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REVIEW

Designing a Dual GLP-1R/GIPR Agonist from Tirzepatide: Comparing Residues Between Tirzepatide, GLP-1, and GIP

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Abstract: Improving type 2 diabetes using incretin analogues is becoming increasingly plausible. Currently, tirzepatide is the most promising listed incretin analogue. Here, I briefly explain the evolution of drugs of this kind, analyze the residue discrepancies between tirzepatide and endogenous incretins, summarize some existing strategies for prolonging half-life, and present suggestions for future research, mainly involving biased functions. This review aims to present some useful information for designing a dual glucagon like peptide-1 receptor/glucose-dependent insulinotropic polypeptide receptor agonist.

Keywords: tirzepatide, GLP-1, GIP, Aib, structure–activity relationship, structure–function relationship

Introduction

From GLP-1 Receptor Agonists to Tirzepatide

Incretins are hormones that are secreted from the gastrointestinal tract into the circulatory system in response to nutrient ingestion, enhancing glucose-stimulated insulin secretion. Incretins are estimated to account for approximately 50–70% of the total insulin secretion after oral glucose administration, and this has been dubbed the "incretin effect". To date, two incretins, glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), have been identified.[1]

GLP-1 receptor agonists are broadly applied in type 2 diabetes mellitus (T2DM) therapy. Exenatide is a non-human peptide analogue originally isolated from the saliva of the Gila monster (*Heloderma suspectum*). Compared to GLP-1, the second residue of exenatide is Gly rather than Ala; thus, it can circumvent the degradation of dipeptidyl peptidase-4 (DPP-4) and has a prolonged intravenous half-life of 30 min and a 2–3 h half-life after subcutaneous administration.[2]

Liraglutide is designed to bind to human albumin via a C16 fatty acid and a spacer covalently attached to Lys^{26}. A residue substitution of Lys^{34} to Arg^{34} occurs to avoid the fatty acid being installed in a wrong place.[3] Liraglutide has an intravenous half-life of 8–10 h and 13–15 h half-life after subcutaneous administration.[2]

Semaglutide converges these principles: the second residue is replaced by α-aminoisobutyric acid (Aib) to avoid DPP-4 degradation, Lys^{34} is replaced by Arg^{34}, and a C18 fatty acid is linked to Lys^{26} via a γGlu-2xOEG spacer (Figure 1) providing higher affinity to albumin.[2] The half-life of semaglutide after subcutaneous administration is up to 183 h.[4]

GIP plays an important role in the incretin effect in healthy people. Unfortunately, its druggability is low. Infusions that achieve supraphysiological GIP concentrations fail to elicit a significant insulin secretory response in patients with T2DMs; thus, GIP infusions cannot rapidly normalize the blood glucose levels of patients with T2DMs.[5] This blunted response is possibly caused by the downregulation of GIP receptors (GIPR) by the high level of circulating glucose.

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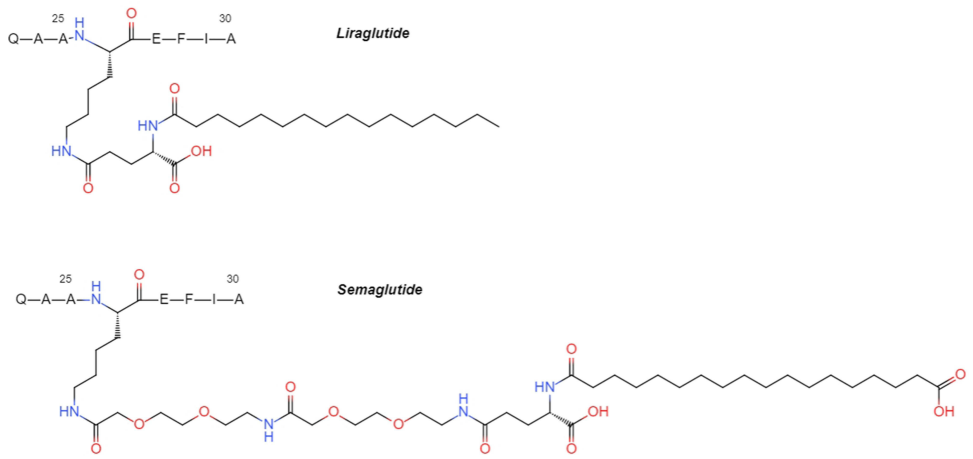

Figure 1 The structures of fatty acid side chains of liraglutide and semaglutide.

However, a substantial body of data suggests that GIP resistance can be largely overcome by agents that lower the circulating glucose level, paving the way for the consideration of GIP as an add-on to glucose-lowering therapies, like GLP-1.[6] Moreover, GIPR signaling blocks emesis and attenuates other negative side effects of GLP-1 receptor (GLP-1R) activation.[7]

Tirzepatide is a unimolecular, bifunctional peptide invented by Eli Lilly and Company (Indianapolis, IN, USA), which simultaneously activates GLP-1R and GIPR. Tirzepatide is composed of 39 amino acids, is amidated at the C-terminal, conjugates a C20 fatty diacid moiety via a spacer connected to Lys^{20}, and has a half-life of 116.7 h. Tirzepatide has a higher affinity with GLP-1R than GLP-1 does (pIC_{50}: 8.90 vs 8.62), and its cAMP activation activity is significantly lower (1–2 decrease in pEC_{50}). Tirzepatide has an obviously lower affinity with GIPR than GIP does (pIC_{50}: 6.70 vs 7.87), and its cAMP activity is approximately equal to that of GIP.[6,8]

To date, tirzepatide has achieved an unprecedented effect on improving Hemoglobin A1c (HbA1c) and body weight in T2DM patients. The drugs used in T2DM were systematically compared by Alan Maloney et al[9] (data updated on the website: https://www.comparediabetesdrugs.com): 15mg tirzepatide lowered HbA1c by 22.8 mmol/mol and weight by 10kg in patients, which were the best data ever. Another meta-analysis showed that HbA1c was reduced 2.1% and body weight 8.6kg with 15mg dose.[10]

To obtain dual activities, which seems to contribute the most in its illustrious clinical effects, tirzepatide not only fuses amino acid residue mainly from GLP-1 and GIP, but also uses some distinctive residues. In the next part, how tirzepatide is built is attempted to analyze based on existing reports.

Analysis of Tirzepatide Residues

Residues of tirzepatide are mainly from GLP-1, GIP and semaglutide and a few residues are unique (Figure 2). The contributions of each substitution are discussed in detail and mainly summarized in Table 1.

Tyr^1

GLP-1 (7–37) His^7 is crucial to the activation of GLP-1R. In vivo, GLP-1 is a substrate for DPP-4 and is rapidly metabolized to GLP-1 (9–37) or GLP-1 (9–36) NH_2 [1]. Among the series of analogues, including GLP-1 (1–37), GLP-1 (6–37), GLP-1 (7–37), GLP-1 (8–37), etc., only those which start with His^7 retain their insulinotropic activity.[11] GLP-1R Arg^{299} and Trp^{306} may interact with GLP-1 His^7.[12] Replacement of GLP-1 His^7 with Phe^7 does not influence either its receptor affinity or cAMP activity, indicating that the aromaticity of His is crucial to its activity. Replacement with Tyr^7 lowers both the affinity (IC_{50}: 0.26 nM→2.7 nM) and activity (EC_{50}: 0.8 nM→5.4 nM). Replacement with Trp^7 lowers the affinity (IC_{50}: 0.26 nM→3.3 nM) and strongly lowers the activity (EC_{50}: 0.8 nM→127 nM).[13]

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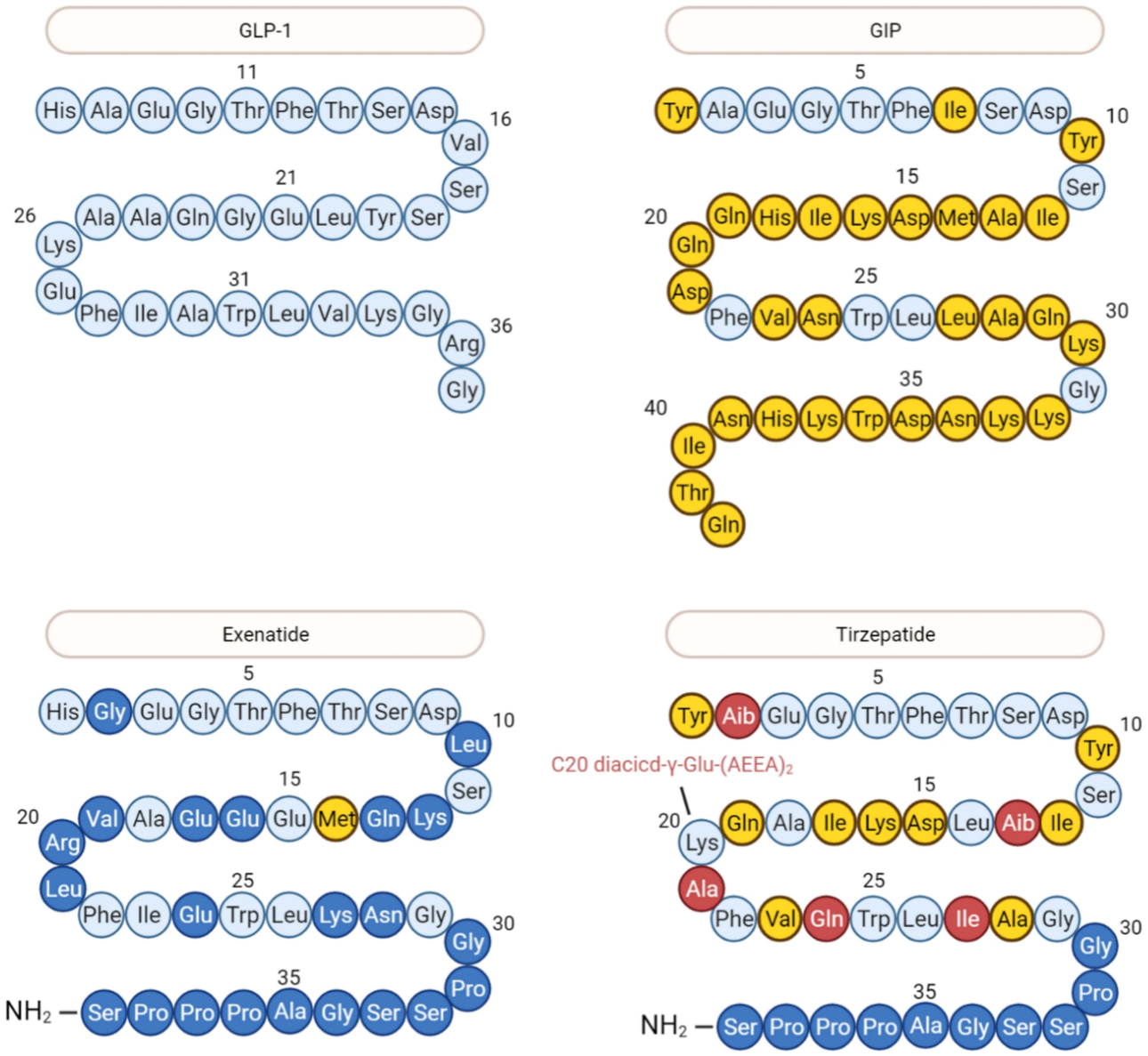

Figure 2 The structures of incretins and analogues. Different color indicates the derivation of each residue. Light blue: GLP-1; Yellow: GIP; dark blue: exenatide; Red: tirzepatide.

The absence of Tyr[1] from the N-terminal of GIP dramatically decreases its activity. GIPR Gln[224], Arg[300], and Phe[357] have been described to interact with GIP Tyr[1].[14]

The adoption of Tyr[1] by tirzepatide may impair its GLP-1 activity while supporting its GIP activity. There were two other dual agonists whose sequence has been reported so far, Tyr[1] has been used in MAR709 (designed by Finan et al in 2013, described by numerous other names: DA5-CH, DA-JC1, NNC0090-2746, RG7697, etc.) and His[1] has been used in CY-5 (Figure 3).[15,16] Additionally, Phe[1] exenatide shows stronger long-term insulin release (which is dependent on β-arrestin recruitment reduction), faster agonist dissociation rates and lower receptor internalization than exenatide.[17] Therefore, when designing a dual agonist, either His or Tyr can be chosen for position 1 by weighing the required GLP-1 and GIP activity, and Phe is also worth considering. Furthermore, several modifications of GIP Tyr[1] that facilitate its insulinotropic activity[18] may also be considered.

Ex. 2035-003

Table 1 Options of Residues in a GLP-1R/GIPR Dual Agonist

Position	Options		
	For GLP-1 Activity	**For GIP Activity**	**Other Plausible Options**
1	His↑, Tyr↓	His↓, Tyr↑	Phe (may reduce receptor internalization)
2	Aib↑, Gly↓	Aib↑, Gly↑, D-Ala↑	Ala (may be feasible in Fc/albumin fused protein)
3	-	-	Gln (is used in CY-5)
7	Thr↑	Thr↓, Ile↑ (will destroy GLP-1 activity)	-
10	Tyr↑	Tyr↑	-
12	-	Ile↑	Leu/Val (is similar to Ile), Lys (is used in exenatide)
13	Aib↓ (influence may be very little), Tyr↑	Aib↑	Gln (is used in exenatide)
14	Leu↑	-	-
15	-	Asp↑	Glu (is from GLP-1 and similar to Asp)
16	Lys↓	Lys↑, Ala↑	Gln, Glu (is used in exenatide), hGlu
17	Gln↑	Ile↑	-
18	Ala↑, Arg↑	Ala↑, Arg↑	-
19	-	Gln↑	-
20	Lys↑	-	Arg (is used in exenatide)
21	Ala↑, Glu↑	Ala↓, Asp↑	Leu (is used in exenatide)/Ile/Val
24	Gln↑	Gln↑	-

Note: ↑ indicates this amino acid would increase activity if is chosen at the position; ↓ indicates decreasing; - means data is lacking.

Aib[2]

GLP-1 (7–37) Ala[8] and GIP Ala[2] are DPP-4 cleavage sites and many studies have attempted to reduce this degradation by substituting or modifying the first three residues. The findings suggest that substitution with Aib or Gly can fully prevent DPP-4 degradation. The affinity, cAMP activity, and insulinotropic activity in vitro all decline when Ala[8] is substituted with Gly[8] (IC_{50}: 44.9 nM→220 nM;EC_{50}: 0.15 nM→1.11 nM).[19] Substitution with Aib[8] hardly influences the cAMP activity and substitution with D-Ala[8] induces little or no change in the affinity or cAMP activity.[11]

Considering GIP analogues, Gly[2] GIP shows improved cAMP activity and insulinotropic activity in vitro,[20] while the cAMP activity of D-Ala[2] GIP matches that of GIP.[21] The introduction of Aib[2] into GIP has also been reported, and it seems that it does not obviously affect the cAMP activity.[15,22]

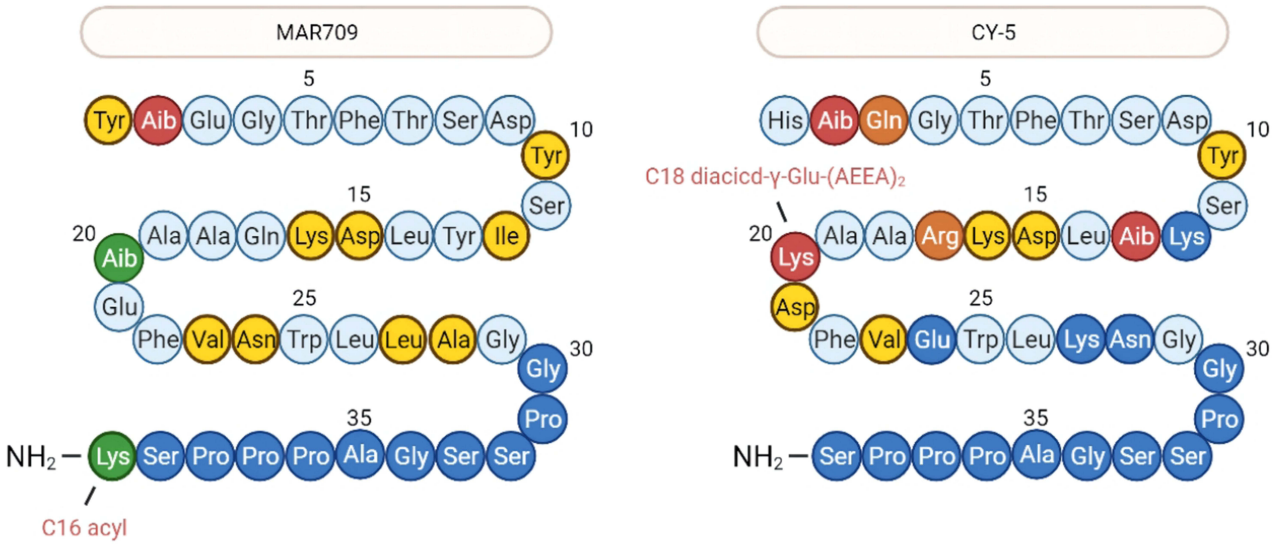

Figure 3 The structures of other two GLP-1R/GIPR dual agonist. Residues in green means they are unique in MAR709 while in orange are unique in CY-5.

Comprehensively, using Aib^2 is likely to only minimally influence the GLP-1 and GIP activities. Although Gly^2 affects GLP-1 activity to some extent, overall, exenatide shows better activity than natural GLP-1. Thus, Gly^2 may be feasible if a dual agonist is biosynthesized. Furthermore, since the instability of Ala^2 is caused by degradation of DPP-4, Ala^2 may not need to be changed if the peptide is fused with albumin or Fc domain (discussed later) for long-term activity, which creates a steric hindrance to prevent the enzyme from getting close.

Thr^7

GLP-1 and GIP are partially homologous, in that GIP can partially activate GLP-1R in extreme doses and GLP-1 may also activate GIPR.[15,23] Tyr^1 and Ile^7 are crucial for the selective activation of GIPR by GIP. Among the possible recombinant GIP analogues, the analogues that simultaneously conserve Tyr^1 and Ile^7 show similar GLP-1R-activating activity to natural GIP, and Ile^7 is more important than Tyr^1.[23] Importing Ile^7 into MAR709 caused a sharp decline in GLP-1 activity. The mechanism remains unclear; Moon et al suggested a hypothesis that GLP-1 Thr^{13} interacts with a binding pocket formed by Ile^{196}, Leu^{232}, and Met^{233} of GLP-1R because Thr^7-containing chimeric peptides are highly sensitive for the Ile^{196} mutation;[24] but it contradicts the model constructed by Zhang et al that Lys^{197} of GLP-1R is highly conserved and is hydrogen bonded to Thr^{13} of GLP-1.[12] The substitution of GLP-1 Thr^{13} or GIP Ile^7 with Ala obviously decreased their receptor-activating activities[25,26] and the substitution of GIP Ile^7 with Thr decreased its cAMP activity (pEC_{50}: 9.76→9.58;Emax: 9.91→8.90).[23] Incorporating Thr^7 from GLP-1 into tirzepatide may lower its GIP activity slightly.

Tyr^{10}

Positions 10, 12, 13, and 14 of exenatide (full GLP-1R agonist) are different from those of GLP-1, suggesting that these residues are unimportant to GLP-1. The mutation of GLP-1 positions 16, 17, 18, and 20 into Ala showed minor effects (IC_{50} from 0.27 nM to 1.7, 0.46, 0.68, and 1.7 nM; maximum EC_{50} varied from 2.6 to 7 nM).[25] Ala substitutions in positions 10 and 11 of GIP had little negative effect on insulinotropic activity while position 12 and 14 mutations had a greater negative effect.[27]

Tyr^{10} and Ile^{12} from GIP are used in MAR709 and Ile^{12} plays a key role in the activation of GIPR [15]. Tyr^{10} and Ile^{12} are also used in tirzepatide. In GLP-1 (7–37) NH_2, both the affinity and activity are slightly promoted when Val^{16} is replaced with Tyr^{16}.[28] Thus, the Tyr^{10} in tirzepatide may have a positive influence on both GLP-1 and GIP activity.

Ile^{12}

Using cryo-electron microscopy, Zhang et al found that a cluster of neighboring serines (Ser^{14}, Ser^{17}, and Ser^{18} of GLP-1) form polar interactions with Thr^{298}, the backbone oxygen of Trp^{297}, and the nitrogen of Arg^{299} in GLP-1R.[12] However, Ala mutations at any of these positions hardly affected activity.[25,29] In contrast, if GLP-1R Thr^{298} was mutated into Ala, it increased affinity to the ligand.[30] Keliher et al ran a series of modifications of Lys^{12} in exenatide, indicating that this position is not important for interactions with GLP-1R and that it is, to some extent, tolerant of bigger groups.[31]

The mutation of GIP Ile^{12} to Ala caused a notable decrease in its insulinotropic effect.[27] Hence, using Ile^{12} in tirzepatide seems not to weaken its GLP-1 activity and to strengthen its GIP activity. In GIP/GIPR binding models, Ile^{12}, which is surrounded by hydrophilic components,[32] might not interact with GIPR. However, the substitution of Ile^{12} with Ala remarkably decreased activity. Therefore, GIP Ile^{12} could function as a spatial obstruction, for example restricting GIPR Arg^{289} so that it interacts with GIP Asp^{15} rather than Asp^9 (as a modeling in RCSB PDB: 7DTY, https://www.rcsb.org). Based on this assumption, Ile^{12} could be replaced with Leu or Val and retain the same effect. Even Phe could be considered as a potential replacement, and Lys from exenatide/CY-5 also seems feasible.

Aib^{13}

GLP-1, GIP, GCG (glucagon), and GLP-2 share high homology so that it ought to be considered that GCGR and GLP-2R should sidestep the activation of the designed GLP-1/GIP dual agonist. In a CY-5 experiment, replacing position 13 with Aib rendered the agonist inactive toward GCGR and slightly altered GLP-1 and GIP activity.[16] Substituting GLP-1 (7–37) Tyr^{19} with Ala notably decreased both the affinity and activity (IC_{50}: 0.27 nM→3.5 nM;EC_{50}: 2.6 nM→55 nM).[25,29]

Ex. 2035-005

In GIP, there is a small chance that activity can be influenced by replacing Ala[13] with Aib. The Aib[13] of tirzepatide seems to lower its GLP-1 activity without affecting its GIP activity, and to destroy its GCG activity. However, this hypothesis lacks supporting evidence. However, Aib[13] is not necessary because other substitutions can fulfill the same role. For instance, in MAR709 Tyr[13] from GLP-1 is retained, and yet it does not activate GCGR. GIP (1–14) is a partial agonist of GIPR. Substitution with Tyr[13] scarcely affects the activity of GIP (1–14) analogues,[33] implying that the use of Tyr[13] in the designed dual agonist may not conspicuously affect GIP activity. Furthermore, considering Gln[13] in exenatide, GLP-1 presumably interact with GLP-1R via a hydrogen bond. Tyr, Gln, Thr, Ser, and Lys are worth trying in this site.

Leu[14]

For certain functions, the Met[14] of exenatide can be substituted with Leu[14], because Met is easily oxidized and has little effect on activity.[34]

Asp[15]

The Glu[21] of GLP-1 mutating into Ala or Gly negatively impacts activity.[25,35] However, as Glu resembles Asp, using Asp[15] from GIP in tirzepatide should not influence its GLP-1 activity.

Lys[16]

Replacing the Gly[22] of GLP-1 with Ala or Aib slightly decreased both its affinity and activity (Ala IC_{50}: 0.27 nM→0.57 nM;EC_{50}: 2.6 nM→4 nM).[25,36] In contrast, replacement with Glu increased both the affinity and activity to some degree.[36] The attachment of long fatty acids relayed by a carboxylic linkage leaves the affinity and activity almost unaffected.[2] A lactam bridge is reported to constrain the turning of the helix. A lactam bridge loaded at Glu[22], either c[Lys[18]–Glu[22]] or c[Glu[22]–Lys[26]], shows lower affinity and activity than a simple substitution of Gly[22] with Glu.[28,36] Theoretically, the substitution of Gly[22] with Ala would make the α-helix more stable, but increments in the affinity and activity were not found; substitution with Glu is likely to stabilize the α-helix as well; however, further increments in the affinity and activity were not found when a more stable lactam bridge was imported; instead, decrements are observed. Thus, the increments in the affinity and activity owing to Glu[22] could be caused by a carboxyl group on Glu adding an extra binding site for the receptor. In this case, the affinity would weaken if Glu was replaced with a lactam bridge because the charge would be dispersed. Moreover, compared to c[Glu[22]–Lys[26]] Gly[8] GLP-1, the affinity and activity of c[Asp[22]–Lys[26]] Gly[8] GLP-1 are lower while those of c[hGlu[22]–Lys[26]] Gly[8] GLP-1 are higher (hGlu: homoglutamic acid),[36] indicating that the length of the amino acid side chain is an important factor.

Tirzepatide Lys[16] may have a stacking interaction with GIPR Phe[127].[8] In CY-5 experiments, if Ser[16] from GCG was changed to Lys from GIP, the GLP-1 activity decreased while the GIP activity increased.[16] Surprisingly, replacing GIP Lys[16] with Ala promotes the insulinotropic effect of GIP.[27] The attachment of fatty acid chains to Lys[16] via an amido bond does not conspicuously influence GIP activity.[37] Thus, Gln seems feasible.

In summary, a carbon side chain of a certain length is favorable, or at least feasible, for interacting with both GLP-1 and GIPR. Apart from this hydrophobic region, it seems that GLP-1R prefers a carboxyl group at the end of the side chain while GIPR can accommodate, but does not favor, an alkaline group. Tirzepatide Lys[16] may slightly decrease GLP-1 activity, but it enhances GIP activity. The following candidates are options: Ala, Gln, or Glu.

Ile[17]

MAR709 experiments have tested the concurrent substitution of Gln[17], Ala[18], and Ala[19] from GLP-1 to Ile[17], His[18], and Gln[19] from GIP, and found that GLP-1 activity decreased (EC_{50}: 0.022 nM→0.140 nM) while that of GIP scarcely increased (EC_{50}: 6.258 nM→5.530 nM).[15] In terms of their overall effect, these changes seem not worthwhile.

Replacing GLP-1 Gln[23] with Ala slightly lowered the affinity and activity (IC_{50}: 0.27 nM→1.1 nM; EC_{50}: 2.6 nM→5 nM).[25] Changing GIP Ile[17] to Ala massively lowered the insulinotropic effect.[27] The Ile[17] from GIP chosen in tirzepatide may be important to GIP activity but could cause a decline in GLP-1 activity.

Ex. 2035-006

Ala^{18}

The mutation of GIP His^{18} to Ala reinforced its insulinotropic effect.[27] In CY-5 experiments, replacing Arg^{18} from GCG with Ala simultaneously improved both the GLP-1 and GIP activity.[16] Using the Ala^{18} from GLP-1 may support GIP activity by chance.

Gln^{19}

GIP (1–30)-NH_2, an active fragment of GIP with affinity and activity that align with those of integrated GIP, dramatically decreased somatostatinotropic activity in isolated perfused rat stomachs. Even when further broken down, GIP (1–14) and GIP (19–30) showed some activity. A study demonstrated that, among GIP (15–30), GIP (16–30), GIP (17–30), and GIP (19–30), GIP (19–30) was the only having activity although it is weak.[38] GIP Gln^{19} may form hydrogen bonds with GIPR Gly^{29} and Thr^{31}.[32] The Gln^{19} adopted in tirzepatide seems to be crucial to GIP activity, but may still affect GLP-1 activity.

Lys^{20}-X

Replacing GLP-1 Lys^{26} with Ala causes both the affinity and activity to decrease slightly.[25] In semaglutide, a fatty acid chain is attached at this site and the affinity and activity are boosted.[2] GLP-1 Lys^{26} may form a polar interaction with the receptor, which is presumably enhanced by Arg^{20} in exenatide, but creates no steric hindrance. GIP is similar, in that GIP Gln^{20} appears to form a hydrogen bond with GIPR Asn^{120} and expose unhindered into the extracellular matrix.[32] A fatty acid chain attached to Lys^{20} in tirzepatide may boost the GLP-1 activity while the GIP activity remains unaffected.

Ala^{21}

The mutation of GLP-1 Glu^{27} to Ala does not change the affinity but slightly increases activity.[25] In CY-5 experiments, Asp^{21} from GIP was replaced by Ala; the GLP-1 activity changed little but slight decreases were observed in the GIP activity. Thus, the Glu^{27} of GLP-1 is not a critical point in the interaction with GLP-1R. GIP (1–42) Asp^{21} may form a hydrogen bond with GIPR Arg^{131} and the mutation of GIPR Arg^{131} to Ala decreases the affinity and activity. Surprisingly, this hydrogen bond between Asp^{21} and GIPR Arg^{131} vanishes in GIP (1–30).[32] Since tirzepatide does not have a GIP (31–42) tail, chosen Ala^{21} was likely to increase the GLP-1 activity, despite it slightly decreasing the GIP activity. In humans, GLP-1 is not only damaged by DPP-4, but also by neutral endopeptidase 24.11 (NEP 24.11), and the main catalytic sites are Glu^{27}–Phe^{28} and Trp^{31}–Leu^{32}.[39] Using the Ala^{21} of tirzepatide can perhaps sidestep this NEP 24.11 degradation to a certain extent. Moreover, the Leu in position 21 of exenatide is unique, and its mutation seems to be unimportant. Thus, the spectrum to consider is expansive, including Glu from GLP-1, Asp similar to Glu from GIP, Ala, and even Leu/Ile/Val (forming a hydrophobic area along with Phe^{22}).

Val^{23}, Ile^{27}

GLP-1 (7–37) Phe^{28}, Trp^{31}, and Leu^{32} are symmetrical with positions 22, 25, and 26 of GIP. GLP-1, GIP, and exenatide seem to have the same patterns here: Phe^{22} is centered to specifically interact with the receptor and the adjacent hydrophobic residues of positions 23, 25, and 26 are used to form a hydrophobic region. The residue of position 24 further away can interact polarly with the receptor and is probably beneficial in locating the hydrophobic region.

GLP-1R Trp^{78}, Pro^{90}, Trp^{91}, and His^{212} could form a hydrophobic region with GLP-1 Phe^{28}, Ile^{29}, Trp^{31}, Leu^{32}, and Val^{33}, and GIPR Leu^{35}, Trp^{39}, Tyr^{36}, Met^{67}, Tyr^{87}, Trp^{90}, and His^{115} could form a hydrophobic region with GIP Phe^{22}, Val^{23}, Trp^{25}, Leu^{26}, and Leu^{27}.[27] GLP-1 Ile^{29} and Val^{33} have similar properties as GIP Val^{23} and Leu^{27}. The mutation of GLP-1 Phe^{28} to Ala causes it to lose nearly all of its affinity (IC_{50}: 0.27 nM→357 nM), and Ile^{29} mutation decreases the affinity notably (IC_{50}: 0.27 nM→25 nM), while Trp^{31}, Leu^{32}, and Val^{33} mutations have little influence, showing that they may be merely subsidiary.[25] The Val^{23} and Ile^{27} used in tirzepatide are likely to have little impact on either GLP-1 or GIP activity because they are the same kind of residue and no specific interaction in these sites has been detected.

Ex. 2035-007

Gln^{24}

Substituting GLP-1 Ala^{30} with Gln decreases the affinity (IC_{50}: 0.27 nM→1.4 nM) but increases activity (EC_{50}: 2.6 nM→0.5 nM).[25] GIP Asn^{24} is similar to Gln; thus, the Gln^{24} adopted in tirzepatide could elevate GLP-1 activity and support GIP activity.

Ile^{27}-

From positions 33–35 of GLP-1, each replacement with Ala increases the IC_{50} about five-fold but somewhat decreases the EC_{50}.[25] Although these replacements lower the affinity, ligands are made to 'correct' the construction. Linking position 30 and 34 with a lactam bridge simultaneously improves affinity and activity.[40] Construction 'correction' could mean forming a more stable α-helix. GLP-1 Arg^{36} replacement with Ala obviously hinders affinity (IC_{50}: 0.27 nM→4.6 nM) and activity (EC_{50}: 2.6 nM→7 nM).[25] Both affinity and activity reduce 10-fold if the Arg^{36} of GLP-1 is cut away,[41] showing that it is crucial for interaction. Moreover, Gly^{35}, Arg^{36}, and Gly^{37} may contribute to selectivity of GLP-1.[42]

Compared to GLP-1, exenatide has nine extra residues in the C-terminal, which constitute a so-called 'Trp-cage' structure, folding and shielding the Trp^{25} indole ring from solvent exposure. Along with truncating the C-terminal, this exposes exenatide to NEP 24.11 degradation.[43] Besides, attaching the GLP-1 C-terminal to exenatide (31–39) can prevent DPP-4 degradation.[44]

With C-terminal curtailing, the insulinotropic activity of exenatide gradually declines. Interestingly, when curtailed to exenatide (1–28), its insulinotropic activity recovers almost all integrity, but when it reaches exenatide (1–26), its insulinotropic activity suddenly disappears.[19] Apparently, the Lys^{27} and Asn^{28} of exenatide play important roles in binding with the receptor. Most previous experiments were comparisons between exenatide (1–30) and GLP-1, thus exploring the function of the extra residues (31–39) in the exenatide C-terminal. However, in the above-mentioned experiments, the comparison should be between exenatide (1–28) and GLP-1, as exenatide (29–39) is truly the extra segment.

Cutting exenatide (29–39) away has a minimal influence on insulinotropic activity in vitro. Moreover, in MAR709 experiments, the attachment of exenatide (29–39) has a limited positive effect on GLP-1 activity (EC_{50}: 0.022 nM→0.020 nM).[15] Although it may decrease the degradation of exenatide in vivo, other strategies to lengthen the half-life can fulfill this effect. Hence, the attachment of an extra tail seems unnecessary.

In vivo, GIP can be scissored between GIP (1–30) and GIP (34–42) by prohormone convertases.[32] Compared to GIP (1–42), GIP (1–30) has a symmetrical IC_{50}, a slightly lower EC_{50}, and a higher E_{max}.[45] In mouse experiments, no variance has been found in the metabolic effects of GIP (1–30) and GIP (1–42).[46] In a simulative binding model of GIP and the receptor, the fragment from position 31–42 neither interacted with the receptor, nor formed any specific structure, but it was able to cling to the receptor.[32] Nevertheless, some aspects of the 31–42 segment of the GIP remain undiscovered, as GIP (3–30) is an antagonist while GIP (3–42) is a partial agonist of GIPR.[14]

GLP-1 (7–36) NH_2, exenatide (1–28) and GIP (1–30), all have two polar residues starting from position 27: GLP-1 has Lys^{28} and Arg^{30}, exenatide has Lys^{27} and Asn^{28}, and GIP has Gln^{29} and Lys^{30}. They seem to have these similar patterns to interact with the receptor. At the initial stage of ligand–receptor binding, the ligand clings to the receptor using its amorphous polar terminal. Then, gradually and thermodynamically, it moves to form the correct interface with the receptor. It further forms a stable helical conformation, with assistance from the receptor. Phe$^{22(28)}$ is inserted to match the hydrophobic region of the receptor, forming a relatively strong interaction. However, as the locations of the two polar residues are distinct, the receptor interaction sites are also different. The tirzepatide arrangements in GLP-1R and GIPR have an angular difference of 8.3°.[8]

An amorphous polar terminal could be implemented for practical applications, such as increasing the odds of contact between ligands and receptors under flowing conditions. In CY-5 experiments, substituting Lys^{27} with Ile^{27}, or Lys^{27}, Asn^{28} with Ile^{27}, Ala^{28} decreased the EC_{50} of both GLP-1R and GIPR, but decreased hypoglycemic efficacies in mice.[16]

Tirzepatide Ile^{27} is akin to GLP-1 Val^{27} and GIP Leu^{27}. Thus, their substitution seems not to affect activity. Using the Ala^{28} from GIP may decrease affinity with GLP-1R and increase the activities of the two receptors in vitro, but it is likely

to decrease these activities in vivo. Moreover, tirzepatide elongated with exenatide (29–39) would most likely show no evident effect.

When designing a neo agonist, the length of the C-terminal may not be as important as previously thought. The crucial point could be that some polarity (such as alkalinity) is required. At least one alkaline Lys residue is contained in the C-terminals of GLP-1, exenatide, and GIP. The replacement of GLP-1 (7–37) Lys^{34} with Arg causes a two-fold reduction in the EC_{50}.[2] Whether the angular variance of tirzepatide binding to GLP-1R and GIP affects its activity has not yet been determined. If the number of polar residues increases, for example using the sequence of $Ile^{27}Lys^{28}Gln^{29}Lys^{30}$, making it possible to match two receptors, will this angular variance be eliminated?

Half-Life Extension Strategies

Apart from DPP-4 enzymolysis, another GLP-1/GIP degradation pathway, renal metabolism, can be overcome by augmenting the drug volume to prolong the half-life. Lipidation additionally induces concentration-independent liraglutide multimerization, resulting in a six- to eight-fold increase in size. However, it is unclear which of these, the size increase or HSA binding, are more pharmacologically relevant and account for the observed extension of the half-life.[47] Another approach is PEGylating which may destroy the activity in vitro but retain the efficacy in vivo.[15]

Human serum albumin (HSA) and IgG have plasma half-lives of around 21 days, owing to HSA and the Fc domain of IgG interacting with the neonatal Fc receptor expressed in blood vessel endothelial cells, which rescues the proteins in the endosomes after pinocytosis, preventing them from entering the lysosomal pathway for destruction. By binding with HSA or IgG-Fc, the half-life of protein drugs could be extended. HSA and IgG-Fc can bind proteins not only through covalent attachments, but also through non-covalent labyrinthine interactions between endogenous albumin and long fatty acid chains, which have been added to GLP-1/GIP in some previous studies.[47] The crucial elements of this strategy are the length of the fatty acid chain and the position of lipidation. Generally, the longer the fatty acid chain, the more readily it binds to HSA. However, excessive length may impede ligand to bind and activate receptor. The fatty acid side chain used in CY-5 is same as that of semaglutide, used in tirzepatide just having two more carbon as C20, used in MAR709 is a simple C16 chain without spacer located at Lys^{40}.[6,15,16] Taken together, the appropriate length is between C16 and C20 and most suitable lipidation position can be chosen from the following range of positions (starting from position 1): 10, 16, 20, 21, 28, and C-terminal.[2,48] Apart from fatty acid chains, there are other molecules that could be used to connect to albumin and could be worth exploring.[49]

The semaglutide development process shows that the lipidation approach is decisive to the selectivity of the protein. Apparently, the intensity of the interaction between albumin and the fatty acid will not change if the length of the fatty acid remains unchanged. However, attaching the fatty acid chain to different sites can diversify the effects extensively. For example, in 2% albumin condition, the IC_{50} is 5.39 when lipidation occurs at position 27, yet at position 26 it reaches 357.[2] It is possible that lipidation at certain sites can create steric hindrance that does not allow GLP-1Rs to capture ligands directly from the albumin, but only to trap free ligands released by the albumin. According to this hypothesis, shortening the fatty acid chain would decrease the affinity of the ligands to albumin while elongating it would impair their selectivity for binding albumin or the receptor, because they would be able to bind both simultaneously.

For biosynthesis, it seems the only strategy to prolong the half-life is a ligand fused with a terminal albumin or Fc domain, whose critical points are length and location. However, whether a protein of such size could infiltrate the blood–brain barrier to bind GLP-1R and GIPR in the brain, and whether receptors in the brain are important to the therapeutic effect, still need to be elucidated.

Plausible Orientations

The contribution of GIP part in dual agonist is still uncertain. Tirzepatide had finished its last Phase 3 clinical trial SURPASS-5 in February 2022,[50] and it favors activating GIPR (for GIPR, native GIP EC_{50}: 0.0334nM, tirzepatide EC_{50}: 0.0224nM; for GLP-1R: native GLP-1 EC_{50}: 0.0705nM, tirzepatide EC_{50}: 0.934nM).[6] MAR709 had finished its Phase 2a clinical trial in 2017,[51] and it is a balanced dual agonist for GLP-1R and GIPR. ① Comparing the results of phase 2 clinical trials of these two drugs (baseline characteristics of populations were similar),[51,52] tirzepatide seemed to be more efficacious in improving T2DM. After 12 weeks treatment, the reductions in HbA1c from baseline were −0.96% with 1.8mg MAR709, −0.9% with

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1mg tirzepatide and −1.7% with 5mg tirzepatide. Other parameters were compared by Marie Bastin and Fabrizio Andreelli.[53] The dose of 5mg MAR709 was determined to be not tolerated[54] while a wide dose range of tirzepatide from 1mg to 15mg was established.[52] The discrepancy of clinical efficacy of these two drugs is due at least partially to their difference of proportion of GLP-1 and GIP activity. In healthy people, postprandial GIP levels are approximately 4-fold higher than GLP-1 levels.[55] The predicted quantity of GIPR occupied by tirzepatide is also more than that of GLP-1R (6.3-fold at 5mg, 4.6-fold at 10mg and 4.1-fold at 15mg).[56] This may be the secret of the extraordinary efficacy of tirzepatide, but it is still possible that the best ratio of GLP-1 and GIP activity in a dual agonist has not been discovered.

Additionally, GIPR antagonists also exhibit improvement of metabolism on obese mice[57] and GIPR agonists or antagonists both show therapeutical effects in combination with GLP-1R agonists.[58] A GLP-1R agonist/GIPR antibody (antagonist) fused protein was reported promoting body weight loss.[59] Nevertheless, whether a GLP-1R agonist/GIPR antagonist can improve T2DM needs more evidences. A GLP-1R agonist/GIPR antagonist peptide is theoretically possible but few full-length GIP-like antagonists were reported,[60] therefore it requires an array of attempts, at least an alanine-scan experiment of GIP with measuring of affinity.

GLP-1R and GIPR belong to class B1 of the G protein-coupled receptors, and share high homology and a similar action pattern. G protein-coupled receptors include an N-terminal extracellular domain (ECD) and a C-terminal transmembrane domain (TMD), linked to each other through a soft sequence. In an inactive state, ECD favors a closed conformation covering the TMD. Subtle dynamics allows the C-terminal of the ligand to access the ECD binding site, and the binding of the ligand triggers further dissociation between the ECD and TMD, allowing the N-terminal to enter the orthosteric pocket in the TMD and activate the receptor. Small populations of open conformations exist as well and can smoothly accommodate the ligand.[61]

GLP-1R activation triggers Gαs-protein coupling, elevating cAMP, modulating calcium mobilization, and inducing β-arrestin recruitment.[62] Different ligands lead to different interaction patterns with the receptor, generating various effects. For example, the P5 exenatide analogue is biased to reduce the recruitment of β-arrestin, resulting in better long-term hypoglycemic effects than those shown by exenatide, under the precondition that P5 has less cAMP activity.[62] Tirzepatide is also biased toward cAMP activation and the activity of β-arrestin recruitment is lower than that of native GLP-1 for GLP-1R.[8,56,63] After binding with a ligand for a certain period, the receptor will trigger the inward sinking of the membrane, forming an endosomal compartment, which is called agonist-induced receptor internalization.[64] Some of these endosomal compartments return to the membrane, while others fuse with lysosomes.

For GLP-1R and GIPR, both tirzepatide and MAR709 showed strikingly decreased receptor internalization compared to mono agonist. GLP-1R internalization is mediated by Gαq signaling and influenced by β-arrestin 1/2; GIPR internalization is mainly influenced by β-arrestin 2.[63] β-arrestin is involved in the desensitization of the receptor, and G-protein coupling will be blocked if the receptor is occupied with β-arrestin. Moreover, β-arrestin mediates receptor internalization and reduces the amount of receptors on the surface of the membrane.

The level of secreted incretin of diabetics commonly resembles that of healthy people, yet the number of incretin receptors is substantially lower. This may be relevant to internalization. Reducing the recruitment of β-arrestin and the residence time of the ligand towards the receptor, thus decreasing internalization, is presumably the key of incretin drugs. The mutation of GIPR Glu^{354} to Gln increases GIP affinity, enhances the agonist residence time by 25%, increases cAMP activity, and facilitates the rate of internalization by 2.1–2.3-fold. The throng with Gln^{354} GIPR has lower bone-mineral density, a > 50% increase in fracture risk, and a slightly increased plasma glucose.[65] Does the paradox that either GIPR agonist or antagonist exhibits benefits in combination with GLP-1R agonist correlates with the likely reduction of internalization, following the decrement of β-arrestin recruitment or the acceleration of the dissociation rate (K_{off}) between the ligand and receptor, owing to competition between used agonists and antagonists in endogenous GIP?

Hence, the dynamic study of incretin drugs may be the center of future research. For the moment, a certain association rate (K_{on}) is required to ensure ligand binding to receptors in blood flow and to compete with endogenous incretins to decrease internalization. A rapid dissociation rate is also required to allow ligands to leave the receptor timeously after activation, to avoid internalization and ultimately maintain the quantity of the incretin receptors in the body.

Apart from the substitution of protein residues, an extra sequence connected to the N-terminal or C-terminal could change the biased interactions between the ligand and receptor. Using rapid selection, Wu et al discovered a surprising

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dual agonist composed of GIP (3–30) and an extra sequence of nine amino acids in the N-terminal.[66] Compared to GIP (1–42), GIP (1–39) has a higher ability to modulate calcium and thus a stronger insulinotropic effect.[67]

Conclusion

GLP-1 analogs were utilized broadly in treatment of T2DM. Another incretin GIP is rather mystery and limited in clinical use, but scientists never stop exploring, tirzepatide is one of the rewards of these efforts.

Compared to the first-generation GLP-1R agonist, tirzepatide has three critical improvements: first, many residues in peptide backbone are changed to obtain GIPR-activating activity; second, C-terminal is prolonged with a sequence of C-terminal of exenatide; third, a fatty acid side chain is conjugated similar to semaglutide to prolong half-life.

By weighing different residues adopted in each position, the activity of a dual agonist could be customized. Initially, main concerns were receptor-activating activity while recently, downstream effects of GLP-1R and GIPR have been more and more revealed that not only receptor activation promotes cAMP and stimulates insulin releasing, but also other factors (β-arrestin recruitment, residence time, etc.) influence receptor internalization, which further elicits long-term effects in vivo. Most existed reports uncovered cAMP activity changes after residue substitutions and they were summarized in part 2. Future research may pay more attention to other activities like β-arrestin recruitment or ligand-receptor affinity.

All of three dual agonist peptides, tirzepatide, MAR709 and CY-5, have a C-terminal same as exenatide but no convincing reasons have been put forward and its use is more likely an "inertia" from MAR709.

Incretins are largely degraded by DPP-4 in vivo, which can be hindered by substitution of first three residues or increasing steric hindrance. The substitution of Aib at position 2 avoids DPP-4 acting and through a fatty acid side chain tirzepatide can adhere to albumin in vivo, whose native half-life is about three weeks, thus can weekly stay in blood circulation. PEGylating or fusing peptide with albumin/Fc domain are also feasible and even the DPP-4 would be resisted by steric hindrance if the latter method is taken.

Although GLP-1 kind has been deeply studied, many GIP aspect fundamental features should be more clarified, such as an alanine scanning, to support development of GLP-1R/GIPR bifunctional drugs. Recent researches suggested the internalization of receptors is the key point and thus more dynamic studies are required eagerly.

Disclosure

The author reports no conflicts of interest in this work.

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