCl₂) saturated with 95% O₂/5% CO₂ and adjusted to pH 7.4 and containing 11 mM glucose. For calcium imaging following desensitisation with GLP1 agonists, islets were beforehand incubated overnight with 100 nM exendin-4 or ex-phe1, and the same concentration of agonist was added to Fluo-2 containing HEPES buffer. Islets were placed in an imaging chamber kept at 37°C and perifused with HEPES-bicarbonate buffer containing 11mM and with addition as stated on the figures. A solid-state 491-nm laser was passed through a Nipkow spinning-disk head (Yokogawa CSU-10) coupled to a 10x/0.3 objective (EC Plan-Neofluar, Zeiss). Emitted signals (510–540 nm) were subsequently captured at a frame rate of 30 images per minutes using a highly-sensitive 16-bit 512×512-pixel electron multiplying charge-coupled camera (Hamamatsu). Fluorescent signals were normalized using the function F/Fbaseline where F is fluorescence at a given time point and Fbaseline is the average fluorescence intensity recorded between 2 and 4 minutes.

**2.18 Caspase assay**

INS-1 832/3 cells, 1000 cells/well in 5 uL, were seeded into low volume 384-well white plates in serum free RPMI with 11 mM glucose. Thapsigargin (target concentration 1 uM) +/- agonist were then added in 5 uL serum free RPMI and cells were incubated overnight at 37°C. After 16 hours, apoptosis was determined by addition of 10 uL of Caspase Glo 3/7 reagent (Promega), and luminescence read after 30 minutes incubation at room temperature. Signal was expressed relative to that of thapsigargin-only wells for that experiment and concentration responses analysed by 4 parameter fitting.
2.19 RNA interference

Silencer Select small interfering RNA targeting rat β-arrestin1 (129662) and β-arrestin2 (129665), or a negative control scrambled sequence were used. Transfection was performed in 96-well plates or T25 flasks using Lipofectamine 2000 and knockdown performed for 72 hours prior to use in experiments.

2.20 Quantitative PCR

To determine degree of silencing in INS-1 832/3 cells, 72 hours after triplicate siRNA transfection in 96-well plates, the Taqman Cells-to-CT kit (Thermo Fisher) was used to extract RNA and obtain cDNA according to the manufacturer’s instructions. Quantitative PCR was then performed using Taqman probes (Arrb1: Rn01648673_m1; Arrb2: Rn01456874_g1), with each sample analysed in triplicate in 384 well plates using an ABI Prism 7900 HT System (Applied Biosystems, USA). Gene expression was then determined relative to the endogenous control gene 18S using the 2^ΔCT method.

2.21 Animal studies

**Mice:** Male C57BL/6J mice (8-10 weeks, Charles River, Margate) were maintained controlled temperature (21–23°C) and light-dark cycles (12:12 hour light-dark schedule, lights on at 0700). Animals were regularly handled to minimise any non-specific stress effects. For high-fat, high-sucrose fed mice, ad libitum access to water and a high fat, high sucrose diabetogenic diet (AIN-76A, TestDiet) was provided unless otherwise stated. For lean mice, RM1 standard chow diet was used. Animals were initially group housed (5 per cage) for >4 months before transfer to single cages for experimental procedures after a further one week acclimatisation period.

**Rats:** Male Wistar rats (8-10 weeks, Charles River) were maintained under equivalent conditions to mice and provided ad libitum access to water and RM1 standard chow diet, unless otherwise stated.

2.22 Dose-finding single injection glycaemia study

Mice were fasted for two hours before intra-peritoneal injection of 50 µL peptide or vehicle (0.9% NaCl) at the indicated weight-adjusted dose. Tail vein bleeds were performed immediately before injection and at indicated timepoints thereafter, and blood glucose measured using a Contour glucose meter (Bayer).
2.23 Intra-peritoneal glucose tolerance tests

Mice were fasted for 14 hours overnight before the procedure. 50 µL peptide or vehicle (0.9% NaCl) was administered by intra-peritoneal (i.p.) injection at the indicated weight-adjusted dose. D-glucose, as 20% dextrose at a weight-adjusted dose of 2 g/kg, was then injected i.p. immediately, four hours, or eight hours after peptide. Tail vein bleeds were performed immediately before glucose administration and at indicated timepoints thereafter, and blood glucose measured using a Contour glucose meter. In separate experiments to obtain larger volume blood samples for insulin measurement, samples were taken immediately before and 10 minutes after glucose injection into lithium heparin-coated microvette tubes. Plasma was obtained by centrifugation (5000g, 8 minutes) and immediately analysed for insulin content by an HTRF assay specific for mouse insulin (Cisbio) or stored at -80°C prior to assay.

In mice undergoing continuous administration of agonist via osmotic minipump, the IPGTT was performed identically except without injection of agonist.

IPGTTs in rats were performed similarly except agonist was injected via the subcutaneous route, and the glucose load was reduced to 1 g/kg.

2.24 Pharmacokinetic study

Ad libitum fed mice were administered agonist by intraperitoneal injection at a weight-adjusted dose of 24 nmol/kg. At indicated timepoints, blood samples were collected by tail vein bleeds into lithium heparin-coated microvette tubes. Plasma was obtained by centrifugation (5000g, 8 minutes) and stored at -80°C. To measure plasma agonist concentration, an ELISA (Phoenix) which detects the C-terminus of exendin-4 was used. As this region of the peptide does not differ between agonists, the assay detects exendin-4 and ex-phe1 equally, as indicated.

2.25 Acute food intake studies

Mice were fasted for 14 hours overnight. 50 µL peptide or vehicle (0.9% NaCl) was administered by i.p. injection at the indicated weight-adjusted dose and access to their normal diet was returned. Diet was then weighed at indicated time points. The experiments were performed similarly was rats except agonist was administered subcutaneously.
2.26 Chronic administration study

Subcutaneous osmotic minipumps (ALZET model 2004, Charles River) filled with agonist or vehicle (0.9% NaCl) to ensure delivery of a weight-adjusted dose of 0.24 nmol/kg/day were inserted under gas anaesthesia. Prophylactic antibiotics and analgesia were administered at the time of surgery.

Mice and diet were weighed day 1 post surgery and body and food weight were weighed at indicated intervals thereafter. Intraperitoneal glucose tolerance tests were performed on day 14.

At the end of the study (day 17), mice were sacrificed by decapitation in the fasting state and blood collected into lithium heparin-coated microtubes, and plasma obtained by centrifugation (5000g, 8 minutes) and stored at -80°C for further analysis. Glucose was measured with a glucometer. Insulin was measured using a mouse insulin HTRF kit as before (Cisbio). HOMA calculations were performed using the HOMA calculator available from the OCDEM website (https://www.dtu.ox.ac.uk/homacalculator/download.php). Lipid profile (total cholesterol, HDL-cholesterol and triglycerides), and liver function tests (ALT, AST) were measured with an Abbott Architect autoanalysers in the Hammersmith Hospital clinical biochemistry laboratory, using Abbott reagents. LDL-cholesterol was calculated using the Friedwald equation.

Body composition was determined by MRI (EchoMRI-100) at the time of death.

Liver tissue was dissected post-mortem and fixed in 4% PFA followed by dehydration in 70% ethanol. Haematoxylin and eosin stained liver sections were scored by a histopathologist blinded to treatment allocation using the Nonalcoholic Activity Score with fat scored 0-3, ballooning 0-2 and lobular inflammation 0-2.

2.27 Statistical analyses

Graphpad Prism 6.0 (Graphpad Software) was used for all data analyses. Curve fitting, including to describe dose response relationships, calculate bias, and determine kinetic binding parameters, was performed as described above in relevant experimental sections. For in vitro experiments, the mean of intra-experimental replicates was treated as a single experimental replicate. ANOVA or two-tailed t-tests were performed throughout, depending on whether two or more treatments were being compared. Randomised block (ANOVA) or paired (t-test) analyses were used if experiments were performed with a matched design. Where an experiment included multiple time-points, two-way ANOVA was used. For ANOVA, post hoc tests were performed according to the primary question of the experiment. Thus, Dunnett’s test was used to specifically compare exendin-phe1 and exendin-asp3
responses with exendin-4, with Tukey's test used when differences between all groups were
compared. Other post hoc tests are indicated in the figure legends.
3 Identification of biased GLP-1R agonists
3.1 Introduction

The premise for this investigation is that the GLP-1R is coupled to more than one intracellular signalling pathway, and that at least one of these can be preferentially activated by different GLP-1R agonists, resulting in bias signalling. Many signalling events are linked to GLP-1R activation, including activation of multiple G proteins (85), recruitment of G protein receptor kinases (102) and β-arrestins (97,98), generation of cAMP (77), and activation of multiple protein kinases including PKA (80), PKC (86), MAPKs such as ERK (191), phosphatidylinositol 3-kinase (PI3K) and Akt (192). Measurement of any two or more of these could theoretically reveal the existence of bias. However, due to substantial crosstalk between signalling pathways (193), it is more useful to measure the proximal events in the signalling chain. Ideally, this would mean measuring proteins which directly interact with the GLP-1R, of which the most commonly considered as having distinct signalling roles are G proteins and β-arrestins. In this study, bias was determined between recruitment of β-arrestin1, β-arrestin2, and cAMP generation, a convenient marker of activation of Gαs, the major G protein responsible for GLP-1R actions in β cells.

Cyclic AMP and β-arrestin signalling have been linked to downstream effects in β cells including insulin secretion and prevention of apoptosis (98,101,194,195). However, basic receptor behaviours including internalisation and functional desensitisation are also G protein- and β-arrestin-mediated events (196,197). Therefore measurement of how biased agonists influence GLP-1R trafficking forms part of this study.

Most known GLP-1R agonists act at the orthosteric site, although positive allosteric modulators displaying inherent agonist activity have also been described (140). Peptide-based agonists which share significant structural homology with the endogenous agonist GLP-1 have proven most effective clinically. Therefore, novel agonists developed in this study are iterations of GLP-1(7-36)NH₂ and the GLP-1 homologue exendin-4, possessing single amino acid sequence substitutions at various locations within the peptide. This approach also provides information which might be useful to aid understanding of the receptor activation mechanism by endogenous ligands.

3.2 Aims

1. Identify novel GLP-1R agonists displaying bias between β-arrestin recruitment and cAMP
2. Determine the effect of GLP-1R bias on receptor behaviours including internalisation and desensitisation
3.3 **Mechanism of agonist-induced GLP-1R activation**

To rationally design peptide analogues of GLP-1 and exendin-4, it is necessary to review the structural basis for binding and activation of the GLP-1R. A full length structure of the agonist-bound GLP-1R is not yet reported, but significant insights have been gained from published structures of GLP-1R ligands, receptor subdomains, more complete structures of related receptors, as well as ligand and receptor mutagenesis studies.

### 3.3.1 Numbering system

To aid comparison with exendin-4, in this study, peptides based on GLP-1(7-36)NH$_2$ are numbered 1-29 from the N-terminal, rather than 7-36 as utilised by some authors.

### 3.3.2 GLP-1R ligand structure

At the macro level, when bound to the GLP-1R, both GLP-1(7-36)NH$_2$ and exendin-4 consist of a flexible N-terminus of several amino acids, followed by a longer region which adopts a predominantly $\alpha$ helical conformation. Notable differences between the two peptides include a) a greater helical propensity of exendin-4 in solution, as determined by circular dichroism (198) and nuclear magnetic resonance (NMR) (199); and b) exendin-4 possesses an 8 amino acid C-terminal extension sometimes referred to as that “Trp-cage” as it forms a interaction with Trp$^{25}$ of exendin-4, stabilising the helix when in solution.

### 3.3.3 GLP-1R structure

The GLP-1R, first cloned in 1992 (71), was classified as a class B GPCR on the basis of sequence similarities with other receptors. This family, also known as the secretin family, comprises 15 receptors including the GLP-1R. Of note, these include the calcitonin, amylin, calcitonin-gene related peptide (CGRP) and adrenomedullin receptors, which function as distinct receptors but are derived from just two parental receptor subunits (calcitonin receptor and calcitonin-like receptor) and rely on the presence or absence of specific receptor activity modifying proteins (RAMPs) to confer ligand specificity (200). This is not the case with the GLP-1R, which does not associate with RAMPs (201).

Class B GPCRs share several common structural elements (202):
1. A large N-terminal extracellular domain (ECD), which consists of two central β-sheets and an α-helix, interconnected and stabilised by disulfide bonds; the ECD serves primarily to facilitate ligand binding
2. A transmembrane region (TMR) comprising 7 α-helices arranged circumferentially and interspersed by intra- and extracellular loops; this region undergoes conformational changes on ligand binding which transduce the presence of a ligand to intracellular signalling cascades
3. An intracellular C-terminal tail, which along with TMR intracellular domains, interacts with effector proteins to initiate signalling, and undergoes post-translational modifications to modulate receptor function.

3.3.4 Two-domain model of GLP-1R ligand binding and activation

A consensus mechanism for how ligands bind and activate class B GPCRs has emerged (203), in which the helical ligand C-terminus interacts with the receptor ECD, orientating the flexible ligand N-terminus so that it interacts with the surface and core regions of the receptor TMR. The TMR helices then undergo ligand-dependent rearrangement in a manner which facilitates interaction with intracellular proteins.

Specific interactions made by both GLP-1 and exendin peptides have been mapped by site-directed mutagenesis (204), photoaffinity cross-linking (205-207), use of chimeric and truncated peptides (72,208), and solving of crystal structures of the GLP-1R ECD bound to both GLP-1 and exendin(9-39) (209,210). These studies have been extensively reviewed by Graaf et al (211). Key hydrophobic residues within the peptide C-terminal helix (Phe\textsuperscript{22}, Ile\textsuperscript{23}, Leu\textsuperscript{26} for both GLP-1 and exendin-4) form a key interaction with a corresponding region in the ECD ligand binding groove. Additional hydrophobic and ionic interactions are also noted, including in particular from Lys\textsuperscript{20} (for GLP-1) or from Arg\textsuperscript{20} and Lys\textsuperscript{27} (for exendin-4). The presence of a more extensive ionic bond network formed by exendin-4 may explain its greater binding affinity than GLP-1 for the isolated GLP-1R ECD (212). The “Trp cage” apparently plays only a minor role in exendin binding (209). The relative lack of binding affinity of GLP-1 for the GLP-1R ECD is mainly rectified by interactions made between the peptide N-terminus and receptor TMR, which, unlike for exendin-4 (213), are required for full binding (72). In particular, alanine scanning of the full sequence of GLP-1(7-36)NH\textsubscript{2} revealed substantial effects on affinity of mutating His\textsuperscript{1}, Gly\textsuperscript{4}, Phe\textsuperscript{6} and Thr\textsuperscript{7} (214), with receptor mutagenesis studies implicating interactions with receptor extracellular loops 1, 2 and 3 (74,215,216).

Once bound, interactions of ligand N-terminus and receptor TMR surface are critical for activation of class B GPCRs (73). A recent comprehensive alanine scan of GLP-1R ECLs and neighbouring regions delineated effects on affinity from those influencing receptor activation, with separate readouts for
cAMP and ERK1/2 phosphorylation; the latter was used in this study as surrogate for β-arrestin recruitment (74). Residues within ECL2 were noted to be particularly important for transducing occupancy to cAMP production, with residues in ECL3 more important for ERK1/2 phosphorylation. In conjunction with a refined homology model based partly on the full length crystal structure of the glucagon receptor (GCCR) (217), some (a minority) of these residues were concluded to form direct interactions with the ligand, with the rest presumably required for the correct tertiary structure of the ECL to enable such interactions. The GLP-1 residues seemingly playing a role in receptor activation beyond affinity effects were reported as Gly\(^4\), Thr\(^5\), Phe\(^6\), Thr\(^7\), and Asp\(^9\), which are conserved in exendin-4.

Transmitting the presence of an agonist to a cellular response requires a change in receptor conformation to facilitate interaction with intracellular signalling molecules. The nature of these conformational changes is not yet known for the GLP-1R and has been best studied in class A GPCRs. Activation of \(G_\alpha_s\) by the \(\beta_2\)AR requires an 14 Å outward movement of the intracellular end of TM6 (218). TM6 of rhodopsin also moves to facilitate the binding of visual arrestin, although to a lesser extent, and this observation, along with differential movement of other TM domains, is hypothesised to play a role in biased coupling to \(G\) proteins vs arrestins (219). Whilst confirmation that similar mechanisms exist for class B GPCRs is awaited, the presence of a network of polar interactions between different transmembrane helices for the GLP-1R, which play an apparent role in stabilising receptor in inactive and pathway-specific active conformations, has been demonstrated (184). It is thought that small agonist-induced changes near to the ligand binding site drive disruption of these hydrogen bonds, culminating in global changes to receptor conformation which can lead to biased signalling.

Not all evidence supports an exclusive role for ligand N-terminus – receptor TMR interaction for GLP-1R activation. Recently, the GLP-1R was shown to require the ECD for activation even when the orthosteric ligand was covalently fused to the ligand binding pocket, or when challenged with the allosteric ligand BETP which binds at a site distant to the ECD (75). Conversely, it was shown that the ECD of the closely related glucagon receptor forms auto-inhibitory interactions with its TMR which can be disrupted by binding of glucagon (220).

3.4 Results

3.4.1 Initial screen for efficacy-determining regions of GLP-1R ligands

Without prior knowledge of ligand structural features which might induce biased GLP-1R signalling, it was decided to perform an initial screen to identify important peptide regions for further detailed
study. A series of peptides with single amino acid substitutions between N- and C-termini were selected from an existing library of agonists developed by this department as part of an obesity drug discovery program. These peptides were all developed as dual GLP-1R/GCGR agonists modelled on the endogenous dual agonist oxyntomodulin (OXM) (221).

First, functional responses to the parent peptide “G1950”, along with “natural” GLP-1R ligands GLP-1(7-36)NH₂, OXM and exendin-4 were determined. Assays were performed to measure β-arrestin2 recruitment and cAMP production in response to agonist stimulation. The PathHunter CHO-GLP-1R system was used to quantify β-arrestin recruitment. This assay is based on enzyme fragment complementation, in which the GLP-1R and β-arrestin are tagged with complementary halves of a β-galactosidase enzyme, which forms a functional enzyme unit only when β-arrestin is recruited to the receptor. Chemiluminescent enzyme substrate added at the end of the stimulation phase allows signal quantitation in a luminescence plate reader. Of note, arrestin – receptor association in this assay is non-reversible, which is not the case with endogenous arrestins, but increases the detection window. Cyclic AMP production was measured in the same cell type with a homogenous time-resolved fluorescence (HTRF) assay used for detection. No phosphodiesterase inhibitors were used as CHO-GLP-1R cells generate large amounts of cAMP. Using a standard curve, cAMP concentration from cell lysates were determined by interpolation. Results are shown in figure 3.1 and table 3.1.
Figure 3.1. Dose response of G1950 in comparison to other GLP-1R ligands in PathHunter CHO-GLP-1R cells.

(A) cAMP response, 30 minute incubation, n=4. (B) β-arrestin2 response, 90 minute incubation, n=4. Data represented as mean ± SEM, after normalisation to maximum GLP-1 response, with 4-parameter logistic fit shown. (C) Typical standard curve from cAMP assay demonstrating how lysate [cAMP] is interpolated.

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<tr>
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<th>cAMP</th>
<th>β-arrestin2</th>
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<td></td>
<td>pEC_{50}</td>
<td>E_{max}</td>
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<tr>
<td>GLP-1</td>
<td>10.27 ± 0.07</td>
<td>100 ± 0</td>
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<td>Ex4</td>
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<tr>
<td>G1950</td>
<td>10.28 ± 0.08</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>OXM</td>
<td>9.16 ±0.10***</td>
<td>101 ± 6</td>
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</table>

Table 3.1. Ligand potencies and efficacies at GLP-1R.

Average of values determined in 4 independent experiments ± SEM. **p<0.01, ***p<0.001 vs GLP-1, by randomised block ANOVA with Dunnett’s post hoc test.

Of note, all ligands achieved a maximal cAMP response, whereas for β-arrestin2 recruitment, E_{max} for exendin-4, G1950 and OXM was reduced in comparison to GLP-1, classing these ligands as partial agonists in this pathway; this has previously been reported for exendin-4 and OXM (102).

In view of β-arrestin2 maximal response discrimination at saturating stimulatory concentrations, it was decided to perform the initial screen of G1950-related peptides using a single high dose of agonist
(1 μM) using β-arrestin2 recruitment as the readout, in order to identify possible differences in agonist efficacy. In each experiment, GLP-1 was used as a positive control against which responses were normalised. The results of this screen are shown in figure 3.2.

Figure 3.2. Effect of point mutations at different peptide regions in G1950 on β-arrestin2 response in PathHunter CHO-GLP-1R cells.

Peptides administered at 1 μM, 90 minute incubation. Data represented as mean of at least two experiments ± SEM, after normalisation to GLP-1 response.

These data indicate substantial reductions in β-arrestin2 response at 1 μM with all tested sequence changes at positions 1, 4 and 9, with variable effects at position 2 and 3. Conversely, sequence changes at positions 5, 15, 16, 25, 28 and 29, were relatively benign. This experiment assumes that the high concentration of ligand applied – approximately 100,000x the molar concentration of physiologically circulating GLP-1(7-36)NH₂ – is likely to achieve a maximal response. This has not been experimentally verified and it is possible that if even higher doses were used, different results would have been obtained. Nevertheless, in comparison to cAMP which is highly coupled and often yields similar maximal responses with strong and weak agonists (and indeed, throughout this project, all GLP-1R agonists tested achieved the same E_{max} for cAMP), β-arrestin recruitment assays frequently reveal wide differences in E_{max} (222).

Consequently, it was decided to further investigate potential GLP-1R bias-mediating sequence changes by synthesising novel analogues of GLP-1 and exendin-4 with a variety of substitutions close to the N-terminus, where the largest differences were observed in the initial screen.
3.4.2 GLP-1 analogues

3.4.2.1 Functional pharmacology of GLP-1 analogues

Nine mutant peptides based on GLP-1(7-36)NH$_2$ were synthesised with single amino acid sequence substitutions at positions 1, 2 and 3 (see appendix A for sequence information). Concentration-response experiments for cAMP and β-arrestin2 were performed to determine agonist activity. Assays were performed with all agonists in parallel, and with both pathways tested in parallel, with the same incubation time (90 minutes) used to avoid artefactual bias resulting from pathway-specific kinetic differences (187). Results are given in figure 3.3 and table 3.2.

![Figure 3.3](image_url)

**Figure 3.3.** Responses to N-terminally substituted GLP-1-derived agonists in PathHunter CHO-GLP-1R cells.
(A), (C) and (E): cAMP responses, 90 minute incubation, n=5. (B), (D) and (F): β-arrestin2 responses, 90 minute incubation, n=5. Data represented as mean ± SEM, after normalisation to maximum response to GLP-1, with 4-parameter logistic fit.

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<td>GLP-1</td>
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<td>6.61 ± 0.05 ***</td>
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<td>9.13 ± 0.11 ***</td>
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<td>7.07 ± 0.03 *</td>
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<td>9.22 ± 0.08 **</td>
<td>97 ± 4</td>
<td>9.21 ± 0.07 ***</td>
<td>7.28 ± 0.05</td>
</tr>
<tr>
<td>ala3</td>
<td>8.40 ± 0.18 ***</td>
<td>95 ± 4</td>
<td>8.60 ± 0.14 ***</td>
<td>6.26 ± 0.13 ***</td>
</tr>
<tr>
<td>leu3</td>
<td>8.66 ± 0.04 ***</td>
<td>91 ± 4</td>
<td>8.71 ± 0.04 ***</td>
<td>6.71 ± 0.09 ***</td>
</tr>
</tbody>
</table>

Table 3.2. Potency and efficacy of N-terminally substituted GLP-1 analogues in PathHunter CHO-GLP-1R cells.

Average of values determined in 5 independent experiments ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs GLP-1, by randomised block ANOVA with Dunnett’s post hoc test. Statistical comparison of E\textsubscript{max} values performed before normalisation.

These results reveal the impact of N-terminal changes to GLP-1 on signalling responses. Virtually all sequence changes resulted in significant reductions in signalling in at least one pathway, either via reductions in potency (EC\textsubscript{50}), or efficacy (E\textsubscript{max}), or both, suggesting the GLP-1 N-terminal region may be optimally adapted for maximal signalling responses. Of note, some changes appeared to exert greater effects on one pathway than the other. For example, substitution of Phe or dHis in place of the usual His at position 1 caused a numerically greater reduction in potency for arrestin recruitment than for cAMP, with an associated reduction in efficacy. Conversely, Asp at position 3 in place of Glu maintained a normal arrestin response, whilst cAMP potency was somewhat reduced. These observations intuitively suggest the existence of pathway bias. To confirm this, formal bias analysis was performed.
3.4.2.2 Quantification of biased signalling with GLP-1 analogues.

A modified version of the operational model of agonism was used to quantify biased agonism, as previously described (185,186). This model aims to generate for each agonist a "transduction ratio", termed $\tau/K_A$, which describes the coupling of the agonist to a particular signalling readout in a particular system. This value is extracted by curve-fitting using the same dose response data used in standard agonist stimulation experiments. The term "$K_A$" is the operational affinity constant for binding of the agonist to the specific form of the receptor whilst it interacts with the relevant downstream effector molecule (for example G protein or β-arrestin), and is therefore distinct from other forms of affinity constant; this is relevant as it is presumed that differential receptor conformations which favour G proteins or arrestins will have different affinities for agonist binding. The term "$\tau$" is effectively a measure of how well the agonist, once bound, is coupled to the signalling response in the system in which it is being measured; this includes contributions from receptor density and availability of cellular response proteins, as well as agonist-specific factors. Of note, $\tau/K_A$ values for full agonists are essentially the same as the EC$_{50}$, but for partial agonists, the reduced $E_{\text{max}}$ is reflected in $\tau/K_A$ although the two are not directly related. Because $\tau/K_A$ is influenced by the cell type in which it is measured, it is usually expressed relative to a reference agonist (often the endogenous agonist for the receptor, as long as this engenders a full response); test agonists are assigned a value of $\Delta \log(\tau/K_A)$ for each pathway, which is simply calculated by subtracting the logarithm of $\tau/K_A$ for the reference agonist from that of the test agonist. Of note, this value is considered to be independent of receptor density (223). To calculate bias, $\Delta \log(\tau/K_A)$ values from two pathways are subtracted to determine $\Delta \Delta \log(\tau/K_A)$ also known as the log bias factor; the anti-logarithm of this parameter is considered to numerically describe the degree of bias, though analyses are usually performed on the logarithms to simplify the statistics. Further details of the calculation are given in the methods section.

Therefore, agonist responses for cAMP and β-arrestin2, as displayed in figure 3.3, were used to determine bias factors for GLP-1 analogues between these two pathways. As each pathway was measured in parallel, bias was calculated on a per-assay basis to minimise error. The results of these analyses are shown in figures 3.4, with $\log(\tau/K_A)$ values previously shown in table 3.2.
Figure 3.4. Quantitation of biased agonism with N-terminally substituted GLP-1 analogues in PathHunter CHO-GLP-1R cells.

Data indicate mean ± 95% confidence intervals of ΔΔLog(τ/K_A) values derived from experiments depicted in figure 3.3. Statistically significant bias inferred if 95% confidence intervals do not cross zero.

These analyses confirm biased signalling induced by changes to the N-terminal region of GLP-1. Substitution of His^1 to dHis or Phe led to bias in favour of cAMP over β-arrestin2 recruitment. Given that both these analogues are weaker agonists in the cAMP pathway than GLP-1, in this case, bias in favour of cAMP indicates specifically that whilst some activity for cAMP is lost, arrestin recruitment is more dramatically affected. In contrast, bias in favour of β-arrestin2 resulted from the relatively conservative substitution (both are negatively charged) of Glu^3 to Asp^3. Of note, a recent GLP-1R homology model described formation of a salt bridge between Glu^3 and R190 in transmembrane helix 2 (224); this residue is believed to play an important role in establishing the inter-helix hydrogen bond networked identified as a fulcrum for biased signalling, and therefore hints at the structural mechanism for the observed results for position 3 mutants. Another recent model has described interactions made by His^1 and Ala^2 with residues in transmembrane helices 5, 6 and 7 (414).

3.4.3 Exendin-4 analogues

Having identified bias-inducing sequence changes in GLP-1, modifications were made to the N-terminal region of exendin-4 to determine whether similar biases result. Biasing the exendin-4 response is of particular interest due to the greater suitability of exendin-4 for subsequent in vivo testing.
3.4.3.1 Functional pharmacology of exendin-4 analogues

11 novel exendin-4 analogues were synthesised with changes at positions 1, 2 and 3. These compounds were tested for cAMP and β-arrestin2 recruitment, with each pathway measured in parallel with a consistent 90 minute incubation, as with GLP-1-derived agonists. As the endogenous agonist, GLP-1 itself was included as a control in each experiment, against which responses were normalised. Results are shown in figure 3.5 and table 3.3.

Figure 3.5. Responses to N-terminally substituted exendin-4-derived agonists in PathHunter CHO-GLP-1R cells.

(A), (C) and (E): cAMP responses, 90 minute incubation, n=5. (B), (D) and (F): β-arrestin2 responses, 90 minute incubation, n=5. Data represented as mean ± SEM, after normalisation to maximum response to...
GLP-1, with 4-parameter logistic fit.

<table>
<thead>
<tr>
<th>cAMP</th>
<th>β-arrestin2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>E&lt;sub&gt;max&lt;/sub&gt;</td>
</tr>
<tr>
<td>Ex4</td>
<td>10.30 ± 0.04</td>
</tr>
<tr>
<td>dHis1</td>
<td>10.35 ± 0.05</td>
</tr>
<tr>
<td>asn1</td>
<td>9.85 ± 0.05 ***</td>
</tr>
<tr>
<td>gln1</td>
<td>9.21 ± 0.04 ***</td>
</tr>
<tr>
<td>tyr1</td>
<td>9.67 ± 0.04 ***</td>
</tr>
<tr>
<td>ala2</td>
<td>9.84 ± 0.04 ***</td>
</tr>
<tr>
<td>A1B2</td>
<td>9.87 ± 0.04 ***</td>
</tr>
<tr>
<td>dTyr1</td>
<td>9.14 ± 0.05 ***</td>
</tr>
<tr>
<td>phe1</td>
<td>9.89 ± 0.05 ***</td>
</tr>
<tr>
<td>asp3</td>
<td>9.93 ± 0.03 ***</td>
</tr>
<tr>
<td>gln3</td>
<td>10.20 ± 0.06</td>
</tr>
<tr>
<td>dGln3</td>
<td>9.94 ± 0.05 ***</td>
</tr>
</tbody>
</table>

**Table 3.3. Potency and efficacy of N-terminally substituted exendin-4 analogues in PathHunter CHO-GLP-1R cells.**

Average of values determined in 5 independent experiments ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs exendin-4, by randomised block ANOVA with Dunnett’s post hoc test.

Significant reductions in potency for cAMP production were seen with the majority of agonists, except where His<sup>1</sup> was changed to dHis, or Glu<sup>3</sup> changed to dGln. For arrestin recruitment, marked differences in the E<sub>max</sub> were observed with a number of analogues, particularly those with changes at position 1. It is clear that the most severe losses in activity tended to apply to both pathways – for example, ex-dTyr1 exhibited a ~15-fold reduction in potency for cAMP, associated with a virtually absent arrestin response. However, as for GLP-1, pathway-specific differences were apparent. For example, ex-dHis1 performed similarly for cAMP to exendin-4, but its arrestin response was blunted. Ex-phe1 was able to recruit very little β-arrestin2, but its cAMP response was only moderately right-shifted. Conversely, ex-asp3 retained a normal arrestin profile but reduced potency for cAMP, with a similar pattern seen with position 2 substitutions from Gly to Ala or AIB. The large differences in both potency and efficacy with these exendin-4 derivatives mandates formal bias analysis to determine pathway preferences.
3.4.3.2 Quantitation of biased signalling with exendin-4 analogues

Bias was quantified as before, and results are shown in figure 3.6, with \( \log(\tau/K_A) \) values previously shown in table 3.3.

![Graph showing quantitation of biased signalling with exendin-4 analogues](image)

**Figure 3.6.** Quantitation of biased agonism with N-terminally substituted exendin-4 analogues in PathHunter CHO-GLP-1R cells.

Data indicate indicate mean ± 95% confidence intervals of \( \Delta \Delta \log(\tau/K_A) \) values derived from experiments depicted in figures 3.5. Statistically significant bias inferred if 95% confidence intervals do not cross zero.

As for GLP-1 analogues, biased signalling was confirmed with several compounds. Changes to position 1 consistently led to bias in favour of cAMP. As with position 1 GLP-1 analogues, in most cases this reflects a disproportionate loss in arrestin recruitment, rather than any increases in cAMP activity, compared to exendin-4. In other words, these are weak agonists for cAMP, but even weaker for \( \beta \)-arrestin2 recruitment. The somewhat wider confidence intervals for ex-gln1 and ex-dTyr1 are likely to reflect the loss of precision of \( \tau/K_A \) estimates when very weak partial agonists are analysed with this model, which is a recognised limitation of this approach. A refinement of this analysis, in which weak partial agonists are used as competitive antagonists, has been suggested as a way to improve accuracy (222). As intuitively predicted from retained arrestin responses but reduced potency for cAMP (figure 3.5), position 2 changes of Gly to Ala or AIB, and from Glu\(^3\) to Asp, resulted in bias in favour of \( \beta \)-arrestin2 over cAMP. Identification of biased exendin-4 analogues provides an opportunity to determine the *in vivo* effects of GLP-1R bias due to the prolonged circulatory half-life compared to GLP-1-derived analogues.
3.4.4 Comparison of bias-inducing changes in GLP-1 and exendin-4

To determine whether equivalent sequence changes led to equivalent bias when instituted in both GLP-1 and exendin-4, data from figure 3.4 and 3.6 are re-plotted in figure 3.7 to allow easier visual comparison.

![Figure 3.7. Comparison of signal bias changes resulting from equivalent N-terminal region substitutions in GLP-1 and exendin-4.](image)

Shown are the mean ± SEM of $\Delta \Delta \log(\tau/K_A)$ values derived from experiments depicted in figures 3.3 and 3.5.

Therefore, at least for these example analogues, the resulting bias direction was consistent where the same sequence change was tested in both GLP-1 and exendin-4.

3.4.5 β-arrestin1 responses

As the GLP-1R recruits β-arrestin1 (98) as well as β-arrestin2, and some differentiation in function for each isoform is recognised (225-227), agonist-induced β-arrestin1 responses were also measured. A single, maximal dose (1 μM) was used to screen all analogues in both pathways. Additionally, full dose responses were repeated for selected representative exendin-4-derived analogues (ex-phe1 and ex-asp3) which were previously shown to exhibit bias between cAMP and β-arrestin2. Results are shown in figure 3.8.
Figure 3.8. Responses to N-terminally substituted GLP-1- and exendin-4-derived agonists in PathHunter CHO-GLP-1R cells.

(A) β-arrestin1 and β-arrestin2 responses at 1 µM with indicated exendin-4 derived peptides, 90 min incubation. (B) as for (A), but GLP-1-derived peptides. (C) Correlation of β-arrestin1 and β-arrestin2 responses with exendin-4 and GLP-1-derived peptides, quantified with linear regression. (D) Web of bias, depicting relative pathway preference for each agonist; data represent the inverse logarithm of normalised log(τ/KA) values derived from (E), (F) and (G), further normalised to a reference agonist (exendin-4) and a reference pathway (cAMP). (E) cAMP response to exendin-4, ex-phe1 and ex-asp3, 90 min incubation, 4-
parameter logistic fit shown. (F) As for (E) but β-arrestin1 response. (G) As for (E) but β-arrestin2 response. N=3-6, data represented as mean ± SEM.

The key observation from this set of experiments is that agonist-induced β-arrestin1 responses are highly correlated with those for β-arrestin2. Consistent responses were seen for both arrestins when agonists were given at a maximal dose, with similar dose response relationships also observed for selected exendin-4 derived peptides. Bias between cAMP and both arrestin pathways for ex-phe1 and ex-asp3 was comparable, with no evidence of bias between β-arrestin1 and β-arrestin2.

3.4.6 Measurement of GLP-1R internalisation

Having identified biased GLP-1R agonists, the challenge was to determine the functional importance of these findings. As well as impacting on downstream cellular responses (considered in chapter 4), β-arrestin recruitment is linked to receptor internalisation. In fact, for the GLP-1R a role for arrestins in internalisation has however not been demonstrated conclusively in the existing literature. Genetic ablation of β-arrestin1 with siRNA or a dominant negative β-arrestin1 construct failed to alter GLP-1R internalisation in INS-1 832/13 cells (98) or HEK293 cells (105), respectively. On the other hand, a GLP-1R-β-arrestin2 fusion protein, and overexpression of GRK5 (which mediates GPCR-arrestin interactions), was internalised faster than wildtype receptor (97).

In view of these discrepancies, it was decided to measure GLP-1R internalisation in response to biased GLP-1R agonists. Two methods were employed – a cell-surface ELISA which measures wildtype GLP-1R remaining at the cell surface after agonist stimulation, and a Förster resonance energy transfer (FRET)-based assay, based on SNAP-tag labelling, which measures GLP-1R internalisation in real time. The SNAP-tag system was also used to for a further endpoint assay to measure both internalisation and recycling of the GLP-1R.

3.4.6.1 Cell surface ELISA

GLP-1R internalisation was measured in response to stimulation with biased GLP-1- and exendin-4-derived agonists in PathHunter CHO-GLP-1R cells. In the cell surface ELISA, cells were stimulated for 90 minutes with a high dose (1 µM) of agonist before fixation with PFA in the plate; an extensively validated monoclonal human anti-GLP-1R antibody (63) was then applied without permeabilisation to bind remaining surface receptor, followed by an HRP-conjugated secondary antibody, allowing detection with a colourimetric substrate. Signal was expressed relative to that of vehicle-treated wells, to determine percentage loss of surface receptor.
Responses to GLP-1- and exendin-4 based agonists is shown in figure 3.9.

Figure 3.9. GLP-1R internalisation with N-terminally substituted analogues in PathHunter CHO-GLP-1R cells.

(A) GLP-1 analogues, n=3. (B) Exendin-4 analogues, n=3. All incubations 90 minutes with agonist at 1 µM. Data represented as mean ± SEM after normalisation to vehicle control. *p<0.05, **p<0.01, & p<0.001 vs GLP-1 or exendin-4, as appropriate, by randomised block ANOVA with Dunnett’s post hoc test.

As for β-arrestin recruitment, changes at position 1 of both peptides were notable for reducing the degree of receptor internalisation, with similar observations at positions 2 and 3. The association between GLP-1R internalisation and β-arrestin recruitment was compared by linear regression by plotting cell surface loss against β-arrestin recruitment at the same 1 µM dose. This analysis is shown in figure 3.10. Clear associations were observed (R²=0.81, p=0.002 for GLP-1 analogues vs β-arrestin1; R²=0.96, p<0.001 for exendin-4 analogues vs β-arrestin1; R²=0.83, p<0.001 for GLP-1 analogues vs β-arrestin2; R²=0.96, p<0.001 for exendin-4 analogues vs β-arrestin2). These results are consistent with a role for β-arrestin1 or 2 recruitment in GLP-1R internalisation (although causality is not determined). Notably, internalisation was greater with exendin-4 analogues than GLP-1 analogues at the same degree of β-arrestin recruitment, implying that whatever role β-arrestins play in GLP-1R internalisation, additional agonist-dependent factors are also important.
Figure 3.10. GLP-1R internalisation with N-terminally substituted analogues as a function of β-arrestin recruitment in PathHunter CHO-GLP-1R cells.

(A) For β-arrestin1 recruitment. (B) For β-arrestin2 recruitment. All agonists administered at 1 µM for 90 minutes; results are derived from experiments shown in figures 3.3, 3.6 and 3.9. Data represented as mean ± SEM after normalisation to control.

3.4.6.2 Measurement of GLP-1R internalisation in real time by DERET

3.4.6.2.1 Assay principle

An assay was adapted from Roed et al (104) in which surface GLP-1R is detected in real time via FRET between N-terminally labelled SNAP-GLP-1R and extracellular buffer containing fluorescein, referred to as "diffusion-enhanced resonance energy transfer" (DERET). SNAP-tag is a genetically encoded tag, based on the enzyme O6-guanine nucleotide alkyltransferase, which has no inherent fluorescence, but can be covalently labelled with small benzyl guanine (BG) substrates coupled to standard fluorophores. For study of GPCRs, one major advantage of this system over using protein tags with endogenous fluorescence, such as green-fluorescent protein (GFP), is that it allows specific labelling of only surface receptors at their N-terminal without labelling of intracellular receptors in the biosynthetic pathway (although internal receptors can be labelled with cell-permeating probes, if desired). Trafficking of this population of surface-labelled receptors in response to agonist stimulation can then be more easily observed. Furthermore, various fluorophores can be selected according to assay requirements. In this case, Lumi4-Tb was used as the donor fluorophore. The fluorogenic moiety in Lumi4-Tb is terbium-cryptate, a lanthanide complex which exhibits very long-lived fluorescence emission, thus enabling detection by time-resolved fluorescence (TRF). TRF improves signal-noise ratio as short-lived autofluorescence e.g. from culture plates is gated out, meaning it is suitable for use in plate reader assays. As Lumi4-Tb has an emission peak at ~485 nm, it is compatible with green FRET acceptors, allowing development of time-resolved FRET (TR-FRET) assays.
In the DERET internalisation assay, when Lumi4-Tb-labelled SNAP-GLP-1Rs at the cell surface are excited at 350 nm, FRET occurs to fluorescein dissolved in the extracellular buffer, increasing the 520 nm signal from fluorescein, whilst quenching the 620 nm signal corresponding to another emission peak for Lumi4-Tb. However, when receptor is internalised into endosomes after addition of agonist, FRET can no longer occur, reducing the 520 nm signal whilst increasing the 620 nm signal. Ratiometrically expressing the two signals (620/520) gives a quantitative measure of internalised receptor. Serial measurements can be taken, allowing measurement of internalisation in real time. The protocol is summarised in figure 3.11.

![Diagram of DERET internalisation assay](image)

**Figure 3.11.** DERET internalisation assay principle. For further details, see text.

### 3.4.6.2.2 Establishment of SNAP-GLP-1R cell line

A monoclonal CHO-K1 cell line stably expressing the N-terminally SNAP-tagged GLP-1R was developed. After initial selection with G418 and FACS sorting of SNAP-labelled single cells to establish clonal populations with good levels of SNAP-GLP-1R expression, 5 clones were selected for evaluation of suitability for use in further experiments. Internalisation in response to exendin-4 was measured by labelling with Lumi4-Tb after agonist stimulation to quantify remaining surface receptor. Results are shown in figure 3.12.
Figure 3.12. Assessment of SNAP-GLP-1R expression and agonist-induced internalisation in CHO-SNAP-GLP-1R clones.

Cells were stimulated with exendin-4 100 nM or vehicle for 60 minutes before labelling with Lumi4-Tb, n=1. Non-SNAP cells were wildtype CHO-K1 cells. Data represent mean signal (616 nm) ± SEM of triplicate wells.

Surface SNAP-GLP-1R was highest with clones 2 and 3, with a slightly greater response to exendin-4 noted with clone 3. Therefore, clone 3 was used for all further experiments. SNAP-GLP-1R internalisation in response to exendin-4 was also confirmed by confocal microscopy, which revealed almost complete loss of surface GLP-1R and the appearance of punctate endosomal structures on stimulation with exendin-4 (figure 3.13).

Figure 3.13. Microscopy images of SNAP-GLP-1R internalisation for exendin-4.

CHO-SNAP-GLP-1R cells labelled with SNAP-Surface 488 (green) and treated with vehicle (A) or exendin-4 (100 nM, 30 mins) (B) before fixation.
3.4.6.2.3 Real time internalisation results

GLP-1R internalisation was measured by DERET in response to the biased exendin-4 analogues ex-phe1, and ex-asp3. Multiple doses were used to establish concentration-responses at different timepoints. Results are shown in figure 3.14 and table 3.4.

Figure 3.14. Real time GLP-1R internalisation in response to biased exendin-4 analogues in CHO-SNAP-GLP-1R cells.

(A) Internalisation timecourse, with each agonist administered at 100 nM, n=6. Basal signal was established by reading a baseline for 15 minutes before agonist addition (arrow) and data are expressed relative to this.

(B) Half-time for internalisation derived from (A), determined by one-phase exponential association analysis of each assay, with t=0 constrained to 1.

(C) Dose-response for internalisation derived from measurements extracted from 15 minute timepoint, n=6.

(D) as for (C) but 30 minutes.

(E) As for (C) but 60 minutes.

**p<0.01 vs exendin-4 by randomised block ANOVA with Dunnett’s post hoc test.
Table 3.4. Potency and efficacy estimates for GLP-1R internalisation induced by biased exendin-4 analogues in CHO-SNAP-GLP-1R cells.

Estimates are derived from data depicted in figure 3.14. Data displayed as mean ± SEM. Note – estimates and statistical analysis derived from 4 parameter logistic fitting of pooled concentration-responses. *p<0.05, **p<0.01, ***p<0.001 vs exendin-4 by two way ANOVA + Sidak’s post hoc test.

<table>
<thead>
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<th></th>
<th>15 mins</th>
<th>30 mins</th>
<th>60 mins</th>
</tr>
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<tbody>
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<td>pEC_{50}</td>
<td>E_{max}</td>
<td>pEC_{50}</td>
</tr>
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<td>8.14 ± 0.17</td>
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<tr>
<td>phe1</td>
<td>ND</td>
<td>ND</td>
<td>7.01 ± 1.57</td>
</tr>
<tr>
<td>asp3</td>
<td>7.68 ± 0.10</td>
<td>2.6 ± 0.1</td>
<td>7.92 ± 0.13</td>
</tr>
</tbody>
</table>

These data revealed that GLP-1R-internalisation induced by exendin-4 and ex-asp3 is more rapid, and more extensive, than for ex-phe1, in keeping with results obtained using the cell surface ELISA results. At 100 nM, exendin-4 and ex-asp3 achieved maximum internalisation within 15 minutes. Modest assay dynamic range (maximum fold change ~3) and intra-experiment timepoint-to-timepoint variability reduced the power of the assay to provide very precise estimates of efficacy and potency (note – it was necessary to perform curve fitting on pooled data to derive these measures, rather than estimates for each experiment). Nevertheless, maximum internalisation with ex-phe1 was clearly reduced in comparison to exendin-4. However, the modest increase in GLP-1R internalisation observed with ex-asp3 vs exendin-4 in the cell surface ELISA was not replicated in this experiment.

Theoretically, these data could be used to derive Δτ/K_A estimates for internalisation, in order to determine bias in relation to signalling pathways. This analysis was not performed as signalling results were not available at timepoints corresponding to those measured in this assay, and bias calculations are more appropriate when time-matched.

Thus, measurement of GLP-1R internalisation revealed a strong association with extent and bias towards β-arrestin recruitment. Whether β-arrestin recruitment is mechanistically implicated in internalisation was not established, as in principle both of these outcomes could stem independently from a common event.
3.4.7 Measurement of differences in GLP-1R recycling

After internalisation into endosomes, GPCRs can either be recycled back to the plasma membrane or targeted to lysosomes for degradation (228-231). Receptor recycling is a resensitisation mechanism as, in addition to returning the receptor to a location accessible to restimulation by extracellular ligand, it allows reversal of post-translational modifications such as phosphorylation, which otherwise render the receptor less responsive (232). Measurements of internalisation presented above in reality indicate "net" cell surface receptor loss, contributed to by rates of both internalisation and recycling.

It has been suggested that agonist-specific factors can influence GPCR recycling rate (233), thereby influencing the overall availability of surface receptors during prolonged incubations. Internalisation and recycling of the GLP-1R was previously measured using DERET (104), which revealed that GLP-1R internalised by exendin-4 or liraglutide recycled more slowly than when GLP-1 itself was used as the ligand. Therefore, it was hypothesised that biased GLP-1R agonists might exhibit different recycling rates.

The low dynamic range of the DERET assay used above to measure internalisation in SNAP-CHO-GLP-1R cells precluded real-time measurement of recycling. However, single time-point measurements were still possible using SNAP-CHO-GLP-1R cells labelled post hoc. In this assay, cells were stimulated with agonist for 60 minutes and then labelled in the cold with Lumi4-Tb, to determine agonist-specific internalisation. In parallel, a separate plate underwent the same internalisation treatment, followed by washout and a further 60 minute incubation in the absence of ongoing agonist (but with exendin(9-39) to prevent rebinding events), to allow recycling to occur. The recycling plate was then labelled as before. The difference in cell surface receptor loss between internalisation and recycling plates is indicative of the extent of recycling. Differences in recycling with biased exendin-4-derived agonists ex-phe1 and ex-asp3 were investigated, and results are shown in figure 3.15.
Figure 3.15. Measurement of internalisation and recycling with exendin-4-derived agonists in CHO-SNAP-GLP-1R cells.

(A) Loss of cell surface receptor with 60 minutes agonist (100 nM) treatment. (B) GLP-1R recycling, expressed as a percentage of GLP-1R internalised, as in (A). Both n=5. *p<0.05, ** p<0.01 vs exendin-4 by randomised block ANOVA with Dunnett’s test. Data displayed as mean ± SEM.

These results confirm the earlier measurement of internalisation, namely that exendin-4 and ex-asp3 lead to similar and extensive loss of SNAP-GLP-1R from the cell membrane, with a much weaker effect from ex-phe1. It is also apparent from these experiments that ex-asp3 leads to slower recycling than exendin-4. This observation may explain why this agonist exhibited slightly higher “net” internalisation in the cell surface ELISA (figure 3.9). Interestingly, ex-phe1 allowed substantially faster GLP-1R recycling than exendin-4 and ex-asp3, which likely contributes to the slower net internalisation rate.

Therefore, on the basis of these two biased agonists, there may be an association with arrestin recruitment for not only GLP-1R internalisation, but also for recycling. These data are however insufficient to mechanistically implicate arrestins in the recycling process, although this has been suggested for the GIP receptor (234).

3.4.8 Measurement of cAMP generation from internalised receptors

Sustained cAMP generation from internalised receptors has been described for a number of GPCRs including PTHR (109), TSHR (235), calcitonin receptor (236), β2-AR (112) and GLP-1R (106). One feature of endosomal cAMP generation is that it is “wash-resistant” in cell-based assays, i.e., on removal of extracellular agonist, cAMP remains elevated despite the action of endogenous phosphodiesterases which ordinarily rapidly degrade cAMP, as internalised agonist is not removed by
washing and continues to stimulate the receptor within endosomes (237,238). This approach was taken to determine whether biased exendin-4 analogues (ex-phe1 and ex-asp3) differ in their ability to generate cAMP from the endosomal compartment.

CHO-SNAP-GLP-1R cells were first stimulated with agonist at a dose which induces a maximal cAMP response at 10 minutes in normal accumulation assays in these cells, before washing to remove extracellular agonist and replacement with buffer containing the antagonist exendin(9-39) to block rebinding to cell surface receptors. No phosphodiesterase inhibitors were used. Cells were lysed at various timepoints up to 24 hours for measurement of cAMP by HTRF. Results of this experiment are shown in figure 3.16.

![Figure 3.16](image-url)

**Figure 3.16. Wash-resistant cAMP generation by exendin-4 analogues in CHO-SNAP-GLP-1R cells.**
(A) cAMP concentration-response, 10 minute incubation, n=3, response expressed relative to GLP-1 max
(B) Wash-resistant cAMP generation when agonist is removed by washing three times after 10 minute incubation at 10 nM and replaced with exendin(9-39) at 10 µM, n=4, with cAMP expressed relative to vehicle pre-treated cells at that timepoint. Data expressed as mean ± SEM. ***p<0.001 vs exendin-4 by two-way randomised block ANOVA with Dunnett’s post hoc test.

These results indicate persistent generation of cAMP with exendin-4 and ex-asp3 after washing, whereas for ex-phe1, this was less pronounced. Strikingly, accumulation of cAMP continued for at least 24 hours after washout. This later timepoint is important as dissociation of high affinity peptide hormones from their receptor is not immediate during washing (239), so it is not possible to exclude with certainty a contribution to signalling from surface receptors bound by agonist which was not washed off. By 24 hours however, residual bound agonist at the surface is very unlikely and the high concentration of exendin(9-39) should prevent rebinding events.

This finding is consistent with the differences in GLP-1R internalisation propensity already recorded, which should lead to a greater number of internalised GLP-1Rs with exendin-4 and ex-asp3, resulting
in greater potential for endosomal cAMP generation. Of note, the somewhat greater internalisation of ex-asp3 observed by ELISA (figure 3.9), but not DERET (figure 3.14), did not translate to longer lasting cAMP generation; this could result from saturation of adenylate cyclase, which places a limit on rate of cAMP accumulation.

3.4.9 Measurement of binding kinetics using TR-FRET

Sustained signalling from internalised receptors, as hinted at in figure 3.16, has gain attention recently as a potential mechanism to extend the duration of drug action, and has been proposed to be linked to receptor binding kinetics (238,240,241). In particular, the rate at which the agonist dissociates from the receptor, referred to as $k_{off}$ (or sometimes indicated as the reciprocal and termed “residence time”), may influence how long the receptor continues to generate intracellular signals. To explore potential differences in receptor binding kinetics with biased agonists identified above, and how they may relate to wash-resistant cAMP responses, a TR-FRET approach was used to measure binding between the SNAP-GLP-1R and fluorescently labelled ligands in real-time.

3.4.9.1 Assay principle

As the GLP-1R ECD interacts directly with agonists, a fluorescently tagged agonist can act as a FRET acceptor for N-terminally SNAP-tagged receptor labelled with a suitable FRET donor, allowing direct detection of agonist-receptor binding in real time. Again, long-lived fluorescence from lanthanide probes such as Lumi4-Tb used the SNAP-tag FRET donor avoids the problems associated with auto-fluorescence from plasticware by time gating of signal collection. The FRET signal is indicative of the degree of binding, and serial measurements allow the kinetics of binding events to be measured with high temporal resolution in comparison to standard radioligand binding assays. Further advantages over the use of radioligands include safety benefits of not having to use radioisotopes, and reduced contribution from non-specific binding as signal is only generated when agonist is specifically bound to receptor. Measurement of ligand binding by TR-FRET has been employed for the growth hormone secretagogue receptor type 1a (242), the PTH receptor (188), the ligand-gated ion channel 5-HT3a (243), amongst others, and commercialised as the “Tag Lite” binding assay.

3.4.9.2 FITC agonists

In order to exploit the CHO-SNAP-GLP-1R cell line to measure binding of biased GLP-1R agonists by TR-FRET, suitable fluorophore-conjugated peptides were required. Tethering fluorophores to proteins can sometimes adversely affect function and compromise binding. Fortunately, recent publications
have explored in detail suitable conjugation points for a variety of fluorophores within exendin-4 (244,245). Depending on fluorophore used, conjugation at position 12, 27 or via a C-terminal extension yields fluorescent peptides with acceptable binding properties. Therefore, position 12 was selected as the conjugation point for fluorescein isothiocyanate (FITC), as previously described for exendin-4 (190). The spectral properties of FITC are suitable for TR-FRET in combination with Lumi4-Tb.

FITC conjugates of exendin-4, ex-phe1 and ex-asp3 were purchased with the fluorophore attached at position 12 in each case. First, the agonist properties of these fluoropeptides were compared with the unmodified peptide by cAMP measurement. These results are shown in figure 3.17 and table 3.5.

![Figure 3.17](image-url)

**Figure 3.17. Concentration-responses of unmodified and FITC-conjugated biased exendin-4 analogues in CHO-SNAP-GLP-1R cells.**

(A) Exendin-4, n=3. (B) Ex-phe1, n=3. (C) Ex-asp3, n=3. 30 minute incubation. Data represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>unmodified</th>
<th>K^{12-}FITC-conjugated</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEC_{50}</td>
<td>9.88 ± 0.05</td>
<td>9.71 ± 0.05</td>
</tr>
<tr>
<td>ex-phe1</td>
<td>9.38 ± 0.10</td>
<td>8.88 ± 0.13**</td>
</tr>
<tr>
<td>ex-asp3</td>
<td>9.70 ± 0.11</td>
<td>9.70 ± 0.08</td>
</tr>
</tbody>
</table>

**Table 3.5. Potency estimates for unmodified and FITC-conjugated biased exendin-4 analogues in CHO-SNAP-GLP-1R cells.**

Average of values from 3 independent experiments shown in figure 3.15. **p<0.01 by randomised block ANOVA with Sidak’s post hoc test.
Thus, FITC conjugation at position 12 had minimal impact on the ability of exendin-4 and ex-asp3 to stimulate cAMP production. The reduced potency of ex-phe1-FITC is unexplained. However, this 3-fold reduction was considered to fall within the acceptable range for use in binding studies.

### 3.4.9.3 Measurement of agonist binding in intact cells

Serial measurement of TR-FRET in SNAP-receptor-expressing cells labelled with Lumi4-Tb provides a measure of rate of association of FITC-ligand with receptor. Conversely, if unbound agonist is removed and replaced with buffer, FRET signal should reduce in parallel with agonist dissociation.

In order to better understand the differences in wash-resistant signalling responses demonstrated in figure 3.16B, an equivalent experiment was performed using FITC-agonists. Labelled CHO-SNAP-GLP-1R cells were exposed to FITC-agonist for 10 minutes, during which FRET was monitored. Unbound agonist was removed, cells washed, fresh buffer added containing the antagonist exendin(9-39) to block rebinding at cell surface receptors, and dissociation of agonist was again monitored by FRET. Binding was quantified as signal at 520 nm expressed as a fraction of that at 620 nm. Agonist / antagonist concentrations were the same as in figure 3.16B, and experiments were conducted at 37°C to mimic the physiological environment. Results are shown in figure 3.18.

![Graph showing association and dissociation of FITC-conjugated exendin-4 analogues](image)

**Figure 3.18. Association and dissociation of FITC-conjugated exendin-4 analogues in CHO-SNAP-GLP-1R cells.**

Agonist (10 nM) applied at t=0 and cold-washed x3 at t=10, after which fresh buffer containing exendin(9-39) (10 µM) was added; n=4, with binding (520 nm signal / 620 nm signal) expressed relative to average baseline. Data expressed as mean + SEM.
These results reveal that agonist dissociation with ex-phe1-FITC was much faster than with Ex4-FITC, with ex-asp3-FITC dissociating even more slowly. To formally quantify association and dissociation kinetics, these data were fit to standard equations to determine the rate constants for association ($k_{on}$) and dissociation ($k_{off}$) in this system. The equilibrium binding constant ($K_d$) was then determined as $k_{off}/k_{on}$. These data are shown in table 3.6.

<table>
<thead>
<tr>
<th></th>
<th>$k_{on}$ (M$^{-1}$min$^{-1}$)</th>
<th>$k_{off}$ (min$^{-1}$)</th>
<th>Log($K_d$) (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex4-FITC</td>
<td>3.1x10$^7$ ± 9.4x10$^6$</td>
<td>0.044 ± 0.006</td>
<td>-8.94 ± 0.15</td>
</tr>
<tr>
<td>ex-phe1-FITC</td>
<td>2.2x10$^7$ ± 8.1x10$^6$</td>
<td>0.235 ± 0.021***</td>
<td>-7.80 ± 0.20 ***</td>
</tr>
<tr>
<td>ex-asp3-FITC</td>
<td>1.5x10$^7$ ± 7.1x10$^6$</td>
<td>0.028 ± 0.005</td>
<td>-8.98 ± 0.12</td>
</tr>
</tbody>
</table>

Table 3.6. Biased exendin-4 analogue rate constants for association and dissociation.

Results represent average of estimates from 4 independent experiments shown in figure 3.18. Equilibrium binding constant ($K_d$) was determined as $k_{off}/k_{on}$ and logarithmically transformed before statistical comparison. ***p<0.001 vs. exendin-4-FITC, by one-way randomised block ANOVA with Dunnett’s post hoc test.

These rate constants confirm what is apparent visually in figure 3.18 – that ex-phe1-FITC dissociates considerably faster than exendin-4-FITC, and ex-asp3 slower still. There was no significant difference in $k_{on}$ in keeping with the appearance of the association phase (although the apparent rate of association is dependent on both $k_{on}$ and $k_{off}$). Comparing agonist binding with cAMP generation measured under identical experimental conditions (figure 3.16B), it is apparent that endosomal cAMP generation is associated with slower dissociation and persistent agonist-receptor interaction after washing.

As the GLP-1R is rapidly internalised in response to exendin-4, it is likely that a significant amount of agonist-bound receptor has reached the endosomal compartment by the beginning of the dissociation phase in this experiment (see figure 3.14C, which indicates detectable surface GLP-1R loss at 10 nM exendin-4 and ex-asp3 after 15 minutes). Spatial constraints, and the absence of exendin(9-39) in endosomes, mean that net dissociation from internalised receptors is unlikely to proceed at the same rate as from the cell surface, where a larger volume and the presence of exendin(9-39) combine to prevent rebinding events. Conversely, pH changes in endosomes likely accelerate dissociation. Therefore, the measured rate constants in table 3.6 represent a composite of association and dissociation events at different subcellular compartments exposed to different conditions. Furthermore, loss of cell surface receptor at a variable and agonist-dependent rate influences binding.
behaviours during the association phase, and recycling back to the cell surface may have an effect during the dissociation phase.

To more specifically measure agonist dissociation at different subcellular locations, modifications were made to this protocol to selectively measure binding at the plasma membrane and at endosomal receptors.

### 3.4.9.4 Measurement of dissociation from the plasma membrane

The possible impact of receptor trafficking on interpretation of binding experiments in intact cells at 37°C means that alternative methodologies are often employed to avoid this potential confounder. Experiments can be conducted at low temperatures which arrest trafficking. Many trafficking events are halted below 20°C (246) although 4°C may be required for full inhibition. Clearly, kinetic parameters estimated from experiments conducted at below-physiological temperatures will underestimate the "true" value at 37°C. Alternatively, ligand binding assays can be, and often are, performed using cell membrane preparations rather than whole cells, which avoids any impact of trafficking (247). Another important factor differentiating whole cell assays from those performed with cell membranes is that in the latter, contributions of intracellular proteins (for example G proteins) can be avoided. In whole cells, agonist efficacy impacts on affinity as interaction between receptor and G protein (or β-arrestin) stabilises different receptor conformational states which themselves have different ligand affinities to the non-G protein bound state. Binding assays with cell membranes can be performed in the presence of guanine nucleotides to inhibit G protein from interacting with receptors (187). Parameter estimates from membrane binding assays can therefore be a truer measure of the ligand association and dissociation from receptor in a single (inactive) state, which most models for mathematical analysis of binding data were designed for. On the other hand, experiments performed in whole cells are a truer measure of functional binding, which for agonists inevitably contains a component of efficacy.

In this study, an alternative method of inhibiting receptor trafficking was used, in order to measure binding at the plasma membrane in intact cells. Trafficking can be pharmacologically inhibited, for example using high concentrations of sucrose (248), metabolic inhibitors which deplete the cell of ATP required for active processes such as internalisation (103), or specific inhibitors of endocytosis such as Pitstop (which inhibits clathrin-coated pit formation, (249)) and dynole derivatives (which inhibits dynamin action, (250)). Using DERET to measure GLP-1R internalisation, a metabolic inhibitor cocktail composed of sodium azide and 2-deoxyglucose was found to prevent endocytosis in response to exendin-4. These results are shown in figure 3.19. The same metabolic inhibitor cocktail was
previously used for radioligand GLP-1R binding experiments (103). Dyngo-4a was found to be unsuitable due to spectral interference, and Pitstop-2 failed to block internalisation (data not shown).

Figure 3.19. Inhibition of GLP-1R internalisation using metabolic inhibitors.
SNAP-GLP-1R cells labelled with Lumi4-Tb and pretreated with NaN₃ (10 mM) and 2-deoxyglucose (20 mM) during 15 minute baseline period before exendin-4 (100 nM) addition, n=2. Data expressed relative to baseline as mean ± SEM.

Therefore, kinetic measurements of biased exendin-4 analogue dissociation were performed in whole CHO-SNAP-GLP-1R cells labelled with Lumi4-Tb and treated with sodium azide and 2-deoxyglucose. TR-FRET was monitored during the dissociation phase after a 30 minute period of exposure to FITC-agonist. Results are shown in figure 3.20, with $k_{off}$ estimates given in table 3.7.

Figure 3.20. Dissociation of FITC-conjugated exendin-4 analogues from surface receptor in CHO-SNAP-GLP-1R cells.
Cells were treated with metabolic inhibitors for 15 minutes before addition of agonist (100 nM) and throughout the experiment. Cells cold-washed x3 after 30 mins agonist association, and buffer replaced with exendin(9-39) 10 μM to prevent rebinding during measurement of dissociation by TR-FRET; n=4, with binding expressed as 520 nm signal / 620 nm signal, after subtraction of non-specific binding signal obtained in presence of saturating concentration (1 μM) of unlabelled agonist. Data expressed as mean ± SEM.
<table>
<thead>
<tr>
<th></th>
<th>$k_{off}$ (min$^{-1}$)</th>
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<tbody>
<tr>
<td>Ex4-FITC</td>
<td>0.121 ± 0.010</td>
</tr>
<tr>
<td>ex-phe1-FITC</td>
<td>0.223 ± 0.020***</td>
</tr>
<tr>
<td>ex-asp3-FITC</td>
<td>0.057 ± 0.005**</td>
</tr>
</tbody>
</table>

Table 3.7. Dissociation rate constant for biased exendin-4 analogues from cell surface.
Results represent average of estimates from 4 independent experiments shown in figure 3.18. **p<0.01, ***p<0.001 vs. exendin-4-FITC, by one-way randomised block ANOVA with Dunnett’s post hoc test.

Of note, greater total binding was achieved with ex-asp3-FITC than with exendin-4-FITC, which was in turn greater than with ex-phe1-FITC – although it cannot be excluded that some dissociation occurred during the wash phases even though these were performed with cold wash buffer. The dissociation timecourse again revealed the fastest dissociation with ex-phe1-FITC and slowest with ex-asp3-FITC. Interestingly, the $k_{off}$ for ex-phe1-FITC determined from this experiment (0.223 min$^{-1}$) closely approximates that obtained in the absence of Mls (0.235 min$^{-1}$), suggesting that most dissociation of ex-phe1 occurs from the plasma membrane even when endocytosis is not inhibited; this is in keeping with the reduced internalisation propensity of ex-phe1. On the other hand, for exendin-4-FITC and ex-asp3-FITC, dissociation from the cell surface was faster than when endocytosis was not inhibited ($k_{off}$ 0.121 min$^{-1}$ vs 0.044 min$^{-1}$ for exendin-4-FITC, and 0.057 min$^{-1}$ vs 0.028 min$^{-1}$ for ex-asp3-FITC). This could be explained by slower net dissociation within endosomes following GLP-1R endocytosis induced by these fast-internalising agonists.

### 3.4.9.5 Measurement of binding within endosomes

Experiments presented so far imply but do not directly demonstrate persistent binding of agonist to receptor within endosomes. To measure internalised agonist-receptor interaction directly, a novel, reversible SNAP-tag labelling protocol was developed in which, after inducing internalisation, residual surface receptor was de-labelled, preventing FRET from plasma membrane without affecting that from endosomes.

Covalent BG binding to SNAP-tag is not easily reversed. However, inclusion of a reducible disulfide bond between BG and fluorescent moieties allows the fluorophore to be released by addition of a reducing agent. This technique been utilised in studies of the β2-AR (251) and glycosylphosphatidylinositol-anchored membrane proteins (252) but not for ligand binding by FRET. As Lumi4-Tb is not commercially available in a cleavable configuration, an alternative two-step labelling strategy was developed based on the streptavidin-biotin interaction. CHO-SNAP-GLP-1R cells
were first labelled with cleavable BG-SS-biotin (253), followed by streptavidin-Tb, thus producing a cleavable terbium cryptate donor suitable for TR-FRET. After exposure to FITC-agonist to induce binding and GLP-1R internalisation, the membrane non-permeating reducing agent sodium 2-mercaptoethane sulfonate (MesNa) was used to release remaining SNAP-biotin-streptavadin-Tb complexes from surface receptor without affecting those within endosomes. Ligand – receptor interaction was then monitored by TR-FRET in the presence of exendin(9-39) to prevent rebinding events at the cell surface from recycled receptor. A graphical depiction of this protocol, and experimental results, are shown in figure 3.21 and table 3.8.

**Figure 3.21. Dissociation of FITC-conjugated exendin-4 analogues from endosomes in CHO-SNAP-GLP-1R cells.**

(A) Cartoon describing the endosomal binding protocol. (B) Cells were treated with agonist (100 nM, 30 minutes) before MesNa treatment. Dissociation measured in presence of exendin(9-39) 10 µM to prevent rebinding; n=5, with binding expressed as 520 nm signal / 620 nm signal. Data expressed as mean + SEM.
### Table 3.8. Dissociation rate constant for biased exendin-4 analogues within endosomes.

Results represent average of estimates from 5 independent experiments shown in figure 3.21. *p<0.05 vs. exendin-4-FITC, by randomised block ANOVA with Dunnett’s post hoc test.

<table>
<thead>
<tr>
<th></th>
<th>k$_{off}$ (min$^{-1}$)</th>
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<tbody>
<tr>
<td>Ex4</td>
<td>0.043 ± 0.005</td>
</tr>
<tr>
<td>ex-phe1</td>
<td>0.057 ± 0.002*</td>
</tr>
<tr>
<td>ex-asp3</td>
<td>0.025 ± 0.005*</td>
</tr>
</tbody>
</table>

These results revealed greater binding of ex-asp3-FITC than exendin-4-FITC, which in turn was more tightly bound than ex-phe1-FITC. Of note, as the FRET signal is expressed ratiometrically in relation to the amount of labelled receptor, which in this case specifically means internalised receptor (as surface receptor is de-labelled), this does not simply reflect differences in internalisation, but avidity of binding. Additionally, dissociation rate constants (table 3.8) point towards slower dissociation with ex-asp3 > exendin-4 > ex-phe1 within endosomes. However, unlike with the surface receptor dissociation experiment (figure 3.20), this likely incorporates rebinding events due to spatial constraints and lack of blocking antagonist within endosomes, so is not a true dissociation constant. Nevertheless, useful information is obtained on differences in degree of binding of agonist to internalised receptors over time, aiding interpretation of corresponding signalling response shown in figure 3.16B.

### 3.4.9.6 Measurement of binding kinetics of non-FITC agonists

As small differences in cAMP potency were noted for FITC-conjugated vs. unmodified agonists, particularly ex-phe1, further TR-FRET kinetic binding experiments were performed using non-FITC conjugated agonists in competition with ex4-FITC. When labelled and unlabelled agonist are added simultaneously, competition between the two alters the apparent rate of association of the labelled form. If the binding kinetics of the FITC-conjugated agonist are known, measurement of responses to several concentrations of unlabelled compound in competition with a fixed concentration of labelled agonist allows calculation of association and dissociation rate constants for the non-FITC agonist by global curve fitting with previously described equations (189). For these calculations, previous determinations of ex-FITC kinetic parameters can be used, but here, to minimise error, they were determined on a per-assay basis by global fitting of binding responses to several concentrations of ex-FITC. Endocytosis was inhibited as before to allow specific measurement of surface binding. Results of these experiments are shown in figure 3.22 and table 3.9.
Figure 3.22. Competitive binding kinetics of unlabelled biased exendin-4 analogues in CHO-SNAP-GLP-1R cells.

Lumi4-Tb-labelled cells were exposed to indicated agonists after endocytosis inhibition with NaN\textsubscript{3}/2-deoxyglucose and binding recorded every 60s as 520/620 nm signal, after subtracting non-specific signal. (A) Kinetic binding of ex4-FITC at indicated concentrations. (B) Kinetic binding of ex4-FITC 10 nM in competition with indicated concentration of unlabelled exendin-4. (C) As for (B) but using unlabelled ex-phe1. (D) As for B but using unlabelled ex-asp3. All n=5. For clarity, data expressed as mean without error bars, with global curve fitting as described in the text to averaged data.

<table>
<thead>
<tr>
<th></th>
<th>(k_{on} (M^{-1}.min^{-1}))</th>
<th>(k_{off} (min^{-1}))</th>
<th>Log((K_d) (M^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex4-FITC</td>
<td>9.8x10(^6) ± 8.1x10(^5) ***</td>
<td>0.052 ± 0.002 ***</td>
<td>-8.23 ± 0.64 *</td>
</tr>
<tr>
<td>Ex4</td>
<td>6.2x10(^5) ± 5.1x10(^5)</td>
<td>0.027 ± 0.001</td>
<td>-8.36 ± 0.04</td>
</tr>
<tr>
<td>ex-phe1</td>
<td>4.1x10(^5) ± 6.0x10(^5) **</td>
<td>0.174 ± 0.017**</td>
<td>-7.36 ± 0.04 ***</td>
</tr>
<tr>
<td>ex-asp3</td>
<td>4.8x10(^5) ± 3.5x10(^5) *</td>
<td>0.015 ± 0.001***</td>
<td>-8.50 ± 0.06 *</td>
</tr>
</tbody>
</table>

Table 3.9. Kinetic binding constants for unlabelled biased exendin-4 analogues.

Results represent average of estimates from 5 independent experiments shown in figure 3.22. Equilibrium binding constant \((K_d)\) was determined as \(k_{off}/k_{on}\) and logarithmically transformed before statistical comparison. *\(p<0.05\) vs. exendin-4, by randomised block ANOVA with Dunnett’s post hoc test.
The most important finding from this experiment is that the rank order for dissociation rate constants calculated here for unmodified agonists matches that of FITC-conjugated agonists presented in tables 3.6 and 3.7, with ex-phe1 dissociating faster, and ex-asp3 slower, than exendin-4. This observation adds confidence to the use of these FITC-modified compounds in other contexts. Other notable points: 1) the $k_{on}$ calculations from this experiment suggest slower association rates than for FITC-conjugated compounds (table 3.6), although experimental differences are also likely as the $k_{on}$ for ex4-FITC was also slower; 2) the $k_{off}$ rates for unmodified agonists were slower than those calculated for FITC-conjugated equivalents (tables 3.6 and 3.7), suggesting that the FITC group to an extent alters binding kinetics. Interestingly, because in this experiment $k_{on}$ for ex4-FITC was faster than for unmodified ex4, the effect of the faster $k_{off}$ for ex4-FITC on binding affinity ($k_{off} / k_{on}$) is partially mitigated against.

In summary, measurement of fluorescent agonist binding by TR-FRET revealed clear differences in binding kinetics, which associate with propensity for β-arrestin recruitment, signal bias, and receptor trafficking. Specifically, the fast-dissociating ex-phe1 recruits little β-arrestin, induces less endocytosis, and binds less well to the GLP-1R even when internalised, recycles faster, and fails to generate a sustained cAMP response after agonist washout. Conversely, slow-dissociating ex-asp3 exhibits an exaggerated β-arrestin response, stimulates rapid GLP-1R internalisation and remains tightly bound within the endosomes, recycles slowly, and continues to generate cAMP from the internal compartment. Although causality cannot be concluded, it is possible to rationalise these observations with a mechanism in which events such as β-arrestin recruitment and receptor endocytosis require relatively prolonged receptor occupancy, and are therefore less likely to occur with a fast-off agonist.

### 3.4.10 Measurement of functional desensitisation via calcium responses

Traditionally, agonist internalisation has been linked to receptor desensitisation. Despite the “in vogue” paradigm of sustained signalling from internalised receptors, extensive loss of cell surface receptor has the potential to reduce cellular responsiveness to repeated agonist challenge. Desensitisation occurs also at the plasma membrane through steric hindrance by β-arrestins of G protein binding. Furthermore, arrestins can recruit phosphodiesterases which induce local degradation of cAMP (254). In view of agonist-specific differences in internalisation and β-arrestin recruitment patterns, a protocol was developed to measure the impact on functional desensitisation in CHO-GLP-1R cells.

Measurement of desensitisation requires an initial period of agonist exposure, followed by washout and rechallenge with a reference agonist. As shown in figure 3.16, CHO-GLP-1R cells exhibit persistent cAMP generation for at least 24 hours after agonist washout, precluding measurement of cAMP as a useful readout for desensitisation. It is likely that the high levels of GLP-1R expression in these cells,
and therefore extensive internalisation of activated receptors, saturates adenylate cyclase required for cAMP generation even after agonist washout. Therefore, a less highly coupled readout, which is not maximally activated by internalised receptors, is required. Measurement of Ca\(^{2+}\) flux fits this criterion in CHO-GLP-1R cells, as it is only activated at high agonist concentrations (255). Transient elevations of cytosolic Ca\(^{2+}\) are a common consequence of GPCR activation, contributed to by Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels and intracellular release of Ca\(^{2+}\) from endoplasmic reticulum (ER) stores. This response is self-amplifying and self-terminating as ER Ca\(^{2+}\) channels, such as the inositol trisphosphate receptor (IP3R) and ryanodine receptor (RyR), are sequentially activated and then inhibited as Ca\(^{2+}\) concentrations rise (256,257). The sensitivity of these channels is modulated by a variety of factors, including intracellular signalling events linked to G protein activation.

### 3.4.10.1 Investigation of pathway dependence of calcium response

Signalling pathway dependence of GLP-1R Ca\(^{2+}\) flux was first determined using specific pharmacological inhibitors in CHO-GLP-1R cells loaded with a fluorescent Ca\(^{2+}\)-responsive dye (Calcium 6). As shown in figure 3.23A, the phospholipase C inhibitor U73122 (258) abolished detectable rise in cytosolic Ca\(^{2+}\), and marked inhibition was noted with ESI09, a specific inhibitor of Epac2 (259). This finding implicates both G\(_{\alpha_q}\) and G\(_{\alpha_s}\) pathways in mediating the Ca\(^{2+}\) response. G\(_{\alpha_q}\) activation leads to activation of phospholipase C, which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to yield diacylglycerol and IP\(_3\); the latter acts on IP3Rs to stimulate Ca\(^{2+}\) release. Interestingly, the IP3R antagonist xestospongin C (260) failed to significantly reduce GLP-1-induced Ca\(^{2+}\) release; this inhibitor has been reported by others to be of limited effectiveness in some cell types (261). Epac2, downstream of G\(_{\alpha_s}\)-mediated cAMP generation, is known to sensitise RyRs to facilitate Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) (262). Conversely, inhibiting PKA (also downstream of cAMP) with the inhibitor H89 extended the duration of Ca\(^{2+}\) elevation in these cells. Typically, PKA is thought to amplify CICR by sensitising RyRs and IP3Rs (256,263); a possible explanation for the opposite finding here is PKA-mediated activation of the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), which aids Ca\(^{2+}\) reuptake from the cytoplasm back to the ER (264) and thus terminates the Ca\(^{2+}\) spark.
Therefore, Ca\textsuperscript{2+} response to GLP-1 requires activation of both G\textsubscript{q} and G\textsubscript{s} pathways. Of note, detectable rises in intracellular Ca\textsuperscript{2+} occur only at agonist concentrations which already maximally stimulate cAMP production as determined biochemically in the same cell type (figure 3.1), raising the possibility that cAMP-mediated Epac2 activity is a permissive factor for CICR but does not dynamically modulate its amplitude. However this comparison ignores different timescales over which these measurements are made (cAMP levels in the first few seconds after agonist addition, when the Ca\textsuperscript{2+} response is measured, have not been determined), as well as any potential differences in spatial localisation of cAMP which cannot be revealed by measuring total cAMP from a population of cells. Therefore, barring any post-receptor desensitisation phenomena which specifically affect one pathway but not another, as a relatively weakly coupled, pan-G protein mediated readout of GLP-1R activation, measurement of Ca\textsuperscript{2+} response appears suitable for detection of receptor desensitisation.
3.4.10.2 Measurement of homologous desensitisation

To measure agonist-specific differences in GLP1-R desensitisation, cells were exposed to biased exendin-4 analogues during Ca\(^{2+}\) dye loading, followed by agonist removal and washing, with a further 30 minute period of dye exposure before measurement of Ca\(^{2+}\) response to rechallenge with GLP-1. This experiment was performed in two ways: a) with varying doses of test agonist followed by a fixed GLP-1 stimulus (to examine concentration-dependence of desensitisation), and b) a fixed dose of test agonist followed by varying doses of GLP-1 stimulus (to examine the effect of desensitisation on both residual efficacy and residual potency). Results of these experiments are shown in figure 3.24 and table 3.10.

Figure 3.24. Agonist-induced functional GLP-1R desensitisation in PathHunter CHO-GLP-1R cells. (A) Schematic of protocol. (B) Ca\(^{2+}\) traces to 100 nM GLP-1 after indicated pre-treatment (1 µM), n=4 (C) Peak Ca\(^{2+}\) response to ATP (10 µM) after agonist (1 µM) pretreatment, n=3. (D) Peak Ca\(^{2+}\) response to 100 nM GLP-1 after varying concentrations of agonist pre-treatment, n=6. (E) Peak Ca\(^{2+}\) to varying doses of GLP-1 after 1 µM agonist pre-treatment, n=5. Data expressed as mean ± SEM after normalisation to baseline.
<table>
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<tr>
<th></th>
<th>pEC$_{50}$</th>
<th>$E_{max}$</th>
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<tbody>
<tr>
<td>vehicle</td>
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<td>6.52 ± 0.49***</td>
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<td>Ex4</td>
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<td>2.75 ± 0.55</td>
</tr>
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</tr>
<tr>
<td>ex-asp3</td>
<td>6.55 ± 0.32</td>
<td>1.72 ± 0.17*</td>
</tr>
</tbody>
</table>

**Table 3.10. Residual Ca$^{2+}$ responses to GLP-1 after prior agonist exposure.**

Efficacy and potency estimates from n=5 experiments represented in figure 3.24E.

These results demonstrate pronounced GLP-1R desensitisation with exendin-4 under the experimental conditions tested, even greater with ex-asp3, but less with ex-phe1. This reveals an association with desensitisation and β-arrestin recruitment, signal bias, and internalisation, in accordance with classical roles of these events as negative modulators of GPCR signalling. Thus, agonists which induce little β-arrestin recruitment and internalisation leave the cell better able to respond to subsequent stimulation. These observations were specifically due to GLP-1R desensitisation, rather than exhaustion of intracellular Ca$^{2+}$ stores, as response to ATP via puronergic P2 receptors was unaffected.

### 3.5 Discussion

The results presented in this chapter describe the identification of regions of GLP-1 and exendin-4 which influence signalling in a pathway specific manner, and examples of amino acid substitutions which lead to formally quantified biased signalling. The majority of changes tested led to some form of reduction in signalling response, with bias resulting when one pathway was more affected than others. Within the limitations of the number of sequences tested, this observation suggests that GLP-1 and exendin-4 exhibit close to optimal structures for full agonist responses in major pathways linked to GLP-1R activation.

A number of sequence changes identified, especially at position 1 of both GLP-1 and exendin-4, reduced β-arrestin recruitment particularly, with less of an impact on cAMP, and can thus be defined as cAMP-biased. Examples including phe$^1$ and dHis$^1$, which induced similar bias when introduced into both exendin-4 and GLP-1. A number of position 3 changes also selectively diminished arrestin responses leading to cAMP bias in GLP-1; one exception was the switch of Glu$^3$ to Asp, which for both exendin-4 and GLP-1 resulted in bias in favour of arrestin recruitment. No marked differences in recruitment patterns of β-arrestin1 vs β-arrestin2 were apparent with any agonist tested. Another interesting observation was that maximal β-arrestin recruitment by exendin-4 was considerably more
susceptible to sequence changes than was GLP-1. This is unexplained but it is tempting to conclude it relates in some way to the different requirement for agonist N-terminal interactions with the GLP-1R for full binding affinity.

These observations are potentially useful in corroborating the recently published mutagenesis studies which identified several GLP-1R regions responsible for biased signalling (74,224). However, crosstalk between signalling pathways, and the impact of signalling kinetics on apparent bias (187) pose challenges for combined interpretation of studies which have taken slightly different experimental approaches. For example, the studies by the Wootten group frequently use ERK phosphorylation as a signalling readout dependent on β-arrestin recruitment; however, GLP-1R-mediated ERK phosphorylation is also dependent on cAMP (101). Additionally, the timepoint at which signalling responses are measured can have major effects on bias quantitation, so if not matched between different studies, conflicting conclusions may arise. Nevertheless, computational modelling of interactions formed between the agonists described in this thesis and current GLP-1R homology models may still be informative in understanding the structural basis of biased agonism.

Whilst only a limited number of exendin-derived FITC-agonists were tested (and corroborated with binding data from non-fluorescent agonists), an association emerged between binding kinetics and biased signalling; namely that slow agonist dissociation is linked to β-arrestin recruitment. Logically, as arrestin recruitment follows receptor phosphorylation by GRKs, and activation of GRKs requires cAMP, it follows that G protein responses kinetically must precede arrestin recruitment, and slow-dissociating agonists might therefore hold an advantage. However, any delay between receptor activation and arrestin recruitment, which is detectable within one second (265), is far shorter than dissociation times of GLP-1R agonists tested here. An association between biased signalling and agonist binding kinetics was recently reported (187), although interestingly, slower dissociation was linked to bias in favour of G protein signalling in that study, in contrast to the GLP-1R agonists presented here. Ligand residence time has been linked to stabilising particular receptor conformations (266), providing a theoretical basis for how agonists with different dissociation kinetics might favour receptor conformations required for pathway-directed signalling.

The effects of biased exendin-4-derived analogues on certain receptor behaviours, namely endocytosis, recycling, sustained signalling from intracellular receptors, and functional desensitisation have also been determined. It was apparent that receptor internalisation occurs at a concentration at which β-arrestin recruitment is also first detectable (~10 nM with exendin-4), and that efficacy for internalisation mirrors quite closely that of arrestin recruitment. However, a mechanistic role for arrestin recruitment in GLP-1R internalisation has not yet been conclusively reported (98,105). Of note, β-arrestin1 and 2 have distinct roles, but may also retain some degree of functional redundancy.
(93,94), as both presumably access the same binding site on the GPCR. Therefore, a lack of an impact on GLP-1R endocytosis in studies in which partial knockdown of individual arrestins is achieved does not mean they have no role in this process. The agonists described in this chapter exhibited comparable loss of recruitment of both β-arrestin1 and 2, which could be more likely to reveal an effect. Dual knockdown of both β-arrestins would be a better way to corroborate any potential role they play in GLP-1R internalisation; even then, compensatory upregulation of alternative internalisation pathways can develop over the transfection period and confound results (267), and cellular behaviour may additionally be altered by effects on other molecules which interact with arrestins. A more specific approach would be to mutate GLP-1R residues required for arrestin binding to determine if this results in altered internalisation. In a somewhat related report a GLP-1R-β-arrestin2 fusion protein was found to be internalised faster than the wildtype receptor (97).

Beyond internalisation, a consistent pattern of agonist characteristics linked to GLP-1R recycling was also noted for exendin-4, ex-phe1 and ex-asp3. In particular, recycling was slowest for ex-asp3 which exhibits long agonist residence times and increased β-arrestin recruitment, with the opposite the case for ex-phe1. Whether β-arrestin recruitment plays an important role for recycling of the GLP-1R is currently unknown, although such a mechanism was recently reported for the glucose-dependent insulinotropic polypeptide (GIP) receptor (234). Furthermore, it seems conceivable that prolonged binding of ex-asp3 once within the endocytic pathway (figure 3.21) could influence interactions with additional proteins implicated in trafficking regulation beyond arrestins, as was previously suggested (268).

Unlike in vitro pharmacological stimulations, in vivo, drug clearance means that receptors are not exposed continually to a fixed dose of agonist. Therefore, washout experiments can be useful as to a certain extent they approximate the effect of drug clearance (as well as being useful to study receptor behaviour). In this study, washouts were used to investigate possible sustained signalling from intracellular receptors, and also to allow measurement of GLP-1R desensitisation with a second challenge of GLP-1. The conclusions from these experiments were not contradictory, but revealed an interesting conundrum, in that the cAMP-biased agonist ex-phe1 was poor at stimulating cAMP production from internalised receptors, but left cells responsive to further stimulation by GLP-1. It is not clear from these results which of these characteristics is dominant in determining agonist efficacy in vivo. In CHO-GLP-1R cells, cAMP generation continued for over 24 hours after washout of an initial brief pulse of exendin-4; presumably cells with lower, physiological levels of GLP-1R expression would not exhibit such a dramatically prolonged response. The proposed role for β-arrestin signalling in coupling GLP-1R activation to insulin release is an additional factor potentially at play. Therefore, the question of how binding kinetics, biased signalling, trafficking, and generation of cAMP from
internalised receptors affects insulin release *in vitro* and *in vivo* is the subject of investigations described in subsequent chapters.

Finally, an interesting recent report highlighted that when β-arrestin1 interacts with the β₂AR, it can adopt either a partially engaged or fully engaged conformation (269), with the former permitting arrestin-mediated internalisation and signalling, but the latter required for desensitisation (270). Whether type of arrestin-GLP-1R engagement differs between biased exendin analogues is not known but would be an interesting subject for further investigation.
Investigation of beta cell effects of biased exendin-4 analogues
4.1 Introduction

The major effect of GLP-1R activation in pancreatic beta cells is to potentiate glucose-stimulated insulin secretion. Additionally, GLP-1R signalling promotes beta cell survival. In this chapter, the novel analogues of exendin-4 identified in chapter 3 are examined for their effects in in vitro beta cell systems.

N-terminal changes to both exendin-4 and GLP-1 led to differences in internalisation, binding kinetics, and signal bias between cAMP and β-arrestin. It was apparent that β-arrestin recruiting-, rapidly internalising- analogues drive sustained signalling (e.g. figure 3.16) but also induce homologous desensitisation (e.g. figure 3.24), with these results respectively in line with both the “modern” (98,106) and “classical” (271) views of regulation of prolonged GPCR signalling by arrestins and receptor trafficking. It is as yet unclear which of these phenomena dominates in a pharmacological setting, either in vivo or with in vitro systems which better approximate target tissue than recombinant CHO cells with unphysiological levels of receptor expression.

4.2 Aims

1. Determine effects of novel exendin-4 analogues in pancreatic beta cells
2. Investigate mechanistic role of arrestins in determining these responses, via alternative approaches to modulate arrestin recruitment

4.3 In vitro assessment of incretin effects

A number of in vitro models exist for studying beta cell biology:

*Immortalised, insulinoma-derived cell lines:* Cell lines derived from rodent insulinomas are prevalent in beta cell research. In particular MIN6 cells (mouse) (272) and INS-1 cells (rat) (273) retain a high insulin content and acceptable glucose-induced insulin secretion. Continual propagation means they are convenient, and reduces reliance on primary tissue, although loss of beta cell characteristics at later passages is common (274). Even at early passage numbers these cells frequently display characteristics of non-beta islet cells, for example secretion of glucagon-derived peptides (275); nevertheless this partially replicates the islet micro-environment where glucagon and related peptides are released by alpha cells. Human-derived clonal beta cells have historically been unavailable, although two cell lines have been produced in the past decade (276,277). Of these, the EndoC-βH1 line more closely approximates primary beta cell behaviour, although slow growth reduces its utility in a high throughput setting.
**Stem-cell derived beta cells:** There is considerable interest in developing techniques to induce differentiation of pluripotent human stem cells into mature beta cells. Primarily, the goal is a virtually unlimited supply of beta cells for therapeutic transplantation (278), but would also serve as a useful resource for beta cell research. Human embryonic stem cells (hESCs) can be progressively differentiated by the addition of various growth factors to ultimately generate cells with islet cell characteristics (279). Historically, the resulting cells have required the final maturation stage to be performed *in vivo* (280), although with recent improvements in *in vitro* protocols this is less necessary (281). As well as hESCs, induced-pluripotent stem cells (iPSCs) can be used, in which differentiated cells from adult humans (e.g. fibroblasts) are first returned to a progenitor state before being reprogrammed as islet cells. This represents an alternative starting point which avoids ethical limitations associated with use of embryonic tissue (282), and facilitates the possibility of using a patient’s own cells as a source of new beta cells for transplantation to avoid the need for immunosuppression. Furthermore, cells might be isolated from patients with disease-implicated genetic variants in order to study pathophysiology in an *in vitro* system. Nevertheless, generating beta cells from stem cells is expensive and even with the latest protocols, only ~ 10% of “mature” cells demonstrate typical beta cell signalling responses (279).

**Rodent islets:** Intact islets can be isolated from rodent pancreata by collagen digestion to separate exocrine and endocrine tissue (283). The primary advantages of using islets over cell lines in an *in vitro* setting are a) the islet cells are fully mature, and b) the 3D architecture of the islet substantially affects responsiveness, which reflects the paracrine action of additional cell types including alpha cells (which produce glucagon), delta cells (somatostatin) and PP cells (pancreatic polypeptide), and also connectivity and hierarchy between cells (190,284) which allows highly coordinated responses. Additionally, with rodents, *in vivo* manipulations including genetic knockouts and drug treatments can be applied before islet isolation to gain better understanding of their islet effects. Where it is necessary to study specific islet cell populations (e.g. only beta cells), islets can be dispersed and cells sorted using a number of techniques (245).

**Human islets:** As stem-cell derived beta cell technology is still in development, primary human islets isolated from cadaveric and surgical donors are the only source of human islet tissue for transplantation. Islets not suitable or required for transplantation are made available for research under appropriate ethical guidelines. The major advantage to human islets over rodent islets is that there are significant inter-species differences in islet microarchitecture which may influence responses (285). In particular, mouse alpha cells are predominantly situated in the islet periphery and beta cells centrally, whereas in humans the organisation is typically described as more homogenous, with implications for paracrine- and local blood flow- mediated intra-islet signalling (286).
Nevertheless, human islets are scarce, and substantially more variable than rodent islets (287), particularly after transport delays. Islets from donors with diabetes, which might be the most suitable for many studies, are even rarer. As with all in vitro beta cell systems, isolated human islets lack major physiological components including blood flow and neural control.

Thus, islet biology can be studied in vitro with a number of approaches. In this study, INS-1 832/3 cultured beta cells, which are highly incretin-responsive and grow rapidly (288,289), were used for higher throughput testing, with key findings replicated with primary human islets.

### 4.4 Results

#### 4.4.1 Insulinotropic effects of biased agonists

##### 4.4.1.1 Sustained secretory responses in INS-1 832/3 cells

*In vitro* insulin secretion experiments are frequently performed with short incubation times (30 minutes to 1 hour), which is appropriate when testing physiological stimuli, as *in vivo* these are subject to rapid homeostatic change. However, rapid DPP-4-mediated degradation of GLP-1(7-36)NH₂ (within minutes) does not apply to exendin-4, with renal clearance the limiting pharmacokinetic factor (126), resulting in a plasma half life of 1.5 hours (290) and allowing twice daily dosing when used clinically. Therefore, a prolonged (overnight) incubation was used to screen agonists for insulinotropic effect as this better replicates *in vivo* drug exposure, with the proviso that a “closed” system with constant drug concentration is not a perfect model as *in vivo* drug elimination and target-mediated ligand depletion are not accounted for (291). Existing insulin secretion protocols (288), which require multiple time-consuming in-plate wash steps resulting in increased variation due to cell loss, were simplified and optimised for a higher-throughput setting. Thus, INS-1 832/3 cells were simply seeded into 96-well plates at the time of agonist addition, with collection of supernatant samples for insulin quantitation after 16 hours incubation. Stimulations were performed at 11 mM glucose as incretin-stimulated insulin secretion is glucose-dependent.

Insulin secretory responses for exendin-4-derived agonists, applied at a single 100 nM dose, are indicated in figure 4.1.
Figure 4.1. Exendin-4 analogue-induced sustained insulin release in INS-1 832/3 cells.

Agonists administered at 100 nM, 16 hour incubation. Certain agonists are colour coded as per chapter 3. Data represented as mean of 5 experiments ± SEM, normalised to 11 mM glucose (G11) response (insulin stimulation index; ISI). *p<0.05, **p<0.01, ***p<0.001 vs. exendin-4, by randomised block ANOVA with Dunnett's post hoc test.

These results reveal substantial differences in insulin secretion between exendin-4 and N-terminally substituted analogues, with a number of compounds exhibiting heightened responses, and others less effective. Therefore, the ability of previously characterised pharmacological properties of each agonist, including cAMP potency, arrestin recruitment and internalisation, to predict insulinotropic capacity was determined (figure 4.2).
Figure 4.2. Relationship between agonist-induced signalling responses in PathHunter CHO-GLP-1R cells and sustained insulin secretion in INS-1 832/3 cells.

Insulin secretion responses as displayed in figure 4.1 against (A) β-arrestin1 response (as in figure 3.8); (B) β-arrestin2 response (as in figure 3.8); (C) GLP-1R internalisation (as in figure 3.9); (D) cAMP potency, represented as ΔpEC_{50}, i.e. negative logarithm of EC_{50} normalised to that of exendin-4 from each experiment (data from figure 3.5); and (E) calculated bias (as in figure 3.6). Relationships quantified by linear regression. Data represented as mean ± SEM.

These results demonstrate a clear inverse relationship between GLP-1R internalisation, β-arrestin1 and β-arrestin2 recruitment (at a single dose), and insulin secretion. Potency for cAMP generation failed to predict insulin secretion capacity. The best predictor was bias between β-arrestin2 and cAMP, with agonists which favoured cAMP generation being the most insulinotropic. The implication of these results is that non-canonical signalling events such as sustained signal generation from internalised receptors, and β-arrestin-mediated pathways, do not in fact augment insulin release under the pharmacological conditions utilised in this experiment. Rather, it appears that the dominant effect of arrestin recruitment and endocytosis is to reduce GLP-1R signalling, which could result from their canonical roles in desensitisation. Furthermore, it is somewhat surprising that potency for cAMP generation, measured acutely in a recombinant system, was not predictive of sustained insulin
secretion, as this metric is commonly used to evaluate experimental GLP-1R agonists, with high potency assumed to be therapeutically desirable.

To better characterise the insulin secretory response of representative analogues from this panel, dose responses were obtained using ex-phe1 and ex-asp3, to compare with exendin-4. In this case, to exclude the possibility of cellular insulin content changes as an explanation for apparent increases in insulin release, cell lysate insulin was also determined, and insulin release quantified as a percentage of cell lysate insulin before normalisation to the glucose-only response as before (figure 4.3, table 4.1).

![Graph](image)

**Figure 4.3. Sustained insulin secretory responses of exendin-4, ex-phe1 and ex-asp3 in INS-1 832/3 cells.**
16 hour incubation; responses normalised to 11 mM glucose response, n=5, with data represented as mean ± SEM, with a 4-parameter logistic fit of averaged data shown.

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<thead>
<tr>
<th></th>
<th>pEC50</th>
<th>E_max</th>
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<tr>
<td>Ex4</td>
<td>9.8 ± 0.2</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>ex-phe1</td>
<td>8.8 ± 0.2***</td>
<td>4.6 ± 0.4***</td>
</tr>
<tr>
<td>ex-asp3</td>
<td>9.9 ± 0.3</td>
<td>1.6 ± 0.7**</td>
</tr>
</tbody>
</table>

**Table 4.1. Potency and efficacy estimates for insulin responses.**
Derived from data shown in figure 4.3, represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs exendin-4, by one-way randomised block ANOVA with Dunnett’s post hoc test.

These data confirm greater maximal insulin secretion induced by an analogue (ex-phe1) which exhibits reduced arrestin recruitment and internalisation, with the opposite observed with ex-asp3. Nevertheless, the potency estimates point in the opposite direction, for example with ex-phe1 achieving half-maximal response at an approximately 10-fold higher concentration than exendin-4, in keeping with the approximate 10-fold reduction in binding affinity (see table 3.9) of this analogue.
4.4.1.2 Acute secretory responses in INS-1 832/3 cells

Having determined that maximal insulin secretion is greater with non-internalising agonists during prolonged incubation, it was decided to also measure responses with shorter incubation periods. In contrast to prolonged incubations, test agonists exhibited no differences in insulin secretion capacity when incubated over 30 minutes or 2 hours (figure 4.4).

![Figure 4.4](image)

Figure 4.4. Acute insulin secretory responses of exendin-4, ex-phe1 and ex-asp3 in INS-1 832/3 cells. (A) 30 minute, and (B) 2 hour incubations, n=6, with data represented as mean ± SEM. No treatment responses are significantly different to exendin-4 by one-way randomised block ANOVA with Dunnett’s test.

4.4.1.3 GLP-1R-dependence of secretory responses

In order to exclude cross reactivity with other receptors expressed in beta cells as an explanation for enhanced insulinotropism with ex-phe1, the prolonged insulin secretion experiment was repeated ± the GLP-1R antagonist exendin(9-39). The insulin secretory response to ex-phe1 was virtually abolished in the presence of GLP-1R blockade, confirming it acts exclusively via the GLP-1R in this system (figure 4.5).
Figure 4.5. GLP-1R specificity of prolonged insulin secretory responses of experimental agonists INS-1 832/3 cells.

Cells exposure to indicated agonist (100 nM) ± exendin(9-39) ("ex(9-39)", 10 µM) for 16 hours, at 11 mM glucose, n=3. Results indicated as mean ± SEM.

4.4.1.4 Secretory responses in human islets

To corroborate these results obtained in rodent cell lines, insulin secretion with exendin-4 and ex-phe1 was also measured in intact human islets from normoglycaemic donors, using both short and long incubations (figure 4.6).

Figure 4.6. Insulin secretory responses of exendin-4 and ex-phe1 in intact human islets. (A) 1 hour (n=8 donors), and (B) overnight (16 hour, n=11 donors) incubations, n=6. Agonists administered at 100 nM. Individual donor responses are shown. *p<0.05 by paired two-tailed t-test.
Thus, as with INS-1 832/3 cells, ex-phe1 exerts a greater insulinotropic effect than exendin-4 when incubated over a prolonged period of time.

In summary, agonist-induced β-arrestin recruitment and GLP-1R internalisation were both associated with reduced maximal insulin secretion during prolonged but not shorter exposure times in INS-1 832/3 beta cells. This finding is at odds with a dominant role for non-canonical signalling mediated by these events, as has been previously suggested (98,106), but compatible with the classical function of arrestins in mediating GPCR sequestration and desensitisation. Furthermore, the apparent advantages of reduced β-arrestin recruitment and GLP-1R internalisation appear to overcome associated reductions in potency for cAMP generation.

4.4.2 Measurement of protection against apoptosis in beta cells

Beta cell apoptosis is proposed as a contributor to progression of T2DM. Islets isolated from patients with T2DM have reduced beta cell mass and increased apoptosis markers (292). Beta cell dedifferentiation is suggested as an alternative explanation for apparent reduction in beta cell mass (293), and the relative importance of each is debated (294-296). Beta cell apoptosis may result from systemic glucolipotoxicity (297) which leads to endoplasmic reticulum (ER) stress (298,299). ER stress represents an initially adaptive response to ER overload causing misfolding of proteins in the biosynthetic pathway, in which protein translation is globally downregulated (300) to reduce demands on protein folding machinery. However, in the diabetic beta cell this ultimately becomes counterproductive as it reduces insulin secretion. Treatment with GLP-1R agonists of isolated islets and in vivo reduces beta cell ER stress (301-303) and apoptosis (67,304). GLP-1-induced cytoprotective effects are proposed as a β-arrestin-mediated effect (101).

Therefore, the ability of experimental GLP-1R agonists ex-phe1 and ex-asp3 to protect against ER stress-induced apoptosis was measured in INS-1 832/3 beta cells in comparison to exendin-4. Cells were treated with thapsigargin to induce ER stress and apoptosis, quantified as caspase-3 activity (305). Results are shown in figure 4.7.
Figure 4.7. Inhibition of apoptosis by GLP-1R agonists in INS-1 832/3 beta cells.
Cells exposed to indicated agonist in presence of 1 µM thapsigargin in serum-free RPMI, 11 mM glucose. Caspase-3 activity quantified using Caspase-Glo assay. Data indicated as mean ± SEM from 6 independent experiments, with 4-parameter fit of averaged data shown (where possible). Note – curve fitting for individual experiments was poor due to significant intra-experimental variability, and therefore parameter (EC$_{50}$, Emax) estimates were not calculated for the purposes of statistical analysis.

These results indicate that ex-asp3 exhibits substantially reduced anti-apoptotic properties, which, in view of the β-arrestin-biased signalling profile of this analogue, does not support the postulated critical role of β-arrestin recruitment in GLP-1R-mediated apoptosis reduction (101). It is presumed that this reflects the net reduction in GLP-1R signalling over time with this agonist due to excessive loss of surface receptor. Nevertheless, in contrast to its potent effects on insulin secretion, ex-phe1 possessed no additional advantage in terms of maximal anti-apoptotic effect over exendin-4.

4.4.3 Agonist-induced cAMP responses in beta cells

To gain further insight into the signalling mechanisms underlying agonist-specific differences in prolonged insulin secretion, duration of cAMP signalling in INS-1 832/3 cells was determined at several timepoints (figure 4.8). Experiments were performed at 3 mM glucose as clonal beta cells, due to their dedifferentiated state, secrete proglucagon-derived peptides under insulinotropic glucose concentrations (275), which can raise cAMP by paracrine GLP-1R activation even in the absence of exogenous ligand. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was used to prevent cAMP degradation for detection purposes.
It is apparent that, relative to the baseline established in the absence of exendin analogues, cAMP responses are characterised by an initial peak, followed by attenuation. This contrasts with responses typically observed in recombinant cell lines, in which cAMP continues to accumulate over long periods of agonist exposure. Such differences likely represent high levels of receptor expression in engineered fibroblast lines, differential coupling to downstream effectors and variable expression of phosphodiesterases which alter the balance between cAMP synthesis and degradation. This highlights a potential issue of translating signalling responses from heterologous systems into natively-expressing cells or tissue. Nevertheless, cAMP generation in response to ex-phe1 was prolonged in comparison to other analogues, such that after several hours incubation there was still a net “positive” effect of this peptide but a net negative effect of exposure to ex-asp3. This could result from receptor desensitisation, sequestration from access by extracellular ligand, or from local recruitment of phosphodiesterases via arrestin recruitment leading to increased cAMP degradation, all of which would be predicted to differ between agonists. The “inverse agonist” appearance of
the ex-asp3 response most likely reflects constitutive or paracrine GLP-1R activation in vehicle-treated cells leading to elevated cAMP, which is lost with high doses of ex-asp3 due to loss of GLP-1R signalling via the above mechanisms. Overall, the increased durability of ex-phe1-induced cAMP response is consistent with its effect on sustained insulin secretion, implying that the processes are linked, as would be expected from the major role played by cAMP in GLP-1R coupling to insulin release.

4.4.4 Measurement of homologous desensitisation in beta cells

4.4.4.1 Desensitisation of cAMP responses in INS-1 832/3 cells

Prolonged cAMP generation and insulin secretion were seen with ex-phe1 in comparison to exendin-4 and, in particular, ex-asp3. This observation is in keeping with reduced receptor desensitisation, such that the beta cells retain for longer the capacity to respond to ongoing presence of extracellular ligand. To investigate this possibility, the ability of each agonist to induce homologous desensitisation was measured by initially exposing INS-1 832/3 cells to experimental agonist, followed by washing and rechallenge with a fixed, acute dose of GLP-1. There was a period of resensitisation between initial exposure and rechallenge, as previously described (307). Results of this experiment are shown in figure 4.9, which revealed that pre-treatment with all agonists reduced response to subsequent GLP-1 treatment, but the extent of desensitisation was least with ex-phe1, and greatest with ex-asp3.

Figure 4.9. Homologous GLP-1R desensitisation in INS-1 832/3 cells.

Cells exposed to indicated agonist (100 µM) for 16 hours in complete RPMI, 3 mM glucose without IBMX, washed twice, resensitised for 60 minutes, and rechallenged with GLP-1 (100 nM) + IBMX (100 µM). Response expressed relative to vehicle pre-treatment, n=7. Data indicated as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 vs. exendin-4 after randomised block ANOVA + Dunnett’s test.
4.4.4.2 Desensitisation of cAMP response in human islets

Homologous desensitisation was also investigated in intact human islets exposed overnight to exendin-4 or ex-phe1 before re-treating with GLP-1 and IBMX, as for INS-1 832/3 cells. In comparison to vehicle pre-treated islets, exendin-4 pre-treatment induced substantial desensitisation, but ex-phe1 very little (figure 4.10).

![Graph showing cAMP response](image)

**Figure 4.10. Homologous GLP-1R desensitisation of cAMP response in intact human islets.**

Human islets (n=5 donors) exposed to indicated agonist (100 nM) for 16 hours before washing and rechallenge with 100 nM GLP-1 + 500 µM IBMX for 30 minutes. Expressed relative to response after vehicle pre-treatment, Data indicated as mean ± SEM, *p<0.05 after two-way paired t-test.

4.4.4.3 Calcium responses in human islets

Increases in cytoplasmic Ca2+ are a major feature of incretin-induced signalling in beta cells, which occurs as a result of the cAMP effectors PKA, which acts on membrane K\text{ATP} channels to cause membrane depolarisation and induce Ca2+ influx via L-type Ca2+ channels (82), and Epac2, which opens ER IP3 channels (81). Furthermore, cellular connectivity via gap junctions induces oscillatory waves across the islet to produce a coordinated response (190,308,309). Therefore, imaging of incretin-induced Ca2+ responses was performed in intact human islets loaded with the Ca2+ sensitive dye fluo-2, to determine whether responses differ depending on prior treatment with exendin-4 or ex-phe1 (figure 4.11). KCl was used as a positive control to determine maximum islet response.
Figure 4.11. \( \text{Ca}^{2+} \) responses induced by exendin-4 or ex-phe1 in human islets.  

(A) Acute responses to 100 nM exendin-4 or ex-phe1, followed by KCl 20 mM, with arrows marking additions; expressed as fold change in fluorescence relative to individual islet baseline.  

(B) As for (A) but response to 100 nM exendin-4 after overnight treatment with 100 nM exendin-4 or ex-phe1. Results from n=2 donors, n= 11-13 islets per condition. AUCs are determined relative to individual islet baselines and compared by two-tailed t-test, with significance indicated by *p<0.05, **p<0.01. Values shown are mean ± SEM.

These results reveal that exendin-phe1 induces cytosolic \( \text{Ca}^{2+} \) rises of smaller magnitude in islets than exendin-4, when applied acutely. However it also induces less desensitisation, as demonstrated in figure 4.11B, where the \( \text{Ca}^{2+} \) response following rechallenge with exendin-4 was less attenuated after ex-phe1 pretreatment compared to exendin-4 pretreatment.

Overall, these results highlight agonist-specific differences in homologous desensitisation which are compatible with the rank order of insulin secretory responses observed over longer incubations.

4.4.5 Measurement of GLP-1R trafficking in islets

Ex-phe1 was previously found to induce less internalisation than exendin-4 in CHO-GLP-1R cells (figures 3.9, 3.14). To investigate agonist-induced differences in surface GLP-1R loss in human islets, which might relate to desensitisation and prolonged insulin secretion as identified above, a protocol was established to determine residual surface GLP-1R after overnight exposure to vehicle, exendin-4 or ex-phe1. The monoclonal anti-GLP-1R antibody used for in-cell ELISA does not perform well for immunohistochemistry, so labelling of remaining surface receptors was performed using exendin-4-FITC after thorough washing. After paraffin embedding, anti-FITC and anti-insulin primary antibodies were used with appropriate secondaries to co-visualise surface GLP-1Rs and beta cells within intact islets. Representative images from this experiment are shown in figure 4.12, which revealed virtually complete loss of surface GLP-1R with exendin-4 but not ex-phe1 treatment.
Figure 4.12. Differential effects of overnight exposure to biased GLP-1R agonists on residual surface GLP-1R expression in human islets.

Human islets were treated with 11 mM glucose (G11) ± 100 nM exendin-4 or ex-phe1 overnight in complete medium. Islets were thoroughly washed and labelled at 4°C to arrest further trafficking with 10 µM exendin-4-FITC before fixation and processing for immunohistochemistry. Representative images indicated from n=3 donors.

In summary, treatment in vitro of beta cells with exendin-4-derived GLP-1R agonists with reduced β-arrestin recruitment and internalisation is associated with greater insulin secretion than with the standard GLP-1R agonist exendin-4, seemingly related to differences in homologous desensitisation and receptor trafficking characteristics. To determine whether agonist-induced β-arrestin recruitment is mechanistically linked to the observed downstream responses, additional approaches were taken to modulate arrestin responses in beta cells.

4.4.6 Enhancement of GLP-1R β-arrestin recruitment via allosteric modulation

Results presented above reveal an inverse relationship between β-arrestin recruitment and insulin secretion after sustained agonist exposure, which contrasts with the previously reported positive role for arrestins in mediating GLP-1R-induced insulin release (98). It is not excluded therefore that reductions in agonist-mediated arrestin recruitment (for example with ex-phe1) might associate with, but not actually be responsible for changes in GLP-1R desensitisation and trafficking. Instead, prolonged activation of downstream pathways could be primarily regulated by other, unmeasured factors. Consequently, alternative approaches to specifically modulate β-arrestin recruitment in the presence of the same orthosteric agonist are potentially useful to determine any causal role.

Allosteric modulation is alteration of receptor activity by a molecule acting at a different site to the orthosteric ligand (310). Allosteric modulators (AMs) may exert positive (PAMs) or negative (NAMs)
effects on signalling. “Pure” AMs act only in the presence of the orthosteric ligand, whereas others (“ago-PAMs” or “ago-NAMs”) possess significant intrinsic agonist or antagonist activity. A number of GLP-1R PAMs have been described over recent years (311). As therapeutics, it is hoped that GLP-1R PAMs will exploit the high circulating concentrations of the major GLP-1 metabolite GLP-1(9-36)NH₂ which are at least 10 times greater than for GLP-1(7-36)NH₂ (312). This truncated product of DPP-4-mediated degradation is usually considered inactive, but may become active at physiological concentrations in the presence of GLP-1R PAMs (139).

One PAM which has attracted considerable attention is 4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine (BETP) (138). This non-peptidic molecule enhances insulin secretion induced by weak GLP-1R agonists including GLP-1(9-36)NH₂ and oxyntomodulin (313). It has since been discovered that BETP is highly electrophilic and acts by covalent modification of Cys347 in the third intracellular loop of the GLP-1R (314). This physicochemical property is non-desirable pharmacokinetically due to reactivity with plasma proteins and rapid disappearance from the circulation (315). Nevertheless, this compound may still be useful for in vitro pharmacological investigations as allosteric enhancement by BETP is pathway-specific, i.e. it induces biased signalling, in a manner dependent on the orthosteric probe (313,316).

4.4.6.1 Effect of BETP on biased agonist signalling and trafficking responses

It was hypothesised that BETP might prove a suitable tool to further bias signalling from orthosteric exendin-4 analogues, and provide support for a mechanistic role of individual pathways in the observed agonist effects in beta cells. Cyclic AMP and β-arrestin responses were measured in PathHunter GLP-1R cells in the absence and presence of BETP using ex-phe1 as the orthosteric probe. Results are shown in figure 4.13 and table 4.2.
Figure 4.13. BETP selectively enhances ex-phe1-induced β-arrestin recruitment in PathHunter GLP-1R cells.

Cells treated with indicated concentrations of ex-phe1 ± 3 µM BETP. (A) β-arrestin1 response, n=5, (B) β-arrestin2 response, n=4, (C) cAMP response, n=5. Values represented relative to GLP-1 max response determined in the same assay as mean ± SEM, with 4 parameter logistic fit shown.

<table>
<thead>
<tr>
<th></th>
<th>βARR1</th>
<th></th>
<th>βARR2</th>
<th></th>
<th>cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E_{max}</td>
<td>pEC_{50}</td>
<td>E_{max}</td>
<td>pEC_{50}</td>
<td>E_{max}</td>
</tr>
<tr>
<td>Ex-phe1</td>
<td>17 ± 1</td>
<td>7.5 ± 0.2</td>
<td>14 ± 2</td>
<td>7.4 ± 0.1</td>
<td>89 ± 8</td>
</tr>
<tr>
<td>Ex-phe1 + BETP</td>
<td>29 ± 4 *</td>
<td>7.2 ± 0.2</td>
<td>40 ± 7 *</td>
<td>7.5 ± 0.3</td>
<td>86 ± 5</td>
</tr>
</tbody>
</table>

Table 4.2. Effect of BETP on ex-phe1-induced responses.

Derived from data shown in figure 4.13, represented as mean ± SEM. *p<0.05 vs. ex-phe1 without BETP, by paired two-tailed t-test.

Thus, at a dose of 3 µM, BETP enhanced recruitment of β-arrestin1 and β-arrestin2, without detectable increases in cAMP production. Of note, this dose of BETP also increased ex-phe1-induced GLP-1R internalisation in PathHunter GLP-1R cells (figure 4.14), as determined by in-cell ELISA. This finding implicates β-arrestin recruitment in GLP-1R internalisation, which has hitherto been disputed (98,105).
4.4.6.2 Effect of BETP on biased agonist-induced insulin secretion

The effect of BETP-induced β-arrestin recruitment and GLP-1R internalisation on sustained insulin release was investigated in INS-1 832/3 cells (figure 4.15 and table 4.3).
### Table 4.3. Effect of BETP on ex-phe1-induced responses.

<table>
<thead>
<tr>
<th></th>
<th>$E_{\text{max}}$</th>
<th>$pE_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex-phe1</td>
<td>$3.6 \pm 0.6$</td>
<td>$8.8 \pm 0.3$</td>
</tr>
<tr>
<td>Ex-phe1 + BETP</td>
<td>$2.5 \pm 0.7^{**}$</td>
<td>$9.0 \pm 0.1$</td>
</tr>
</tbody>
</table>

Parameter estimates derived from data shown in figure 4.15. represented as mean ± SEM. **p<0.01 vs. ex-phe1 without BETP, by paired two-tailed t-test.

It is readily apparent that maximum insulin secretion was reduced in the presence of BETP at a concentration demonstrated to selectively enhance β-arrestin recruitment by ex-phe1. This provides some support for a causal role of β-arrestin recruitment in GLP-1R desensitisation during prolonged agonist exposure, is in keeping with the canonical effects of arrestins which has up till now not been demonstrated for the GLP-1R. Such a conclusion remains with the proviso that the desensitising effect of BETP might yet result from other signalling events which have not been measured in this study. Additionally, this observation does not contradict published findings of enhanced insulinotropism with BETP, which were made in the context of acute incubations with endogenous GLP-1R agonists, unlike the prolonged incubations with novel exendin-4-derived peptides described here.

### 4.4.7 Beta cell effects of β-arrestin silencing by RNA interference

Increased β-arrestin recruitment by orthosteric (e.g. ex-asp3 and similar analogues) and allosteric (e.g. BETP) GLP-1R activation thus far appears to exert a net inhibitory effect on insulin secretion in beta cells under pharmacological conditions. However, whether the agonist-specific patterns of β-arrestin recruitment are responsible for this effect, rather than “bystanders” for the true causal cellular event, has not been excluded. Therefore, it was decided to specifically deplete cells of β-arrestins by RNA interference (RNAi) to observe if this replicates the beta cell phenotype of agonists with reduced arrestin recruitment such as ex-phe1.

RNAi involves introducing genetic material into cells with a complementary sequence to a specific target gene, which is then able to bind to the target mRNA and promote its degradation by the cytoplasmic RNA-induced silencing complex (RISC), abolishing or substantially reducing protein synthesis (317). RNA can be introduced to the cell directly in the form of short RNA strands known as small interfering RNA (siRNA), or encoded in a DNA vector from which interfering RNA subsequently transcribed and processed (e.g. small hairpin RNA approach). The latter has the advantage, given a suitable selection marker within the plasmid, of allowing establishment of cell lines with stable target knockdown. On the other hand, prolonged silencing may lead to compensatory upregulation of other processes which disguise or confuse the direct phenotype of the knockdown itself.
4.4.7.1 Validation of small interfering RNA sequences

In this study, as well as individual silencing of each arrestin, dual knockdown of both β-arrestin1 (gene symbol: Arrb1) and β-arrestin2 (Arrb2) was used to recapitulate the loss of recruitment of both arrestin isoforms by ex-phe1 and related agonists. Commercially available siRNA against rat Arrb1 and Arrb2 sequences were transfected using Lipofectamine 2000, with the extent of knockdown confirmed at 72 hours confirmed by quantitative polymerase chain reaction (qPCR) analysis (figure 4.16). This duration of knockdown was chosen to ensure adequate time for β-arrestin protein degradation to occur. A negative control siRNA which targets no known mammalian sequence was used in parallel to control for non-specific effects of introducing foreign RNA into cells.

![Graph A](A) ![Graph B](B)

**Figure 4.16.** siRNA-mediated silencing of Arrb1 and Arrb2 in INS-1 832/3 cells.
Cells transfected with negative control siRNA, siRNA targeting Arrb1 (“b1 siRNA”), siRNA targeting Arrb2 (“b2 siRNA”), or both. Gene expression at 72 hours determined using 2^{ΔΔCT} method and normalisation to control siRNA value, n=3. Data represented as mean ± SEM. **p<0.01, ***p<0.001 vs ctrl siRNA by one-way ANOVA with Dunnett's test.

Thus, adequate knockdown of both targets was achieved, allowing assessment of the functional effects of arrestin silencing on agonist-induced GLP-1R activation.

4.4.7.2 Effects of RNA silencing on signalling responses in INS-1 832/3 cells

Cyclic AMP responses to exendin-4 was measured in INS-1 832/3 cells with and without arrestin depletion (figure 4.17). Interestingly, this revealed a small reduction in cAMP response after β-arrestin1 knockdown at early timepoints, in keeping with a role for β-arrestin1 in mediating some of the GLP-1R cAMP response, as previously described (98) but not fully explained. Conversely, silencing
of β-arrestin2 and, in particular, both arrestins together potentiated cAMP responses, although statistical difference was no longer apparent at the later timepoint. The contrasting effects of silencing each arrestin could imply they have different roles - β-arrestin1 exerting a positive signalling effect, but β-arrestin2 negatively modulating GLP-1R signalling. On the other hand, depletion of both arrestins simultaneously did not mean that individual effects were “cancelled out”. This could imply a degree of redundancy, in which β-arrestin1 can play a role in desensitisation in the absence of β-arrestin2, which is unmasked when both arrestins are lost. Importantly, this situation best replicates the situation with ex-phe1 and similar agonists, with which reductions in recruitment of both arrestins is seen.

Figure 4.17. Cyclic AMP responses in INS-1 832/3 cells after dual β-arrestin knockdown.
After 72 hours siRNA treatment, cells treated with 100 µM IBMX ± 100 nM exendin-4 for indicated incubation period in RPMI, 3 mM glucose. Response normalised to timepoint-specific IBMX-only reading for each siRNA group. Data displayed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. ctrl siRNA by two-way randomised block ANOVA with Dunnett’s test.

4.4.7.3 Effect of RNA silencing on insulin secretion in INS-1 832/3 cells

The effect of arrestin knockdown on coupling of GLP-1R activation to insulin secretion was determined in INS-3 832/3 cells (figure 4.18). Again, a trend towards reduced acute insulin secretion was observed with β-arrestin1 siRNA, in keeping with previous reports (98). There was no statistically significant increase in insulinotropic response after β-arrestin2 or dual silencing at early timepoints, but with overnight agonist exposure, dual arrestin ablation led to increased insulin release. Overall, this series of results parallels those of ex-phe1, which was similarly insulinotropic to exendin-4 when applied acutely, but achieved greater insulin release when incubated for prolonged periods.
After 72 hours siRNA treatment, cells were treated ± 100 nM exendin-4 at 11 mM glucose for (A) 1 hour, n=6, or (B) overnight, n=5. Insulin secretory responses expressed relative to siRNA-specific glucose-only value. Values indicated as mean ± SEM. *p<0.05 vs. control siRNA by randomised block ANOVA with Dunnett’s test.

4.5 Discussion

This chapter describes the assessment of the potential therapeutic utility of novel biased GLP-1R agonists using in vitro models which approximate the target in vivo tissues better than recombinant systems, and addresses an important question regarding the effect of biased GLP-1R signalling on insulin secretion and beta cell apoptosis. Three recent studies have described biased GLP-1R agonists, none of clearly identified a link with insulinotropism (183,318,319). Therefore, this is the first study to identify a positive role for GLP-1R bias on insulin secretion using experimental agonists.

As reiterated several times above, the revelation that β-arrestin1 recruitment couples GLP-1R activation to insulin secretion and pro-survival effects has raised hopes that specific pharmacological approaches which exploit this non-canonical pathway will be advantageous in T2DM treatment. Sustained signalling by internalised receptors has also been proposed as a mechanism to prolong drug duration of action (238), and is a feature of GLP-1R signalling (106). On the basis of these emerging paradigms in GPCR action, an agonist such as ex-asp3, which induces maximal β-arrestin responses and is extensively internalised, ought to be ideally suited to maximise downstream effects of GLP-1R activation, and an agonist such as ex-phe1, which is poorly internalised and recruits little β-arrestin, should be non-ideal. However, when tested for insulin secretion in vitro over an extended period of time which approximates dosing schedules of GLP-1R agonists when used clinically, the opposite pattern was observed. Similarly, anti-apoptotic effects of ex-asp3 were virtually absent in an
established model of ER stress. At least *in vitro*, ex-phe1 appears to possess desirable characteristics for treatment of T2D, with replication of key findings with isolated human islets strengthening the case for treatment of human disease.

These findings caution against over-interpreting the therapeutic importance of non-canonical signalling events, which might simultaneously act to negatively regulate net cellular activation. Of course, this does not negate possible physiological relevance of β-arrestin-mediated and endosomal signalling, particularly as physiological elevations of GLP-1 are short lived whereas the pharmacological treatments applied in this study took several hours to reveal the “advantages” of reduced arrestin recruitment. Indeed, knockdown of β-arrestin1 in this study reduced cAMP production acutely (figure 4.17), in keeping with the seminal earlier finding which linked β-arrestin1 recruitment to incretin-mediated insulin release (98). However, the combination of orthosteric agonist mediated responses, allosteric modulation of arrestin recruitment by BETP, and dual arrestin silencing by RNAi reported here all point towards a net desensitising effect of GLP-1R arrestin recruitment during prolonged treatment, which dominates over any positive signalling role.

Furthermore, it is noteworthy that the allosteric modulator BETP, developed to enhance insulin secretion by weak GLP-1R agonists such as the endogenous metabolite GLP-1(9-36)NH₂, negatively impacted on ex-phe1-induced insulin secretion. Whilst BETP has been abandoned for clinical development, GLP-1R PAMs with improved physicochemical properties remain a pharmacological goal (140). As GLP-1 metabolites remain elevated for longer than active GLP-1(7-36)NH₂, the possibility that PAMs could result in counterproductive GLP-1R desensitisation should be considered during their development.

Emerging understanding of the dual role of β-arrestins, endocytosis and recycling in GPCR signalling and desensitisation may shed light on the pharmacological action of the biased agonists described herein. For example, for the class A β₂AR, arrestins can adopt either “partially engaged” or “fully engaged” conformation when recruited to the receptor (269), which determines function and is linked to biased agonism (270). Notably, the partially engaged conformation allows signalling and endocytosis but does not lead to desensitisation. It is unknown whether this paradigm is relevant to class B GPCRs such as the GLP-1R.

Two of the three recent studies to describe biased GLP-1R agonists investigated their insulino tropic properties, but did not draw the same conclusions as reported here. The first (183) described an analogue based on exendin-4 but with the first 8 amino acids scrambled and expressed as a library of tethered peptides in a recombinant system; the most potent peptides were selected by an automated flow cytometric enrichment procedure and subsequently tested for bias, with the preferred
compound, termed “P5”, found to possess similar pharmacological characteristics to ex-phe1 (i.e. severely blunted arrestin recruitment). No studies were performed in beta cells or islets, and whilst this peptide was highly effective at lowering blood glucose in animal models of T2DM, it was concluded to be poorly insulinotropic in vivo on the basis of intraperitoneal glucose tolerance tests. However, the in vivo experimental approach taken most closely approximates the acute incubations described here, in which ex-phe1 exhibited no advantage over exendin-4. No experiment was performed to investigate the possibility of enhanced insulinotropism developing after several hours exposure to P5, and therefore whether this is a feature this compound’s pharmacology cannot be excluded. The other study to investigate biased GLP-1R agonists (319) described a series of analogues of GLP-1 with amino acid substitutions introduced into the mid-portion of the peptide to enhance peptide helicity. Formal bias quantification was not performed but discrepancies between cAMP accumulation and β-arrestin2 recruitment were highlighted. Acute insulin secretion experiments were performed using INS-1 cells and it was concluded that increased potency for cAMP was the key determinant of insulin secretion, contrasting with results reported here in which cAMP potency was inversely related to insulin secretion (albeit with sustained rather than acute incubations). Of note, only EC50 values for arrestin recruitment were reported, suggesting there may not have been substantial differences in maximal arrestin recruitment with these analogues, which appears likely to be important as a negative regulator of maximal insulin release. The third study to investigate novel biased GLP-1R agonists (318) did not examine insulin secretion.

In summary, a consistent series of observations, based on orthosteric agonist signalling profiles, selective allosteric pathway enhancement, and genetic pathway ablation, support the idea that for greater pharmacological insulin secretion and beta cell cytoprotection, agonists with reduced arrestin recruitment and internalisation are preferable. However, as these experiments have all been conducted with closed in vitro systems where agonist concentration remains constant, it is unknown whether this translates into greater in vivo efficacy. Indeed, it was previously suggested that exendin-4-induced homologous desensitisation is an in vitro – only artefact (307). This question is the subject of chapter 5.
5 Anti-diabetic effects of biased exendin analogues \textit{in vivo}
5.1 Introduction

Multiple physiological roles for GLP-1 in vivo have been identified (320). The major source of circulating GLP-1 is enteroendocrine L-cells, which release GLP-1 in response to meal ingestion (321). Thus, the most notable effects of GLP-1 relate to its role in postprandial metabolism. In particular, GLP-1 potentiates glucose-stimulated insulin secretion, i.e. acts as an incretin (322), promotes satiety resulting in reduced food intake (64), and also delays gastric emptying (323); the latter contributes indirectly to glycaemic and satiety effects. These actions have clear application in treatment of T2DM, in which insulin secretion is reduced and insulin resistance results partly due to calorie excess. Long acting GLP-1R agonists are now an established treatment for T2DM, with multiple clinical trials demonstrating clear glycaemic benefits and weight reduction (128,324-327). Furthermore, reductions in adverse cardiovascular outcomes, the main cause of mortality in T2DM patients, were recently shown for both liraglutide and semaglutide (131,134). Liraglutide is additionally licensed for weight loss in the absence of diabetes (328), and may have benefits in non-alcoholic fatty liver disease (329). GLP-1R agonists are also under evaluation for other indications such as Parkinson’s disease (330), Alzheimer’s disease (331), and myocardial ischaemia (332).

The earlier finding that GLP-1R bias conferred by N-terminal modifications to exendin-4 can prolong signalling responses by avoiding desensitization poses the question as to whether such effects will also be replicated in vivo, and if so, whether they are of potential clinical utility. Increases in sustained insulin secretion by agonists with reduced β-arrestin recruitment and internalisation, such as ex-phe1, might be advantageous in T2DM. However, a previous study concluded that desensitisation induced by exendin-4 was an in vitro phenomenon without in vivo manifestations. In that study, normal glycaemic responses to exogenous exendin-4 were retained in transgenic mice which endogenously express exendin-4 in multiple tissues (307). Furthermore, a recently described biased GLP-1R agonist, which displays similar signalling characteristics to ex-phe1, was concluded to be poorly insulinotropic in vivo (183). However, the experimental approach taken in that study was not designed to detect enhanced insulinotropism developing after several hours, as is seen with ex-phe1. Therefore, whether biased GLP-1R agonists exert distinct in vivo effects, particularly with regard to insulin secretion, remains an open question.

5.2 Aims

1. Determine the metabolic consequences of single dose administration of biased GLP-1R agonists
2. Determine whether ex-phe1 possesses improved anti-diabetic efficacy compared to exendin-4 when delivered chronically
5.3 Rodent models of T2DM

Cellular systems provide a convenient, reproducible model for initial investigations in the search for new pharmacological agents. Commonly, recombinant cells, optimized for fast growth and clear signalling responses are used to identify lead compounds, which can then be tested in a more relevant *in vitro* model which approximates better the target tissue, such as an immortalised cell line or primary cell isolate. Ultimately, even the most sophisticated *in vitro* models are highly limited for prediction of clinical response, as they lack several factors present *in vivo* such as the presence of multiple target tissues, inter-cell and inter-organ communication, neural innervation, the presence of endogenous competing or modulating substances, variations in agonist concentration over time determined by pharmacokinetics (including the complexities of drug-tissue distribution), as well as inter-individual variation due to genetic and environmental factors. Therefore, studies using animal models are an essential component of drug development as they provide essential information on efficacy and safety.

Rodents, in particular mice and rats, are commonly used for T2DM research due to clear similarities to human metabolism. In general, rodent models fall into four categories (333): 1) chemically-induced diabetes; 2) monogenic diabetes; 3) polygenic diabetes; and 4) diet induced dysglycaemia.

**Chemically-induced diabetes.** Streptozotocin (STZ) (334) is an alkylating agent which is taken up via GLUT2 glucose transporters specifically expressed by pancreatic beta cells. High dose STZ administration induces rapid beta cell destruction and severe hyperglycaemia, which biochemically is more akin to type 1 diabetes. Lower dose STZ leads to less severe beta cell damage, with partial insulin deficiency resulting (335). In both cases, the associated metabolic features of T2DM, such as obesity and dyslipidaemia are lacking, although appropriate diets (see below) can be used in combination with the STZ approach (336).

**Monogenic diabetes models.** Some strains of mice are genetically susceptible to development of a T2DM-like phenotype. Mice with defects in the leptin system, either lacking leptin itself (*ob/ob*) or the leptin receptor (*db/db*), are the commonest examples (337-340). After birth they rapidly become obese and develop associated metabolic disturbances. Zucker fatty diabetic rats harbour homologous mutation to *db/db* mice (341,342) and also develop hyperglycaemia when fed an appropriate diet. The severe phenotype of these rodent models makes them useful for studying new treatments, as large changes can be observed. However, the monogenic basis for disease is clearly distinct from human T2DM, which is polygenic in origin and does not usually require extremely severe obesity to develop.

**Polygenic diabetes models.** Selective breeding to favour metabolic phenotypes has produced a variety of rodent strains with genetic predisposition to diabetes due to inheritance of multiple genes,
rather than a single, severe gene defect. This scenario is closer to human T2DM, in which small effects from a large number of different genes are thought to underlie the strong heritable component of diabetes risk (18). By definition, individual animals from these strains are genetically homogenous, and each model possesses its own pathophysiological characteristics. For example, KK mice spontaneously become overweight, insulin resistant and hyperglycaemic, but also develop pituitary, adrenal and parathyroid abnormalities (343). These factors need to be born in mind when choosing an appropriate rodent model for diabetes research.

**Diet-induced metabolic disturbances.** C57BL/6J mice are a commonly used strain of mouse in metabolic research. With appropriate dietary manipulation, they have increased susceptibility to development of metabolic phenotypes than related C57BL/6 (non-J) strains. High fat feeding for several months induces obesity, insulin resistance, and glucose intolerance (344). More recently, diets including a high sucrose content, as well as high fat, have been advocated, as this better represents a typical "Western"-type diet (345). The degree of hyperglycaemia is modest in comparison to monogenic models, which can limit scope for observing treatment-relating differences, but provocative testing (see below) can be used. The role of environment, in particular diet, in human T2DM, means that this approach may be the best approximation of human disease.

### 5.4 Results

#### 5.4.1 Acute *in vivo* glycaemic effects

Glycaemic improvements in patients treated long term with GLP-1R agonists reflect the combined effects of enhanced insulin secretion, improvements in overall beta cell function e.g. due to reduced glucotoxicity, and increased insulin sensitivity due to weight loss. Whilst therapeutically important, the confounding effect of weight loss can introduce complexities if interpretation of mechanism is required. Therefore, single dose studies, whilst they do not provide a full description of long term treatment effects, are frequently performed as they can identify beta cell effects before weight change clouds the picture. Whilst it is unclear whether endogenous GLP-1, with its short circulatory half life, exerts its incretin effect via direct beta cell stimulation or indirectly via a neural gut-brain-pancreas circuit, it is generally agreed that long-lasting synthetic GLP-1R agonists act directly on the pancreatic islet (69,70).
5.4.1.1 Pilot experiment in HFHS mice

Therefore, to investigate the potential in vivo therapeutic effect of biased exendin analogues, an initial single dose administration pilot experiment was performed. C57BL/6J mice were fed a high fat, high sucrose (HFHS) diet for 4 months to induce obesity and glucose intolerance, providing a rodent model relevant to human T2DM (346). Treatments compared were the reference agonist exendin-4, biased agonist ex-phe1, both at two doses, and vehicle. Weight adjusted doses for both agonists, 2.4 nmol/kg and 0.24 nmol/kg, were chosen as the former is a commonly used dose in rodents with pronounced effects (347), and the latter closer approximates the human dose after allometric scaling (348). Both these doses were used to evaluate the recently described biased exendin analogue "P5" (183). The experiment was designed to detect differences in glucose-lowering potency independent of changes to food intake, which are indicative of changes to beta cell function. Therefore, mice were lightly fasted (2 hours) at the beginning of the light phase before intraperitoneal (i.p.) injection of agonist or vehicle (50 μL 0.9% saline), with serial monitoring of blood glucose, obtained by tail bleed and read with a glucometer. Results are shown in figure 5.1.

**Figure 5.1. Dose finding glycaemia study.**

HFHS-fed mice (n=9-10/group) were fasted overnight and injected i.p. with indicated dose of agonist or vehicle. (A) Blood glucose responses. (B) AUC for (A), determined relative to glucose at t=0. *p<0.05, ***p<0.001, by one-way ANOVA with Tukey’s post hoc test. Data represented as mean ± SEM.

Therefore, ex-phe1, when given at the higher dose of 2.4 nmol/kg, led to significantly greater and longer-lasting glucose lowering than exendin-4 at the same dose. The effect size was not very large, which may partly reflect the mild baseline hyperglycaemia in this rodent model and therefore limited scope for glucose reduction before dropping below the permissive level of glucose required for incretin action. The result is nevertheless consistent with the observed in vitro profile of this agonist, for which insulin secretion was greater over prolonged periods due to reduced desensitisation.
However, as insulin secretion was not measured in this study, the findings could equally result from differences in acute modulation of insulin sensitivity.

5.4.1.2 Intra-peritoneal glucose tolerance tests

The above experimental approach has previously been used to study exendin-4 glycaemic effects in monogenic diabetes rodent models (ob/ob and db/db mice, Zucker fatty diabetic rats) with severe hyperglycaemia (349). However, when baseline glycaemia is not so marked, it is more common to use provocative glucose testing to challenge the beta cell to secrete insulin. The hyperglycaemic clamp approach, in which glucose is infused at a variable rate to maintain a high plasma glucose concentration, is the gold standard for evaluating beta cell function, although is technically challenging to perform (350). Oral glucose tolerance tests, or “mixed-meal” tolerance tests are a more physiological investigation, but are influenced by changes to gastric emptying, so less useful when evaluating beta cell-specific effects. Administering a glucose bolus parenterally avoids this confounder, and in rodents, the intra-peritoneal route is most commonly used as it is technically simple and allows rapid glucose absorption. When insulin is measured as well as glucose, it can be indicative of beta cell effects rather than changes to insulin sensitivity (although a hyperglycaemic clamp is more definitive). Therefore, intraperitoneal glucose tolerance tests (IPGTTs) were performed in HFHS-fed mice to further evaluate the glycaemic effects of exendin-4 vs ex-phe1.

5.4.1.3 IPGTT glycaemic responses in HFHS-fed mice

In this study, thought was given to the timing of glucose challenge during IPGTT in relation to the agonist injection. Typically, acute IPGTTs are performed immediately or up to 30 minutes (to allow time for absorption) after agonist administration (349,351,352). In this case, it was hypothesized that, as the in vitro advantages of non-desensitising agonists such as ex-phe1 take several hours to appear, ex-phe1 would become relatively more effective than exendin-4 over a similar timeframe. Some support for this concept was provided by the initial pilot investigation, in which glycaemic differences after exendin-4 and ex-phe1 developed over several hours. Therefore, it was decided to perform IPGTTs immediately, 4 hours, and 8 hours after injection of agonist. Each experiment included a single IPGTT (i.e. not several performed sequentially during the same experiment). Mice were fasted overnight before the experiment. The higher dose (2.4 nmol/kg) was used due to positive results from the pilot experiment at this dose. In an initial series of experiments, for manpower reasons, only blood glucose (rather than other analytes) was measured at several timepoints after glucose injection, as this requires only a small blood sample volume which can be obtained rapidly. Glucose was administered at a weight-adjusted dose of 2 g/kg. Results are shown in figure 5.2.
Figure 5.2. IPGTTs in HFHS mice at different post-dose intervals.

HFHS-fed mice (n=9-10/group) were fasted overnight and injected i.p. with agonist (2.4 nmol/kg) or vehicle. Glucose (2g/kg) was then injected immediately, at 4 hours, or 8 hours, as indicated. (A) Blood glucose responses, with significance vs. exendin-4 determined by two-way ANOVA with Dunnett's test. (B) AUC for (A), determined relative to glucose at t=0, with significance vs. “0hr GTT” determined by two-way ANOVA with Dunnett's test. *p<0.05, **p<0.01, ***p<0.001, by test indicated above. Data represented as mean ± SEM.

It is apparent from these results that exendin-4 and ex-phe1 are both similarly effective at reducing blood glucose excursions when glucose is administered shortly after i.p. injection. However, when the IPGTT is performed 4 hours after agonist injection, exendin-phe1 is significantly better than exendin-4 at reducing hyperglycaemia. At 8 hours, the effect size is even larger.

In case results using the 2.4 nmol/kg agonist dose, which is commonly used in rodent studies but is higher than the clinically approved human dose even after allometric scaling (348), reflects a high dose-only phenomenon, the delayed (8 hour) IPGTT was repeated in HFHS-fed mice using a 10-fold lower dose of 0.24 nmol/kg using the same protocol. Results are shown in figure 5.3 and reveal that the ex-phe1 at this dose remained substantially more effective at reducing hyperglycaemia than exendin-4.
5.4.1.4  IPGTT insulin responses in HFHS-fed mice

These results are compatible with enhanced beta cell function (or at least, reduced beta cell desensitisation) following ex-phe1 vs exendin-4 treatment. However, acute reduction in insulin resistance would also explain this finding. Therefore, a second series of IPGTTs were performed in which a larger blood volume was collected for measurement of plasma insulin. For animal welfare and manpower reasons, only baseline and a single post-glucose timepoint were selected to measure insulin. As insulin secretion is dynamically modulated by circulating glucose concentration, an early timepoint (10 minutes post glucose injection) was chosen as this reduces the extent of treatment-related differences in blood glucose, and therefore gives a better representation of beta cell functionality before substantial glucose disposal has occurred. Results of this experiment, in which agonist was injected at 2.4 nmol/kg, are shown in figure 5.4.
HFHS-fed mice (n=9-10/group) were fasted overnight and injected i.p. with agonist (2.4 nmol/kg) or vehicle. Glucose (2g/kg) was then injected immediately, at 4 hours, or 8 hours, as indicated. Plasma insulin concentrations immediately before and 10 minutes after glucose administration are indicated. **p<0.01, ***p<0.001, by two-way ANOVA with Sidak’s test. Data represented as mean ± SEM.

Comparison of “pre vs. post” insulin responses reveals that both exendin-4 and ex-phe1 potentiate glucose-induced insulin secretion during the “immediate” IPGTT. However, exendin-4 is apparently no longer as insulinotropic when the IPGTT is performed at 4 hours, but ex-phe1 remains effective; the difference is again magnified at 8 hours. This provides evidence that the single dose glycaemic effects of ex-phe1 relate to changes to beta cell function, and is relevant as the recently reported exendin-derived biased agonist “P5” was stated to be poorly insulinotropic both when given as a single dose and or administered chronically (183). It is likely that this apparent difference between P5 and ex-phe1, which both display similar bias between cAMP and β-arrestin in vitro, partly results from differences in the experimental approach taken, as the single dose experiment for P5 involved an IPGTT performed immediately following agonist injection without any testing at later timepoints. Nevertheless, it is notable that, even in the “immediate” GTT, ex-phe1 was as insulinotropic as exendin-4, whereas P5 under similar conditions was markedly less insulinotropic.

5.4.1.5 Measurement of plasma drug concentration

An obvious potential alternative explanation for the improved anti-hyperglycaemic effect of ex-phe1 vs. exendin-4 at later timepoints would be pharmacokinetic differences, i.e. if ex-phe1 had a longer circulating half life than exendin-4, the apparent longer duration of action would be expected without invoking differences in desensitisation as the mechanism. The N-terminal region of GLP-1 is highly susceptible to degradation by DPP-4 (117), and whilst exendin-4 is DPP-4 resistant due to the presence of a glycine rather than alanine at position 2 (121), the N-terminal sequence substitution in ex-phe1 vs. exendin-4 raises the possibility of differences in DPP-4 degradative susceptibility. Furthermore, whilst the major route of clearance for exendin-4 is glomerular filtration (126), a partial
role for GLP-1R endocytosis as a route for elimination has also been proposed, on the basis of persistent exendin-4 disappearance despite bilateral nephrectomy (353). This phenomenon, known as target-mediated drug disposal (TMDD), is widely recognized in other fields, such as clearance of monoclonal antibodies (354) and has also been modelled for exendin-4 (355). An endocytosis-deficient GLP-1R agonist such as ex-phe1 might thus be expected to undergo less clearance via this route, potentially extending the pharmacokinetic profile. Therefore, an experiment was performed to measure plasma concentrations of exendin-4 and ex-phe1 after a single i.p. injection. An ELISA which recognizes the C-terminal region of exendin-4 and ex-phe1 after a single i.p. injection. An ELISA which recognizes the C-terminal region of exendin-4 and ex-phe1, was used for this purpose, with identical recovery for both peptides first confirmed with known quantities of each peptide. Mice were injected with a single dose of each agonist and blood samples taken at 4 and 8 hours, to correlate with the IPGTT timings in figures 5.2 and 5.3. As exendin-4 is relatively rapidly cleared by renal filtration with a half life of approximately 2 hours (353), a higher dose of each agonist (24 nmol/kg) was used in this experiment to ensure levels did not fall below the limit of detection of the assay. Results are shown in figure 5.5.

**Figure 5.5. Validation of C-terminal exendin ELISA and pharmacokinetic measurement.**

(A) Measured exendin from 100 nmol/L exendin-4 or ex-phe1 spiked into assay buffer, demonstrating equivalent recovery when measured with C-terminal exendin ELISA. (B) Plasma exendin concentration at indicated timepoint after i.p. injection of HFHS mice (n=4/group) with 24 nmol/kg exendin-4 or ex-phe1. No significant different as determined by one- or two-way ANOVA. Data represented as mean ± SEM.

Therefore, there are no differences in circulating agonist concentration at each timepoint, excluding this as an explanation for differences in gluco-modulatory effects. It is nevertheless intriguing that ex-phe1 appeared more potent for *in vivo* insulin secretion at 8 hours than at earlier timepoints, despite markedly reduced plasma drug concentration.
5.4.1.6 Confirmation of bias effect on glycaemia with additional agonists

To determine if the observed effects of biased agonist ex-phe1 were peculiar to that particular agonist or a general manifestation of GLP-1R bias, additional biased exendin-4-derived agonists were also tested for their acute glycaemic effects using the same “delayed” (8 hour) IPGTT protocol, in lean mice. Glucose profiles, and their relationship with differences in in vitro β-arrestin recruitment and GLP-1R internalisation, as determined in chapter 3, are indicated in figure 5.6.

![Graph A](image1.png)

**Figure 5.6. IPGTTs in lean mice.**

(A) Lean mice, n=4/group were injected with indicated agonist at 2.4 nmol/kg. Blood glucose responses to 2 g/kg i.p. glucose, injected 8 hours after agonist, are indicated. Also shown are relationship of AUC from (A), determined relative to baseline glucose at t=0, to agonist-related (B) β-arrestin1 recruitment, (C) β-arrestin2 recruitment, and (D) GLP-1R internalisation. R² determined by linear regression. Note: AUC for vehicle treatment = 618.1 ± 76.1 mM.min. Data indicated as mean ± SEM.

It is apparent there are marked agonist-related differences in the plasma glucose response to a fixed glucose challenge several hours after a single agonist dose. Ex-phe1, the “lead” agonist with the greatest insulinotrophic effect in vitro, also exhibited the greatest anti-hyperglycaemic effect in vivo (note – the glucose AUC for ex-dGln3 was marginally lower than for ex-phe1 due to a higher baseline glucose at t=0). Several other compounds with reduced β-arrestin recruitment were also effective in minimising glycaemic excursions, such as ex-dHis1 and ex-dTyr1. When the in vivo response was
plotted against β-arrestin recruitment, there was a clear inverse relationship between the two, as previously noted for in vitro insulin secretion. Of note, exendin-4, and particularly ex-asp3, which both induce large β-arrestin recruitment responses, were the least effective as anti-hyperglycaemic agents in this experiment, and indeed, tended towards worsening glucose tolerance compared to the vehicle control. This paradoxical observation might speculatively be explained by a permissive role for beta cell GLP-1Rs in ensuring normal beta cell responses to glucose even in the absence of pharmacological GLP-1R agonist treatment (356). If beta cell glucose competence depends partly on “tonic” GLP-1R stimulation, perhaps by low circulating levels of the hormone, or at least from islet-derived GLP-1 or cross-reacting peptides such as glucagon (275), extensive and long-lived loss of surface GLP-1Rs might be counterproductive. This mechanism would be consistent with the trafficking “phenotypes” of exendin-4 and ex-asp3 (figure 3.15), which both allowed only slow GLP-1R recycling. However, the apparent deleterious effect of exendin-4 (and here, ex-asp3) found in lean mice was not observed in HFHS-fed mice (figure 5.2) under the same experimental conditions (IPGTT performed 8 hours after agonist injection).

5.4.1.7 Measurement of glycaemic responses in rats

To gain confidence that an observed in vivo effect from an animal study might be translated to a therapeutic effect in humans, consistent effects are usually sought in other species. Therefore glucose tolerance testing was next performed in lean Wistar rats, using a similar protocol to with mice, i.e. including a “delayed” experiment to determine if reduced GLP-1R desensitisation leads to improved effectiveness of ex-phe1. Male rats were fasted overnight and injected with vehicle, exendin-4 or ex-phe1 at a weight-adjusted dose of 1 nmol/kg, with i.p. glucose (1 g/kg) then administered either straight away (“immediate”) or after a 6 hour delay, with serial monitoring of blood glucose. Results from this experiment are shown in figure 5.7.
Figure 5.7. Immediate and delayed IPGTTs in lean Wistar rats.

Rats (n=8/group) were fasted overnight and injected s.c. with agonist (1 nmol/kg) or vehicle. (A) Blood glucose response to 1 g/kg glucose challenge administered immediately after agonist injection. (B) AUC for blood glucose from (A), determined relative to glucose at t=0. (C) and (D): as for (A) and (B), but glucose challenge administered 6 hours after agonist. *p<0.05, ** p<0.01 by one- or two-way ANOVA with Dunnett’s test vs. exendin-4 response. Data represented as mean ± SEM.

These results contrast with the single dose experiments performed with mice, in which exendin-4 and ex-phe1 were both similarly effective during the “immediate” IPGTT but ex-phe1 was substantially better after a several hour delay post-injection. Here, during the “immediate” IPGTT, exendin-4 failed to improve glucose tolerance compared to vehicle control, whereas ex-phe1 exerted a modest anti-hyperglycaemic effect. However, when the IPGTT was performed 6 hours after agonist injection, both exendin-4 and ex-phe1 appeared to worsen glucose tolerance compared to vehicle treatment, with ex-phe1 being particularly deleterious.

The implication of these results is unclear. The moderate glycaemic improvements from t=40 minutes during the “immediate” IPGTT with ex-phe1 compared to exendin-4 are broadly in keeping with previous results, suggesting increased insulinotropism with non-desensitising agonists, although the time course is much faster than previously observed. However, apparent detrimental effects on glucose tolerance during the delayed IPGTT are not consistent with this. Acute exendin-4 treatment in non-diabetic rats was previously shown to induce a paradoxical hyperglycaemic response, dependent on the sympathetic nervous system (357). Here, the same phenomenon was apparent with exendin-4 during both the acute and delayed IPGTTs, and with ex-phe1 during the delayed IPGTT only. Given the apparent decline in glucose tolerance shown above in lean mice 8 hours after a single injection of exendin-4 and ex-asp3 (but not ex-phe1 and similar agonists), the question remains whether excessive GLP-1R internalisation and consequent loss of beta cell competence is a contributing factor to the hyperglycaemia induced here in rats by exendin-4. This explanation is not entirely compatible with a
similar phenomenon being seen with ex-phe1, which evidence so far suggests internalises more slowly. On the other hand, differences in trafficking of the rat vs. human GLP-1R are possible; for example, ex-phe1 might still induce substantial internalisation in rat beta cells by 6 hours, if the rat GLP-1R is more susceptible to internalisation. The difference between exendin-4 and ex-phe1 at later timepoints during the “immediate” IPGTT in rats provides some possible evidence that internalisation of the rat GLP-1R is faster.

In summary, in vivo testing of glycaemic responses to exendin-4-derived agonists with varying degrees of signalling bias revealed substantial advantages of reduced β-arrestin recruitment in mice, including in a model of T2DM. On the other hand, in lean rats, any glycaemic benefits of ex-phe1 over exendin-4 were short-lived, and ultimately reversed at later timepoints, which may reflect a recognized exendin-4-induced hyperglycaemic phenomenon.

5.4.2 Acute in vivo appetite responses

GLP-1 is an anorectic hormone which acutely inhibits food intake when injected into the peripheral circulation (64). The appetite-suppressing effects of GLP-1 can be replicated by direct injection into the central nervous system (CNS) (358,359). Central injection of the antagonist exendin(9-39) increases food intake, implying a physiological role for CNS GLP-1 in appetite regulation (358). However, as for its function as an incretin, rapid GLP-1 degradation raises the question whether gut-derived GLP-1 persists for long enough in the circulation to enter the CNS. Overall evidence favours peripherally released GLP-1 mediating its appetite effects via nearby vagal afferent nerve fibres, in particular via the nodose ganglion (68,360). GLP-1 is also produced within the brain stem (361,362) and this is the presumed source of physiologically active central GLP-1. Therapeutic GLP-1R agonists with prolonged half-lives can nevertheless access the brain. Satiety and weight-lowering effects of liraglutide are lost in brain-specific GLP-1R knockout mice (363). The precise brain regions involved are not clarified, with various studies implicating the arcuate nucleus of the hypothalamus (147), ventral tegmental area (364), nucleus accumbens (365), and parabrachial nucleus (366).

5.4.2.1 Appetite suppression in mice

As different in vivo potencies for glycaemic and insulinotropic responses were noted above for differentially biased exendin analogues, acute effects on appetite was also measured in mice. Single-caged, male, HFHS-fed C57BL/6J mice were fasted overnight during the dark phase, when they would usually eat, and then injected with exendin-4, ex-phe1, or vehicle. Food was then returned and weighed at regular intervals to determine intake. The experiment was performed at the same two
agonist doses as used in glycaemic testing (2.4 nmol/kg and 0.24 nmol/kg). Results are shown in figure 5.8.

![Figure 5.8. Acute appetite suppression in HFHS-mice.](image)

Mice (8-10/group) were fasted overnight and administered agonist at (A) 0.24 nmol/kg or (B) 2.4 nmol/kg by i.p. injection and cumulative food intake (FI) was monitored at regular intervals. No significant difference between agonists by two-way repeat measures ANOVA with Dunnett’s test vs. exendin-4. Data represented as mean ± SEM.

As indicated, both exendin-4 and ex-phe1 acutely reduced food intake over 8 hours compared to vehicle. However, there was no notable difference between each agonist at either dose. This result contrasts with the considerable differences in glycaemic responses observed at 8 hours and suggests the effects of biased agonism diverge between these two physiological effects.

### 5.4.2.2 Appetite suppression in rats

Appetite suppression effects were also tested in male, lean Wistar rats. Using a similar protocol to as with mice, rats were fasted during the dark phase and then their diet was returned at the beginning of the light phase after injection of exendin-4, exendin-phe1 or vehicle. Results are shown in figure 5.9, with cumulative and timepoint-specific food intake indicated. A dose-response curve for cumulative food intake reduction at different timepoints was constructed from the several agonist doses trialled.
Figure 5.9. Acute appetite suppression in Wistar rats.
Rats (8/group) were fasted overnight and administered a agonist at the indicated dose by s.c. injection, with serial monitoring of subsequent food intake (FI). (A) – (E) show cumulative total food intake at the indicated dose. (F) and (G) show food intakes between specific timepoints at several doses using data from (A) – (E). (H) – (K) show cumulative total food intake at the indicated timepoint, relative to the food intake from the equivalent vehicle group, with 4-parameter logistic fit of data indicated. *p<0.05, **p<0.01, ***p<0.001 by two-way repeat measures ANOVA with Dunnett’s test vs. exendin-4. Data represented as mean ± SEM.

Overall, exendin-4 at all doses achieved greater cumulative food intake reduction measured after 8 hours than did ex-phe1, at all doses above 0.3 nmol/kg. This contrasts with results from HFHS-fed mice in which there was no clear difference between treatments. Notably, a consistent time-related effect was observed. At 1 hour, the difference in cumulative food intake effect of exendin-4 vs. ex-phe1 was most marked, with some subsequent convergence in difference at 2 and 4 hours (especially 1 nmol/kg and 3 nmol/kg [figures 5.9C,D]). However, at 8 hours, exendin-4 was again most effective. This effect is illustrated by considering timepoint-specific food intakes – between 0 and 1 hour, exendin-4-treated mice ate less than ex-phe1-treated, whereas, between 2 and 4 hours, the difference

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was less marked or even reversed (figures 5.9F, G). Constructing a 4-parameter dose-response curve of data illustrates this finding in an alternative way, with the curve for ex-phe1 demonstrably right shifted at 1 hour (figure 9H) but less so at 2 or 4 hours (figures 9I and J).

The explanation for this interesting effect is unclear. It can be speculated that agonist-related differences in GLP-1R trafficking play a role. Of potential relevance, it was shown that in GLP-1R knockout mice, uptake of liraglutide into the brain is absent (147). Furthermore, leptin access to appetite regulatory hypothalamic centres is dependent on leptin receptor-mediated endocytosis by specialised glial cells known as tanycytes, which then export the hormone into the CSF (367). If a similar mechanism exists for the GLP-1R, differences in endocytosis propensity might manifest as changes to rate of onset of the anorectic effect of biased agonists. This would explain why ex-phe1, which is only slowly internalised, is relatively ineffective at reducing food intake in the first hour after injection, but “catches up” within hours 2 – 4. Relatively fast renal clearance of both compounds adds an extra level of complexity; between 4 and 8 hours, the circulating concentration of each falls 10-20-fold, as demonstrated earlier in the pharmacokinetic study in mice (figure 5.5). As GLP-1R endocytosis rate is dependent on agonist concentration, it might be imagined that by 8 hours, uptake of ex-phe1 ceases, explaining why by this later timepoint, ex-phe1 is once again less effective than exendin-4. Further studies are required to investigate this possibility. It would be interesting to measure CSF concentrations of each peptide over time after peripheral injection to provide insight into the differences in exposure of neuronal GLP-1Rs to each agonist, which might partially explain the apparent divergence between glucose and appetite modulatory effects.

5.4.3 Chronic administration study

The results from single dose testing so far suggest glycaemic benefits from agonists with reduced β-arrestin recruitment and internalisation, albeit with the strongest evidence in mice rather than rats. Conversely, the same pattern was not seen for suppression of food intake over the same time period, with no difference between exendin-4 and ex-phe1 in mice, and an advantage to exendin-4 in rats despite some indication of ex-phe1 “catching up” after 2 hours. However, whilst these results provide useful insight into the acute (several hours) effects of GLP-1R desensitisation on physiological behaviours, they do not provide a complete picture of the longer term effects of sustained administration of each agonist in a disease-relevant model of T2DM. GLP-1R desensitisation has not been studied extensively in vivo, but was claimed to be unimportant physiologically due to the persistence of response to GLP-1R agonists in transgenic mice which endogenously express exendin-4 in multiple tissues (307). Data presented so far in this chapter suggest that in vivo desensitisation plays a significant role in determining response to single doses of GLP-1R agonists, but a longer term study is required to address whether this can be translated into improved therapeutic effect.
Therefore, a study was performed to compare the metabolic effects of exendin-4, ex-phe1 or vehicle, when administered to HFHS-fed mice, a model of T2DM, over 2 weeks. This study primarily aimed to determine the effect of chronic administration on glucose tolerance, assessed by an IPGTT at the end of the study, with additional outcomes relating to food intake and body weight, as well as other metabolic parameters discussed below.

Consideration was given to mode of agonist administration. Due to extensive renal clearance, exendin-4 is traditionally given to patients and in experimental animal studies by twice-daily injections (subcutaneous in humans, usually i.p. in mice). Alternative formulations of exendin-4 have been developed which allow less frequent dosing. These include Bydureon, which is injected as a subcutaneous depot from which the active drug exendin-4 is slowly released (145), and most recently ITCA 650, in which exendin-4 encased in an osmotic minipump, which gradually releases its contents at a constant rate and is intended for once-yearly placement (136). Osmotic minipumps function by gradually absorbing fluid from the subcutaneous space, leading to gradual increase in pressure on the pump contents, resulting in steady drug extrusion via a small aperture. In research studies, such pumps are already in routine usage for drug delivery including with GLP-1R agonists (368-370).

Clearly, even when adjusted to achieve the same total drug exposure, differences in pharmacokinetic profile will exist with twice-daily dosing of a rapidly absorbed and rapidly cleared drug, compared to when the same drug is delivered continuously delivery; the former causes significantly higher peak levels interspersed with troughs during which drug levels are much lower. This is of potential relevance when considering the proposed mechanistic differences between exendin-4 and the biased analogue ex-phe1. GLP-1R recycling after exendin-4 treatment was found to be slow, with pronounced retention of intracellular receptor after agonist washout; combining this observation with the in vivo single injection results presented in this chapter, it could be reasoned that the peak plasma drug concentration achieved after injection is important in determining the extent of GLP-1R internalisation, with lasting effects even after much of the drug is cleared from the circulation. This would support frequent, short acting injections as the preferred mode of administration to reveal a difference between agonist effects dependent on GLP-1R desensitisation. On the other hand, it could also be argued that periods between injections when circulating levels fall below that required to induce GLP-1R endocytosis could allow resensitisation, potentially negating “beneficial” effects. For practical reasons, with twice-daily dosing, inter-injection intervals would likely exceed 12 hours during the dark phase, which is the period during which agonist action is of greatest importance, as most feeding occurs during this time. It has been suggested that short-acting GLP-1R agonists lead to more frequent nausea due to reversal of tachyphylaxis whilst plasma drug levels are low between injections (146,371). Continuous minipump-mediated administration would avoid these issues by ensuring a constant stimulus to tissue GLP-1Rs, more akin to in vitro conditions in which agonist
concentration remains static for the duration of the experiment. Recognising that each approach offers theoretical and practical advantages, it was decided to use subcutaneous minipumps to deliver a constant infusion of agonist, which additionally avoids the need for regular injection-related stress and associated welfare concerns.

5.4.3.1 Measurement of peptide stability under pump conditions

ITCA 650, under investigation as a minipump-driven exendin-4 product for clinical use, includes a proprietary peptide stabilisation formulation to avoid peptide degradation. As this is not available for research use, the stability of exendin-4 and ex-phe1 in osmotic minipumps was first determined by incubating known concentrations of each peptide in the device for two weeks in a 37°C incubator, and comparing the bioactivity with that of freshly prepared peptide by cAMP generation in CHO-GLP-1R cells. These results are shown in figure 5.10, and reveal moderate loss of activity after 2 weeks. However, as apparent degradation was similar with each peptide, valid comparisons of in vivo results should still be possible rather than reflecting differences in peptide stability.

![Figure 5.10. Stability of peptides at 37°C.](image)

Peptides were incubated at 37°C in Alzet pumps for two weeks and then used to determine cAMP response in PathHunter CHO-GLP-1R cells at the same nominal concentrations as with freshly prepared peptide. Each peptide concentration was used in triplicate wells with a 10 minute incubation with IBMX. A two-fold EC50 shift, reflecting similar loss of biological activity, is seen with each peptide after 2 weeks incubation at 37°C. Data represented as mean ± SEM.

5.4.3.2 Study design

HFHS-fed mice were allocated to receive exendin-4, ex-phe1 or vehicle (saline) at a weight-adjusted dose of 0.24 nmol/kg/24 hours, i.e. the same dose as was given by single injection, spread over a longer period. This dose was selected for a number of reasons. Firstly, the higher dose used in acute
studies (2.4 nmol/kg) is considerably higher than the equivalent dose in humans even after allometric scaling (348) (equivalent to ~65 µg in an 80kg human, compared to a clinically approved dose range in humans of 5 – 10 µg, albeit twice daily). Secondly, it was reasoned that the enhanced insulinotropic effect seen with a single injection of ex-phe1 might allow differences to emerge at a lower dose potentially associated with fewer adverse events. Thirdly, in the recently published study the “P5”, a biased exendin-4-derived agonist with similar signalling characteristics to ex-phe1 (although apparently reduced insulinotropism), benefits were seen at lower agonist doses; in this study treatments were however given by twice daily injection (183).

A summary of the study protocol is shown in figure 5.11. After randomisation, Alzet osmotic minipumps containing the indicated treatment were inserted into the subcutaneous space of HFHS-fed mice under general anaesthesia. For the next 14 days, body weight and food consumption was serially monitored. On day 15 an intraperitoneal glucose tolerance test was performed in overnight-fasted mice, with the Alzet pump in situ. After 2 further recovery days, the study was terminated and mice culled, with blood and tissues collected for further analyses. Body composition was also measured by magnetic resonance imaging (MRI) at this time.

Figure 5.11. Chronic study protocol.
FI = food intake, BW = body weight, IPGTT = intra-peritoneal glucose tolerance test.

5.4.3.3 Food intake and body weight responses

Food intake and body weight responses to each treatment are shown in figure 5.12. Interestingly, at this dose, neither treatment was effective at reducing food intake or body weight at this dose, compared to vehicle. A mild degree of weight loss relative to baseline weight was observed, including with vehicle treatment, which is a common result of minipump use and may reflect a non-specific minor stress effect (372). Whilst weight loss is usually considered therapeutically desirable with GLP-1R agonists, from an experimental perspective, lack of body weight differences are potentially useful
when interpreting other metabolic results for which treatment-related weight loss is frequently a confounding factor. Furthermore, the lack of a significant effect on food intake suggests that at this dose, nausea is unlikely to result.

![Figure 5.12](image)

**Figure 5.12. Food intake and body weight effects of chronic administration of exendin-4 analogues.** HFHS-fed mice (10/group) were treated with continuous exendin-4, ex-phe1 (both 0.24 nmol/kg/day) or vehicle by subcutaneous minipump. (A) Cumulative food intake over the course of the study. (B) Body weight changes, relative to baseline weight. No significant differences between treatments by two-way repeat measures ANOVA with Dunnett’s test vs. exendin-4. Data represented as mean ± SEM.

### 5.4.3.4 Glucose tolerance tests after 14 days agonist treatment

On day 15 an IPGTT was performed in overnight-fasted mice and glycaemic responses measured. Results are shown in figure 5.13, and demonstrate that, at this dose, exendin-4 lacks anti-hyperglycaemic properties compared to vehicle, whereas ex-phe1 remains effective. Note – reductions in fasting glycaemia with exendin-4 were still noted (figure 5.15). Considering this result in combination with the effect on food intake and body weight, it can be concluded that ex-phe1 leads to glycaemic benefits without a major effect on appetite modulation or weight-related changes to insulin sensitivity. This is consistent with earlier single dose findings in mice, where ex-phe1 performed better in the “delayed” IPGTT than exendin-4, but similarly for acute appetite suppression.
Figure 5.13. Effect of chronic exendin analogue treatment on glucose tolerance in HFHS-fed mice.

HFHS-fed mice (n=10/group), treated for 15 days with exendin-4, ex-phe1 (both at 0.24 nmol/kg/day) or vehicle, were fasted overnight and injected with 2 g/kg glucose. (A) Blood glucose response, two-way repeat measures ANOVA with Dunnett’s test indicating significance for ex-phe1 vs exendin-4. (B) AUC from (A), determined relative to baseline glucose at t=0, one-way ANOVA with Tukey’s test. *p<0.05, by test indicated above. Data represented as mean ± SEM.

5.4.3.5 Body composition at study end

After a further 48 hours to recover from the IPGTT, the study was completed and mice culled. At the time of death, body composition was determined by MRI. Tissues were weighed, including liver, heart and epidydimal white adipose fat pad. Results are indicated in figure 5.14. In keeping with the lack of an effect on body weight, there were no significant differences between treatments for any of the parameters tested. There were non-significant trends for ex-phe1 treatment to decrease body fat %, and a trend towards lower liver weight with ex-phe1 vs. exendin-4.
Figure 5.14. Effect of chronic exendin analogue treatment on body composition and organ weight in HFHS-fed mice.

HFHS-fed mice (n=10/group) were treated for 17 days with exendin-4, ex-phe1 (both at 0.24 nmol/kg/day) or vehicle. (A) Body fat %. (B) Lean mass. (C) Liver weight. (D) Epidydimal white adipose tissue (eWAT) weight. (E) Heart weight. No significant difference between treatments by one-way ANOVA. Data represented as mean ± SEM.

5.4.3.6 Blood parameters at study end

Terminal blood samples, in the fasting state, were also collected at the completion of the study. Biochemical analysis was performed to determine any effects on gluco-modulatory analytes and lipid parameters. Results are shown in figure 5.15. Fasting glucose was lower in agonist-treated mice than with vehicle, with the magnitude of the reduction greater with ex-phe1 treatment, although the difference to exendin-4 treatment was not significant. Aside from this, there were no significant differences between vehicle and either active treatment for any of the parameters measured. There was a trend towards increased plasma insulin with ex-phe1 treatment vs. exendin-4 treatment. Insulin and glucose results were analysed by HOMA assessment (373) to provide estimates of beta cell function (HOMA-B) and insulin resistance (HOMA-IR). Both agonists non-significantly increased beta cell function, and there was a non-significant trend towards improved insulin sensitivity with ex-phe1 vs. exendin-4. Little difference was seen in the lipid profile at the end of the study, with the exception of triglycerides, which were non-significantly lower in the active treatment groups.
**Figure 5.15. Effect of chronic exendin analogue treatment on plasma metabolic parameters.**

HFHS-fed mice (n=10/group) were treated for 16 days with exendin-4, ex-phe1 (both at 0.24 nmol/kg/day) or vehicle. (A) Blood glucose, (B) plasma insulin (C) beta cell function, calculated as HOMA-B from from (A) and (B), (D) insulin resistance, calculated as HOMA-IR from (A) and (B). (E) Total cholesterol, (F) HDL-cholesterol, (G) LDL-cholesterol, calculated using the Friedwalde equation, (H) triglycerides. **p<0.01, ***p<0.001 by one-way ANOVA with Tukey's test. All other comparisons non-significant. Data represented as mean ± SEM.

Therefore, ex-phe1, compared to exendin-4 treatment over two weeks, had at most modestly beneficial effects on fasting metabolism according to the parameters tested. This contrasts with the clear difference in treatment responses as assessed by parenteral glucose challenge in the same chronically-treated mice in figure 5.13. It is likely that with a relatively brief two week treatment period, provocative testing is required to reveal benefits which might otherwise be more obvious with more prolonged treatment (374,375). Furthermore, some of the metabolic benefits from GLP-1R agonist treatment undoubtedly result from weight loss and improvements to insulin sensitivity. At the dose tested here, no additional weight loss compared to vehicle treatment was observed.

### 5.4.3.7 Histological evaluation of hepatic steatosis

GLP-1R agonist treatment is effective in treatment of non-alcoholic fatty liver disease (329,376,377). The biased agonist "P5" (183) outperformed exendin-4 in steatosis resolution. Therefore, livers from mice treated chronically with exendin-4 and ex-phe1 were examined histologically for signs of fatty
liver disease. This work was done in collaboration with Prof. Robert Goldin, Centre for Pathology, Imperial College London. A histopathologist blind to treatment allocation scored haematoxylin-eosin-stained liver sections according to the Non-alcoholic Activity Score (NAS) (378). Plasma transaminase activities were also measured as potential biochemical markers of liver damage. Results and representative histological images are shown in figure 5.16.

**Figure 5.16. Effect of chronic exendin analogue treatment on liver histology and biochemistry.**
A) Representative haematoxylin-eosin sections from livers obtained from HFHS-fed mice treated for 2 weeks with exendin-4 or ex-phe1 (both 0.24 nmol/kg), or vehicle, scale bar 10 µm. (B) Non-alcoholic score (NAS) determined histologically, indicative of severity of fatty liver pathology, comparison by Kruskall-Wallis test for non-parametric data vs. vehicle treatment shown. (C) Alanine aminotransferase (ALT) from terminal plasma sample, (D) Aspartate aminotransferase level from terminal blood sample. *p<0.05. Data represented as individual scores, or mean ± SEM.

Histological assessment therefore revealed that ex-phe1 treatment led to a significant reduction in severity of fatty liver disease, whereas exendin-4 was ineffective. This provides evidence of end-organ protection, which is a more powerful outcome than biomarker improvement. In fact, liver biochemical markers were unchanged between groups. However, liver function tests are well known to be insensitive markers of hepatic steatosis.
In summary, chronic administration of ex-phe1 to HFHS-fed mice led to some metabolic improvements compared to an equal dose of exendin-4, most notably manifest as improved responses during an IPGTT, and greater resolution of hepatic steatosis. There was no significant treatment-related suppression of food intake, or weight loss at this dose. This confirms the divergence in glucoregulatory and appetite effects of biased GLP-1R agonism, which were apparent in single dose studies. Other biochemical improvements, for example in fasting lipid parameters and were non-significantly improved, which may reflect the lack of weight change or the relative short treatment period.

5.5 Discussion

Results presented here describe the in vivo effects of single dose and continuous administration of a biased GLP-1R agonist in comparison to the clinically approved compound exendin-4, from which it is derived. Critically, pharmacokinetic properties of each compound were identical, meaning that response differences can plausibly be linked mechanistically to previous in vitro characteristics rather than altered stability in plasma.

Single dose studies in mice revealed striking differences in duration of anti-hyperglycaemic effect, associated with greater insulin release with ex-phe1. This is the first demonstration of enhanced insulinotropism with a biased GLP-1R agonist, as the “P5” agonist claimed to be poorly insulinotropic. P5 might behave similarly to ex-phe1 but this was not apparent from the different experimental approach taken by the authors of that study. This finding dovetails with previous in vitro results, which showed that ex-phe1 induces a greater maximum insulin response over prolonged incubation than exendin-4. A clear pattern linking agonist-induced β-arrestin recruitment and receptor trafficking behaviour to in vivo glycaemic effectiveness emerged when a wider selection of agonists were trialled in lean mice, thereby excluding an idiosyncratic result with ex-phe1. These results bolster the proposed mechanism of action, in which agonists with reduced β-arrestin and internalisation responses induce less GLP-1R desensitisation and are thus able to signal for longer. It is of interest that some agonists with demonstrable greater insulinotropic capacity in vivo in fact would ordinarily be classed as weakly potent agonists on the basis of their EC50 values for cAMP, the most widely used metric to assess GLP-1R agonists in vitro.

The time course of the effects observed with a single injection are of interest when considered in the light of the pharmacokinetic profile. Injection of 24 nmol/kg of exendin-4 or ex-phe1 resulted in a plasma concentration of <1 nmol/L at 4 hours and ~50 pmol/L at 8 hours. With a 100-fold lower dose (0.24 nmol/kg), when circulating levels would be expected to be significantly lower and likely below the threshold for significant GLP-1R activation (even in highly-overexpressing recombinant GLP-1R
cells this is in the low pM range, especially for ex-phe1 for which potency is ~3-5-fold reduced vs. exendin-4), marked glycaemic differences between exendin-4 and ex-phe1 remained at 8 hours. Therefore, the persistence in in vivo effect implies the plasma concentration is not entirely predictive of biological efficacy. One possible explanation relates to sustained signalling from internalized receptors (106,109), as discussed in chapter 3. Exendin-4 achieved longer-lasting signalling responses in the agonist washout experiment than did ex-phe1, but ex-phe1 retained some capability to continually signal for several hours after removal of extracellular agonist, when measured in a recombinant system with high levels of GLP-1R. Conceivably, in beta cells with physiological GLP-1R expression, exendin-4 overstimulation might lead to eventual desensitisation of these internalised receptors, with ex-phe1 achieving a more balanced level of stimulation which allows ongoing signalling but avoids desensitisation. Additionally, agonists are unlikely to be uniformly distributed through the body, with distribution between plasma and tissue compartments influenced by target (GLP-1R) availability, as well as the kinetics of binding which affect the likelihood of rebinding vs. diffusion away from the target after agonist dissociation (291). Therefore, the plasma levels may not be a good guide to biological effect.

Analogous glycaemia studies in lean rats failed to replicate the anticipated benefits of ex-phe1, with paradoxically worsened glucose tolerance observed several hours after agonist injection, although during the "immediate" IPGTT, ex-phe1 performed better than exendin-4. This phenomenon has previously been reported in rats with exendin-4 (357) and linked to sympathetic nervous system activation. It might be argued that this reflects a stress response to acute nausea. Of note, in the experiments presented in this chapter, in lean mice there was also a trend towards worsened glucose tolerance with exendin-4 and in particular ex-asp3 during the delayed IPGTT. Ex-asp3 displays opposite in vitro characteristics to ex-phe1, leading to extensive retention of the GLP-1R within the cell after internalisation. The acute deleterious effect of this peptide might therefore relate to GLP1-R bias and trafficking. On this basis, sympathetic nervous system activation might be a manifestation of GLP-1R internalisation through unknown mechanisms. Alternatively, loss of beta cell competence might result from excessive GLP-1R sequestration leading to an inability to respond to endogenous or intra-islet GLP-1. If this proceeds at a faster rate in rats than mice, it could explain why ex-phe1 in rats outperformed exendin-4 at the late timepoints of the "immediate" GTT. In either case, it is in general thought that this paradoxical hyperglycaemia is only relevant to non-diabetic conditions, as exendin-4 given acutely to hyperglycaemic animals and humans leads to rapid reductions in blood glucose (349,379). Therefore, the results from HFHS-mice, in which a single dose of ex-phe1 led to greater insulin release and improved glucose tolerance several hours after administration, should be given more weight than those from lean animals.
The disparity between effects on glycaemia and food intake are of interest. To recap, in HFHS-fed mice, exendin-4 and ex-phe1 lead to identical degrees of food intake reduction at both high and low doses, despite marked differences in IPGTT responses. In rats, appetite suppression with exendin-4 was greater than with ex-phe1, although the difference was most marked at early timepoints, with ex-phe1 partially “catching up” between two and four hours after injection. Overall, both sets of results broadly support a relative lack of effect for ex-phe1 in modulating appetite. An intriguing candidate mechanism for this observation reflects differential brain penetration of each peptide, dependent on GLP-1R-mediated endocytic uptake and transport by specialist glial tanyocytes (147), as was previously observed with leptin (367). Albiglutide, a new GLP-1R agonist now approved for clinical use, consists of a GLP-1-type peptide fused to albumin, which reduces renal clearance leading to prolonged pharmacokinetics. This large molecule, which might be expected to exhibit reduced brain penetration, is less effective than other, smaller GLP-1R agonists for weight loss, and this is associated with a favourable gastrointestinal side effect profile (380). Therefore, ex-phe1 might share similar therapeutic properties.

A chronic study in HFHS-fed mice, a model of T2DM, was performed to determine whether the single dose differences between exendin-4 and ex-phe1 might be translated into clinical benefits. After two weeks of continuous treatment, the headline effects from acute administration studies, namely improved glycaemia during IPGTT with ex-phe1, with no differential effects on appetite, were recapitulated. The magnitude of the glycaemic benefits was less than with single doses. There are a number of possible explanations for this. The mode of administration, i.e. continuous subcutaneous delivery by osmotic minipump in the chronic study, resulting in stably elevated plasma drug concentrations, might induce different levels of GLP-1R desensitisation than with a single, rapidly absorbed dose which leads to far higher peak plasma levels. A side-by-side comparison of twice daily injections and continuous administration to achieve the same total drug exposure would be interesting to perform. In the “P5” biased agonist study, agonists were given by twice daily injection during the chronic study. Interestingly, results from that study (no difference in weight, improved anti-hyperglycaemic effect of P5 with a dose of 0.24 nmol/kg twice daily) were comparable to those presented here for ex-phe1 vs. exendin-4. A consideration is the stability of the peptides within the pump over the duration of the chronic study. Some apparent degradation was observed, as determined by loss of biological potency for in vitro cAMP response, corresponding to approximately 50% degradation, with the caveat that this degree of change is close to the assay variability. The amount of apparent degradation was similar between peptides, and is therefore not likely to be a confounder for outcome differences in the chronic study. However, it may have led to somewhat reduced effectiveness of both peptides later in the study. An additional possible reason for relatively smaller effects observed in the chronic study are adaptive changes which result with longer term treatment. As well as acute differences to receptor trafficking and desensitisation, downregulation of
receptors and signalling pathways may result from transcriptional changes or mRNA degradation (381).

The lack of effect on body weight at this dose is of uncertain significance from a clinical point of view. In general, beneficial effects of GLP-1R agonist treatment are partly attributable to improvements in insulin sensitivity due to loss of adipose tissue. On the other hand, nausea is a frequent adverse event with most existing GLP-1R agonists (144), and furthermore, at an individual level, weight loss does not correlate with glycaemic improvements in clinical trials (382). In the chronic study presented in this thesis, glycaemic benefits resulted from ex-phe1 treatment without weight loss. Therefore, it is conceivable that greater metabolic benefits might result for the same degree of weight loss (or nausea) compared to exendin-4 treatment, increasing the “therapeutic window”. The greater reduction in liver steatosis with ex-phe1 vs. exendin-4 treatment supports clinical potential for this compound, particularly as non-alcoholic fatty liver disease a major health concern at present due to the anticipated increase in obesity-related prevalence and its association with cirrhosis and liver cancer (383). Several further indications of GLP-1R agonist therapy are under investigation, including protection against myocardial ischaemia (384), Alzheimer disease (331) and Parkinson’s disease (330). If prolonging GLP-1R activation by avoiding desensitisation is beneficial in these scenarios, ex-phe1 might provide additional advantages over existing molecules. It is not totally clear whether the proposed GLP-1R agonist-mediated benefits in these conditions result from direct action at GLP-1Rs in the tissues of interest, or via global metabolic improvements resulting from weight loss and improved glycaemia. If due to direct tissue activity, differences in brain penetration which are hypothesised (but not yet demonstrated) with ex-phe1 might render it less suitable for treatment of neurodegenerative diseases.
6  General conclusions and future perspectives
6.1 Introduction

Interest in biased agonism has increased substantially over recent years. This likely reflects greater understanding of the multiple intracellular signalling pathways triggered by many GPCRs, in particular “non-canonical” pathways such as β-arrestin recruitment, along with advances in analytical approaches to quantify bias. Demonstrating that a receptor can engage in biased agonism potentially has substantial implications:

1. It may aid discovery of structural mechanisms of receptor activation and/or receptor conformations promoting interaction with particular intracellular proteins
2. Biased agonists can be used as tools to probe the role of intracellular signalling pathways on downstream cellular events
3. Biased agonists might be therapeutically advantageous if they can avoid signalling outputs associated with adverse effects

In this project, a series of biased orthosteric GLP-1R agonists have been identified, and the major functional implications of this investigated at a cellular and whole body level, with a particular focus on pancreatic beta cells and glucose homeostasis. The key observations are summarised in figure 6.1.

![Diagram of cAMP-biased and β-arrestin-biased signalling](image)

**Figure 6.1.** Summary of key findings from this project.
In the subsequent sections, the findings are reiterated, along with their implications, experimental limitations, and questions for future study.

6.2 Implications of from structure-activity relationship studies of biased GLP-1R agonists

During this study, a variety of N-terminally substituted analogues of GLP-1 and exendin-4 were generated and tested to determine pathway bias between cAMP generation and β-arrestin recruitment. It was apparent that in almost all cases, mutations away from the natural amino acid at position 1 (His), 2 (Ala or Gly) or 3 (Glu) led to loss of activity in one or both pathways (figures 3.3 and 3.5). This observation suggests that the natural sequence of each agonist approaches that which is optimal for maximal activation of these pathways, and that with orthosteric experimental GLP-1R agonists, bias is more likely to result from relative and selective loss of activity rather than augmentation. As per figure figure 3.7, a consistent bias effect of individual amino acid substitutions was observed irrespective of whether the “base” agonist was GLP-1 or exendin-4. These findings appear to implicate specific interactions between the N-terminal region of the peptide and the receptor in inducing preferential activation of particular pathways. Extensive mutagenesis studies have already revealed a number of GLP-1R residues which, when mutated, functionally affect the generation of signalling bias with “natural” agonists GLP-1, exendin-4 and oxyntomodulin (74). Of these, only a minority of receptor residues were predicted to form direct interactions with the agonist, with others assumed to be required for propagation of bias-inducing conformational changes downwards to the intracellular surface of the receptor. One of these, R190, was validated by reciprocal mutagenesis of position 3 of GLP-1 to replicate the receptor mutant’s signalling phenotype. A clear extension of the work described in this thesis would be to use a similar approach to examine structural mechanisms by which the novel biased GLP-1R agonists preferentially activate particular pathways. With the aid of existing computational GLP-1R models, such as that described in the above study by Wootten et al, it may be possible to identify a restricted set of candidate receptor residues with the potential to directly interact with the ligand N-terminal region. Measuring the signalling consequences of targeted mutagenesis at these residues would provide experimental verification of structural models which aim to understand “biased” receptor conformations (224,385).

Exendin-4 and GLP-1-derived peptides with sequence substitutions at positions 1, 2 and 3 were synthesised in this study. However, it is apparent from an initial screen of GLP-1R-active oxyntomodulin analogues, based on the proprietary compound “G1950” but with individual amino acid substitutions throughout the molecule (figure 3.2), that other residues can significantly impact on β-arrestin recruitment. These could therefore be candidates for further study. In particular, some changes at positions 4 and 9 were capable of almost abolishing arrestin recruitment. Changes at
regions more distant from the N-terminus appeared to have a less dramatic impact on arrestin recruitment, although in several cases it was still moderately reduced compared to the base agonist. The apparent primacy of N-terminal changes in modulating bias is generally in keeping with the consensus model for activation of the GLP-1R and other class B GPCRs (203), in which the ligand C-terminal and helical section are primarily required for binding affinity, with the N-terminal primarily engaged in the activation process. Nevertheless, recent evidence suggests this may be an oversimplification, with a possible role for the ligand C-terminal in GLP-1R activation now advocated (75,386). From these recent studies, evidence in favour of a proposed novel activation mechanism derived in particular from the observation GLP-1 analogues lacking the entire N-terminal region can still activate the receptor when covalently fused to it. On the basis of this and other findings, it was proposed that the GLP-1R ECD forms auto-inhibitory contacts with the surface of the trans-membrane region (TMR), as previously reported for the glucagon receptor (220); on agonist binding, the ECD might maintain contact with the TMR but via different interactions which induce an active receptor conformation. Biased agonism was not explicitly studied in those reports, but it raises questions as to whether agonist differences towards the C-terminal of the molecule might induce bias. The in-house peptide library examined in the preliminary screen for this thesis would potentially be a valuable resource to study this in more depth.

Several potential limitations can be identified in the examination of biased signalling presented here. Firstly, only three pathways (cAMP, β-arrestin1 and β-arrestin2) were investigated. Several other signalling pathways have been linked to GLP-1R activation, such as Gαq signalling (86) and ERK1/2 phosphorylation (191). Indeed, robust GLP-1-induced Ca2+ responses, albeit at high doses only, were elicited in PathHunter CHO-GLP-1R cells in a manner which was Gαq-dependent, as demonstrated by diminished responses in the presence of the PLC inhibitor U73122 (figure 3.23). Test agonist-induced Ca2+ responses were however not incorporated into bias measurements for three reasons: 1) inhibition by ESI09 indicated the response is also partly dependent on Epac2 activation, and is thus not a “pure” Gαq readout as it also requires Gαs activation; 2) Ca2+ responses are measured over the course of seconds, compared to the 90 minute incubations used for the other readouts, meaning that bias estimates could be misleading due to their time-dependence (187). The same issues apply to ERK1/2 phosphorylation, which is usually peaks at ~5 minutes before rapidly diminishing (387) and is dependent on both G protein signalling and β-arrestin recruitment (101).

Conceptually, bias is best described using more proximal readouts, preferably direct measurements of proteins recruited to the receptor, as these are most clearly linked to bias-inducing receptor conformations. In this project, cAMP was measured in place of Gαs recruitment (or activation); the HTRF cAMP assay used has a number of advantages including simplicity and suitability for a medium-high throughput setting, but corroborative experiments could be performed to measure G protein
responses directly. Arrestin recruitment was measured directly using the PathHunter reporter system; recent studies have highlighted however that the functional effect of β-arrestin recruitment depends on mode of engagement (270) and conformational changes (265). It would be interesting to study whether these behaviours apply to the GLP-1R and if they are agonist-dependent.

Additionally, beyond prototypical GPCR interactors such as G proteins and β-arrestins, biased agonists might alter direct interactions of the GLP-1R with other proteins, or facilitate indirect interactions via protein scaffold formation. For example, the tyrosine kinase c-Src is known to interact with the GLP-1R via β-arrestin1, and plays a role in beta cell mass (225). Thus, its recruitment might differ in an agonist-dependent manner. Co-immunoprecipitation is a standard technique to probe such interactions, but is low throughput, semi-quantitative and provides information on known interactors only. Mass spectrometric methods are more powerful in providing unbiased proteomic measurements and can be adapted to measure protein-protein interactions. This approach was recently taken for the glucagon receptor (388). Alternatively, further studies could be performed on more “distal” signalling readouts to produce a “signature” of biased GLP-1R activation. Kinase arrays, which measure activation of several selected kinases are a well validated and targeted approach (389); alternatively, mass-spectrometry-based phosphoproteomics allows the measurement of agonist-related changes to the whole phosphoproteome (390). Due to the potential for cell-dependent differences in protein expression, these experiments would be most informative if performed in primary beta cells, or a beta cell-like cell line such as the human insulinoma-derived EndoC-βH1 (276) if sufficient primary tissue is not available.

Another important limitation to highlight is that bias was only measured at a single timepoint (90 minutes for all pathways). This was selected as the arrestin assay gives an excellent signal-to-noise ratio at this timepoint, with the incubation for cAMP matched to this due to the kinetic nature of bias (187). Of note, one feature of the PathHunter β-arrestin assay is that once recruited, the arrestin reporter cannot dissociate from the receptor, unlike endogenous arrestins. These 90 minute incubations are also relatively long in comparison to what is known about the kinetics of recruitment events; when measured directly, for example using resonance energy transfer assays, G protein and β-arrestin recruitment can be detected within seconds (265) and often peak after a few minutes (391), depending on receptor and cell system. Given the role of β-arrestin recruitment in mediating desensitisation, it is unclear what impact different arrestin recruitment patterns could have had on cAMP generation by 90 minutes. It is therefore not necessarily trivial to conclude that bias identified here necessarily correlates with promotion of G protein- or arrestin-specific receptor conformations. This should be born in mind if these results are used to probe the structural basis of biased signalling.

A number of “biosensors”, usually based on BRET or FRET, have been generated which allow real-time measurement of recruitment events (G proteins, β arrestins, etc) (392) or activation of downstream
kinases such as PKA (393) and ERK (394). The dynamic range of these assays is unfortunately less good than with the endpoint techniques used in this project, but they have the significant advantage of allowing measurement of signalling responses sequentially from the same well over time, so could be used to acquire early readouts of pathway activation. Nevertheless, irrespective of whether biased receptor conformations can be concluded from the signalling data presented here, bias toward β-arrestin recruitment was strongly and consistently associated with a series of receptor behaviours and downstream consequences, namely internalisation, desensitisation, and reduced sustained insulin secretion both in vitro and in vivo. A mechanistic role for this bias appears likely, on the basis of partial replication of some of these effects by RNA interference and other methods.

6.3 Implications of GLP-1R receptor trafficking findings

The GLP-1R undergoes rapid agonist-mediated endocytosis (103), which was confirmed here using a variety of methods to measure loss of surface GLP-1R, including a cell-surface ELISA to detect wildtype GLP-1R and various techniques based on labelling of N-terminally SNAP-tagged GLP-1R. Agonist-specific GLP-1R trafficking differences in primary human islets were measured by immunofluorescence. Large differences in capacity to induce internalisation were noted with biased GLP-1- and exendin-4-derived agonists. These findings are in keeping with the known role of β-arrestins in GPCR endocytosis, although manipulation of arrestin recruitment by genetic means such as RNA interference is required to confirm this. As well as internalisation, GLP-1R recycling was measured and found to inversely correlate with arrestin recruitment, albeit only in the limited subset of agonists tested (exendin-4, ex-phe1, ex-asp3; see figure 3.15). Whilst arrestins are well known to mediate endocytosis for several receptors, their role in recycling is less clear. Amongst GPCRs closely related to the GLP-1R, recycling of the GIP receptor was recently reported to be slowed by agonist-induced β-arrestin2 recruitment (234), whereas in contrast, the glucagon receptor was found to recycle in a β-arrestin manner (229). Measurement of recycling of a larger number of biased GLP-1R agonists with variable β-arrestin recruitment patterns, and modulation of arrestin recruitment by other means, are needed to gain confidence as to what role arrestin recruitment plays in GLP-1R recycling.

Substantial interest in the interplay of GPCR trafficking and signalling has arisen recently following the observation, repeated by several groups for different receptors (109,111,112), that internalisation does not automatically result in termination of receptor signalling. These observations broadly fit within the larger theme of spatial encoding of signalling within subcellular microdomains (395,396), a process which is required to ensure different stimuli can engender specific responses despite using the same messenger systems (for example cAMP). The overall effect of prolonged stimulation with biased GLP-1R agonists on cAMP generation was investigated in this study (figure 4.8), with an overall
increase in total cellular cAMP resulting from treatment with agonists with minimal arrestin recruitment, leading to greater insulin secretory responses from cell populations. However, these macroscopic behaviours do not preclude more subtle but still relevant signalling differences. For example, in a recent study, distinct transcriptional responses were found with optogenetically generated cAMP from plasma-membrane vs. endosome-localised light sensitive adenylate cyclase isoforms (113). The identification of differently trafficking GLP-1R agonists therefore provides useful tools to study the impact of GLP-1R signalling from different regions of the cell, with the potential for long term treatment of differentially trafficking agonists to substantially alter the transcriptional landscape. Genetically encoded signal-activated biosensors which record activity of PKA and ERK could be targeted to particular cellular domains using localisation sequences (397,398). Resulting alterations to patterns of gene expression would be best measured using microarrays, or preferably RNA-seq (399).

The SNAP-GLP-1R cellular system established during this project provides a further useful tool for studying receptors at particular subcellular locations. In particular, reversible labelling, for example using cleavable SNAP-probes, provides a means to isolate cell surface vs. endosomal receptor populations. The BG-SS-biotin probe used in figure 3.21 could be used in combination with a streptavidin-based pull down systems (253) to purify endosomal or membrane-resident receptors, depending on what point the probe was applied and cleaved. This could then be used to identify receptor-interacting proteins at each location, either in a targeted way by immunological detection, or using mass-spectrometry to capture all potential interactors.

### 6.4 Implications of binding kinetics of biased GLP-1R agonists

Binding kinetics of exendin-4, ex-phe1 and ex-asp3 were investigated in this project using a FRET approach which detects, in real time, specific interactions between SNAP-GLP-1R labelled with a TR-FRET donor, and FITC-conjugated agonist equivalents (figures 3.18 and 3.20). Findings were corroborated with unlabelled agonists in case the FITC moiety affect binding properties (figure 3.22). With these representative agonists, the greatest differences were noted with the dissociation rate constant, which was consistently faster for ex-phe1 and slowest for ex-asp3. An alternative way to consider this is in terms of the agonist "residence time" (238), which is the reciprocal of the dissociation rate, and represents the "average" time the agonist remains bound to the receptor. Association rate constants were in fact slower with both ex-phe1 and ex-asp3, although this was less marked. Association rates are often considered less influential, at least in determining in vivo action, although this has been questioned (291). It is interesting that, with these 3 agonists, residence time was associated with efficacy for β-arrestin recruitment, receptor internalisation and recycling. Binding assays were performed with and without metabolic inhibitors demonstrated to prevent detectable
internalisation in this assay system, with comparable results, suggesting the differences in binding kinetics were not a consequence of co-internalisation of agonist and receptor, which might otherwise interfere with the dissociation process. It is unclear whether the duration of agonist residence itself is wholly or partly responsible for the observed signalling bias generated by these peptides, or whether distinct, bias-favouring receptor conformations are stabilised by particular agonists in a manner independent of duration of agonist residence. This should be investigated in more detail with additional compounds to determine if the effect is consistent, and to corroborate this finding, the impact on binding kinetics of receptor mutagenesis at key residues predicted to interact with biased agonists could be measured.

Agonist binding kinetics were measured here in whole cells over a similar time course to functional assays. However, the derived “macroscopic” rate constants are in reality likely to represent the average of several microscopic rate constants relating to different aspects of the binding process. The simplest model of receptor binding describes joining of the agonist and receptor to form a complex. However, agonist binding leads to receptor conformational change, with the resulting conformation possessing different affinity characteristics than the receptor in its “ground state”. This is commonly described as the “induced-fit” or “two-step” model (400,401) and has been described for the PTH receptor (402) but not yet the GLP-1R. As receptor activation is a rapid event, these measurements require a high degree of time resolution which was not possible with the measurement system used in this project, but can be more easily performed with live cell FRET imaging approaches. It would be interesting to determine whether biased agonists with different receptor activation profiles exhibit differences in binding kinetics relating to receptor activation.

### 6.5 Possible therapeutic effects of biased GLP-1R agonists

If biased agonists are to be used as drugs, they need to generate a therapeutically useful output. Beta cell effects of standard GLP-1R agonists, including GLP-1 itself, exendin-4, liraglutide and others, have been investigated in depth at the cellular level. The major effects on beta cells of potential clinical importance are potentiation of glucose-stimulated insulin release, and preservation of beta cell mass. Both cAMP signalling (77) and β-arrestin1 recruitment (98) have been implicated in both of these processes, so what effect biased agonist treatment, if any, might have on these events was difficult to predict.

Insulin secretion and apoptosis prevention were therefore measured with a selection of exendin-4-derived agonists to identify potential bias-related differences. Biased agonists had little incremental effect on insulin secretion compared to exendin-4 when measured acutely (figure 4.4). Short term incubations are typically used to measure insulin secretion in vitro, and are appropriate for modelling
physiological exposure to endogenous GLP-1. However, clear differences emerged when the incubation was extended by several hours to more closely approximate exposure to a drug which is given either twice per day (Exenatide standard release) or once per week (Exenatide extended release). Under these conditions, biased agonists with reduced β-arrestin recruitment were substantially more insulinotropic than those with greater arrestin responses (such as exendin-4 itself – see figures 4.1 – 4.3). This is despite the fact that agonists with reduced arrestin response also displayed in some cases 10-fold or greater loss in potency for cAMP generation. Nevertheless, a ceiling for loss of arrestin recruitment was apparent, as beyond a ~70% reduction compared to exendin-4, insulin secretion started to decline. Ex-phe1 was the most insulinotropic peptide tested and fell in this “sweet spot”. Ex-asp3, which was biased towards arrestin recruitment, was the least insulinotropic compound tested despite high affinity binding (figure 3.22). Protection from apoptosis, measured as changes to caspase-3 activity induced by the ER stressor thapsigargin, was virtually lost with ex-asp3 (figure 4.7), although no incremental advantage was seen with ex-phe1 compared to exendin-4.

Functional effects of GLP-1-derived agonists with similar biases were not investigated due to the lack of suitability of GLP-1 itself for in vivo use application due to short circulatory half-life. It would however be interesting to incorporate these changes into compounds such as liraglutide, dulaglutide etc, which retain most of the GLP-1 peptide sequence but exhibit prolonged pharmacokinetics due to addition of a lipid side chain to enhance plasma albumin binding, or conjugation to an antibody Fc fragment, respectively. Some N-terminally substituted GLP-1-derived peptides utilised in this project have previously been reported, such as Phe1, Gly2 and Asp3 (403). Historical interest in the GLP-1 N-terminal pertains mainly to circumventing DPP-4 degradation, and in that study, effects on glucose homeostasis were not evaluated in such a way to permit any conclusions on differential effects relating to biased signalling.

The pharmacology of biased exendin-4 analogues was investigated further to aim to understand why apparently weak agonists were more insulinotropic. The presumptive explanation was elicited by measurement of homologous desensitisation, using calcium responses in CHO-GLP-1R cells and human islets, and cAMP generation in INS-1 832/3 cells and human islets, which revealed that biased agonists such as ex-phe1 allow the cell to retain responsiveness to restimulation. Furthermore, simultaneous genetic ablation of both β-arrestin isoforms by RNA interference led to augmentation of sustained exendin-4-induced insulin secretion (figure 4.18), supporting a mechanistic role for β-arrestins as negative regulators of GLP-1R activity under pharmacological conditions. These results contrast with the prevailing view of an important role for β-arrestin1 recruitment in mediating GLP-1-induced responses in beta cells (98,101). Of note, in this thesis, single knockdown of β-arrestin1 led to a reduction in exendin-4-induced cAMP generation at the earliest timepoint (figure 4.17), as well as a non-significant reduction in acute insulin secretion (figure 4.18). β-arrestin2 knockdown did not affect
insulin secretion acutely but did slightly augment cAMP response. Furthermore, a recent study found β-arrestin2 knockout does not affect acute insulinotropic responses to GLP-1 in isolated mouse islets (404). Together, these findings allow for a role for β-arrestin1, but not β-arrestin2, in mediating responses to brief exposures to GLP-1, but with negative regulatory effects emerging with longer incubations; the latter approach has not been tested in previous studies. The greater effects seen with dual vs. individual arrestin knockdown may mean that some functional redundancy exists between isoforms. Incomplete knockdown with RNA interference, along with the possibility of functional redundancy, means that more advanced techniques such as CRISPR could be usefully employed to induce total silencing of one or other arrestin to more clearly delineate their roles at a cellular level (405).

From a clinical perspective, the most important finding of this study is that the insulinotropic effects of biased GLP-1R are recapitulated in vivo, including in a mouse model of T2DM (chapter 5), validating biased agonism as a potential means to enhance therapeutic insulin secretion by GLP-1R agonists. In figure 5.2, the anti-hyperglycaemic effect of exendin-4 clearly “wears off” after 8 hours, yet ex-phe1 remains highly effective. Insulin secretion was increased with ex-phe1, suggesting a beta cell effect. Consistent effects with lower doses, and with other biased GLP-1R agonists, are shown in figures 5.3 and 5.6. As the pharmacokinetic profiles of these compounds are unchanged compared to exendin-4, the observed differences in homologous desensitisation could explain the effect on insulin secretion. It is interesting however that this effect persists beyond a point that might be expected from the measurements of plasma drug levels, which fell to <1 nM by 4 hours after injection when using a dose >100-fold greater than the lowest effective dose tested (figures 5.3, 5.6). After intra-peritoneal drug injection, peak plasma concentrations are rapidly achieved and this initial spike might induce GLP-1R desensitisation or downregulation which persists despite the steady loss of extracellular ligand. Additionally, distribution of drug within islets may not mirror that of plasma. One potential contributory factor could be that the apparently faster association rate constant for exendin-4 vs. ex-phe1 (tables 3.6 and 3.9) increases rate of “rebinding” (i.e. after dissociation from the receptor, the drug binds a nearby receptor rather than diffusing away), functionally increasing the local drug concentration (291,406). Different routes of administration (such as subcutaneous injection or infusion, intravenous injection, etc) matched to the same total drug exposure but higher or lower peak plasma concentrations could be systematically compared to determine if this is material in determining biological response. Furthermore, theoretical analyses of Exenatide pharmacokinetics and pharmacodynamics which explicitly incorporate binding kinetics and endocytosis rates could be adapted for biased GLP-1R agonists to determine if pharmacodynamics effects are indeed reflective of these parameters (355,407-409). As Exenatide and related peptides are predominantly cleared by the renal route (126), another intriguing possibility is whether biased agonists could be further modified, for example by lipidation or antibody fragment-coupling, to reduce renal clearance and increase the
relevance of receptor endocytosis as a clearance mechanism; agonists such as ex-phe1 which are less prone to internalisation might then possess further pharmacokinetic advantages over fast internalisers such as exendin-4.

The lack of correspondingly increased appetite reduction (figures 5.8 and 5.9) with ex-phe1 to match the glycaemic effects is both interesting mechanistically and potentially important in determining clinical applicability of biased GLP-1R agonists. The precise site(s) of action for appetite reduction by peripherally-administered GLP-1R agonists is still under investigation, but appears at least to be within the central nervous system (363). A mechanism for agonist entry into the CNS is therefore required. This could be achieved by passive diffusion, but an alternative possibility is via GLP-1R-mediated endocytosis. The lack of uptake of liraglutide into the brain of GLP-1R knockout mice (147) hints at this possibility, with a similar mechanism more conclusively demonstrated for leptin uptake into the brain by the leptin receptor (367). Investigating this possibility using biased agonists with differential endocytosis propensities, including the fluorescently conjugated biased peptides used in this study to allow direct visualisation, is a logical future step to understand the mechanisms for differential glycaemic and appetite effects.

From a therapeutic perspective, a lack of improved CNS penetration and appetite suppression could alternatively be viewed as a disadvantage or a benefit. GLP-1R agonists cause weight loss and are now a major area of development for the treatment of obesity with or without diabetes (130,328). With several GLP-1R agonists already in clinical use or in late stages of development, greater efficacy for appetite reduction or weight loss would clearly be therapeutically attractive for this indication. On the other hand, there are a number of reasons why excessive appetite suppression might not be desirable in novel GLP-1R agonists. Firstly, nausea is experienced by 30-50% of patients in GLP-1R clinical trials despite careful dose escalation, and this is strongly associated with drug discontinuation (410). Compounds with greater CNS penetration might therefore be less tolerable, although formal behavioural experiments designed to measure rodent equivalents of nausea, such as pica (411) or conditioned taste aversion (365) should be performed to test this. Secondly, metabolic improvements with GLP-1R agonists are, perhaps surprisingly, poorly correlated with weight loss (382). Thirdly, it appears that ex-phe1 is not less effective for appetite suppression than standard therapies, but achieves greater glycaemic benefits for the same degree of acute food intake reduction. In the era of "personalised medicine" (371), this may make it particularly suitable when weight loss is not the primary treatment goal.

A chronic administration study was performed to determine if the acute glycaemic benefits of the cAMP-biased GLP-1R agonist ex-phe1 translate to better performance over a longer period. In this study, agonists were delivered by osmotic minipump at a daily dose corresponding to the lowest dose
single injection trialled acutely. The main finding was broadly in agreement with the acute administration studies, i.e. that ex-phe1 improved glucose tolerance relative to exendin-4 (figure 5.13) despite no difference in weight or food intake (figure 5.12). The improved metabolic actions of ex-phe1 were further supported by evidence of greater resolution of hepatic steatosis compared to ex-phe1 (figure 5.16), although reductions in transaminase levels were not observed. The positive histological finding suggests weight loss is not essential for beneficial hepatic effects of GLP-1R agonist treatment. Interestingly, in a recent study, steatosis reduction in patients treated with liraglutide did not correlate with weight loss (329), suggesting additional mechanisms.

To determine the suitability for clinical development of the biased GLP-1R agonists described in the project, further preclinical studies need to be undertaken. Firstly, the lack of congruence between mouse and rat glycaemic responses raises the possibility of a species specific effect, although positive in vitro experiments using both human and rat GLP-1R systems imply that any differences are not likely to result from receptor specificity. The lack of glycaemic benefits in lean rats (figure 5.7) may well represent a known rat-specific phenomenon in which acute exendin-4 treatment leads to paradoxical hyperglycaemia (357). Exendin-4 is an effective anti-hyperglycaemic agent in diabetic rats (349), which may be a suitable model to address the issue. In future chronic administration studies, it would be appropriate to investigate different routes of administration (e.g. continuous vs. twice daily injections) and additional doses (e.g. a higher dose with the aim of achieving weight loss), with a longer study duration to allow potential treatment-related differences to emerge. Comparisons would ideally be made with other clinically approved GLP-1R agonists, such as Liraglutide, Semaglutide, Dulaglutide, etc, although differences in plasma protein binding and pharmacokinetics would make it difficult to achieve dose equivalence. An extra interesting head-to-head comparison would be with the biased exendin-4 agonist “P5” (183) which appears to show similar pharmacological properties to ex-phe1. Furthermore, differences in diabetes-related microvascular complications, including retinopathy and neuropathy, should specifically be evaluated as emerging evidence suggests GLP-1R agonists can act directly on the retinal endothelium (412) and peripheral nerves (413) i.e. benefits may not derive exclusively from reductions in glycaemia.

Following further preclinical testing, the effects of ex-phe1 should be evaluated in humans. To answer the question of what role biased signalling plays in mediating GLP-1R actions in humans, a physiological trial could be performed in which "delayed" glycaemic effects of exendin-4 versus ex-phe1 (analogous to the mouse study in figure 5.2) are compared in healthy volunteers. However, to determine therapeutic effects, clinical trials in relevant patient populations would need to be conducted.
6.6 Summary

In summary, this project has identified novel biased agonists based on simple modifications to the N-terminal region of GLP-1 and exendin-4. Pharmacological profiling revealed a link with receptor binding kinetics and trafficking. The major downstream consequence of biased signalling was found to relate to the canonical role of β-arrestins as negative regulators of receptor activity, an important finding relevant to how the functional consequences of bias are evaluated. Importantly, similar effects were observed in vivo in preclinical models of T2DM, suggesting that GLP-1R biased agonism might be exploitable therapeutically.
## Appendix A (peptide sequences)

### Unmodified agonists

| Glp-1 | H | A | E | G | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| OxM | H | S | Q | G | P | Y | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| Ex-DQG50 | H | S | Q | G | P | Y | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |

### GLP-1-derived peptides

| GLP-1 | H | A | E | G | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| GLP-1 dHs1 | D | H | A | E | G | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| GLP-1 ph1 | F | A | E | G | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| GLP-1 gly2 | H | G | E | G | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| GLP-1 glr3 | H | A | Q | G | E | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| GLP-1 dGln3 | H | A | Q | G | E | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| GLP-1 his3 | H | A | H | G | E | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| GLP-1 asp3 | H | A | D | G | E | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| GLP-1 ala3 | H | A | A | G | E | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |
| GLP-1 leu3 | H | A | L | G | E | T | F | T | S | D | V | S | Y | L | E | G | Q | A | A | K | E | F | I | A | W | L | V | K | G | R | NH2  |

### Exendin-4-derived peptides

| Ex4 | H | G | E | G | T | F | T | S | D | S | K | Q | M | E | E | E | A | V | R | L | F | E | W | L | K | N | G | P | S | S | G | A | P | P | P | S | NH2  |
| ex-dHs1 | D | H | G | E | G | T | F | T | S | D | S | K | Q | M | E | E | E | A | V | R | L | F | E | W | L | K | N | G | P | S | S | G | A | P | P | P | S | NH2  |

### FITC-conjugated peptides

Appendix B (human islet donors)

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