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A Novel Human-Based Receptor Antagonist of Sustained Action Reveals Body Weight Control by Endogenous GLP-1

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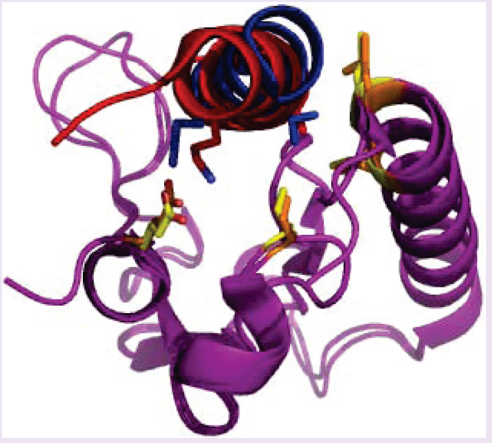
Supporting Information

ABSTRACT: Ex-4 (9-39)a is a well characterized GLP-1 receptor antagonist that suffers from two notable limitations, its nonhuman amino acid sequence and its relatively short *in vivo* duration of action. Comparable N-terminal shortening of human GLP-1 lessens agonism but does not provide a high potency antagonist. Through a series of GLP-1/Ex-4 hybrid peptides, the minimal structural changes required to generate a pure GLP-1-based antagonist were identified as Glu16, Val19, and Arg20, yielding an antagonist of approximately 3-fold greater *in vitro* potency compared with Ex-4 (9-39)a. The structural basis of antagonism appears to result from stabilization of the α helix combined with enhanced electrostatic and hydrophobic interactions with the extracellular domain of the receptor. Site-specific acylation of the human-based antagonist yielded a peptide of increased potency as a GLP-1 receptor antagonist and 10-fold greater selectivity relative to the GIP receptor. The acylated antagonist demonstrated sufficient duration of action to maintain inhibitory activity when administered as a daily subcutaneous injection. The sustained pharmacokinetics and enhanced human sequence combine to form an antagonist optimized for clinical study. Daily administration of this antagonist by subcutaneous injection to diet-induced obese mice for 1 week caused a significant increase in food intake, body weight, and glucose intolerance, demonstrating endogenous GLP-1 as a relevant hormone in mammalian energy balance in the obese state.



Glucagon-like peptide-1 receptor (GLP-1R) activation has been observed to stimulate glucose-dependent insulin secretion (the incretin effect), decrease gastric motility, inhibit glucagon secretion, and increase β-cell mass.[1] GLP-1 and its paralog exendin-4 (Ex-4) are both α helical peptides capable of activating the GLP-1 G protein-coupled receptor.[2−4] These homologous peptides (∼50% sequence similarity) share a common hydrophobic face but differ at several amino acids including alternating charged residues in Ex-4 that appear to function in stabilizing the α helix (Supplementary Figure 1).

Ex-4 (9-39)a is an established antagonist of the GLP-1 receptor.[5−7] Eng and colleagues have demonstrated that GLP-1 agonism is sensitive to N-terminal truncation but, unlike Ex-4, without any evidence of *in vitro* antagonism.[7] Through analysis of hybrid GLP-1/Ex-4 antagonists, a sequence within the central to C-terminal region of Ex-4 was identified that appreciably strengthened receptor affinity. The specific molecular determinants of the increased binding interaction were not identified and could provide a route to GLP-1-based antagonists more suitable for human study than the reptilian, Ex-4-based antagonist. Consequently, continuous pump infusion of Ex-4 (9-39)a is the only pharmacological route to achieve persistent GLP-1R antagonism. Preclinical studies commonly utilize GLP-1R knockout mice as an alternative approach.[8] While these animals have been of utmost importance for our understanding of GLP-1 biology, a pharmacological approach is required for clinical studies. A long-acting human sequence antagonist would be preferred since the reported immunogenicity of Ex-4[9,10] is a likely limitation to chronic human use of an exendin-4-based antagonist, especially given the projected higher dose in comparison to agonist peptides.

Antagonism at the GLP-1R has been observed to correct blood glucose in *SUR-1*$^{-/-}$ mice, suggesting that antagonism might be suitable therapy for treating congenital hyperinsulinism and the resultant hypoglycemia.[11] GLP-1R antagonists may also have therapeutic utility in management of hypoglycemia that has been observed subsequent to bariatric surgery.[12] The prevalence of such observations is steadily increasing with the increased popularity of surgical management of obesity. Blocking GLP-1 receptor activity abolishes excessive insulin secretion, thus elevating glucose levels.[11,13] Paradoxically, GLP-1R antagonism may also prove useful in preventing the development of adult-onset diabetes where pancreatic hyperstimulation precedes β-cell

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exhaustion.[14] Restoration of reduced insulin secretion is hypothesized to restore peripheral insulin sensitivity and promote glucose homeostasis.

Recently, the crystal structures of GLP-1 and the antagonist Ex-4 (9-39)a bound to the GLP-1R extracellular domain were solved.[15,16] The structures reveal that antagonist binding spans the peptide helix from positions 15 to 27, making both hydrophobic and hydrophilic interactions. It is surmised that the conserved N-terminus of the peptide extends into the core domain of the receptor to facilitate signal transduction.[17] The extracellular portion of the full-length GLP-1 receptor has been reported to be responsible for selectivity between GLP-1 and the structurally related hormone glucagon, through interactions at the C-terminus of the peptide.[18,19] What is less understood is how GLP-1 binds with comparable affinity as Ex-4 to the full-length receptor but exhibits a dramatically reduced affinity for the extracellular domain.[20−22] The ability to more favorably bind the GLP-1R extracellular domain could therefore constitute the means by which Ex-4 (9-39)a successfully functions as an antagonist.

We have designed a series of hybrid GLP-1/Ex-4 N-terminally truncated peptides to elucidate the structural requirements for binding the GLP-1R. Experimental results in this report identify three Ex-4 residues that enable high affinity antagonism in truncated GLP-1 analogues (Table 1). The specific amino acid substitutions include two hydrophilic changes and one hydrophobic enhancement. Simultaneous substitution of GLP-1 (9-30)a with amino acids Glu16, Val19, and Arg20 bestowed superior potency relative to Ex-4 (9-39)a antagonism in a functional *in vitro* assay by approximately 3-fold. The GLP-1-based antagonist proved equally effective to Ex-4 (9-39)a when assessed in an acute *in vivo* mouse provocative test of glucose tolerance against challenge with native agonist. Application of site-specific C-terminal fatty acid acylation further enhanced receptor antagonism and prolonged *in vivo* pharmacology, such that daily subcutaneous administration suitable for chronic therapy was made possible. One week of daily administration of the sustained-acting antagonist to diet-induced obese (DIO) mice demonstrated a progressive increase in food intake, body weight, and impaired glucose tolerance.

RESULTS

Probing the Agonist to Antagonist Transition. A series of hybrid GLP-1/Ex-4 peptides that introduced distinctive features of Ex-4 (Glu16 and/or C-terminal sequence extension of exendin-4 as noted in Supplementary Figure 1 (Cex)) to the GLP-1 sequence were synthesized and biochemically analyzed (Table 1 and Supplementary Table 1). Ex-4 (9-39)a (**4**) fully blocked GLP-1R-mediated cAMP induction in human embryonic kidney (HEK) 293 cells overexpressing the GLP-1 receptor with an IC_{50} of 220 nM (Figure 1, panel a and Table 1). There was no evidence of cAMP induction observed when Ex-4 (9-39)a was incubated in the absence of GLP-1, confirming antagonism without any apparent agonism (Figure 1, panel b). Analogous N-terminal truncation of GLP-1 yielding GLP-1 (9-30)a (**5**) decreased potency but did not enable antagonism at the GLP-1R (Figure 1, panel a). Quite to the contrary, full receptor agonism was maintained but reduced in potency approximately ten thousand-fold (Figure 1, panel b). Appendage of Cex to GLP-1 (9-30)a to form GLP-1-Cex (9-39)a (**6**), lowered maximal cAMP induction (Figure 1, panel a), but the peptide still functioned as a weak agonist (Figure 1, panel b). Further modification with introduction of Glu16 provided analogue **7**, which demonstrated partial agonism and slightly enhanced antagonism (Figure 1). The comparable behavior of analogues **6** and **7** encouraged the synthesis of the C-terminally shortened Glu16-substituted GLP-1 (9-30)a analogue (**8**). This peptide displayed partial antagonism much like that of **7**, which establishes Glu16 as one amino acid difference between exendin-4 and GLP-1 that contributes to the antagonism of truncated analogues. Comparative binding analysis of peptides **6**−**8** revealed a 2-fold improvement in potency attributable to Glu16 and no further enhancement through Cex (Table 2). It is clear from these results that Ex-4 residues other than Glu16 and Cex contribute sizably to GLP-1R antagonism.

To further investigate the structural basis for antagonism, a series of hybrid GLP-1/Ex-4 peptides were prepared with linear portions of the Ex-4 sequence substituted into the GLP-1-Cex (9-39)a Glu16 peptide. Three hybrid peptides of the C-terminal (residues 21−30; Jant-1), central (residues 17−21; Jant-2), and N-terminal (residues 10−14; Jant-3) regions of the hormone were studied. Though Cex was not contributing differential activity, it was utilized to enhance biophysical character, which improved synthetic yield and aqueous solubility. The Jant-1 (**17**) and Jant-3 (**19**) hybrid peptides proved to be partial antagonists with similar biochemical activity as the parent peptide **7** (Figure 2). In contrast, Jant-2 (**18**) antagonized the GLP-1 receptor with a nearly 3-fold enhancement in potency, IC_{50} 90 nM, relative to Ex-4 (9-39)a (**4**) (Figure 2, panel a and Table 1). As observed for Ex-4 (9-39)a (**4**), no detectable GLP-1R agonism was apparent in peptide **18** (Figure 2, panel b). Thus, it appears the amino acids in addition to Glu16 that bestow antagonism and eliminate residual agonism are located within the sequence 17−21 of Ex-4.

Since Ex-4 antagonists transition toward agonism with extension at the N-terminus, Jant analogues were also synthesized as (6-39)a peptides (Supplementary Figures 2−4). Maximal agonism of all peptides truncated by at least five residues was lower than that of the full-length agonists. Ex-4 (6-39)a (**11**) displayed partial antagonism where the maximal suppression ranged from 50% to 85% throughout multiple analyses. The analogous Glu16 GLP-1-based peptide (**12**) was an agonist at the GLP-1R (Supplementary Figure 3, panel b). Notably, all peptides where the first two amino acids were deleted to mimic DPP-IV cleavage products (**10**, **13**, **15**, **31**) proved to be weak but full agonists in our *in vitro* assay using engineered cells that overexpress the human GLP-1 receptor (Supplementary Figure 2 and Table 1). This is consistent with the initial study of peptide **13**[7] but incongruent with the report of Knudsen and Pridal[23] where low potency *in vitro* antagonism was reported but the physiological relevance was questioned. More recently, human study of this peptide has revealed no biological activity suggestive of a GLP-1 antagonist or agonist.[24] Introduction of Ex-4 residues 17−21 to the GLP-1 (6-39)a Glu16 peptide to yield **21** conferred high potency antagonism as previously observed with the slightly shorter analogous (9-39)a peptide **18** (Supplementary Figure 4, panel a). Of additional importance was the lesser residual agonism displayed by **21** relative to **11**, Ex-4 (6-39)a (Supplementary Figure 4, panel b). These experimental findings with N-terminally lengthened antagonists validate that substitution of the central amino acids of Ex-4 provides a potent GLP-1-based antagonist devoid of any apparent agonism.

Site-Specific Basis for Antagonism. The further definition of the molecular basis for GLP-1R antagonism bestowed by residues 16−21 was investigated through a series of Jant-2-based

Table 1. Summary of antagonist and agonist functional assays[a]

No.	Peptide	Antagonism, IC_{50} (nM)	Agonism, EC_{50} (nM)
1	GLP-1 (1-30)a[b]	n/a	0.03 ± 0.01
2	Ex-4 (1-39)a	n/a	0.009 ± 0.004
3	Ex-4 (1-30)a	n/a	0.009 ± 0.005
4	Ex-4 (9-39)a	220 ± 50	n/a
5	GLP-1 (9-30)a	n/a	300 ± 90
6	GLP-1-Cex (9-39)a	5,600 ± 1,500	800 ± 40
7	GLP-1-Cex (9-39)a Glu16	1,600 ± 500	350 ± 90
8	GLP-1 (9-30)a Glu16	2,200 ± 900	320 ± 80
9	Ex-4 (9-30)a	230 ± 50	n/a
10	Ex-4 (3-39)a	n/a	9 ± 1
11	Ex-4 (6-39)a	190 ± 80	9 ± 2
12	GLP-1-Cex (6-39)a Glu16	n/a	10 ± 3
13	GLP-1 (3-30)a	n/a	140 ± 10
14	GLP-1 (6-30)a	n/a	570 ± 90
15	Ex-4 (3-30)a	n/a	8 ± 1
16	Ex-4 (6-30)a	n/a	10 ± 2
17	Jant-1 (9-39)a	n/a	860 ± 110
18	Jant-2 (9-39)a	90 ± 20	n/a
19	Jant-3 (9-39)a	870 ± 200	180 ± 70
20	Jant-1 (6-39)a	670 ± 260	n/a
21	Jant-2 (6-39)a	80 ± 10	n/a
22	Jant-3 (6-39)a	n/a	20 ± 3
23	Jant-2a (9-39)a	370 ± 70	n/a
24	Jant-2b (9-39)a	490 ± 70	330 ± 100
25	Jant-2c (9-39)a	130 ± 50	n/a
26	Jant-2d (9-39)a	130 ± 40	n/a
27	Jant-2e (9-39)a	190 ± 40	n/a
28	Jant-4 (9-39)a	70 ± 10	n/a
29	Jant-4 (9-39)a Ala19	250 ± 30	n/a
30	Jant-4 (9-39)a Lys20	200 ± 50	n/a
31	Jant-4 (3-30)a	n/a	20 ± 2
32	Jant-4 (6-30)a	80 ± 20	30 ± 2
33	Jant-4 (9-30)a	90 ± 20	n/a
34	Jant-4 (9-30)a Gly16	340 ± 30	n/a
35	Jant-4 (9-30)a Aib16	70 ± 10	n/a
36	Ex-4 (9-30)a Gly16Ala19Lys20	n/a	880 ± 280
37	Jant-4a (9-30)a	110 ± 10	n/a
38	Jant-4b (9-30)a	270 ± 50	n/a
39	Jant-4c (9-30)a	430 ± 100	n/a
40	Ex-4 (9-40)a Lys40-C16	20 ± 6	n/a
41	Jant-4 (9-40)a Lys40-C16	20 ± 4	n/a

[a] Antagonist assays were normalized to the Ex-4- or Jant-4-based antagonist IC_{50} values, and the same normalization was performed with agonist assays using the EC_{50} value of GLP-1. [b] GLP-1 numbering is consistent with Ex-4, positions (1-30)a corresponding to GLP-1 (7-36)a.

peptides. The design held residues Glu16, Ala18, and Leu21 constant while Ex-4 derived amino acids at 17 (Glu), 19 (Val), and 20 (Arg) were substituted to the GLP-1-Cex (9-39)a sequence (Supplementary Table 1). Ala18 is conserved between Ex-4 and GLP-1. Leu21 was held constant as it is common to Jant-1, 2 and to prevent a potential negative charge conflict with Ex-4 Glu17 or GLP-1 Glu21 (**23**). Leu21 decreased residual agonism as was observed in peptides **4** and **18** but did not improve potency (Supplementary Figure 5). Glu17 (**24**) did not appear to contribute to antagonism (Table 1). Substitution of either Val19 (**25**) or Arg20 (**26**) enhanced the potency of antagonism beyond that of Ex-4 (9-39)a (**4**). However, neither of these single substitutions yielded potency equivalent to that of **18** (Supplementary Figure 5, panel a and Table 1), where both changes were resident.

To explore the putative interaction of Glu17 with Arg20, the single change of Gln17 in **26** was studied. No enhancement in antagonism appeared in this analogue (**27**) relative to peptide **26** (Table 1). We reasoned that Val19 and Arg20 were additively strengthening the potency of the Jant-2 analogue (**18**), which led to the analysis of the triple substitution Glu16, Val19, Arg20 analogue (**28**) to define the minimal changes to yield a human-based GLP-1

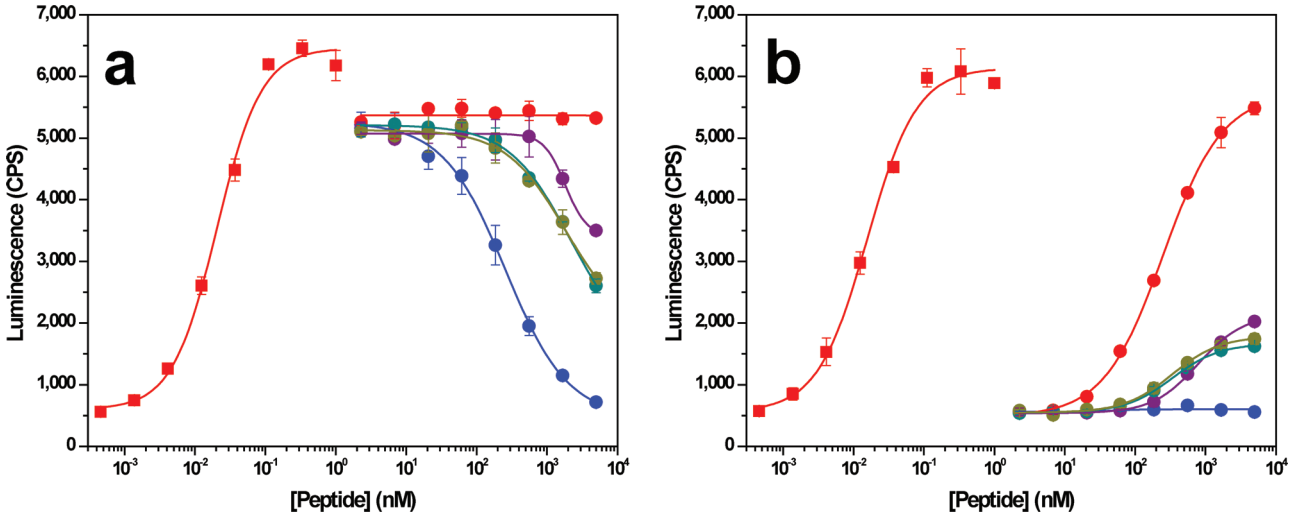

Figure 1. *In vitro* measurement of GLP-1R-mediated cAMP of Glu16 and/or Cex substitution on N-truncated GLP-1 peptides. GLP-1R cAMP induction was monitored in antagonist (a) and agonist (b) assay formats by measuring luminescence output as described in Methods. (red ■) GLP-1 (1-30)a; (blue ●) Ex-4 (9-39)a; (red ●) GLP-1 (9-30)a; (purple ●) GLP-1-Cex (9-39)a; (cyan ●) GLP-1-Cex (9-39)a Glu16; (mustard ●) GLP-1 (9-30)a Glu16.

Table 2. Summary of GLP-1 receptor binding[a]

No.	Peptide	Binding, IC_{50} (nM)
1	GLP-1 (1-30)a	0.8 ± 0.3
2	Ex-4 (1-39)a	0.6 ± 0.2
4	Ex-4 (9-39)a	14 ± 2
6	GLP-1-Cex (9-39)a	120 ± 40
7	GLP-1-Cex (9-39)a Glu16	60 ± 15
8	GLP-1 (9-30)a Glu16	60 ± 10
28	Jant-4 (9-39)a	4 ± 1
33	Jant-4 (9-30)a	5 ± 1

[a] [^{125}I]-GLP-1 competitive ligand binding was detected in a scintillation proximity assay as detailed in Methods.

antagonist. Peptide **28**, Jant-4, antagonized the GLP-1 receptor (IC_{50} 70 nM) with equivalent potency as **18** and was superior to the well-established Ex-4 (9-39)a antagonist, peptide **4** (Figure 3 and Table 1). Single substitution of Ala19 (**29**) or Lys20 (**30**) to peptide **28** decreased potency (Figure 3, panel a and Table 1). GLP-1R binding analysis correlated with functional tests as Jant-4-based analogues **28** and **33** were observed to be of higher affinity relative to **4** (Supplementary Figure 6 and Table 2). These results confirm the seminal importance of Glu16, Val19, and Arg20 in achieving high potency antagonism.

Additional analogues were designed to identify how changes at the C-terminus of our Jant-4 antagonist influenced activity. The ligand-bound structures of Ex-4 (9-39)a and GLP-1, as well as receptor mutagenesis studies, suggest that this region of the peptide is important for binding the extracellular domain.[15,16] Jant-4a (9-30)a (**37**) incorporates additional substitutions at Leu21 and Glu24 to extend the alternating charge network as is present in Ex-4. A marginal loss of activity was observed for this peptide (Supplementary Figure 7 and Table 1). Next, the importance of the positive charge inversion at positions 27 and 28 of Ex-4 and GLP-1 was tested through peptide **38**, which proved to be similarly effective (Supplementary Figure 8). The substitution to Arg27 (**39**), which was a preferred change at position 20, caused an approximate 2-fold loss in potency (Supplementary Figure 7). Intriguingly, the influence of these related C-terminal changes on the Jant-4 backbone could explain why the potency of the previously reported Chimera 6 analogue did not surpass the antagonist activity of Ex-4 (9-39)a.[7]

Reversal of Antagonism and Helicity Function. We examined the inverse effect of GLP-1 substitutions to Ex-4 for validation of the differential basis of Ex-4 antagonism relative to GLP-1. Jant-4 (9-30)a (**33**) was used as the reference peptide to eliminate any contribution of Cex. The inverse substitutions to Ex-4 (9-30)a yielded the Gly16, Ala19, Lys20 analogue (**36**). Weak agonism was observed in this triply substituted Ex-4 analogue (Figure 4), comparable to that previously noted for GLP-1 (9-30)a (Figure 1, panel b). This led us to explore the importance of amino acid 16 as an anchor for the observed biochemistry, through its purported effect on the helicity of GLP-like peptides. Jant-4-based antagonists were designed with Gly16 to destabilize the helix or, conversely, 2-aminoisobutyric acid (Aib)16 to promote helix. Substitution with Aib16 (**35**) was well tolerated, comparable to Glu16 of Jant-4 and Ex-4. The Gly substitution (**34**) decreased the potency of antagonism approximately 4-fold (Figure 4, panel a and Table 1). Although the inherent potency decreased with Gly16, of greater importance was the fact that there was no inversion from antagonist to agonist for **34**. Subsequently, consistent with our other observations, helicity mediated by Glu16 appears to enhance the potency of antagonism but is not central to the inhibitory nature of the pharmacology.

Three peptides were studied *in vivo* to determine the level of GLP-1 antagonism relative to *in vitro* observations. Figure 5 shows the glucose excursion of DIO mice treated with peptides derived from Ex-4 (**4**), GLP-1 (**7**), or Jant-4 (**28**). The results are consistent with *in vitro* measurements as peptide **7** does not antagonize the GLP-1 challenge. Peptides **4** and **28** are similar in their effectiveness for elevating glucose beyond what is observed in a normal glucose tolerance test, where endogenous GLP-1 serves to lessen the excursion. These observations confirm the comparable effectiveness of the Jant-4-based sequence to the Ex-4-based peptide as a fully effective antagonist, but of enhanced human GLP-1 sequence. With this as the foundation, we set to

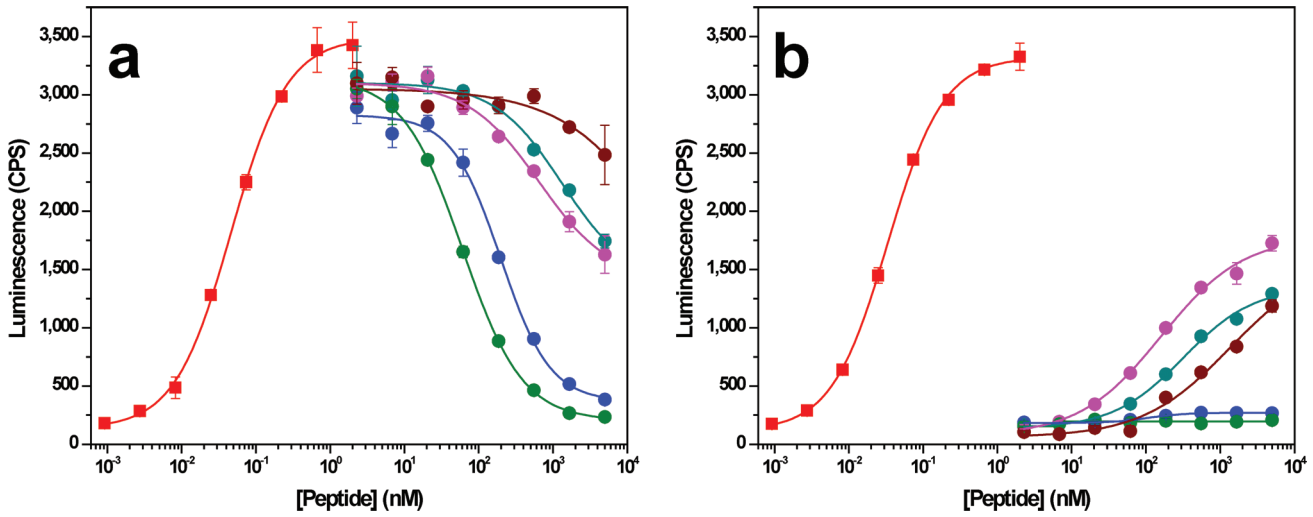

Figure 2. *In vitro* measurement of GLP-1R-mediated cAMP of N-truncated GLP-1/Ex-4 hybrid peptides in antagonist (a) and agonist (b) assays. (red ■) GLP-1 (1-30)a; (blue ●) Ex-4 (9-39)a; (cyan ●) GLP-1-Cex (9-39)a Glu16; (red ●) Jant-1 (9-39)a; (green ●) Jant-2 (9-39)a; (wine ●) Jant-3 (9-39)a.

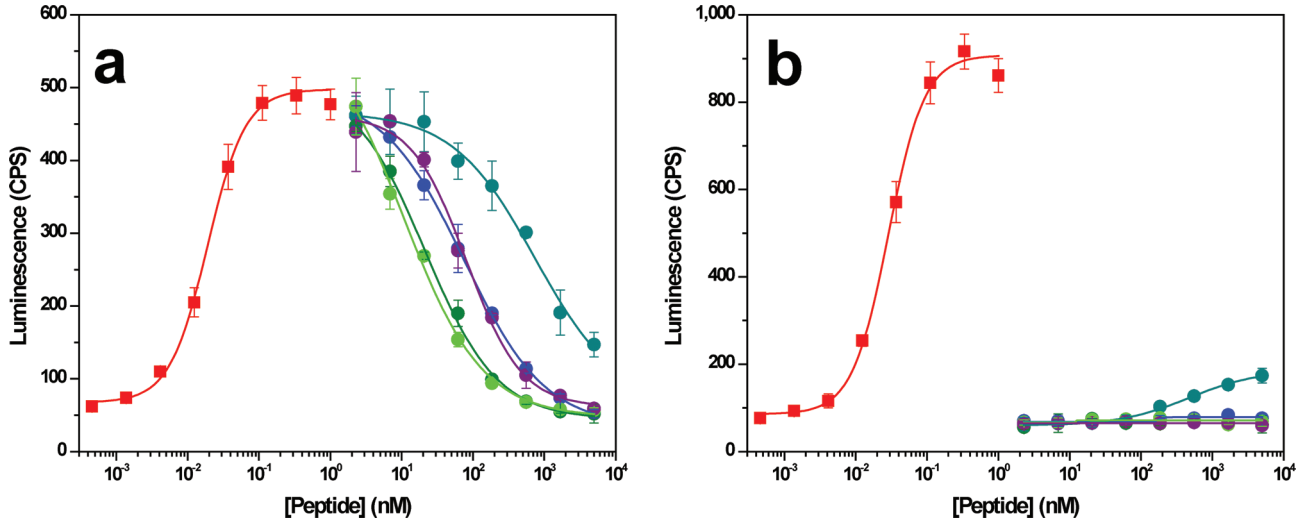

Figure 3. *In vitro* measurement of GLP-1R-mediated cAMP of central Ex-4 residues on N-truncated GLP-1 peptide antagonism. GLP-1R cAMP induction was monitored in antagonist (a) and agonist (b) assay formats. (red ■) GLP-1 (1-30)a; (blue ●) Ex-4 (9-39)a; (green ●) GLP-1-Cex (9-39)a Glu16; (green ●) Jant-2 (9-39)a; (light green ●) Jant-4 (9-39)a; (purple ●) Jant-4 (9-39)a Ala19.

explore the development of a longer-acting form of our humanized antagonist.

Fatty Acid Acylation Enhances Antagonism. Ex-4 and Jant-4 antagonists acylated with palmitic acid were synthesized with the objective of extending serum half-life by facilitating binding to albumin.[25] Synthesis of acylated analogues was achieved on-resin with *tert*-butyloxycarbonyl (Boc) chemistry using orthogonal 9-fluorenylmethyloxycarbonyl (Fmoc) side-chain protection at an additional C-terminal lysine, position 40. Acylation of Ex-4 (9-40)a and Jant-4 (9-40)a at Lys40 improved the potency by approximately 10-fold and 3-fold, respectively (Figure 6). Both Ex-4 and Jant-4 (9-40)a Lys40-C16 (**40** and **41**) displayed comparable inhibitory potency, eliminating the differential advantage of Jant-4 in the nonacylated peptides. Acylated peptide binding to albumin was detected by a plasma shift assay (Supplementary Figure 8). Further examination of C8, C14, and C18 fatty acids showed similar improvements in potency (unpublished results). An examination of GLP-1R receptor selectivity revealed that Ex-4 (9-39)a had low level inhibitory activity at the related glucose-dependent insulinotropic polypeptide (GIP) receptor (GIPR) (Supplementary Figure 9) as previously reported.[26] Jant-4 (**28**) was observed to be slightly more selective than Ex-4 (**4**). The acylated forms of the antagonists had 10-fold preferential activity at the GLP-1 receptor, demonstrating that the fatty acid increases inherent potency but lessens selectivity.

Figure 7 reports the glucose excursion in DIO mice over a period of 2 h following provocative glucose challenge 24 h after administration of the acylated GLP-1R antagonist **41** at one of two doses (0.5 or 2 μmol kg^{-1} body weight). The antagonist was effective at both doses with the higher dose slightly increased in the magnitude of the glucose excursion. The magnitude of the difference was quantified by measurement of the glucose elevation throughout the glucose tolerance test in animals administered an antagonist relative to animals receiving vehicle (Supplementary Figure 10). Additionally, as we previously

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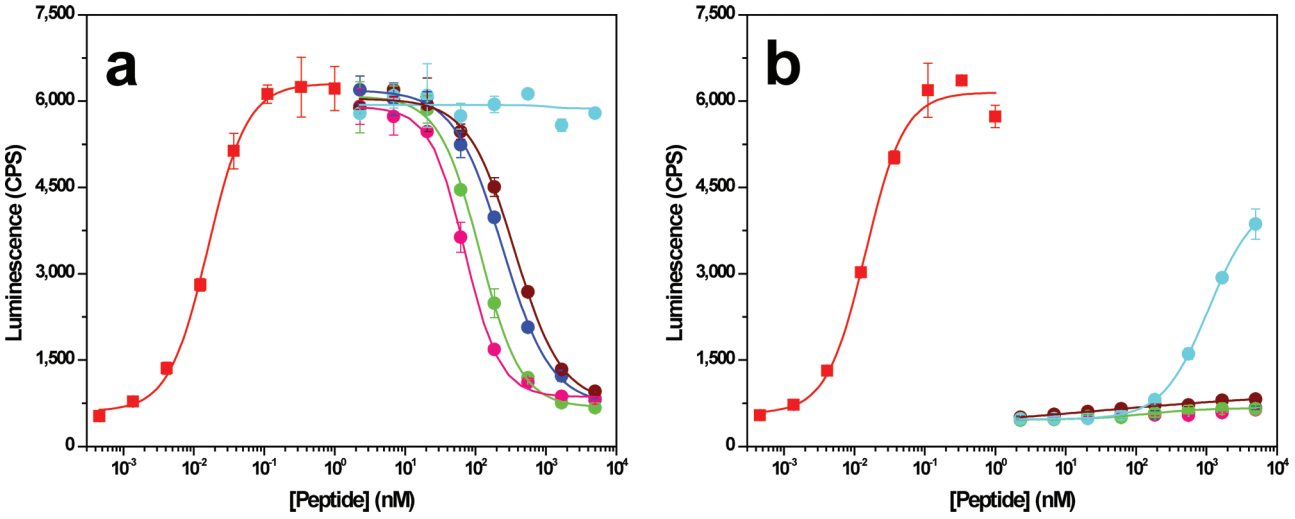

Figure 4. *In vitro* measurement of GLP-1R-mediated cAMP of substitution at positions 16, 19, and 20 on N-truncated GLP-1 and Ex-4 analogues in antagonist (a) and agonist (b) assays. (red ■) GLP-1 (1-30)a; (blue ●) Ex-4 (9-30)a; (light green ●) Jant-4 (9-30)a; (wine ●) Jant-4 (9-30)a Gly16; (magenta ●) Jant-4 (9-30)a Aib16; (light blue ●) Ex-4 (9-30)a Gly16Ala19Lys20.

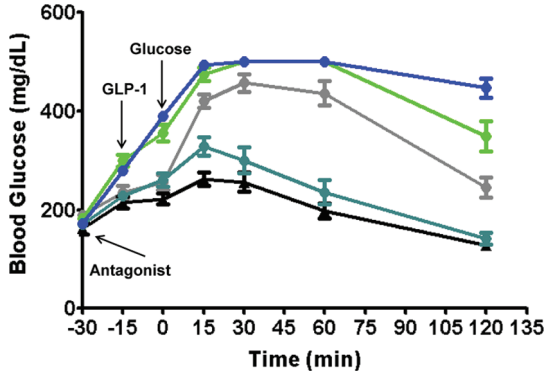

Figure 5. Acute intraparenteral glucose tolerance test of (gray ●) 0.01 N HCl; (▲) 0.01 N HCl + GLP-1; (blue ●) Ex-4 (9-39)a; (cyan ●) GLP-1-Cex (9-39)a Glu16; and (light green ●) Jant-4 (9-39)a in DIO mice. Peptides (0.2 μmol kg^{-1}) were administered 30 min prior to the glucose challenge, and the GLP-1 agonist (0.65 nmol kg^{-1}) 15 min after the antagonist. All treatment groups received GLP-1 agonist with the exception of (gray ●) 0.01 N HCl.

observed (Figure 5), there was a statistically greater increase in glucose levels in animals receiving the antagonist relative to those that did not receive GLP-1 agonist or an antagonist prior to glucose challenge. This illustrates the contribution of endogenous GLP-1 agonism to minimizing glucose elevation through the course of a glucose challenge, and the fact that the antagonists 24 h after administration are capable of blocking such action in addition to that of the exogenously administered agonist.

Antagonism Further Increases Body Weight in Diet-Induced Obesity. To explore the pharmacological effect of persistent antagonism of endogenous GLP-1, we administered the sustained-acting peptide **41** each day for a period of 1 week to DIO mice. All animals exhibited normal health and behavior throughout the course of the experiment. The antagonist treated group demonstrated a steady increase in body weight relative to vehicle-treated animals (Figure 8, panel a). The total relative increase in body weight over 1 week was approximately 3%, and it was associated with a proportional increase in food consumption. One day after the end of treatment a measurement of glucose tolerance was performed. Animals did not differ in starting glucose concentration, but again, antagonist-treated mice were noticeably less glucose tolerant than the vehicle-treated control animals (Figure 8, panel b). The magnitude of the observed difference in glucose excursion was considerably enhanced relative to that observed 1 day after administration of a single similar dose of antagonist (Figure 7). Our results demonstrate the utility of this novel long-acting GLP-1R antagonist to the study of endogenous GLP-1 in the control of mammalian energy balance and glucose metabolism.

DISCUSSION

GLP-1 and Ex-4 are homologous incretin hormones that activate the GLP-1 receptor. As agonists, the two peptides differ appreciably in their *in vivo* pharmacology with Ex-4 being more potent and longer in its duration of action. This differential pharmacology has largely been attributed to the increased backbone stability of Ex-4 derived from Glu16 and the C-terminal sequence (Cex).[20,27] A much less studied observation given the greater medicinal interest in agonism is the fact that Ex-4 can be converted to a high potency antagonist by truncation of the N-terminus, whereas GLP-1 cannot.[7] The uncertainty of immunogenicity in human use warrants limiting non-native amino acid changes to those essential to antagonism. Therefore, definition of the molecular basis for antagonism to develop a human-based GLP-1R antagonist was the primary objective of this work.

Through an iterative structure−activity study using peptides hybridized with varying degrees of Ex-4 and GLP-1, we have identified residues Glu16, Val19, and Arg20 as central determinants for GLP-1R antagonism. GLP-1-based peptides with these three substitutions (**28**, **33**, and **41**) were observed to be highly potent GLP-1R antagonists. The Cex terminal sequence extension was found not to be an integral element to *in vitro* receptor antagonism. Additionally, the nature of the amino acid at position 16 had an independent influence on potency but was also not observed to be the central element to antagonism. Introduction of the GLP-1 amino acids Gly16, Ala19, and Lys20 to the Ex-4-based antagonist yielded weak agonism (**36**), supporting the observations that these amino acids function in concert to bestow the differential ability of Ex-4 relative to GLP-1 to be N-terminally shortened to an antagonist.

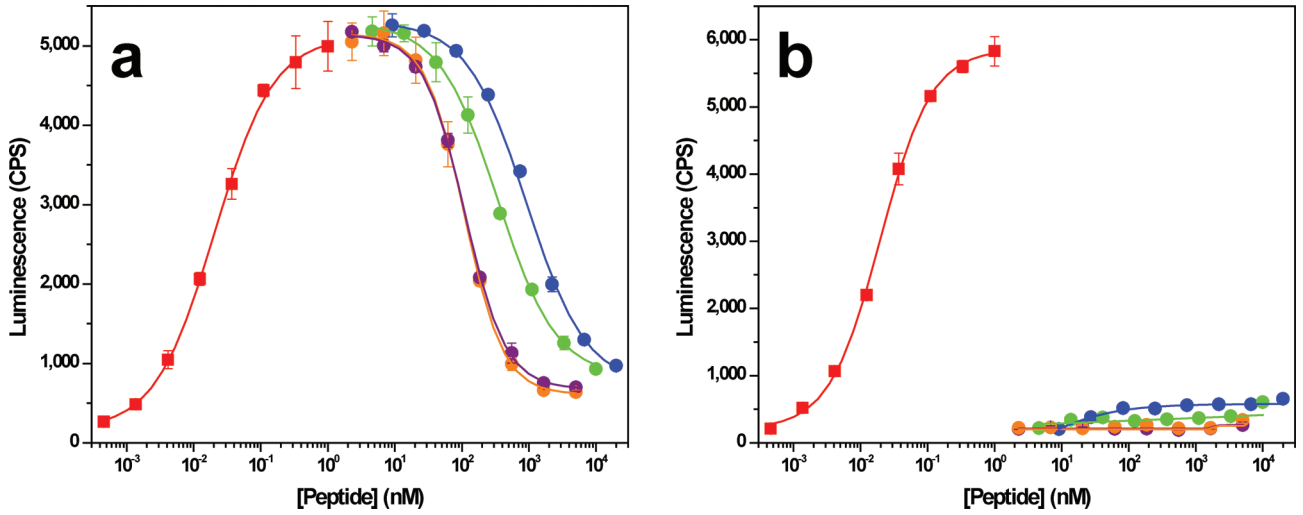

Figure 6. *In vitro* measurement of GLP-1R-mediated cAMP of palmitic acid acylated antagonists at Lys40 of Ex-4 and Jant-4. GLP-1R cAMP induction was monitored in antagonist (a) and agonist (b) assay formats. (red ■) GLP-1 (1-30)a; (blue ●) Ex-4 (9-39)a; (light green ●) Jant-4 (9-39)a; (purple ●) Ex-4 (9-40)a Lys40-C16; (orange ●) Jant-4 (9-40)a Lys40-C16.

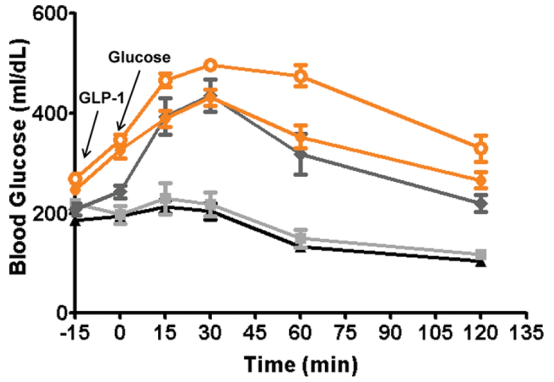

Figure 7. Intraparenteral glucose tolerance test of (♦) PBS, (■) PBS + GLP-1, (▲) 0.01 N HCl + GLP-1, and Jant-4 (9-40)a Lys40-C16 (● 0.5 or ○ 2 μmol kg^{-1}) in DIO mice. Antagonist was administered twenty-four hours before the GTT, and the GLP-1 agonist (0.65 nmol kg^{-1}) fifteen minutes prior to the glucose challenge. All treatment groups received GLP-1 agonist with the exception of (♦) PBS.

GLP-1-based Jant-4 (**28**) proved to be a potent and highly effective antagonist when studied by *in vitro* (Figure 3) and *in vivo* methods (Figure 5). The homologous GLP-1 analogue **7** without the two additional substitutions at positions Val19 and Arg20 was ineffective as an antagonist validating the seminal importance of these changes. Furthermore, the application of fatty acid acylation yielded higher potency antagonists (Figure 6) that provided fully effective *in vivo* GLP-1 antagonism 1 day following administration (Figure 7). However, acylation increased the relative potency at the related GIP receptor, but without any apparent activity (submicromolar) at the glucagon receptor. The selectivity of the acylated antagonists at the GLP-1R relative to the GIPR is approximately 10-fold (Supplementary Figure 9 and Supplementary Table 2) and consistent with prior characterization of Ex-4 (9-39)a.[26] The humanization of Jant-4 relative to Ex-4 and the application of acylation to simplify chronic *in vivo* studies should render peptide **41** a preferred choice for human studies.

The use of this long-acting peptide as a subcutaneous injection to persistently antagonize endogenous GLP-1 in metabolically compromised obese mice has demonstrated a further impairment of body weight and glucose tolerance. Previous study with exendin-4-based antagonism has demonstrated suppression of insulin secretion in glucose tolerance testing.[11,13,28] We have similarly observed a correlation of increased glucose excursion with suppression of insulin secretion when animals treated with acylated antagonist are glucose-challenged the following day. Whether the low level of *in vitro* GIP receptor antagonism contributes to the observed pharmacology was not determined, but if so it would be limited by the low level of cross-reactivity and should function to buffer against weight gain.[29] However, Ex-4 (9-39)a was incapable of blocking GIP-induced insulin secretion in rats[28,30] and humans,[31] suggesting the antagonist's actions *in vivo* are mediated by the GLP-1 pathway. Previous studies reporting icv administration of the short-acting exendin-4 antagonist have revealed similar increases in food intake, body weight, and glucose intolerance.[32,33] In contrast, daily administration of Ex-4 (9-39)a peripherally to normal and *ob*/*ob* mice for 11 days was reported to have little influence on these metabolic parameters.[13] It is plausible that direct central administration or the sustained pharmacokinetics of these acylated peptides yields enhanced pharmacology demonstrating an inherent virtue for *in vivo* study.

We performed structural analysis by circular dichroism to examine if residues Glu16, Val19, and Arg20 were enabling antagonism through stabilization of the α helix or by positional interactions with the receptor (Supplementary Table 3). All peptides adopted highly helical character when analyzed in 30% TFE, but relative differences were observed in the absence of helix-promoting solvent (Supplementary Figure 11). Substitution of helix-stabilizing amino acids, such as Glu16 and Aib16,[34] to Ex-4- and Jant-4-based peptides increased helicity, whereas Gly16 diminished it. These results demonstrate that the degree of helicity is not strictly correlated with potency in antagonism, as the most helical Ex-4-based peptides are no more effective than the Jant-4-based antagonists. The Gly16 peptides were of lesser helicity, and only one (**34**) was an antagonist of lesser potency, showing that helicity is not a requirement for antagonism but is associated with enhanced potency. The acylated peptides that were of increased potency relative to their nonacylated forms also displayed enhanced helicity. Consequently, we conclude that

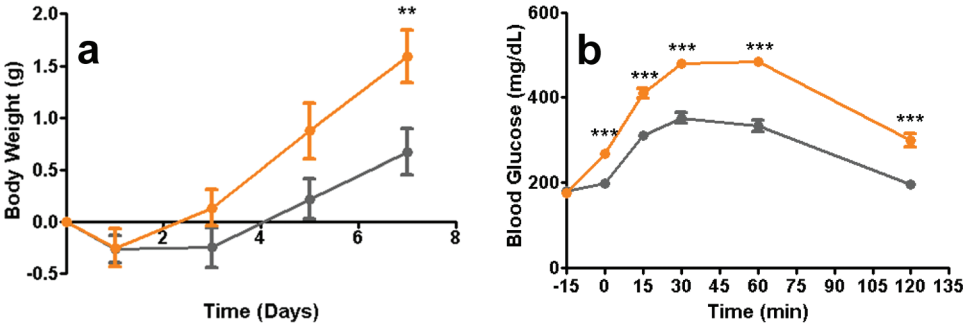

Figure 8. One week daily injection of vehicle (gray ●) 0.01 N HCl or (orange ●) Jant-4 (9-40)a Lys40-C16 at a dose of 0.5 μmol kg^{-1}. (a) Body weights were measured on days 0, 1, 3, 5, and 7. (b) A glucose tolerance test was performed on day 7, 24 h after the last administration of vehicle or antagonist. **denotes $P < 0.01$, and ***$P < 0.001$.

stabilization of the α helix alone is not singularly sufficient for GLP-1R antagonism but can serve to enhance potency in peptides of proper amino acid sequence.

Examination of the reported crystal structures for GLP-like peptides bound to the extracellular domain of the receptor provides a model for postulating a molecular basis for antagonism.[15,16] These crystal structures show both GLP-1 and Ex-4 (9-39)a making hydrophobic contacts with the receptor, but exendin-4 makes additional ionic contacts not present in the native peptide. Ex-4 displays enhanced hydrophobic interaction with residue Val19, one of the central amino acids we observed to be of vital function to antagonism. This valine binds near the GLP-1R extracellular domain residues Leu32*, Thr35*, and Pro90*, which comprise part of the hydrophobic face of the receptor's extracellular domain[15] (Figure 9). Receptor mutagenesis of Leu32* to Ala caused an approximate 10-fold reduction in Ex-4 (1-39)a activity,[16] supporting the importance of this molecular interaction as a source of enhanced ligand−receptor interaction.

The ligand-bound extracellular domain structures of GLP-1 and Ex-4 (9-39)a advocate the additional importance of extracellular receptor domain residues Glu127* and Glu128*. Receptor mutagenesis implicates Glu127* as the amino acid that discriminates between GLP-1 and Ex-4 binding through differential affinity for residues Glu24 and Lys27 of Ex-4.[16] The Jant-1 analogues are GLP-1-based but possess this C-terminal region of Ex-4. They did not demonstrate any improvement in potency, which indicates the inability to translate this interaction for constructive purposes to our GLP-1-based antagonist. This stands in contrast to the enhanced potency of Jant-2 and Jant-4 (Figure 3 and Supplementary Figure 4). Receptor position Glu128* appears to interact through ionic contact with position 20 of GLP-1 and Ex-4 (9-39)a (Figure 9). Our observations support the importance of this receptor−ligand ion contact with Arg20 being approximately 3-fold more potent than lysine (**28** and **30**). We hypothesize that the dispersed cationic nature of Arg20 allows more effective coordination of the Glu128* carboxylate. Moreover, we have observed the importance of GLP-1R residue Glu128* for discrimination between GLP-1 and glucagon binding,[35] and here we present additional evidence that this site is of central importance to achieving high affinity GLP-1R antagonism.

In conclusion, our results elucidate the molecular basis for *in vitro* and *in vivo* antagonism by a nearly human-based GLP-1 peptide to be a function of substitution at amino acids 16, 19, and 20. The application of site-specific fatty acid acylation increases potency and provides an antagonist suitable for simplified daily *in vivo* subcutaneous administration. The molecular basis for antagonism appears to be a function of optimized interactions of Arg20

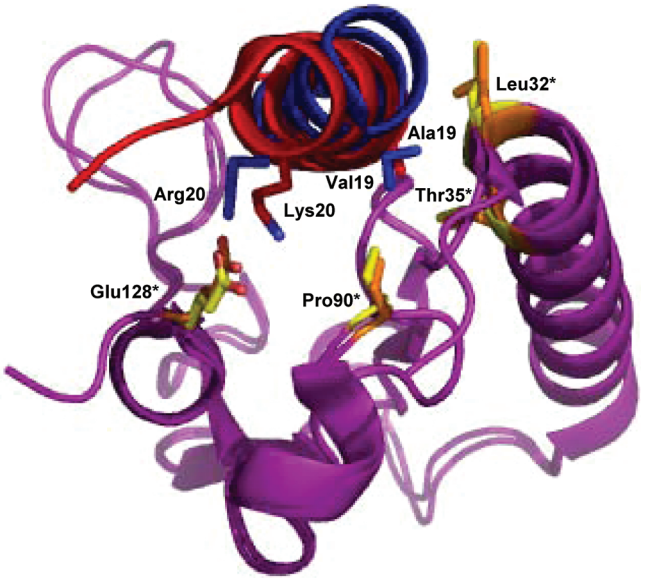

Figure 9. Molecular basis of GLP-1R antagonism. GLP-1 (red, Protein Data Bank code 3IOL) and Ex-4 (9-39)a (blue, Protein Data Bank code 3C5T) are overlaid with the extracellular domain (purple). C-Terminal Ex-4 amino acids 30−33 were eliminated for clarity. Residue 19 is in close proximity of extracellular domain residues Leu32*, Thr35*, and Pro90*, and position 20 is shown interacting with Glu128*. Receptor residues implicated in antagonism are shown in orange for the Ex-4 crystal structure and GLP-1 in yellow.

with receptor residue Glu128* and Val19 with the hydrophobic extracellular domain residues Leu32*, Thr35*, and Pro90* (Figure 9), combined with an enhanced backbone helicity supported by Glu16. The discovery of these antagonists supports the importance of endogenous GLP-1 to proper appetite, body weight, and glucose control in DIO mice. Extension of these observations to nonrodent species and identification of molecules with enhanced and/or expanded receptor selectivity for the purposes of delineating GLP-1 action relative to related hormones such as GIP, glucagon, and oxyntomodulin remains a target for future study.

METHODS

Boc Synthesis. Peptides were generated by solid-phase peptide synthesis. An *in situ* neutralization protocol for Boc chemistry was followed as described by Kent and colleagues.[36] Peptide synthesis was performed using 0.2 mmol 4-methylbenzhydrylamine resin (Midwest

Biotech) on a modified Applied Biosystems 430A synthesizer. Amino acids (Midwest Biotech) were side-chain protected with the following groups: Arg(Tos), Asp(OcHex), Asn(Xan), Glu(OcHex), His(BOM), Lys(2-Cl-Z), Ser(Bzl), Thr(Bzl), Trp(CHO), and Tyr(Br-Z). Activation of amino acids (2 mmol) was performed with 0.5 M 3-(diethoxyphosphoryloxy)-3*H*-benzo[*d*][1,2,3]triazin-4-one in dimethylformamide (DMF) and *N*,*N*-diisopropylethylamine (DIEA) (4:1 v/v). Cleavage of the peptides from the solid support used HF/*p*-cresol, 95:5 v/v, for 1 h at 0 °C followed by *in vacuo* evacuation of the HF. Peptides were precipitated in diethyl ether and collected using a 50 μm Teflon filter funnel. Acetic acid (10%) was used to solubilize the cleaved peptides, which were then lyophilized and stored at 4 °C.

Antagonist Acylation. Palmitic acid was introduced to Ex-4 and Jant-4 antagonists using an orthoginal solid-phase protection scheme. Boc synthesis was utilized for peptide synthesis, allowing selective introduction of base-sensitive side-chain protected Lys(Fmoc)-OH at Lys40. The fully protected peptides were treated on resin with 20% piperdine in DMF (v/v) for 30 min to remove the Lys40 side-chain Fmoc group. Amide bond formation was facilitated with excess fatty acid and 5 equiv of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (Fluka) in DMF/DIEA (4:1 v/v) for approximately 18 h. Ninhydrin testing was used to monitor reaction progress, and acylation was confirmed after peptide cleavage by ESI mass spectrometry.

Peptide Purification. Reversed-phase HPLC (RP-HPLC) was used for peptide purification. A C_{18} stationary phase (Vydac 218TP, 250 mm × 22 mm, 10 μm) was employed with a linear acetonitrile gradient in 0.1% trifluoroacetic acid during the preparative RP-HPLC purification. Analytical analysis was performed on peak fractions by employing RP-HPLC with a C_8 column (Zorbax 300SB, 4.6 mm × 50 mm, 3.5 μm). Peptide identity and purity was assessed by analytical RP-HPLC and ESI- or MALDI-mass spectrometry. All peptides were found to have the correct molecular weight and were approximately 95% pure. Lyophilized peptides were stored at 4 °C.

GLP-1 Receptor-Mediated cAMP Induction. The ability of peptides to stimulate or block cAMP induction at the GLP-1 receptor was examined by a luciferase-based reporter gene assay. Cotransfection of HEK 293 cells with the human GLP-1 receptor (Open Biosystems) and a cAMP-inducible (cAMP responsive element) luciferase gene constituted the cellular construct where receptor activation could be measured. Bioassays were performed by first serum depriving the cells for 16 h in 0.25% bovine growth serum (HyClone)-supplemented Dulbecco's modified Eagle's medium (Invitrogen) and then adding serial dilutions of the peptides over the appropriate concentration ranges in 96-well poly-D-lysine-coated plates (BD Biosciences). For antagonism assays, a constant concentration of GLP-1 (0.05 nM) was added to the assay plate after the diluted peptides. Incubation continued for 5 h at 37 °C, 5% CO_2 and was followed by the addition of an equivalent volume (100 μL) of LucLite luminescence substrate reagent (Perkin-Elmer). MicroBeta 1450 liquid scintillation counting (Perkin-Elmer) quantified the luminescence signal in counts per second (cps) after shaking the plate at 600 rpm for 3 min. Data was plotted using Origin software (OriginLab) and the effective concentration 50 (EC_{50}) or inhibitory concentration 50 (IC_{50}) was determined by sigmoidal fitting. Potency was determined by comparative analysis of relative EC_{50} or IC_{50} values. Each experiment was repeated at least three times with each sample assayed in duplicate.

GLP-1 Receptor Binding. The affinity of peptides for the GLP-1 receptor was measured in a competition binding assay using scintillation proximity technology. Three-fold serial dilutions of the peptides were prepared in binding buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% w/v bovine serum albumin) and mixed in 96-well plates (Corning Inc.) with 0.05 nM [^{125}I]-GLP-1 (Perkin-Elmer). An aliquot of 1−6 μg of plasma membrane fragments prepared from cells overexpressing human GLP-1 receptor were present in each well, and 0.25 mg per well polyethyleneimine-treated wheat germ agglutinin-coated type A scintillation proximity assay beads (Perkin-Elmer) was added. After 5 min of shaking at 800 rpm, the plate was incubated for 6 h at RT, and the amount of radioactivity was measured with a MicroBeta 1450 liquid scintillation counter (Perkin-Elmer). Nonspecifically bound (NSB) radioactivity was measured in the wells with a 4-fold concentration excess of "cold" native ligand in relation to the highest concentration in test samples. Total bound radioactivity was detected in the wells with no competitor. Percent specific binding was calculated as followed: % specific binding = (bound-NSB/total bound-NSB) × 100.

Animals. C57Bl/6 mice were obtained from Jackson Laboratories and fed a diabetogenic diet from Research Diets (D12331), a high-sucrose diet with 58% kcal from fat. Mice were single- or group-housed on a 12:12 h light-dark cycle at 22 °C with free access to food and water. All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati.

Glucose Tolerance Test (GTT). For the determination of glucose tolerance, mice were subjected to 6 h of fasting and injected intraperitoneally with glucose. Injections consisted of 1.5 g glucose per kg body weight (25% w/v D-glucose (Sigma) in 0.9% w/v saline). Tail blood glucose levels (mg dL^{-1}) were measured using a hand-held glucometer (FreeStyle Freedom Lite) before 0 min and at 15, 30, 60, and 120 min after injection.

Acute *In Vivo* Study. Mice received an intraperitoneal injection of peptide (in 0.01 N HCl) either 1 h (0.2 μmol kg^{-1}) or 24 h (0.5 or 2.0 μmol kg^{-1}) before being challenged with an IP injection of dipeptidyl peptidase-IV-protected Aib2, GLP-1 (0.65 nmol kg^{-1}). A GTT was performed fifteen minutes after the GLP-1 injection. Tail blood glucose values were obtained as described above.

One Week *In Vivo* Study. Animals received a subcutaneous injection of peptide **41** daily at a dose of 0.5 μmol kg^{-1} in 0.01 N HCl. Body weights and food intake were measured on days 0, 1, 3, 5, and 7. On day 0 the mice were fasted for 6 h before taking blood glucose values. On day 7 body composition was measured, and the mice were fasted 6 h for a GTT. Whole-body composition (fat and lean mass) was measured using NMR technology (EchoMRI).[37]

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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