* **Peptide-based multi-agonists: a new paradigm in metabolic pharmacology**
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* **Abstract**
* Obesity and its comorbidities, such as type 2 diabetes, are pressing worldwide health concerns. Available anti-obesity treatments include weight loss pharmacotherapies and bariatric surgery. While surgical interventions typically result in significant and sustained weight loss, available pharmacotherapies are far less effective, typically decreasing body weight by no more than 5 – 10%. An emerging class of multiagonist drugs may eventually bridge this gap. This new class of specially-tailored drugs hybridizes the amino acid sequences of key metabolic hormones into one single entity with enhanced potency and sustained action. Successful examples of this strategy include multiagonist drugs targeting the receptors for glucagon-like peptide-1 (GLP-1), glucagon, and the glucose-dependent insulinotropic polypeptide (GIP). Due to the simultaneous activity at several metabolically relevant receptors, these multiagonists offer improved body weight loss and glucose tolerance relative to their constituent monotherapies. Further advancing this concept, chimeras were generated that covalently link nuclear acting hormones such as estrogen, thyroid hormone (T3), or dexamethasone to peptide hormones such as GLP-1 or glucagon. The benefit of this strategy is to restrict the nuclear hormone action exclusively to cells expressing the peptide hormone receptor, thereby maximizing combinatorial metabolic efficacy of both drug constituents in the target cells while preventing the nuclear hormone cargo from entering and acting on cells devoid of the peptide hormone receptor, in which the nuclear hormone might have unwanted effects. Many of these multiagonists are in preclinical and clinical development, and may represent new and effective tools in the fight against obesity and its comorbidities.
* **Introduction**
* Obesity is a growing public health problem that imposes a large economic burden on our society. In 2015, 107.7 million children and 603.7 million adults worldwide were classified as obese [1]. Obesity is one of the most important and modifiable risk factors for the development of metabolic complications such as type 2 diabetes (T2D), cardiovascular diseases, and certain malignancies [2, 3]. Prevention and early treatment of excess body weight therefore serves as an important strategy to decrease the clinical and economic consequences of obesity. In line with this notion, weight loss of even 5 – 10% significantly improves impaired glucose tolerance in patients with T2D, decreases cardiovascular risk factors, lowers intra-abdominal and hepatic fat accumulation, improves β-cell function, and enhances insulin sensitivity in liver, muscle and adipose tissue [4-6].
* Conventional weight loss strategies built upon dietary interventions and exercise are failing to tackle the global obesity pandemic [7, 8]. In addition, most historically used weight loss pharmacotherapies display an unfavorable imbalance between efficacy and safety. For example, several quite effective anti-obesity drugs such as fenfluramine/phentermine (“Fen-Phen”) or rimonabant have been withdrawn from the market due to unacceptable adverse effects [9, 10]. Currently approved drugs for weight management, such as the gastric and pancreatic lipase inhibitor orlistat [11, 12], the serotonin receptor agonist lorcaserin [13-15], or the combination of the opioid antagonist naltrexone with the antidepressant bupropion [16-18] cause only moderate weight reductions. So far, the best weight lowering effect by pharmacotherapies (approximately 7% body weight loss from baseline) is achieved by the injectable glucagon-like peptide-1 mimetic Saxenda® (liraglutide, 3mg) [19], which is discussed later in this review.
* Currently the most effective anti-obesity therapy is a group of bariatric surgeries, including Roux-en-Y gastric bypass (RYGB), vertical sleeve gastrectomy (VSG), and biliopancreatic diversion. In contrast to the existing pharmacotherapies, bariatric surgery causes profound and sustained weight loss of 13–27 % in severely obese patients (BMI > 35-40 kg/m2), with follow-up for as many as 15 years [20]. It can further ameliorate the majority of obesity-related comorbidities, including a full remission from T2D in approximately 80% of the patients [21-23]. The anti-diabetic mechanisms are weight independent, which has prompted considerations of applying these surgeries to T2D patients with only mild (stage 1) obesity (BMI 30-35 kg/m2) [24]. Initial studies where RYGB surgery was applied to diabetic patients with stage 1 obesity indeed revealed significant but inconsistent remission rates between 25% and 88% [25, 26].
* Despite the advantages of bariatric surgery, the highly invasive and irreversible nature of the surgeries, the underlying financial costs, and the risk for severe adverse outcomes such as dumping syndrome, postprandial hyperinsulinemic hypoglycemia, and the long-term risk of micronutrient deficiencies prevent the use of bariatric surgery as a widespread tool to tackle obesity and its comorbid sequale.
* Novel pharmacotherapies aim to mimic the complex and multi-target beneficial effects of bariatric surgery on body weight and glycemic control. Extensive research aims to uncover the molecular mechanisms that are driving the body weight and blood glucose lowering effects following the surgical interventions. It is now appreciated that that the success of bariatric surgery is not solely due to mechanical aspects such as restriction in food intake and malabsorption but also involves physiological effects including altered gastrointestinal hormone secretion [27]. One of the most significant hormonal changes after bariatric surgery is the marked postprandial elevation of circulating glucagon-like peptide 1 (GLP-1), a powerful insulinotropic and anorectic hormone [28-30]. Although data from GLP-1 receptor (GLP-1R) knock out mice suggest that enhanced endogenous GLP-1 action is not the only driver for the metabolic benefits of bariatric surgery [31], it has been proposed that either more potent GLP-1 analogues or the combination of GLP-1 with other peptide hormones could serve as putative superior therapeutics for obesity and T2D. In line with this notion, the pharmacological inhibition of either GLP-1 or PYY after RYGB does not affect food intake. However, when both GLP-1 and PYY are blocked together, food intake is increased in patients with RYGB by as much as 20% [32]. Together, these data suggest that GLP-1, when acting in concert with other gut hormones, may play a causal role in the metabolic effects of bariatric surgery, and this has inspired the development of several GLP-1 analogues and GLP-1 combination therapies, as discussed in this review.
* **The endogenous GLP-1 system**
* GLP-1 is a member of the glucagon peptide family. Together with at least four other bioactive peptides, including GLP-2, glucagon, oxyntomodulin (OXM) and glicentin, it is derived from the proglucagon gene, which is expressed in the alpha-cells of the endocrine pancreas, the L-cells of the intestine, and neurons of the caudal brainstem and hypothalamus [33, 34]. The proglucagon mRNA is translated into a 180 amino acid precursor protein and postranslationally processed by cell type specific prohormone convertase enzymes resulting in different organ-specific peptide profiles [35]. During the fasting and interprandial state, low levels of bioactive GLP-1(7-37) and GLP-1(7-36) amide are continuously secreted from the intestinal cells into the circulation. Following food intake, the secretion is rapidly increased and circulating GLP-1 levels rise by several fold [36]. The receptor for GLP-1, a class B G-protein coupled receptor, was originally cloned from pancreatic β-cells [37]. The lack of specific antibodies against the GLP-1 receptor (GLP-1R) hampered several attempts to identify the cellular targets of GLP-1 action. By crossing *glp1r*-cre mice and fluorescent reporter strains resulted in an antibody- independent method for the identification of GLP-1R expressing organs [38]. This model revealed major expression sites of GLP-1R expression in pancreatic β- and δ-cells, vascular smooth muscle, cardiac atrium, gastric antrum/pylorus, enteric neurons, and vagal and dorsal root ganglia [38]. In the murine central nervous system*,* GLP-1R expression was evident in the circumventricular organs, amygdala, arcuate nucleus, paraventricular nucleus, and ventromedial hypothalamus and the ventrolateral medulla [38, 39].
* The best-described target organs for GLP-1 mediated biological actions are the pancreas, the gastrointestinal- and the central and peripheral nervous systems [34].
* In pancreatic β-cells GLP-1 receptor agonism stimulates glucose-dependent insulin secretion [29, 40-43]. GLP-1 further induces insulin biosynthesis [43] and promotes β-cell proliferation and survival in rodents [44-47]. GLP-1 also suppresses glucagon secretion from α-cells [48, 49]. Single cell RNA sequencing revealed only very low levels of GLP-1R expression α-cells [50], suggesting that the inhibitory effect of GLP-1 on glucagon secretion occurs indirectly. One possible mechanism involves the binding of GLP-1 to its receptor on pancreatic δ-cells and the subsequent release of somatostatin, which in turn inhibits the release of glucagon from somatostatin receptor 2 (SSTR2) expressing α-cells. Evidence comes for instance from an experiment in isolated perfused rat pancreas, where co-infusion with a SSTR2 antagonist (PRL-2903) completely abolished the GLP-1-induced suppression of glucagon secretion [51]. GLP-1 may inhibit glucagon secretion through β-cell-derived products, such as insulin, GABA, zinc or amylin [52].
* In the gastrointestinal system, GLP-1 receptor agonism exhibits a potent inhibitory effect on gastric emptying that attenuates the meal associated increase in blood glucose [53, 54].
* Besides its glucometabolic effects, additional observations suggest that GLP-1 is also relevant for appetite regulation and weight maintenance. In rats, central administration of GLP-1 analogues causes a dose-dependent, albeit short-lived reduction in food intake, independent of the presence of food in the stomach or gastric emptying [55, 56]. Similarly, peripheral administration of GLP-1 affects the regulation of feeding [57, 58]. These and other findings suggest a synergistic action of GLP-1 on both central and peripheral receptors in the regulation of satiety with the ultimate result of promoting weight loss. A more recent study investigated the GLP-1 analog liraglutide in mice deficient in GLP-1R expression in either the vagal afferent/effect nerves or the central nervous system [59]. In this study, deletion of GLP-1R signaling in the central nervous system (CNS) ablates the action of the peripherally administered liraglutide on food intake and body weight, whereas deletion of GLP-1R signaling in the peripheral nervous system does not.
* Additional GLP-1 mediated metabolic effects include the inhibition of hepatic gluconeogenesis and subsequent glucose output, an effect potentially mediated via GLP-1’s ability to decrease glucagon secretion [60-62]. In the skeletal muscle, GLP-1 enhances glucose uptake [63] and glycogenesis [64].
* All of these findings support GLP-1-based therapies for the effective treatment of obesity and T2D. However, the clinical applicability of native GLP-1 as an anti-diabetic and anti-obesity therapy is limited by its short circulating half-life of 1-2 minutes in humans, which results from its deactivation by endopeptidase dipeptidyl peptidase-4 (DPP-4) and neutral endopeptidase 24.11 (NEP 24.11), also known as neprilysin [65-68]. Pharmacological inhibitors of DPP-4 have been developed with the aim to enhance the biological activity of endogenous GLP-1 [69]. Since 2006, several DPP-4 inhibitors including sitagliptin, saxagliptin, and linagliptin have been approved for clinical use. However, when administered as monotherapies, DPP-4 inhibitors are weight neutral. Moreover, glycated hemoglobin A1C (HbA1C), a surrogate measure that reflects glycemic exposure over the erythrocyte life time and current gold standard in assessment of metabolic control [70] is only modestly decreased (typically between 0.5 - 1%) after DPP-4 inhibitor treatment [71]. This suggests that supraphysiological levels of active GLP-1 are required to achieve a body weight lowering effect and further improvements in glycemic control. Pharmacological GLP-1 agonists can overcome this limitation. They are chemically modified in order to enhance the stability and optimize pharmacokinetics to achieve a prolonged half-life compared to the endogenous GLP-1. This maximizes efficacy at low concentrations reduces the dosing frequency to improve patient convenience.
* **From gila monster venom to GLP-1 analogues**
* In 1992 a systematic investigation of the composition of the salivary secretions from the Gila monster (*Heloderma suspectum*) by John Eng and colleagues revealed a 39-amino acid peptide designated as exendin-4. Exendin-4 only shares 53% sequence homology with bioactive GLP-1(7-36) and is therefore considered a GLP-1 paralogue **(Figure 1)**, as opposed to a synthetic analog of the type discussed throughout this review. Similar to GLP-1, exendin-4 is a α-helical peptide that interacts with the GLP-1 receptor, albeit with a much higher binding affinity [72]. The enhanced stability of exendin-4 results from a Leu21–Ser39 span, which builds a compact tertiary structure (“Trp cage”) that protects Trp25, Leu26 and Lys27 from aqueous solvent exposure and supports stabilization of secondary structure upon receptor binding [73, 74]. The receptor binding affinity of exendin-4 binding is further enhanced by a nine-residue extension at the C-terminal extension (CEX) of exendin-4 [75], **Figure 1**.
* The discovery of exendin-4 has led to its experimental and clinical evaluation as an anti-diabetic agent. In 2005, the first synthetic version of exendin-4, ***exenatide BDI*** ***(ByettaTM from Amylin, now BMS)*** was approved for distribution and broad patient use. It has a prolonged half-life of 2.4 hours, which results from the chemical benefits of the C-terminal Trp-cage and an additional alanine- to glycine exchange at position two of the peptide, which increases resistance to DPP-4 mediated degradation [76].
* The efficacy and safety of exenatide BDI have been evaluated in the AMIGO phase III clinical trials, where metformin, sulfonylurea or a combination of metformin and sulfonylurea at maximal effective doses were combined with either 5 μg or 10 μg of exenatide or placebo treatment. All studies were multicenter, randomized and triple blinded studies that enrolled more than 1.400 T2D patients with inadequate glycemic control by metformin, sulphonylurea or the combination thereof. As a primary outcome, the addition of exenatide to any of the three conventional treatments resulted in a more significant reduction of HbA1C from baseline to week 30 compared to the matching placebo groups [77, 78]. The most common side effect of exenatide, nausea, was dose dependent and resulted in a drop out rate in the study of 1.8 - 4.0% [79]. Along with the improvements in glycemic control, exenatide treatment caused a significant but moderate body weight reduction following 26 weeks of treatment with 10 μg exenatide twice daily [80].
* ***Lixisenatide (LyxumiaTM/AdlyxinTM, Sanofi/Zealand)*** is a second synthetic analogue of exendin-4, which has been modified by extending the C-terminus of native exendin-4 to possess 6 lysine residues with deletion of one C-terminal proline. The additional chemical modification slightly increased its half-life to 3-4 hours allowing for once daily subcutaneous administration, and, more importantly, it results in a four-times more potent GLP-1 receptor binding [81]. Clinical efficacy of lixisenatide compared to exenatide BDI has been assessed in the 24-weeks GetGoal-X trial (Table 1), a randomized open label actively controlled study in T2D patients that were inadequately controlled by metformin therapy [82]. In this study, add-on lixisenatide (20 µg) demonstrated noninferior improvements in HbA1c, with slightly lower mean weight loss, but better gastrointestinal tolerability and lower incidence of hypoglycemia compared with twice daily exenatide (10 µg). Lixisenatide was approved by the European Commission in 2013 and received FDA approval in 2016.
* Despite the stabilization against DPP-4 mediated degradation, the half-life of GLP-1 analogues is still very short, due to its rapid renal clearance. One strategy to increase the plasma half-life was the development of slow release preparations, such as ***exenatide LAR (BydureonTM, Amylin, now BMS)****.* In this preparation, exenatide is persistently and slowly released from poly(D,L-lactide-co-glycolide) forming microspheres [83]. This strategy increases the median plasma half-life from a few hours to 2 weeks. Approved in 2011, exenatide LAR represents the first registered, once-weekly injectable drug against hyperglycemia. The DURATION-1 clinical trial (Table 1) compared the once weekly exenatide LAR (2 mg) with the twice-daily exenatide BDI (10 µg). After 30 weeks of treatment, the once weekly formulation resulted in a significantly greater reduction of the HbA1C compared to the twice-daily formulation, while the body weight reduction remained similar between both groups [84].
* Other strategies to improve peptide pharmacokinetics favored the conjugation of GLP-1 analogues to long-chain fatty acids in order to achieve enhanced albumin binding and to hinder renal clearance. One example is ***liraglutide* *(VictozaTM or SaxendaTM Novo Nordisk)***. Liraglutide lacks the alanine- to glycine exchange as seen in exendin-4 based GLP-1 analogues. Instead, an arginine residue replaces a lysine residue at position 28 and an additional glycine at position 31. Another lysine at position 20 is conjugated to a C16 palmitic acid via a gamma, glutamic acid spacer [85].
* These chemical modifications lead to a self-association of the peptide into a heptameric structure, which delays the absorption from the injection site. In the bloodstream, extensive binding to albumin reduces its susceptibility to DPP-4 and NEP mediated cleavage, resulting in significant reduction in renal clearance. Liragutide has a plasma half life of 13 hours [86]. In the LEAD-6 clinical trial (Table 1), a head-to-head comparison of once daily liraglutide (1.8mg) and twice-daily exenatide BDI (10 µg) added to a background treatment of metformin, sulphonylurea or a combination of both, liraglutide demonstrated a statistically significantly greater decrease in hemoglobin HbA1C than exenatide BDI [87]. Similarly, in the DURATION-6 trial, once weekly exenatide LAR resulted in improvements in glycaemic control, with greater reductions as achieved with daily liraglutide [88], (Table 1). Liraglutide is now prescribed under two different brand names. *VictozaTM* (FDA approval in 2010) is available in 1.2 mg and 1.8 mg doses and leads to an average HbA1c reduction of approximately 1.6% [89]. It is marketed for the treatment of type 2 diabetes but has only a subtle effect on body weight at these concentrations. Saxenda*TM* (FDA approval in 2017) comes in a 3 mg doseand has been FDA approved for the treatment of obesity. In a clinical trial, Saxenda *TM* resulted in average body weight loss of 8.5 kg over the course of the 56 week study with mild or moderate nausea and diarrhea being the most reported side effect [19].
* A similar strategy to prolong the half life of a peptide is its direct conjugation to recombinant albumin. In ***albiglutide* *(TanzeumTM, GlaxoSmithKline)*** two copies of the GLP-1(7-37) peptide are fused as a tandem repeat to the N-terminus of recombinant albumin. A single alanine to glycine exchange at the DPP-4 cleavage site increased resistance to DPP-4 mediated cleavage. Albiglutide has a half-life of 6-8 days [90] and is administered once weekly at doses of 30–50 mg. However, as shown by the HARMONY- 7 trial (Table 1), the average HbA1c reduction and weight lowering effects of albiglutide were less when compared to liraglutide, thus not meeting the noninferiority criteria [91]. In August 2017 and only three years after its FDA approval, GlaxoSmithKline announced that albiglutide will be withdrawn from market by July 2018 for economic reasons.
* Other recombinant GLP-1 fusion peptides have been developed. ***Dulaglutide (TrulicityTM, Eli Lilly and Company, FDA approval in 2014)*** is a long-acting GLP-1 analogue in which a GLP-1(7-37) analog has been covalently linked to each Fc arm of human immunoglobulin G4 (IgG4) to form a dimeric agonist, with the goal to prolong plasma circulation. Additional amino acid substitutions were made to increase resistance to DPP-4 mediated clearance (alanine to glycine at position 2). An exchange of Gly16 with Glu enhanced the secondary structure and potency of the peptide and an Arg30 to Gly exchange further enhances stability [85]. Overall this led to an increased half-life of dulaglutide of approximately 4 days. In the AWARD-6 clinical trial (Table 1), dulaglutide (1.5mg once a week) met the predefined non-inferiority criteria by causing a significantly greater reduction of HbA1c compared to liraglutide (1.8 mg once daily). However, weight reduction was significantly greater in the liraglutide treatment compared to the dulaglutide group, while the adverse side effects were comparable [92].
* At present, liraglutide seems to be one of the most effective anti-glycemic and weight-lowering GLP-1 analogues. In December 2017, a next-generation liraglutide variant, named ***semaglutide (OzempicTM, NovoNordisk)*** was approved for commercial distribution. This chemically optimized version of liraglutide includes two modifications. A glycine in position 2 is replaced by the non-natural amino acid aminoisobutyric acid (Aib) to increase resistance to degradation by DPP-4, and other serine proteases. The C16 fatty - acid side chain conjugated to lysine at position 20, as present in liraglutide, has been exchanged with a dicarboxlic-stearic acid (C18:0) and a lengthier molecular spacer [85]. Both modifications further increase the half-life of the peptide in humans to 165 hours [93]. Further modifications relative to endogenous GLP-1 are the lysine to arginine exchange at position 28 and the addition of a glycine at position 31.
* The efficacy of once-weekly semaglutide has been assessed in clinical trials. In the SUSTAIN-1 trial, 30 weeks of a once per week semaglutide monotherapy resulted in a significant reduction of HbA1c of -1.43% (0.5 mg), and -1.53% (1.0 mg) compared to the placebo group. Simultaneously, in these T2D patients treatment with semaglutide was associated with a significant weight loss −3.73 kg (0.5 mg semaglutide group) and −4.53 kg (1.0 mg group) compared to placebo [94]. In an additional head-to-head clinical trial (SUSTAIN-7, Table 1) semaglutide was superior to dulaglutide in improving glycemic control and reducing body weight [95]. Other clinical trials are currently investigating oral vs. subcutaneous administration routes of semaglutide for the treatment of T2D.
* When comparing all head-to head clinical trials, daily liraglutide, particularly when used at the highest doses, still appears to be the best, verified HbA1C and weight reduction pharmacotherapy [96]. Liraglutide remains the clinical standard for future advances, of which daily semaglutide therapy has been purported, but yet to be peer-reviewed to deliver superior outcomes. While the GLP-1 analogue field is continuously growing there is still a need for optimization. At present gastrointestinal side effects such as nausea and gastrointestinal discomfort limit the tolerability of GLP-1 analogues and their applicability at higher and maximal efficient doses. In view of these limitations and the still unprecedented benefits of bariatric surgeries it was hypothesized that combining GLP-1 with other insulinotropic and/or anorectic peptide hormones could result in an enhanced efficacy and reduction of the dose limiting toxicities. Intense efforts have been made to develop GLP-1 based combination therapies, as discussed further below.
* **GLP-1 based combination therapies**
* Previous studies have investigated GLP-1 analogues in combination with other weight lowering drugs, such as PYY (3-36) [97-101], salmon calcitonin [102], leptin [103] or with an MC4R agonist (setmelanotide, RM-493) [104]. Although most studies demonstrated additive or synergistic effects of the drug combinations, the clinical application of these combinations is often challenging due to the different pharmacokinetic and pharmacodynamic profiles of the constituents, and the risk of unwanted drug-drug interactions. More recently, efforts have been made to combine two or more peptide hormones into one functional molecule, exerting only one pharmacokinetic profile and one targeted site of dual action, ideally resulting in a synergistic or complementary pharmacological action. The relative potency at each hormone receptor (either balanced or preferential) can be used to leverage efficacy and potency relative to unwanted dose dependent side effects.Two different strategies have been used for the development of GLP-1 based unimolecular therapies: (A) Fusion molecules, where GLP-1 is appended with another mono-agonist to form a bivalent molecule, and (B) hybrid molecules with comparable size to the native peptides [105]. The resulting molecules are summarized below.
* **GLP-1/ Glucagon:** Originally,glucagon was discovered as a peptide hormone with counter-regulatory effects to insulin [106]. Glucagon is secreted from pancreatic α-cells in response to hypoglycemia, and stimulates glycogenolysis and gluconeogenesis in the liver [85]. Patients with glucagonoma, a rare malignant tumor of the pancreatic α-cells, experience hypergluconemia and as a result exhibit diabetes-like symptoms such as severe hyperglycemia [107]. In contrast, postprandial glucose-mediated inhibition of glucagon secretion is impaired in patients with diabetes [108, 109]. Later studies demonstrated that blocking glucagon action through application of glucagon receptor antagonists or blocking antibodies significantly lowered fasting and postprandial glucose levels in different laboratory animal species [110-112], healthy subjects, and diabetic patients [113, 114]. Apart from its hyperglycemic actions, glucagon has several beneficial effects on energy and lipid metabolism **(Figure 2).** For instance, administration of glucagon lowers circulating levels of cholesterol in multiple species [115-118] and affects lipid metabolism through the inhibition of lipogenesis and stimulation of lipolysis [119-121]. In addition, glucagon is secreted during meals and acts in the central nervous system as a satiety signal to reduce food intake in humans [122-124] and rodents [125] [126]. Glucagon also stimulates energy expenditure and thermogenesis [127, 128], likely through the activation of brown adipose tissue (BAT) [128-130] and other, BAT-independent pathways [131]. The energy expenditure-stimulatory, hypolipidemic, and satiating effects suggest glucagon as an attractive therapy against obesity. Despite the plethora of positive effects, the acute hyperglycemic effect of glucagon argued against the pharmacological application of glucagon as an anti-obesity drug [52].
* Stemming from the same precursor protein, glucagon and GLP-1 exhibit considerable amino acid similarity. Like the peptides, the GLP-1 and glucagon receptors are closely related, with an overall sequence homology of 58% [37, 132]. Structure-function analyses using truncated GLP-1 and glucagon peptides revealed specific residues throughout the length of the peptide that are important for receptor binding and activation. In 1994, Hjorth et al., investigated a series of glucagon/GLP-1 chimeric peptides for their ability to bind and activate both receptors [133]. The study revealed that residues located at the opposite ends of both peptides determine the receptor selectivity. A chimera containing N-terminal residues of glucagon and C-terminal residues of GLP-1 had high affinity for both receptors, but has not been tested *in vivo.*
* In 2009 the research groups of Richard DiMarchi and Matthias Tschöp engineered a more complex dual GLP-1R/GCGR agonist by modifying the native glucagon sequence [134]. The authors hypothesized that dual agonism at both receptors would synergistically lower body weight by reducing food intake and stimulating energy expenditure, while the insulinotropic actions of GLP-1 would counter the hyperglycemic liability of glucagon **(Figure 3)**. Combined agonism was achieved by a stepwise introduction of GLP-1 residues into the glucagon backbone to bolster GLP-1R activity. GCGR activity was further enhanced by introduction of a lactam bridge between the glutamic acid at position 16 and the lysine at position 20, which stabilizes the alpha helix required for GCGR activation. An aminoisobutyric (Aib) acid at position 2 increased resistance to DPP-4 degradation, and a 40 kDa PEG attached via the cysteine at position 24 enhanced the pharmacokinetics of the molecule [85]. The result was a soluble and chemically stable glucagon-based peptide with nearly balanced activity at both the GLP-1R and GCGR, and only slightly diminished potency compared to the natural ligands [134].
* In DIO mice, a single high-dose injection of the PEGylated co-agonist (325 nmol/kg) induced a drop in body weight of 26% over the course of one week, primarily through the loss of fat mass, with an observed decrease in food intake. Longer-term treatment of DIO mice with lower doses (70 nmol/kg) of the same GLP-1R/GCGR agonist resulted in a weight loss comparable to the acute high-dose study. Notably, weight loss was associated with increased energy expenditure and thermogenesis, in line with glucagon’s thermogenic capabilities, while no differences were observed in food intake or locomotor activity [134]. The importance of the glucagon moiety for the body weight loss was demonstrated in mice lacking the GLP-1R, in which the dual agonist maintained a significant weight-lowering capacity. Importantly, the GLP-1R KO mice did not show the previously observed benefits in glucose tolerance, underlining the importance of GLP-1 agonism to regulate glucose metabolism [134]. In addition to these findings, GLP-1R/GCGR receptor dual agonism provided benefits in lipid metabolism, normalization of liver lipid contents [134] and restored leptin sensitivity in DIO mice chronically maintained on a 58% HFD [135]. The beneficial effects of single molecule GLP-1R/GCGR co-agonism were also demonstrated in Lepob/ob mice, where the co-agonist enhanced glucose stimulated insulin secretion and improved glucose tolerance [136].
* Recently, the efficacy of a similar dual agonist of the GLP-1 and glucagon receptors (MEDI0382) was tested in rodents and cynomolgus monkeys. When compared to matched doses of liraglutide, both compounds reduced blood glucose to similar extents. The key differentiator from liraglutide was the superior weight loss in both species [137]. MEDI0382 has entered Phase II trials, in which patients with controlled T2D received a once daily subcutaneous injection of the dual agonist (300 μg for 22 days or 200 µg for 41 days) or a placebo treatment. MEDI0382 treatment resulted in a significant reduction of the glucose area under curve (AUC) following a mixed meal tolerance test. The bodyweight reduction was significantly greater with MEDI0382 than with placebo, suggesting its potential as a disease-modifying therapy for T2D [138].
* Simultaneously to the development of the GLP-1/glucagon receptor co-agonist by Day et al., the research group of Pocai et al, developed a oxyntomodulin (OXM) analogue termed “Dual AG” [139]. Oxyntomodulin is a peptide hormone derived from proglucagon cleavage by PC1. Oxyntomodulin binds to both the GLP-1 and glucagon receptors, albeit with 10-100 fold reduction in potency compared to the native hormones [140-143]. The OXM analogue “Dual AG” includes an amino acid exchange at position 2, which increases resistance to DPP-4 cleavage. A cholesterol moiety is conjugated via a cysteine side chain at the C-terminus resulting in longer plasma retention. Daily subcutaneous injections of Dual AG in diet-induced obese (DIO) mice for 2 weeks lowered body weight by 25%, primarily through the loss of fat mass and a fractional decrease in food intake [139]. In addition, treatment with Dual AG also improved glucose tolerance, reduced plasma cholesterol and triglycerides, and decreased hepatic steatosis [139]. Receptor knock out studies show reduced efficacy when either GLP-1R or GCGR is knocked out, indicating that both GLP-1R and GCGR agonism contribute to the metabolic actions of Dual AG [139].
* Based on these observations, GLP-1R/GCGR dual agonists may become effective metabolic therapies. Several pharmaceutical companies are developing GLP1-R/GCGR dual agonists to treat diabetes and obesity, and many of these potential therapeutics have progressed to clinical trials [144].
* **GLP1/Amylin:** Amylin (AYM), also known as islet amyloid polypeptide (IAPP), is co-secreted with insulin from the secretory granules of the β-cells. In contrast to insulin, which stimulates peripheral glucose uptake, amylin’s glucose lowering effect is primarily mediated by suppressing pancreatic glucagon secretion. Amylin slows gastric emptying and promotes satiety, thus decreasing food intake [145] without causing food aversion [146]. Chronic amylin treatment elicits sustained weight loss in diet-induced obese rats and mice [147, 148].
* Davalintide, a stable amylin analogue with 49% homology to the native hormone [149], was shown to cause significantly enhanced weight loss in rodent models when compared to rat amylin [149]. To further enhance the weight lowering properties, the effects of incretin and amylin classes of therapeutic hormones were combined by ligating davalintide and the GLP-1 analogue exenatide 1-28 into a single chemical entity using either a Gly-Gly-Gly (AC164204) or a β-Ala-β-Ala (AC164209) spacer, creating two new, so-called “phybrids” [150]. In obese and diabetic Lepob/ob mice, infusion with either phybrid reduced blood glucose and HbA1c levels to a similar extent as detected in the exenatide treated group. However, both phybrids had a greater body weight lowering effect compared to exenatide or davalintide monotherapies. In DIO rats, both phybrids caused a dose-dependent reduction of food intake and body weight [150]. The phybrid effect exceeded the exenatide or davalintide monotherapies, but was equal to the co-infusion of both single hormones. In another approach the authors linked davalintide and exenatide 1-28 by a large intervening 40kD PEG spacer [151]. This phybrid provides enhanced glycemic control and weight loss in DIO rats and mice, along with a prolonged *in vivo* half-life of 27 hours, compared to a side-chain PEGylated phybrid [151].
* **GLP-1/CCK**: Cholecystokinin (CCK) and gastrin together constitute a family of structurally and functionally related peptide hormones. Both hormones share five terminal amino acids at the active carboxyl terminus (Gly-Trp-Met-Asp-Phe-NH2). CCK is released from enteroendocrine I-cells in the mucosal lining of the duodenum when fatty- and amino acids leave the stomach and enter the small intestine [152]. There are several different forms of CCK, with the octapeptide CCK8 being the most abundant in the brain [152]. This peptide has been implicated in satiety, as acute administration of CCK8 reduces meal size in rodents, although this effect is counterbalanced by an increase in the number of meals, which mitigates the initial meal size reduction [152].
* There are two CCK receptors, termed CCKA and CCKB, or more recently, CCK1 and CCK2. CCK1 is abundantly expressed in the brain areas mediating satiety, such as the solitary nucleus (NTS), area postrema (AP), and the dorsal medial hypothalamus (DMH). CCK1 mediates the inhibitory effects of CCK on food intake [152]. The CCK2 receptor, identical to the gastrin receptor, is also present in the CNS. Both gastrin and the C-terminal amidated form of CCK bind to this receptor [153]. Recently, the co-administration of CCK and a GLP-1R analogue resulted in synergistic weight loss in rodents [154, 155], paving the way for a stable (pGlu-Gln)-CCK-8/exendin-4 hybrid in which the key amino acid sequences of the well-characterized, stable, and specific CCK-8 and GLP-1 analogues (pGlu-Gln)-CCK-8 and exendin-4 were ligated through a (2-[2-aminoethoxy]ethoxy)acetic acid linker [156]. The fusion peptide demonstrated decrease energy intake and lowering of body weight in NIH Swiss mice fed a high-fat diet, with metabolic improvements that were not seen with a matched dose of exendin-4 alone [156]. Compared to the monotherapies, the conjugate also improved glucose tolerance and insulin sensitivity [156]. In a recent study, another fusion peptide (C2816) comprised of a stabilized GLP-1R agonist (AC3174) and a CCKR1-selective agonist (AC170222) exerted a superior reduction in body weight compared to co-administration of AC3174 and AC170222 in DIO mice [157].
* **GLP-1/gastrin:** The structural and functional similarity between CCK-8 and gastrin naturally suggested the combination of GLP-1R and gastrin. Gastrin is synthesized by the G cells in the stomach and duodenum, is released in response to meal ingestion, and binds to the CCK2 receptor [153]. A dual agonist of the GLP-1 and CCK2 receptors, ZP3022, lowers body weight and improves glucose tolerance in male db/db mice [158]. ZP3022 also increases pancreatic β-cell mass, without increasing the number of pancreatic islets, while simultaneously increases insulin levels in these mice [158]. The exact mechanism of action remains unknown, but it is speculated that GLP-1 action on β-cells, in combination with indirect gastrin action, is responsible for the observed pancreatic islet expansion. In a more chronic, 8 week study in ZDF rats, ZP3022 significantly reduced body weight and blood glucose, and increased the pancreatic β-cell fraction compared to vehicle treated controls [159], suggesting that this peptide has potential as an anti-diabetic pharmacotherapy.
* **GLP-1 / GIP:** The glucagon backbone was used as a template to generate another hybrid peptide with dual agonism at the receptors for GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), **Figure 3** [134]. GIP is another member of the glucagon peptide family. It is derived from a 153-amino acid proprotein encoded by the GIP gene and is secreted from the intestinal K-cells in response to a meal, and promotes insulin secretion in a glucose-dependent manner [160, 161]. Beyond its insulinotropic action, GIP also stimulates the release of glucagon under conditions of hypoglycemia [162], **Figure 2**. Therefore, as a bifunctional hormone, it may be doubly capable of stabilizing blood glucose levels [163, 164]. Despite these clear glycemic benefits, the application of GIP analogues as pharmacological targets against T2D has long been hindered by its suggested role as an obesogenic, lipogenic and adipogenic peptide in rodents and humans [165-169]. However, more recent studies show that overexpression of GIP as well as GIP agonism improved glucose metabolism in DIO mice, without detrimental effects on body weight [170, 171]. Similarly, transgenic pigs expressing a dominant-negative GIP receptor in pancreatic islets developed a diabetic phenotype without apparent changes in body weight [172]. In rats, central delivery of GIP even decreased the body weight compared to the vehicle injected control animals [173].
* Since both GLP-1 and GIP are insulinotropic, the combination of GLP-1R and GIPR agonism was hypothesized to result in additive or even synergistic effects on insulin secretion and glucose tolerance **(Figure 3)**. Moreover, the anorectic effect of GLP-1 could buffer the alleged obesogenic effect of GIP. Indeed, the combination of liraglutide and an acylated GIP was more potent at lowering blood glucose and stimulating insulin secretion in leptin-deficient Lepob/ob mice than the single compounds [174]. Similarly, in healthy human volunteers, co-infusions of synthetic GLP-1 and GIP analogues additively increased the insulinotropic action relative to the monotherapies [175]. Interestingly, in T2D patients, adding GIP to GLP-1 did not further enhance the insulinotropic activity of GLP-1 but antagonized the GLP-1 mediated suppression of glucagon [176].
* Although GLP-1 and GIP only share 37% of their amino acid sequence, their receptor binding domains are very similar. Therefore, while designing dual agonists with affinity for both incretin receptors is possible, it is much more challenging than what was initially achieved with GLP and glucagon. Recently, a series of unimolecular GLP-1/GIP peptides has been developed to achieve potent and balanced co-agonism at both receptors with negligible cross-reactivity at the glucagon receptor [177]. In one example, amino acids were introduced stepwise to the glucagon sequence to impart GLP-1R and GIPR activity. To extend its in vivo activity and plasma half life, an Aib residue at position 2 increased resistance to DPP-4 degradation, and the nine amino acid CEX extension provided additional stability and aqueous solubility. To prevent unwanted GCGR activity, an additional Aib residue was incorporated at position 20, which partially stabilizes the secondary structure of the molecule and minimizes GCGR activity. Finally, a cysteine at position 24 or lysine at position 40 were included to serve as unique sites for for subsequent conjugation to fatty acyl, or PEG polymers. While both peptide versions demonstrated balanced receptor activities, acetylation at Lys40 resulted in slightly increased receptor potency and PEGylation at Cys24 diminished receptor potencies when compared to the natural peptide hormones. In DIO and leptin-receptor deficient db/db mice, the fatty acylated and PEGylated versions of the co-agonist resulted in superior anti-hyperglycemic and insulinotropic efficacy, with profound body weight lowering relative to a pharmacokinetically-matched GLP-1 monoagonist, such as exendin-4 and liraglutide [177]. Notably, the apparent safety and insulinotropic efficacy of the acylated version of the unimolecular GLP-1/GIP co-agonist translated from rodent models of obesity to cynomolgus monkeys [177]. The same compound (formerly NNC0090-2746, now RG7697) was also tested in human patients with type 2 diabetes on a metformin background therapy using a dose comparable to liraglutide [178]. Daily subcutaneous injections of 1.8 mg of the co-agonist for 12 weeks decreased HbA1c by 0.96 % and fasting glucose by 38.2% relative to placebo, and reduced body weight in an absolute sense by nearly 3% over the twelve-week trial [178]. Notably, RG7697 significantly decreased total cholesterol, compared to the placebo group, whereas liraglutide alone had no effect, suggesting an additional benefit of the dual GLP-1/ GIP receptor agonist. Other independently derived GLP-1R/GIPR co-agonists are in development and are being investigated in preclinical and phase 2 clinical trials [144].
* **GLP-1 / Glucagon / GIP:** The success of the incretin co-agonists naturally led to the hypothesis that a triagonist, with agonism at both incretin hormone receptors and the glucagon receptor, would result in even more effective metabolic improvements **(Figure 3)**. Optimally, the glycemic benefits arising from both incretin hormones would oppose glucagon’s diabetogenic actions, while the weight lowering properties of GLP-1 and glucagon would synergistically suppress any potential obesogenic character residing in GIP agonism.
* In 2013, three different peptides with triple agonism at the GLP-1R, GCGR and GIP-R were developed. The first example replaces the initial 11 N-terminal residues of OXM with D-Ala-GIP to generate a GIP-OXM peptide ([DA2]GIP-Oxm) [179]. A second triagonist, [DA2]GLP-1-glucagon ([DA2]GLP-1/GcG), was created as a fusion of key amino acid sequences from GLP-1, GIP, and glucagon [136]. The third triagonist, YAG-glucagon, was derived by manipulating the glucagon peptide sequence [180]. All three peptides stimulated cAMP production in GIP-R, GCGR and GLP-1R transfected cells to a comparable or lesser extent than the native peptides, demonstrating triple agonism *in vitro*. *In vivo*, all peptides significantly reduced glycaemia, while only [DA2]GIP-Oxm and [DA2]GLP-1/GcG significantly decreased body weight. Since both GLP-1R and GCGR agonism results in body weight loss, the inability of YAG-glucagon to lower body weight may be a result of unbalanced agonism towards the GIP receptor.
* Similarly, in 2015, the research groups of Matthias Tschöp and Richard DiMarchi developed another novel triagonist, beginning with a validated GLP-1R/GIPR co-agonist and introducing amino acids known to confer GCGR agonism in a stepwise fashion [181]. Further modifications included an Aib at position 2 to increase resistance to DPP-4 degradation, a lysine at position 10 (Lys10) as an attachment site for a palmitic acid, and the CEX extension to improve the solubility of the peptide [181]. The triagonist displayed full GLP-1R agonism in pancreatic mouse β-cells (MIN6), full GIPR activity in mouse 3T3-L1 adipocytes, and full GCGR activity in rat hepatocytes [181]. In addition, compared to [DA2]GLP-1/GcG, this triagonist was at least 1,000-fold more potent at all three receptors *in vitro* [181]. In DIO mice, daily treatment at 3 nmol/kg of this triagonist lowered body weight by 26.6% over a 20 day period, compared to only 15.7% loss with a dose matched GLP-1/GIP co-agonist. Body weight loss was mainly the result of loss in fat mass and not lean mass. The triagonist induced superior glycemic control and reduction in hepatic lipid content, all greater than a matched dose of liraglutide [181]. These effects are not gender-specific, as similar reductions in body weight and hepatic steatosis were observed in female DIO mice [181, 182]. Compared to [DA2]GLP-1/GcG, this triagonist induces body weight loss and metabolic improvements at much lower doses.
* Chronic treatment with this triagonist in lean mice resulted in no reduction of body weight, lean mass, or food intake [181], suggesting that the triagonist does not impair normal metabolism and only acts to improve metabolic dysregulation. Moreover, there were no instances of hypoglycemia in either DIO or lean mice treated with the triagonist, demonstrating that the hypoglycemic liability of glucagon receptor agonism is safely managed. The triagonist preserved pancreatic islet architecture in ZDF rats and db/db mice, suggesting that the triagonist has potential as both an anti-obesity and an anti-diabetic therapy [181]. Meanwhile, several triagonist peptides have entered pre-clinical trials [144]. First results are published for the compound HM15211, developed by Hanmi Pharmaceuticals, a triple agonist based on a modified glucagon analogue with activity at all three receptors [183]. This triple-agonist is modified with a human glycosylated Fc fragment to prolong the half-life. In rodent models, every other day treatment with HM15211 decreased body weight and glycaemia, while increasing energy expenditure to a significantly greater extent than a daily administration of liraglutide. In addition, HM15211 reduces hepatic steatosis and plasma cholesterol in a mouse model of NASH, indicating therapeutic potential beyond weight loss and glycemic control [183]. HM15211 is currently being investigated in phase 1 clinical trials.
* In summary, the triagonists developed so far have demonstrated unmatched pre-clinical efficacy in improving metabolic dysregulation that may recapitulate many benefits of bariatric surgeries. Moreover, based on the multi-organ receptor expression, the triagonists could have great potential to treat a number of other diseases. Indeed first results have demonstrated that the triagonist HM15211 exerted neuroprotective effects against Parkinson’s disease by reducing microglia activation [184] and is effective against NASH [183].
* **GLP-1 based nuclear hormone delivery**
* Similar to the peptide hormones reviewed to this point, certain nuclear hormones such as estrogen, thyroid hormone (T3) and dexamethasone are potent and beneficial modulators of energy metabolism [185-188]. However, their medical utility is restricted due to notable unwanted side-effects. A novel approach to avoid an impact on off-target tissues was to covalently link nuclear hormones to peptide hormones such as GLP-1 and glucagon. Peptide hormones promote their biological action via binding and activation of receptors located on the cell surface, followed by internalization of the ligand-receptor complex and activation of downstream signaling pathways. In the context of metabolic therapy, GLP-1 is an ideal nuclear hormone conjugation partner since GLP-1 targets mainly the endocrine pancreas and central nervous system, thus potentially delivering nuclear hormones preferentially to these tissues. So far GLP-1 has been conjugated to estrogen [189] and dexamethasone [190] **(Figure 4)**. In addition, T3 has been conjugated to glucagon [191] **(Figure 4)**.Conjugation to GLP-1 has resulted in targeted benefits of the respective nuclear hormones. Treatment of male and female DIO mice with a stable GLP-1/estrogen conjugate induced synergistic weight loss and metabolic improvements, which were dependent on the presence of the GLP-1R in the central nervous system [189]. The estrogen effect of the conjugate was limited to GLP-1R expressing tissues and did not cause any estrogen-related gynecological or tumor promoting effects in tumor-bearing mice, nor did it affect bone mineral density.
* A conjugate of GLP-1 and dexamethasone utilizes the anti-inflammatory properties of dexamethasone to target the chronic, low-grade inflammation that is typically observed under conditions of obesity [192, 193]. Unaltered dexamethasone induces hyperglycemia, hyperphagia, and reduces bone density [190]. In mice, a GLP-1/dexamethasone conjugate at a dose of 100 nmol/kg for two weeks reduced food intake and induced a 25% body weight loss, relative to baseline, predominately a result of loss in fat mass. The metabolic benefits of the conjugate are due in part to an increase in energy expenditure, since the conjugate increases oxygen consumption, reduces the respiratory exchange ratio (RER), and induces greater body weight loss relative to pair-fed controls. In addition, the anti-inflammatory action of dexamethasone was apparent both in the hypothalamus and in plasma, where the conjugate reduced cytokine levels and other markers of inflammation. The lack of GLP-1 receptors in the liver precluded dexamethasone related effects on hepatic glucose output and hyperglycemia. Nevertheless, the conjugate improved glucose tolerance and increased glucose stimulated insulin secretion, indicating positive glycemic effects. In addition, the conjugate does not appear to affect bone density, as whole-body and spine bone mineral density were unaltered by treatment [190].
* Another approach for targeted nuclear hormone delivery was the covalent binding of T3 to glucagon. Since GCGR expression is largely restricted to the liver, this hybrid molecule was designed to accentuate the hepatic effects of T3, which include clearing of circulating LDL via stimulation of reverse cholesterol transport and enhanced production of bile acids. Abnormally high cholesterol and dyslipidemia is a major health concern, and dyslipidemia is often associated with type 2 diabetes, coronary heart disease, and non-alcoholic steatohepatitis (NASH) [194]. Most dyslipidemia drugs, such as statins, lower cholesterol but do not affect body weight. It was thus hypothesized that glucagon-directed hepatic T3 action would synergistically improve hepatic lipid and cholesterol metabolism and simultaneously counteract the hyperglycemic actions of glucagon, and that targeted delivery to glucagon receptor expressing cells would assure that T3 does not reach and act on tissues such as the heart, skeletal muscle, and bone to cause unwanted side-effects such as cardiotoxicity.
* Indeed, in DIO mice, the conjugate lowered blood glucose, improved glucose tolerance, and dose-dependently prevented the development of hyperglycemia or glucose intolerance [191]. The glucagon/T3 conjugate moderately lowered body weight by reducing food intake and increasing energy expenditure. Moreover, in various mouse models of dyslipidemia, the glucagon/T3 conjugate lowered total plasma cholesterol and decreased circulating and hepatic levels of LDL. These mice also display lowered hepatic cholesterol and a decrease in hepatocellular vacuolation. These effects were lost in global GCGR knockout mice and in liver-specific thyroid hormone receptor-β knockout mice [191], demonstrating the necessity of both the glucagon receptor and thyroid hormone action in the liver. The glucagon/T3 conjugate was demonstrably safer than either of its individual components, with only limited T3 effects in heart and bone. The full magnitude of the improvements in the therapeutic index will nevertheless require more extensive studies, extension of these initial reports from rodents to primates, followed by refinement of the chemical structure to suit drug development with completion of preclinical safety studies supportive of translation to clinical study.
* **Conclusion**
* Combining the actions of multiple hormones into single molecular entities has resulted in multi-action agonists that display superior efficacy and safety when compared to the constituent monotherapies.
* Multi-agonism results in enhanced activity through synergistic agonism at multiple receptors. Many of these multi-agonists have proven more effective than either the monotherapies or the co-injection of the hormones. This enhanced activity allows for lower dosing strategies, which decreases the risk of dose-dependent adverse effects. In addition, a multi-agonist approach can overcome inherent liabilities of individual hormones, by targeting nuclear hormones to specific tissues and providing counter-regulatory buffering activity.
* The multi-agonist pharmacotherapies in this review constitute great translational potential and promising preclinical results are emerging. It is premature to conclude the magnitude of the pharmacology that might be achieved in human patients. However, it feels inevitable that within this broad set of agonists that function by multiple, differentiated mechanisms that a meaningful enhancement to the efficacy rendered by GLP-1 specific pharmacology should emerge through continued research and refinement of those entities that prove most effective.

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**Table 1: Head to head comparison of different types of glucagon-like peptide 1 receptor agonists**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Clinical trial program** | **Comparator 1** | **Comparator 2** | **Background Therapy** | **HbA1c reduction**  **(Comparator 1 vs. 2)** | **Body weight loss (Comparator 1 vs. 2)** | **Ref.** |
| GetGoal-X | Lixisenatide  (20 µg once daily) | Exenatide BDI  (10 µg twice daily) | Metformin | -0.80% vs. -0.96%  (95% CI 0.033 to 0.297) | -2.96 kg vs. -3.98 kg  (95% CI 0.45 to 1.58) | [82] |
| DURATION-1 | Exenatide LAR  (2 mg once weekly) | Exenatide BDI  (10 µg twice daily) | Naive, or one or more oral anti-diabetics | -1.9% vs. -1.5%  (p-value < 0.0023) | -3.7 kg vs. -3.6 kg  (p-value = 0.89) | [84] |
| LEAD-6 | Liraglutide  (1.8 mg once daily) | Exenatide BDI  (10 µg twice daily) | Metformin, Sulfonylurea or both | -1.12% vs. -0.79%  (p-value < 0.0001) | -3.24 kg vs. -2.84 kg  (p-value = 0.22) | [87] |
| DURATION-6 | Liraglutide  (1.8 mg once daily) | Exenatide LAR  (2 mg once weekly) | Metformin, Sulfonylurea or both or Metformin and pioglitazone | −1.48% vs. -1.28%  (p-value < 0.02 | −3.57 kg vs. −2.68 kg (p -value <  0.0005) | [88] |
| HARMONY-7 | Albiglutide  (50 mg once weekly) | Liraglutide  (1.8 mg once daily) | Metformin, pioglitazone, sulfonylurea or any combination thereof | -0.78% vs. -0.99%  (p-value < 0.0846) | -0.64 kg vs. -2.16 kg  (p-value < 0.001) | [91] |
| AWARD-6 | Liraglutide  (1.8 mg once daily) | Dulaglutide  (1.5 mg once weekly) | Metformin | -1.36% vs. -1.42%  (p-value < 0.0001) | -3.61 kg vs. -2.90 kg  (p-value = 0.011) | [92] |
| SUSTAIN-7 | Semaglutide  (1.0 mg once weekly) | Dulaglutide  (1.5 mg once weekly) | Metformin | -1.8% vs. -1.4%  (p-value < 0.0001) | -6.5 kg vs. 3.0 kg  (p-value < 0.0001) | [95] |

**Figure Captions**

**Figure 1:** Comparison of amino acid sequences for native human GLP-1 (left sequence) and exendin-4 (right sequence), which provide the basis for human GLP-1 analogues (Liraglutide, Semaglutide, Dulaglutide and Albiglutide) and exendin-4 derivatives (Exenatide, Lixisenatide).

**Figure 2:** Effects of GLP-1 (blue arrows), glucagon (red arrows) and GIP (green arrows) on energy metabolism in key metabolic tissues. Small arrows in boxes pointing upwards indicate an increase or improvement of the respective metabolic function, while arrows pointing downwards indicate a decrease.

**Figure 3:** Effects, working principles and target tissues of dual agonists GLP-1/glucagon (upper panel) and GLP-1/GIP (middle panel), and triple agonist GLP-1/GIP/glucagon (lower panel). The most predominant metabolic effects are indicated in bold letters.

**Figure 4: (A)** Schematic for thepeptide hormone-mediated delivery of small molecules and nuclear hormones via receptor internalization. **(B)** Metabolic effects and major target organs of GLP-1/estrogen, GLP-1/dexamethasone (GLP-1/Dexa), and glucagon/T3 hybrid molecules and bypass of established adverse side effects of estrogen, dexamethasone or T3 by their targeted delivery to GLP-1 receptor (GLP1-R) or glucagon receptor (GCGR) expressing organs (lower boxes).

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**Conflict of Interest**

S J B, T D M and K S declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review. R.D.D. are current employees of Novo Nordisk. R.D.D. is a cofounder of Marcadia, a company that pioneered the discovery of glucagon mixed agonists. It was acquired by Roche and later Novo Nordisk. M.H.T. is a scientific advisor for Novo Nordisk and Erx Biotech.