Design and Synthesis of Conformationally Constrained Glucagon-Like Peptide-1 Derivatives with Increased Plasma Stability and Prolonged in Vivo Activity

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A series of conformationally constrained derivatives of glucagon-like peptide-1 (GLP-1) were designed and evaluated. By use of [Gly^8]GLP-1(7–37)-NH$_2$ (**2**) peptide as a starting point, 17 cyclic derivatives possessing *i* to *i* + 4, *i* to *i* + 5, or *i* to *i* + 7 side chain to side chain lactam bridges from positions 18 to 30 were prepared. The effect of a helix-promoting α-amino-isobutyric acid (Aib) substitution at position 22 was also evaluated. The introduction of *i* to *i* + 4 glutamic acid−lysine lactam constraints in *c*[Glu^{18}-Lys^{22}][Gly^8]GLP-1(7–37)-NH$_2$ (**6**), *c*[Glu^{22}-Lys^{26}][Gly^8]GLP-1(7–37)-NH$_2$ (**10**), and *c*[Glu^{23}-Lys^{27}][Gly^8]GLP-1(7–37)-NH$_2$ (**11**) resulted in potent functional activity and receptor affinities comparable to native GLP-1. Selected GLP-1 peptides were chemoselectively PEGylated in order to prolong their in vivo activity. PEGylated peptides [Gly^8,Aib^{22}]GLP-1(7–37)-Cys$^{(PEG)}$-Ala-NH$_2$ (**23**) and *c*[Glu^{22}-Lys^{26}][Gly^8]GLP-1(7–37)-Cys$^{(PEG)}$-Ser-Gly-NH$_2$ (**24**) retained picomolar functional potency and avid receptor binding properties. Importantly, PEGylated GLP-1 peptide **23** exhibited sustained in vivo efficacy with respect to blood glucose reduction and decreased body weight for several days in nonhuman primates.

Introduction

The worldwide prevalence of type 2 diabetes continues to increase.[1] Patients with type 2 diabetes have elevated blood glucose levels (fasting plasma glucose of >126 mg/dL) as a result of insulin resistance and a deficit in insulin secretion.[2] Glucagon-like peptide-1 (GLP-1[a]) is a glucoincretin hormone that is secreted from intestinal L-cells in response to food intake, and its predominant physiological role is to maintain blood glucose levels via a glucose-dependent mechanism.[3] GLP-1 has two well-established, naturally occurring, and equipotent isoforms, GLP-1(7–36)-amide and GLP-1(7–37), representing ∼ 80% and 20% of the circulating bioactive GLP-1, respectively (Figure 1).[4] GLP-1 has multiple desirable effects including increased insulin secretion (glucose dependent) from pancreatic *β*-cells, stimulation of insulin biosynthesis (glucose-dependent), decreased glucagon secretion (glucose-dependent), and delayed gastric emptying.[5]

Administration of native GLP-1 in humans suggests that GLP-1 enhances insulin secretion not only in normal subjects but also in those with type 2 diabetes mellitus.[6,7] GLP-1 binds to family B of the seven transmembrane G-protein-coupled receptors (GPCR),[8] making the GLP-1 receptor an attractive target for the treatment of hyperglycemia resulting from type 2 diabetes.[9] The clinical application of native GLP-1 is, however, restricted by its short circulating lifetime. After endogenous secretion of GLP-1, circulating levels of its bioactive forms are rapidly lowered via the action of dipeptidyl peptidase IV (DPP-IV),[10] which inactivates GLP-1 via cleavage between alanine^8−glutamic acid^9 and as a result of high clearance. The elimination half-life of GLP-1 in humans after intravenous administration has been estimated to be less than 2 min.[11,12] Efforts to increase GLP-1 half-life through the preparation of DPP-IV resistant analogues, such as [Gly^8]GLP-1(7–36)-amide, have only marginally increased in vivo half-life to approximately 4–5 min.[13] The discovery of more physiologically stable and efficacious GLP-1 receptor agonists is needed and actively being pursued.[14–16]

The structure–activity[17–19] and conformation[20–23] of GLP-1 have been studied by many researchers. Circular dichroism (CD) studies indicate that GLP-1 largely adopts a helical conformation in helix-favoring aqueous conditions or at high concentrations when helix bundle formation occurs. NMR studies show that GLP-1 gradually assumes a stable, single-stranded helical structure when lipid micelles or fluoro alcohols are increased from 0% to 35% (v/v). In a DPC micelle-associated state, the major GLP-1 conformations represent structures with two helical regions, where glycine[22] appears to serve as the N-cap for the C-terminal helix domain. It has been proposed that the GLP-1 folding process is initiated by the formation of the C-terminal helical segment that is gradually extended toward the N-terminus.[21] In comparison to GLP-1, exendin-4, a 39-amino acid peptide that shares approximately 50% sequence identity but differs by the presence of an extended C-terminal domain, displays a greater intrinsic propensity toward the formation of a monomeric helix than GLP-1.[23]This is attributed, at least in part, to the presence of a helix-favoring glutamic acid residue in exendin-4 at the corresponding position rather than a helix-interrupting glycine[22] residue of GLP-1.[20,23] The high overall helical structure in exendin-4 has also been partly attributed to

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[a] Abbreviations: Aib, α-amino-isobutyric acid; Boc, *tert*-butyloxycarbonyl; Cpa, *p*-carboxylphenyalanine; Dab, α,*γ*-diaminobutyric acid; DPP-IV, dipeptidyl peptidase IV; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylforamide; Fmoc, 9-fluorenylmethyloxocarbonyl; GLP-1, glucagon-like peptide-1; GPCR, G-protein-coupled receptor; HPLC, high-performance liquid chromatography; PEG, polyethylene glycol; PyBOP, benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid.

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Figure 1. Primary structure of GLP-1(7–37) using single letter amino acid codes. Residue numbering and the DPP-IV cleavage site are indicated.

the leucine[21]−proline[38] span forming a compact tertiary fold (Trp cage),[20] providing efficient C-capping of the helix and potentially reducing the entropic cost associated with receptor binding that requires a central and C-terminal helix conformation.

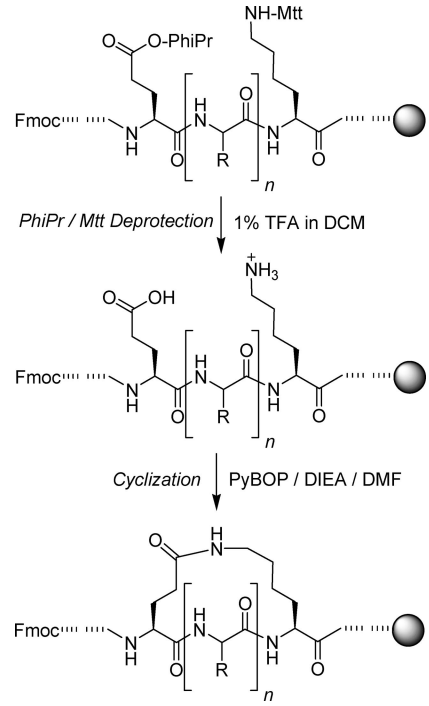
With the objective of identifying potent GLP-1 receptor agonists with improved metabolic stability, we have evaluated the effect of helix favoring amino acid residue substitutions, as well as the incorporation of a lactam bridge involving a series of side chain groups along the GLP-1 chain (Table 1). Side chain to side chain bridges (*i* to *i* + 4, *i* to *i* + 5, and *i* to *i* + 7) have been successfully used as constraints in the design of amphipathic or model α-helical peptides,[24–26] oxytocin antagonists,[27] melanotropin analogues,[28] glucagon,[29] and small stromal cell-derived factor-1 (SDF-1) peptide analogues.[30] Depending on the space between the cyclization sites and choice of residues, lactam bridges can be used to induce and stabilize turn[31,32] or helical[24,26] conformations. In this article we report the design, synthesis, conformation, and plasma stability of constrained GLP-1 derivatives and report their ability to bind and activate the human GLP-1 receptor in vitro. The efficacy of conformationally constrained, PEGylated, GLP-1 analogues in normal and *db/db* mouse models as well as in nonhuman primates is also reported.

Results and Discussion

GLP-1 analogues were prepared by solid-phase synthesis using a N^{α}-Fmoc/*tert*-butyl strategy[33,34] on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy (Rink) resin.[35] Side chain to side chain lactam formation (*i* to *i* + 4, and *i* to *i* + 5) was carried out on the assembled N-terminally Fmoc-protected resin-bound peptide (Scheme 1). At cyclization sites, side chain amino (lysine, ornithine, α,γ-diaminoabutyric acid) and carboxyl (aspartic acid, glutamic acid, homoglutamic acid, and β-homoglutamic acid) functionalities were protected with 4-methyltrityl (Mtt) groups and 2-phenylisopropyl ester (2-PhiPr) groups, respectively.[36,37] After deprotection of these groups with dilute TFA, PyBOP-mediated cyclization was used to form the chemically stable side chain to side chain lactam constraint.[38] Typically, the lactam cyclized peptide, in comparison to the linear uncyclized peptide, increased the reversed phase high-performance liquid chromatography (RP-HPLC) retention time by approximately 0.5−1 min. Electrospray mass spectrometry was used to confirm the condensation (−18 Da) of the involved side-chain groups. GLP-1 analogues were then purified by preparative RP-HPLC and assessed by analytical HPLC and electrospray mass spectrometry (see Supporting Information).

To compare the potency of test compounds with GLP-1, reporter CHOK1 cell lines expressing human GLP-1 receptors were generated.[8,39] Increased cAMP levels were measured through enhanced expression of a luciferase reporter gene.[40–42] The functional potency and receptor affinity of GLP-1(7–37) (**1**) and related derivatives are listed in Table 1. All tested compounds were full GLP-1 receptor agonists. Dose–response curves for GLP-1 receptor binding and activation of selected

Scheme 1. Synthesis of *i* to *i* + 4, and *i* to *i* + 5 Constrained GLP-1 Analogues via Orthogonally Protected Peptides[a]



[a] For *i* to *i* + 4 constraints *n* = 3, and for *i* to *i* + 5 constraints *n* = 4. R indicates the protected side chain group of L-amino acid residues spanning the cyclization site. Dash lines represent the remaining protected peptide segments of GLP-1.

analogues are shown in Supporting Information. As previously reported, the substitution of alanine[8] in GLP-1(7–37)-NH_2 (**1**, IC_{50} = 2 nM, EC_{50} = 6 pM) with glycine[8] improves resistance to DPP-IV degradation[10] and resulted in similar potent functional activity and binding affinity (**2**, IC_{50} = 3 nM, EC_{50} = 14 pM) (Table 1).

***i* to *i* + 4 Cyclized GLP-1 Analogues.** A total of nine cyclic GLP-1 analogues (**6**−**14**) with lactam bridges between glutamic aid residues at position *i* and lysine residues at position *i* + 4 were prepared (Figure 2). The *i* to *i* + 4 lactam constraints were designed to induce and stabilize helical conformations in the GLP-1 peptide from residue positions 18−30. Compared to the strong binding affinity of [Gly[8]]GLP-1(7–37)-amide (**2**, IC_{50} = 3 nM, EC_{50} = 14 pM), we found that the introduction of *i* to *i* + 4 glutamic acid−lysine lactam constraints between residues 18 and 22 (**6**), 22 and 26 (**10**), and 23 and 27 (**11**) was very well tolerated with respect to both GLP-1 binding affinity (IC_{50} = 4, 2, and 2 nM, respectively) and functional activity (EC_{50} = 6, 10, and 8 pM, respectively) (Table 1). Reversal of the direction of the side chain lactam constraint in **10** by the introduction of an *i* to *i* + 4 lysine−glutamic acid constraint at positions 22 and 26 to give **15** had little effect on the tolerability of cyclization (IC_{50} = 3 nM, EC_{50} = 8 pM). However, the introduction of an *i* to *i* + 4 glutamic acid−lysine lactam constraint between residues 19 and 23 (**7**), 20 and 24 (**8**), 21 and 25 (**9**), 24 and 28 (**12**), 25 and 29 (**13**), and 26 and 30 (**14**) did result in significant reduction in binding affinities for the GLP-1 receptor (Table 1). The large loss in potency for **12** and 13 is consistent with previous structure–activity studies on GLP-1,[17] where phenylalanine[28] and isoleucine[29] have been shown to be important for receptor binding and activity.

Given the *i* to *i* + 4 lactam constraint in **10** substitutes and spans the helix-interrupting Gly[22] residue of GLP-1, further

Table 1. List of All Peptides and in Vitro Activities Using Recombinantly Expressed Human GLP-1 Receptor

compd	peptide	binding affinity, $IC_{50} \pm SD$ (nM)	CRE/Luc activation, $EC_{50} \pm SD$ (nM)
1	GLP-1(7–37)-NH_2	1.7 ± 0.6	0.006 ± 0.003
2	[Gly^8]GLP-1(7–37)-NH_2	2.8 ± 2.2	0.014 ± 0.006
3	exendin-4	0.66 ± 0.17	0.004 ± 0.002
4	[Gly^8,Glu^{22}]GLP-1(7–37)-NH_2	1.1 ± 0.1	0.003 ± 0.0002
5	[Gly^8,Aib^{22}]GLP-1(7–37)-NH_2	2.9 ± 1.1	0.027 ± 0.018
6	*c*[Glu^{18}-Lys^{22}][Gly^8]GLP-1(7–37)-NH_2	3.6 ± 2.8	0.006 ± 0.0003
7	*c*[Glu^{19}-Lys^{23}][Gly^8]GLP-1(7–37)-NH_2	17 ± 10	0.077 ± 0.074
8	*c*[Glu^{20}-Lys^{24}][Gly^8]GLP-1(7–37)-NH_2	26 ± 9	0.45 ± 0.29
9	*c*[Glu^{21}-Lys^{25}][Gly^8]GLP-1(7–37)-NH_2	60 ± 12	0.14 ± 0.08
10	*c*[Glu^{22}-Lys^{26}][Gly^8]GLP-1(7–37)-NH_2	2.1 ± 1.7	0.010 ± 0.004
11	*c*[Glu^{23}-Lys^{27}][Gly^8]GLP-1(7–37)-NH_2	1.9 ± 1.1	0.008 ± 0.007
12	*c*[Glu^{24}-Lys^{28}][Gly^8]GLP-1(7–37)-NH_2	290 ± 130	0.74 ± 0.095
13	*c*[Glu^{25}-Lys^{29}][Gly^8]GLP-1(7–37)-NH_2	>1000	15 ± 9
14	*c*[Glu^{26}-Lys^{30}][Gly^8]GLP-1(7–37)-NH_2	16 ± 4.7	0.024 ± 0.004
15	*c*[Lys^{22}-Glu^{26}][Gly^8]GLP-1(7–37)-NH_2	2.7 ± 0.78	0.008 ± 0.007
16	*c*[Glu^{22}-Orn^{26}][Gly^8]GLP-1(7–37)-NH_2	6.4 ± 2.2	0.019 ± 0.018
17	*c*[Asp^{22}-Lys^{26}][Gly^8]GLP-1(7–37)-NH_2	64 ± 41	0.14 ± 0.13
18	*c*[hGlu^{22}-Lys^{26}][Gly^8]GLP-1(7–37)-NH_2	6.2 ± 4.5	0.004 ± 0.003
19	*c*[βhGlu^{22}-Lys^{26}][Gly^8]GLP-1(7–37)-NH_2	>500	1.8 ± 1.2
20	*c*[Glu^{21}-Lys^{26}][Gly^8]GLP-1(7–37)-NH_2	210 ± 98	0.47 ± 0.31
21	*c*[Cpa^{19}-Lys^{26}][Gly^8]GLP-1(7–37)-NH_2	2000 ± 600	9.0 ± 0.06
22	[Gly^8]GLP-1(7–37)-$Cys^{(PEG)}$-Ser-Gly	140 ± 9.9	0.23 ± 0.22
23	[Gly^8,Aib^{22}]GLP-1(7–37)-$Cys^{(PEG)}$-Ala-NH_2	86 ± 58	0.13 ± 0.06
24	*c*[Glu^{22}-Lys^{26}][Gly^8]GLP-1(7–37)-$Cys^{(PEG)}$-Ser-Gly NH_2	54 ± 21	0.17 ± 0.15
25	[Gly^8,Aib^{22}]GLP-1(7–37)-$Cys^{(PEG)}$-Ser-Gly	>100	0.32 ± 0.17

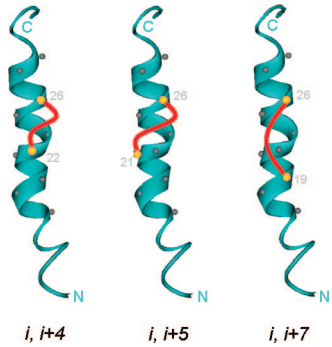


Figure 2. General connectivity of *i* to *i* + 4, *i* to *i* + 5, and *i* to *i* + 7 lactam constraints on GLP-1(7–37). The structure was obtained from a single NMR energy minimized conformer from PDB coordinate file 1D0R and is represented as a solid ribbon. Cα atoms between residues 16 and 30 are indicated as spheres.

analogues were also prepared. In addition, contraction of the side chain to side chain lactam constraint was examined. Shortening of the amino side of the lactam by a methylene unit was achieved through the incorporation of an *i* to *i* + 4 glutamic acid−ornithine lactam constraint (**16**) and resulted in an approximate 3-fold reduction in binding affinity (IC_{50} = 6 nM). Shortening of the carboxyl side of the lactam by a methylene unit with the incorporation of an *i* to *i* + 4 aspartic acid−lysine lactam constraint (**17**) resulted in a >30-fold reduction in binding affinity (IC_{50} = 64 nM). Attempts to prepare more highly constrained lactams with two less methylene units, such as *c*[Glu^{22}-Dab^{26}][Gly^8]GLP-1(7–37)-NH_2 and *c*[Asp^{22}-Orn^{26}]-[Gly^8]GLP-1(7–37)-NH_2 were not successful, even after prolonged cyclization reaction times with forcing conditions. Conversely, expansion of the carboxyl side of the lactam through the incorporation of an *i* to *i* + 4 homoglutamic acid−lysine lactam constraint (**18**) resulted in a marginal reduction in binding affinity to 6 nM but still retained good functional activity (Table 1). However, expansion of the GLP-1 peptide backbone by one methylene unit through the incorporation of a *i* to *i* + 4 β-homoglutamic acid−lysine lactam constraint was not well tolerated (**19**, IC_{50} > 500 nM, EC_{50} = 2 nM).

***i* to *i* + 5 and *i* to *i* + 7 Cyclized GLP-1 Analogues.** The *i* and *i* + 7 residues in an α-helical peptide are proximal in space but separated by two turns of the helix rather than one as with *i* and *i* + 4 residues (Figure 2). In light of the published NMR structure[20–23] and known structure–activity for GLP-1, it appeared tyrosine[19] and lysine[26] residues would be reasonable candidates for *i* to *i* + 7 cyclization spanning the helix-interrupting Gly^{22} residue of GLP-1. Starting from [Gly^8]GLP-1(7–37)-amide (**2**), tyrosine[19] was substituted with a *p*-carboxylphenyalanine (Cpa) residue to provide complementary functionality to facilitate *i* to *i* + 7 cyclization to lysine[26] to give **21**. This GLP-1 analogue was prepared using N^{α}-Boc/Bzl-based solid-phase synthesis[43] strategy with Fmoc-*p*-carboxylphenylalanine(O^{t}Bu)-OH and Boc-Lys(N^{ϵ}-Alloc)-OH residues at the cyclization sites. After the addition of Fmoc-*p*- carboxylphenylalanine(O^{t}Bu)-OH at residue 19 during the synthesis, the side chain ^{t}Bu group was removed with neat TFA, and the Alloc group from lysine[26] was removed using $Pd(PPh_3)_4/PhSiH_3$ in DCM.[44] After deprotection of these groups, PyBOP-mediated cyclization was used for lactam ring formation, and after Fmoc-deprotection of the N-terminus the remaining stepwise synthesis of the GLP-1 peptide was completed using standard protocols. In our assays, the GLP-1 receptor affinity and activity for **21** were found to be poor, approximately 700-fold and 600-fold lower than **2**, respectively.

Even though GLP-1 generally adopts an overall helical structure, the impact of incorporating a turn-inducing *i* to *i* + 5 lactam constraint between glutamic acid[21] and lysine,[26] respectively, was examined. The *i* to *i* + 5 derivative, **20**, was prepared and found to have relatively poor receptor affinity and activity (IC_{50} = 210 nM; EC_{50} = 470 pM).

Position 22 GLP-1 Analogues. The helix-interrupting glycine[22] residue in [Gly^8]GLP-1(7–37)-amide was substituted with a helix-favoring glutamic acid residue, which is present in the corresponding position in exendin-4. This modification resulted in good binding affinity (**4**, IC_{50} = 1 nM) and potent functional activity (3 pM) comparable to **2**. When aminoisobutryic acid

(Aib), an α,α-dialkylamino acid with strong helical propensity,[45,46] was introduced at position 22, binding affinity was retained, and the analogue still displayed potent functional activity (**5**, $IC_{50} = 3$ nM, $EC_{50} = 27$ pM) (Table 1). In comparison to the parent GLP-1 peptide **2**, the introduction of the Aib residue, **5**, increased apparent helical content as determined by circular dichroism analysis (see Supporting Information).

Plasma Stability and Conformation of GLP-1 Analogues. The plasma stability of the most potent conformationally constrained GLP-1 receptor agonists (**5**, **6**, **10**, and **11**) was evaluated and compared to GLP-1(7–37)-NH_2 **1** and [Gly^8]GLP-1(7–37)-NH_2 **2**. In this study, GLP-1(7–37)-NH_2 **1** had an approximate half-life of 30 min in human plasma at 37 °C, while as expected, the DPP-IV resistant analogue [Gly^8]GLP-1(7–37)-NH_2, **2**, had a 10-fold longer half-life ($t_{½} \approx 300$ min). Importantly, we found GLP-1 analogues **5**, **6**, **10**, and **11** were each in turn more stable than analogue **2** (plasma stability graph shown in Supporting Information). In general, the introduction of a lactam bridge into GLP-1 (**6**, **10**, and **11**) led to increased plasma stability compared to Aib^{22} substitution (**5**). In particular, c[Glu^{22}-Lys^{26}][Gly^8]GLP-1(7–37)-NH_2, **10**, displayed the highest plasma stability, with ∼75% of the compound remaining intact after 5 h in human plasma at 37 °C. The increased metabolic stability is attributed to the incorporation of either an α,α-dialkylamino acid (**5**) or side chain to side chain lactam constraint (**6**, **10**, and **11**), which serves to promote intramolecular hydrogen bonding associated with helix formation and reduce protease recognition.

Circular dichroism analysis of **1**, **6**, **10**, and **11** showed all peptides contained peak minima centered at 208 and 222 nm and a positive peak at 195 nm that are indicative of α-helical structure (see Supporting Information). The i to $i + 4$ cyclized peptides exhibited more helical structure compared to the linear native GLP-1 peptide **1**. Helical content rank ordering as determined by the Chou−Fasman method is as follows: **10** (∼70% helical) ∼ **6** (∼68% helical) > **11** (∼60% helical) > **1** (∼44% helical). Importantly, although increased helical content is observed with cyclized GLP-1 peptides, such as **6**, **10**, and **11**, the benefit of the cyclization modification centers on improved metabolic stability rather than increased potency. It is envisaged that the introduction of divergent residues into helix-favoring GLP-1 derivatives could lead to superior ligand–receptor interactions and greater affinity to the GLP-1 receptor. Nevertheless, side chain to side chain i to $i + 4$ cyclization is very well tolerated in **6**, **10**, and **11**, and in terms of binding and functional activity, these compounds are equivalent to their highly potent parent peptide, **2**.

In Vivo Activity of GLP-1 Analogues. The in vivo efficacy of conjugates derived from linear GLP-1, **2**, and conformationally constrained GLP-1 analogues with increased plasma stability and helical content, **5** and **10**, was examined. For conjugation to polyethylene glycol (PEG) the GLP-1 peptides were extended at their C-terminus. Interestingly, the nature of the C-terminus extension was found to effect the PEGylation yield as well as their in vivo efficacy. In general, C-terminal extensions with cysteine−serine−glycine or cysteine−alanine segments were found to be effective. Accordingly, GLP-1 compounds **2**, **5**, and **10** were extended at their C-terminus with cysteine−serine−glycine and PEGylated using 20 kDa monomethoxypolyethylene glycol maleimide[47,48] to give compounds **22**, **25**, and **24**, respectively, in order to prolong their in vivo circulation. PEGylation was carried out in pH 6 sodium phosphate buffer for 2 h, and mono-PEGylated products were purified by cation-exchange chromatography. The functional activity of compounds **22**, **25**, and **24**

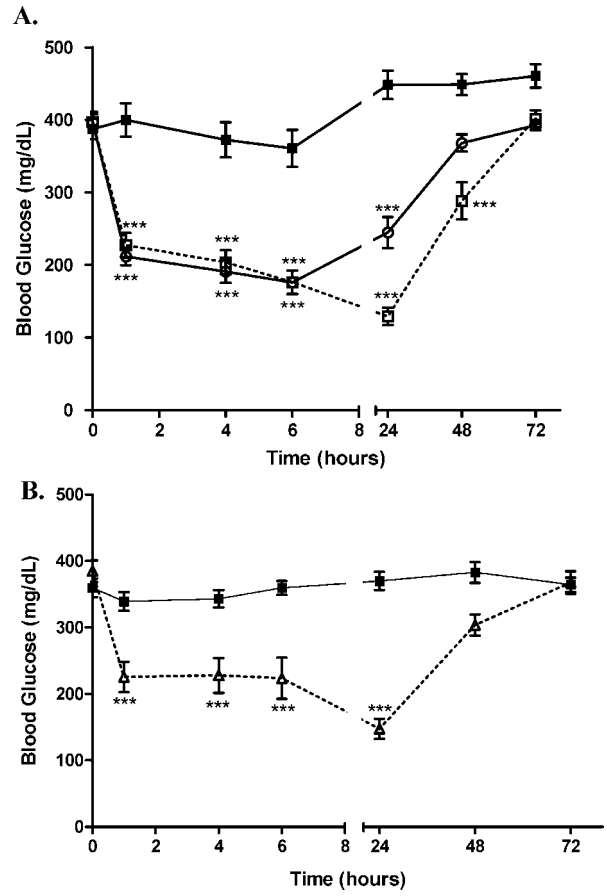


Figure 3. Effect of three PEG-GLP-1 analogues on blood glucose levels in *db/db* mice. Peptides dosed at 10 μg/mouse were compared: $n = 9$–10/group. (A) Filled squares are the mean ± SEM of the vehicle group. Open circles are **22**, and open squares are **25**. (B) Filled squares are the mean ± SEM of the vehicle group. Open triangles are **24**: (***) $p < 0.001$ compared to baseline blood glucose levels.

was 230, 320, and 170 pM, respectively (Table 1). GLP-1 compound **5** was also PEGylated in an analogous manner with a C-terminus cysteine−alanine extension to give **23** and had 130 pM functional activity. The PEGylated derivatives were formulated at 1 mg/mL in 10 mM sodium acetate, 5% sorbitol, pH 4, or phosphate buffered saline (PBS). The PEGylated peptides remained unchanged after incubation at −80 °C, 4 °C, room temperature, or 37 °C for >1 week as determined by ion-exchange HPLC (IEX-HPLC) and RP-HPLC analysis. *db/db* diabetic mice[49] were used to examine the effects of compounds **22**, **24**, and **25** in lowering blood glucose levels. Baseline blood glucose levels were measured immediately prior to each compound administration (time = 0) and at 1, 2, 4, 6, 24, 48, and 72 h or until blood glucose levels return to baseline levels. Mice treated with the PEGylated GLP-1 analogues containing Aib^{22} (**25**) and i to $i + 4$ glutamic $acid^{22}$−$lysine^{26}$ (**24**) constraints showed significantly lowered blood glucose levels for at least 48 and 24 h, respectively (Figure 3, (***) $p < 0.001$ versus baseline blood glucose levels). Blood glucose levels of the animals treated with compounds **25** and **22** returned to baseline levels at 72 and 48 h, respectively. At 24 h, blood glucose levels of the animals treated with GLP-1 analogue **22**, which does not contain conformational constraints, was not decreased to as low levels as blood glucose levels of animals treated with compounds **25** and **22**, and returned to baseline blood glucose levels by 48 h. In other in vivo experiments, where PEGylated GLP-1 analogues containing the Aib^{22}

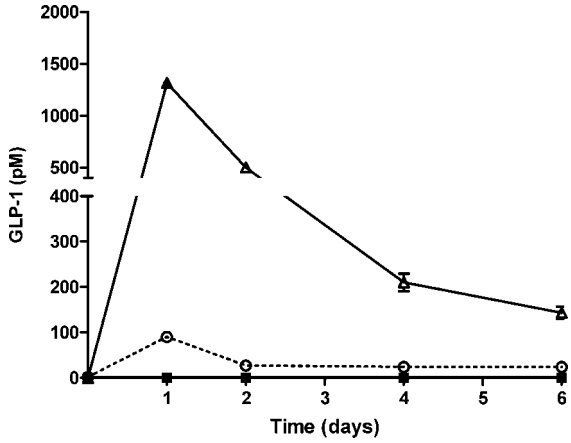


Figure 4. Measurement of GLP-1 levels in monkey serum after a single injection on day 0 of vehicle or analogue **23** at 0.2 or 2 mg/kg. GLP-1 levels were assessed from day 0 to day 6 by ELISA. Filled squares are the mean ± SEM of the vehicle group. Open circles are **23** dosed at 0.2 mg/kg, and open triangles are **23** at 2 mg/kg. $N = 5$/group.

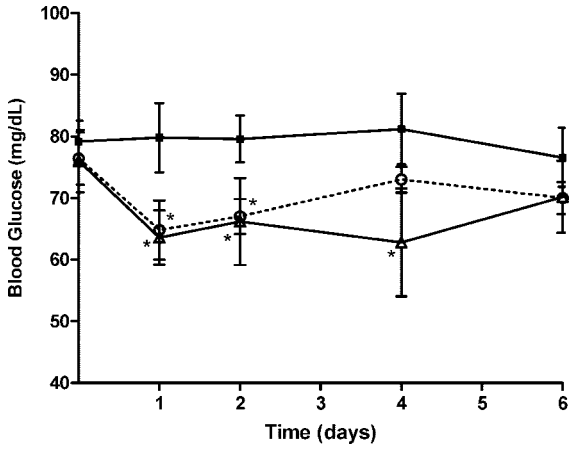


Figure 5. Effect of a single injection of analogue **23** on fasted blood glucose levels in nonanesthetized monkeys. Fasted blood glucose were measured on day −10 (prior to vehicle or compound administrations) and on days 1, 2, 4, and 6 (after vehicle or compound administrations). A single injection of vehicle or **23** at 0.2 or 2 mg/kg was performed on day 0. Filled squares are the mean ± SEM of the vehicle group. Open circles are **23** dosed at 0.2 mg/kg, and open triangles are **23** dosed at 2 mg/kg. $N = 5$/group, (∗) $p < 0.05$ compared to vehicle group.

substitution, **23** and **25**, were tested in *db/db* mice, **23** was one of the most efficacious analogue (data not shown).

The effects of GLP-1 analogue **23** were evaluated in male cynomolgus monkeys. Compound **23** was administered subcutaneously in a single bolus injection at 0.2 or 2 mg/kg on day 0. Plasma levels of **23** were measured on days 1, 2, 4, and 6 using a commercially available ELISA[50] for active GLP-1. Estimated half-life of compound **23** at 2 mg/kg was 2.2 ± 0.5 days (Figure 4). For the 0.2 mg/kg dose, fasted blood glucose levels were decreased for the first 2 days (Figure 5). On day 4, fasted blood glucose levels of animals treated with 2 mg/kg of **23** were still significantly decreased (∗, $p < 0.05$) and returned to baseline glucose levels on day 6. Fed blood glucose levels were statistically significantly decreased to the same extent with both doses on day 1. On day 3 only the high dose statistically significantly showed decreased fed blood glucose levels, and in this group fed blood glucose levels returned to baseline glucose values on day 5 (data not shown). In addition to reduced glucose levels in nonhuman primate models, GLP-1 receptor agonism with other ligands, such as exendin-4, have also led to reduced body weight in nonhuman primates and humans apparently through changes in meal sizes.[51,52] Consequently, after a single administration of **23**, body weight was measured every week and percentage change of body weight was calculated for each monkey. Body weight decreased in the monkeys treated with both doses in the first week after the injection, but only body weights of monkeys treated with 2 mg/kg of compound **23** were still decreased on week 2 and their body weights were back to normal on week 3 (Supporting Information).

We found that a single administration of **23** in diabetic (*db/db*) mice or cynomolgus monkeys lowered blood glucose levels between 24 h and 6 days. We anticipate that such compounds could be used to lower blood glucose levels in patients with type 2 diabetes with once a week subcutaneous administration.

Conclusion

We found that the introduction of i to $i + 4$ glutamic acid−lysine lactam constraints between residues 18 and 22 (**6**), 22 and 26 (**10**), and 23 and 27 (**11**), or an Aib substitution at position 22 (**5**) in the GLP-1 peptide is very well tolerated and increases helical content. These peptides are highly potent GLP-1 receptor agonists with receptor affinity comparable to native GLP-1, but are distinguished by their improved plasma stability. PEGylated GLP-1 peptides **23** and **24** retained high potency and strong receptor binding properties but importantly exhibited prolonged in vivo efficacy with respect to blood glucose and body weight decrease for several days in rodents and nonhuman primates.

Experimental Section

Materials. N^{α}-Fmoc protected amino acids and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin were purchased from Novabiochem (San Diego, CA) and Bachem (Torrance, CA). The following compounds were purchased: trifluoroacetic acid (TFA, Burdick and Jackson); *N,N*-diisopropylethylamine (DIEA), piperidine, acetic anhydride, and phenylsilane (Aldrich, Milwaukee, WI); dichloromethane (DCM, Mallinckrodt Baker, Inc.); *N,N*-dimethylforamide (DMF, Burdick and Jackson); 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, Matrix Innovation, Montreal, CA; HPLC-quality water and acetonitrile (Burdick and Jackson); [^{125}I]Tyr-GLP-1 (PerkinElmer Life Sciences, Boston, MA); luciferase assay system (Promega Corporation, Madison, WI); Tissue-Tearor (Biospec Products). Protease-free bovine serum albumin (BSA), sucrose, sorbitol, sodium azide, and Tris-HCl were from Sigma, St. Louis, MO. STI and Pefabloc SC were purchased from Roche Applied Sciences (Mannheim, Germany). Active GLP-1 ELISA was purchased from Linco Research (St. Charles, MO).

Peptide Synthesis. GLP-1 analogues were prepared by solid-phase methods employing N^{α}-Fmoc/*tert*-butyl chemistry. N^{α}-Fmoc, side chain protected amino acids, Wang resin, and Rink amide resin were used. The following side chain protection strategies were employed for standard amino acid residues: Asp(O^{t}Bu), Arg(Pbf), Cys(Acm), Glu(O^{t}Bu), His(Trt), Lys(N^{ϵ}-Boc), Ser(O^{t}Bu), Thr(O^{t}Bu), and Tyr(O^{t}Bu). Lys(N^{ϵ}-Mtt), Glu(O2-PhiPr), Lys(N^{ϵ}-Alloc), and Cpa(O^{t}Bu) were used at cyclization sites. GLP-1 peptide derivatives were synthesized in a stepwise manner on a Ranin Symphony synthesizer by SPPS using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU)/*N,N*-diisopropylethylamine (DIEA)/*N,N*-dimethylformamide (DMF) coupling chemistry at 0.2 mmol equiv resin scale (Fmoc Rink amide resin). For each coupling cycle, 1 mmol of N^{α}-Fmoc-amino acid, 4 mmol of DIEA, and 1 mmol equiv of HBTU were used. The concentration of the HBTU-activated Fmoc amino acids was 0.2 M in DMF, and the coupling

time was 45 min. Fmoc deprotections were carried out with two treatments using a 30% piperidine in DMF solution first for 2 min and then for an additional 20 min.

General Procedure for the Cyclization via Lactam Formation. Side chain to side chain lactam formation was carried out on the assembled N-terminally Fmoc-protected resin-bound peptide. At cyclization sites, side chain amino and carboxyl functionalities were protected with 4-methyltrityl (Mtt) groups and 2-phenylisopropyl ester (2-PhiPr) groups, respectively. In a typical example of cyclic lactam formation on the resin, following assembly of the fully protected peptide on the solid support, the resin was washed with DCM (3 × 2 min). The Mtt and 2-PhiPr groups (protecting group at the specified lactam bond forming site) were removed by repeated treatment with 1% TFA in DCM solution containing 5% TIS. Treatment of the peptide−resin with the 1% TFA in DCM solution was repeated eight times in 30 min increments, and each treatment was followed by extensive DCM washes. The resin was then washed with DMF (3 × 1 min). The liberated carboxyl and amino groups were then condensed by the addition of 5 equiv of 0.5 M benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) and 10 equiv of DIEA in DMF to the peptide−resin and left for 24 h. This process was repeated until a negative Kaiser ninhydrin test. The resin was then wash thoroughly with DMF, DCM, and DCM/MeOH and dried.

Side Chain Deprotection and Cleavage from Resin. Following synthesis and modification, the resin was then drained and washed with DCM, DMF, DCM and then dried in vacuo. The resin bound peptide (0.2 mmol) was placed in a disposable 50 mL polystyrene tube. The peptide−resin was deprotected and released from the resin by treatment with a 10 mL solution of trifluoroacetic acid (TFA)/1,2-ethanedithiol (EDT)/triisopropylsilane (TIS)/H_2O (92.5:2.5:2.5:2.5 v/v) at room temperature for 90 min. The combined solution was concentrated and the crude peptide precipitated with cold diethyl ether (40 mL). The peptide was collected by centrifugation to remove the ether and washed with another 40 mL of diethyl ether. The peptide was centrifuged and dried in vacuo.

Reversed-Phase HPLC Analysis, Purification, and Mass Spectrometry. Reversed-phase high-performance liquid chromatography was performed on Waters 2795 analytical RP-HPLC system using a 1% per min linear gradient (0–60% buffer B in 60 min; A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile) on a Kromasil C_{18}, 5 μm, 300 Å, 0.46 cm × 5 cm) column at a flow rate of 1 mL/min with UV monitoring at 214 nm. Preparative peptide purification was performed on a Water FractionLynx HPLC system typically using 5–95% gradient of buffer B versus buffer A in 120 min on a Kromasil C_{18}, 10 μm, 300 Å, 2.2 cm × 25 cm column at 20 mL/min with monitoring at 214, 230, and 280 nm. Mass analysis of GLP-1 products and HPLC fractions were performed on a Waters Acquity UPLC-LCT Premier system (Z-spray ionization coupled time-of-flight (TOF) mass spectrometer. Samples (typically 2 μL) were chromatographed on an Agilent Eclipse column (XDB-C18 2.1 mm × 50 mm, 1.8 μm) using a 5–95% gradient of buffer B versus buffer A in 10 min. Molecular masses were derived from the observed *m*/*z* values from the LCT Premier TOF mass spectrometer.

PEGylation. Monofunctional methoxyPEG-maleimide (Nektar, CA) was reacted chemospecifically with cysteine−GLP-1 peptide analogues. The peptide was dissolved at 2 mg/mL in 50 mM sodium phosphate buffer containing 5 mM EDTA at pH 6. Solid 20 kDa monomethoxyPEG−maleimide was added in 1.2- to 1.5-fold molar excess and allowed to react for 2 h at room temperature. The reaction was monitored by reverse phase HPLC, quenched with 5 mM β-mercaptoethanol, allowed to incubate at room temperature another 30 min, and then purified. Purification was achieved by preparative cation-exchange chromatography using SP Sepharose HP (GE Healthcare) and eluting with a linear 0–500 mM sodium chloride gradient. The eluted PEG−peptide was monitored by RP-HPLC and SDS−PAGE, pooled, then concentrated and dialyzed into 10 mM sodium acetate containing 5% sorbitol at pH 4.

Circular Dichroism. Far-UV CD spectra were recorded on a Jasco J-840 spectropolarimeter at ambient temperature using a cuvette with 0.02 cm path length. The peptide concentrations were 0.5 mg/mL, and individual spectra obtained were the average of 10 accumulations. Helical content was determined by the Chou−Fasman method.[54]

NMR. NMR experiments were performed at 311K using a Bruker DRX-600 spectrometer equipped with an inverse-detection cryoprobe. Chemical shift assignments were based on $^1H-^1H$ TOCSY, $^1H-^1H$ NOESY, and $^1H-^{13}C$ HSQC experiments. $^1H-^1H$ dipolar interactions were measured by $^1H-^1H$ NOESY experiments, with 100 ms mixing times. Samples contained 1 mM peptide, 300 mM DPC-d_{38}, and 50 mM sodium phosphate buffer at pH 5.5.

Plasma Stability. HPLC grade water and methanol were obtained from Burdick and Jackson (Muskegon, MI), and formic acid was from J. T. Baker (Philipsburg, NJ). Human plasma was obtained from Bioreclamation Inc. (East Meadow, NY). In vitro stability of GLP-1 peptides was carried out with initial concentration of 1000 ng/mL of each peptide in human plasma at 37 °C. A 100 μL of plasma was aliquoted from the incubation solution at 0, 0.5, 1, 1.5, 2, 3, 4, and 5 h time points followed by solid phase extraction on an Oasis HLB 96-well plate (Waters, Milford, MA). Then 20 μL of the extract was injected into the LC−MS/MS system. Mass spectrometry was carried out on an API4000 triple quadrupole mass spectrometer from Applied Biosystems (Foster City, CA) with Turbo ESI source. The signal of GLP-1 peptides was monitored through multiple reaction monitoring (MRM). Reverse phase liquid chromatographic separation was performed on a Polaris C18A column (5 μm, 2.0 mm × 75 mm, Varian). The mobile phase consisted of 5% methanol in water with 0.1% formic acid (solvent A) and 95% methanol in water with 0.1% formic acid (solvent B). The linear gradient elution was 5–95% B in 6.5 min at a flow rate of 400 μL/min.

Cloning of the Human GLP-1 Receptor. The human GLP-1 receptor was isolated from a human heart cDNA library (Clontech, Palo Alto, CA) by nested PCR. PCR primers were designed to hybridize to 5′ and 3′ untranslated regions of the GLP-1R cDNA. The sequence of the cloned GLP-1R was verified by double-stranded sequencing and comparison to Genbank Accession numbers U01156.1 and U10037.1.[8,53] One polymorphism was noted (G to S at aa 168), for which SNP analysis reported an allele frequency of 15%. The cloned receptor was ligated into EcoR1/Xba1 (New England Biolabs, Ipswich, MA) digested pcDNA3.1*Zeo*(+) (Invitrogen, Carlsbad, CA).

Cell Culture and Expression of GLP-1R. The human GLP-1 receptor expression construct was transfected into CHOK1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacurer's protocol. Stably transfected cells were selected using 333 μg/mL Zeocin (Invitrogen, Carlsbad, CA) in F-12 medium, supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Individual subclones were screened for [^{125}I]-GLP-1 binding in order to select a cell line for receptor binding studies.

For functional assays, the human GLP-1 receptor was transfected into a CHO cell line harboring a luciferase reporter construct. The CHO K1 (46–24 LUC) parental cell line contains a luciferase reporter gene regulated by a SV40 promoter element (pGL3 reporter vector, Promega, Madison, WI). Transcriptional activity is enhanced via multiple (6) repeats of 5′ proximal cAMP response element (CRE) binding sites. In addition to selection for Zeocin resistance, subclones were screened for GLP-1 induced luciferase activity.

Membrane Preparation. Membrane preparations of cells expressing the human GLP-1 receptor were harvested from 150 mm culture dishes using PBS. Cells were sedimented at 1500 rpm for 10 min. The resulting pellets were homogenized in 15 mL of ice-cold sucrose buffer (25 mM Tris-HCl, 0.32 M sucrose, 0.25 g/L sodium azide, pH 7.4) with a motorized, glass-fitted Teflon homogenizer. The homogenate was centrifuged at 48,000*g* at 4 °C for 10 min, resuspended in 25 mL of assay buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mg/mL protease-free BSA, 0.1 mg/mL STI, and 0.1 mg/mL Pefabloc, pH 7.4) with a Tissue-Tearor (Biospec Products), then centrifuged again at 48000*g* for 10 min. The pellets were homogenized for a third time in 15 mL of assay buffer using

the Tissue-Tearor and again centrifuged at 48000*g* for 10 min. Membrane pellets were stored at −80 °C until needed.

Ligand Binding Assay. Cell membrane pellets were resuspended in assay buffer at a wet weight concentration of 4 mg/mL. Ligand binding assays were performed in 96-well U-bottom plates. Two hundred micrograms of prepared tissue per well was incubated at room temperature for 2 h in assay buffer containing 0.2 nM [^{125}I]-GLP-1 (PerkinElmer Life Sciences, Boston, MA) and a range of concentrations of test compound or GLP-1 in a total volume of 100 μL. In addition, nonspecific binding was assessed in the presence of 1 μM unlabeled GLP-1. The reaction was terminated by rapid filtration through Unfilter-96 GF/C glass fiber filter plates (FilterMate 196 Packard Harvester, PerkinElmer, Shelton, CT) presoaked in 0.5% polyethylenimine, followed by three washes with 300 μL of cold 50 mM Tris-HCl, pH 7.4. Bound radioactivity was measured using a TopCount microplate scintillation and luminescence counter (Packard Instrument Company, PerkinElmer, Shelton, CT). Nonlinear regression analyses of resulting concentration curves were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The "IC_{50}" represents the concentration of compound that reduces the maximal specific [^{125}I]-GLP-1 binding by 50%. Assays were performed in triplicate and repeated at least three times.

Cell-Based GLP-1R Functional Assay. To compare the potency of test compounds with GLP-1, reporter cell lines expressing human GLP-1 receptors were used to measure compound induced increases in cAMP levels through enhanced expression of the luciferase reporter gene. Recombinant cells were plated 2 days prior to the assay, then cultured at 37 °C, 5% CO_2, and saturating humidity. During the evening prior to assay, the cells were washed and the medium was replaced with serum-free medium containing 0.5% protease-free bovine serum albumin (BSA) and then cultured overnight. Cells were exposed to a range of concentrations of test compound or GLP-1 for a period of 6 h at 37 °C in medium containing 0.5% protease-free BSA and 100 μM IBMX. Cell lysates were assayed for luciferase activity using the luciferase assay system (Promega Corporation, Madison, WI). Luciferase activity was measured using a Luminoskan Ascent (Thermo Electron Corporation, Marietta, OH). Nonlinear regression analyses of resultant compound concentration curves were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The "EC_{50}" represents the concentration of compound at which 50% of the maximal activity is achieved.

Mouse Husbandry. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee of Amgen Laboratory Animal Resources. *db/db* male mice at 8 weeks of age were acquired from Jackson Laboratory and delivered to Amgen animal facility for acclimation. Animals were maintained under controlled conditions of 21 °C and 12 h light/dark cycles (6:30 a.m./6:30 p.m.) with free access to food (no. 8640, Harlan Teklad 22/5 rodent diet) and water. Mice were used for experiments at 10–11 weeks of age.

Mouse in Vivo Experiments. *db/db* diabetic mouse model was used in this screen to further examine the effect of GLP-1 compounds on fed blood glucose lowering. Blood glucose levels were measured immediately before the tested compound intraperitoneal administration at time 0 and 1, 2, 4, 6, 24, 48, and 72 h (or until blood glucose levels returned to baseline levels) after the tested compound administration. The criterion for selection for each mouse to enter the study was blood glucose levels of at least 250 mg/dL. On the day of the experiment, mice were bled at 9 a.m. (time 0 blood glucose level) and then immediately handed over to the injector, who then injected the appropriate GLP-1 compound or vehicle. Mice were then placed in a fresh cage without any chow to limit any variability in blood glucose levels associated with eating behaviors. Normal chow was given back after the 6 h time point blood glucose levels were collected.

Cynomologus Monkey Husbandry. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee of SNBL USA Animal Resources. The animals, which have previously been quarantined, were acclimated to the study room at the SNBL USA facility for a minimum of 42 days prior to initiation of dosing. Animals were maintained under controlled conditions between 24 and 26 °C and 12 h light/dark cycles (6:30 a.m./6:30 p.m.) with free access to food.

Efficacy of Compound 23 in Cynomolgus Monkey. Vehicle or compound **23** at 0.2 or 2 mg/kg was subcutaneously administered on day 0. Fasted blood glucose levels from nonanesthetized animals were measured on day −10 (prior to vehicle or compound administrations) and days 1, 2, 4, 6 (after vehicle or compound administrations). Body weights were measured weekly. Active GLP-1 levels were measured using the GLP-1 (Active) ELISA kit (catalogue no. EGLP035K, Linco Research, St. Charles, MO) following the manufacturer's protocol.

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Supporting Information Available: Displacement of [^{125}I]-GLP-1 with **2**, **5**, **6**, **10**, **17**, and **18**; functional dose response curve of **22**−**25**; far-UV CD spectrum of **1**, **2**, **6**, **10**, and **11**; NMR data for **6**; percent change in monkey body weight after dosing compound **23**; plasma stability of **1**, **2**, **5**, **6**, **10**, and **11**; and list of purity data, k', and calculated and experimentally observed molecular weights of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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