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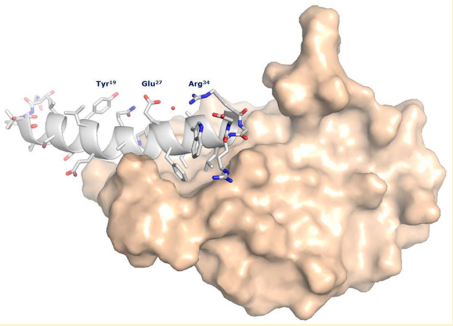
Discovery of the Once-Weekly Glucagon-Like Peptide-1 (GLP-1) Analogue Semaglutide

Jesper Lau,* Paw Bloch, Lauge Schäffer, Ingrid Pettersson, Jane Spetzler, Jacob Kofoed, Kjeld Madsen, Lotte Bjerre Knudsen, James McGuire, Dorte Bjerre Steensgaard, Holger Martin Strauss, Dorte X. Gram, Sanne Møller Knudsen, Flemming Seier Nielsen, Peter Thygesen, Steffen Reedtz-Runge, and Thomas Kruse

Global Research, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark

Supporting Information

ABSTRACT: Liraglutide is an acylated glucagon-like peptide-1 (GLP-1) analogue that binds to serum albumin *in vivo* and is approved for once-daily treatment of diabetes as well as obesity. The aim of the present studies was to design a once weekly GLP-1 analogue by increasing albumin affinity and secure full stability against metabolic degradation. The fatty acid moiety and the linking chemistry to GLP-1 were the key features to secure high albumin affinity and GLP-1 receptor (GLP-1R) potency and in obtaining a prolonged exposure and action of the GLP-1 analogue. Semaglutide was selected as the optimal once weekly candidate. Semaglutide has two amino acid substitutions compared to human GLP-1 (Aib[8], Arg[34]) and is derivatized at lysine 26. The GLP-1R affinity of semaglutide (0.38 ± 0.06 nM) was three-fold decreased compared to liraglutide, whereas the albumin affinity was increased. The plasma half-life was 46.1 h in mini-pigs following i.v. administration, and semaglutide has an MRT of 63.6 h after s.c. dosing to mini-pigs. Semaglutide is currently in phase 3 clinical testing.



INTRODUCTION

GLP-1 receptor agonists (GLP-1 RAs) have become a successful treatment for type 2 diabetes providing effective glucose control, improved beta-cell function, body weight loss, and lowering of systolic blood pressure.[1] Exenatide for twice daily administration was the first approved compound in 2005, followed by once-daily liraglutide in 2009.[2,3] Exenatide is a non-human peptide analogue originally isolated from the saliva of the Gila monster and has an i.v. half-life of 30 min and a half-life of 2−3 h after s.c. administration.[4−6] Liraglutide is a close analogue of human GLP-1 designed to bind to human albumin via a fatty acid and a spacer covalently attached to the peptide backbone.[7,8] Liraglutide has an i.v. half-life of 8−10 h and 13−15 h after s.c. administration, making it suitable for once-daily administration.[7,9,10] There is a strong PK/PD relationship in the GLP-1RA class where short-acting GLP-1RAs display a marked ability to reduce gastric emptying, whereas long-acting GLP-1RAs with pharmacologically relevant exposure more than 24 h have better glucose lowering effect and less effect on gastric emptying.[1,11] The next generation of GLP-1RAs are mainly aimed for once weekly administration and albiglutide was approved as the first once-weekly GLP-1 analogue. Albiglutide has two Gly[8] GLP-1 molecules fused in tandem to human serum albumin. The time to maximum plasma concentration, T_{max}, is 2−4 days and the half-life is around 6−8 days.[12] Recently, a second once weekly analogue dulaglutide, was approved. Dulaglutide is a GLP-1 analogue fused to a F_c-fragment, Gly[8]Glu[22]Gly[36]-GLP-1(7−37)-$(Gly_4Ser)_3$Ala-Ala[234,235]Pro[228]-IgG4-F_c.[13] There is still a need for optimization in the once-weekly GLP-1 analogue field because both albiglutide and dulaglutide have been shown to be less efficacious than liraglutide with respect to weight loss. For albiglutide, weight loss in a 26-week trial was 0.6 kg, whereas it was 2.2 kg for liraglutide.[14] For dulaglutide, weight loss was 2.9 kg, whereas it was 3.6 kg for liraglutide ($p < 0.01$).[15]

We report a series of acylated GLP-1 analogues where increased albumin affinity relative to liraglutide has led to a profile suitable for once-weekly administration. The attachment of long fatty acids to peptides has successfully extended the half-life of native short-acting GLP-1 to the once daily profile of liraglutide. As albumin has a half-life of several weeks, it was hypothesized that an increased albumin affinity could extend the circulating half-life beyond the 13−15 h seen with liraglutide.[16,17] The main focus of the studies reported here was to generate GLP-1RA analogues with an extended pharmacokinetic profile and efficacy suitable for once-weekly s.c. administration without compromising the potency. We selected semaglutide for clinical development, and the molecule is currently in phase 3 clinical development. The half-life in humans is reported to be 165 h.[18]

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Table 1. Derivatives of Arg[34] GLP-1 (7-37) with C16 to C20 Acids and C16 to C18 Diacids and Linkers Attached to Lys[26] and Compared to GLP-1 (7−37) and Arg[34] GLP-1 (7−37)[a]

analogue	sequence modification	protractor	linkage	acylation position	GLP-1R binding (IC_{50}, nM) (SEM) 0% HSA	GLP-1R binding (IC_{50}, nM) (SEM) 2% HSA	ratio 2%/0% HSA	GLP-1R potency (EC_{50}, pM) (SEM) 0% HSA
(GLP-1)7−37		none	none		0.19 (0.03)	0.10 (0.02)	0.5	16.2 (0.9)
1	Arg[34]	none	none		0.25 (0.01)	0.14 (0.01)	0.6	7.6 (1.1)
liraglutide	Arg[34]	C16	γGlu	Lys[26]	0.11 (0.02)	4.78 (1.01)	43	8.5 (0.7)
2	Arg[34]	C18	γGlu	Lys[26]	0.60 (0.04)	1.95 (0.04)	3	17.1 (1.3)
3	Arg[34]	C20	γGlu-2xOEG	Lys[26]	0.16 (0.03)	0.97 (0.05)	6	3.7 (0.8)
4	Arg[34]	C16 diacid	none	Lys[26]	0.87 (0.06)	74.0 (5.8)	85	70.9 (10.2)
5	Arg[34]	C18 diacid	none	Lys[26]	2.83 (0.37)	295 (52)	104	238 (42)
6	Arg[34]	C18 diacid	γGlu-2xOEG	Lys[26]	0.28 (0.04)	148 (15)	527	3.8 (0.4)

[a]GLP-1 receptor binding at 0% and 2% albumin (HSA) using BHK cells expressing the human GLP-1R and *in vitro* potency measured in BHK cells that express both the human GLP-1R and a luciferase reporter system.

RESULTS AND DISCUSSION

Several series of derivatized GLP-1 analogues were prepared (Tables 1−4). The design of these peptides was mainly inspired by liraglutide, which was developed as the first GLP-1 applicable for once daily dosing with full daily efficacy. One of the important design criteria was to keep the analogues structurally similar to native GLP-1 and not introducing unnecessary amino acid changes in order to avoid immunogenicity responses similar to those shown for exenatide and taspoglutide. In the latter case the program was terminated following phase 3 clinical development, whereas liraglutide has shown a very low immunogenic profile.[19,20] For exenatide, a significant amount of patients developed antibodies, some were neutralizing, and the antibody numbers and titers were higher with the once weekly formulation than the twice daily simple formulation.[21] The design space included investigation of the structural impact on *in vitro* human GLP-1 receptor binding affinity and potency by modifying the "side-chain" of liraglutide (Figures 1 and 2). The results showed that GLP-1 receptor affinity measured in the absence of albumin was in general only marginally affected by attachment of an albumin binding moiety (protractor) to a lysine residue in positions 16, 22, 26, 37, or 38, and most derivatives had IC_{50} values below 0.5 nM. In some cases, the receptor affinity was significantly increased, as seen for analogues such as **13** (0.04 nM), **38** (0.06 nM), and **35** (0.06 nM) compared to native GLP-1 and to the nonderivatized analogues Arg[34]GLP-1 (7−37) and Aib[8], Arg[34] GLP-1 (7−37). The affinities of these analogues were significantly increased compared to the binding affinity of 0.11 nM for liraglutide. However, it was also possible to reduce the receptor affinity as exemplified by **31**, which had a poor binding affinity in the absence of albumin. In addition to receptor binding, an *in vitro* functional assay was used to estimate the agonistic potencies of the various GLP-1 analogues (Tables 1−4). Native GLP-1 had an EC_{50} value of 16.2 pM, whereas Arg[34]GLP-1 (7−37) and Aib[8], Arg[34]GLP-1 (7−37) are slightly more potent (7.6 and 6.2 pM, respectively).

In order to further improve the pharmacokinetic profile and obtain a product that is suitable for once weekly administration, the N-terminus was substituted to protect against DPP-4 degradation. As albumin has a serum half-life of approximately 3 weeks in humans,[22] an increased affinity to albumin could increase the systemic half-life beyond the 13 h seen for liraglutide after s.c. administration.

One major risk of increasing the albumin binding affinity is that the free active fraction would significantly decrease leading to a diminished *in vivo* potency and an increased dose needed to achieve acceptable efficacy. Based on the experience from various analogues,[8,10] as well as for fusion proteins,[23] it was a realistic concern whether it was at all possible to obtain sufficient albumin binding combined with high receptor affinity. The negative effect on receptor binding affinity and potency with increasing length and lipophilicity of the fatty acids has earlier been described[8,24] and is also observed here. Comparison of the data for liraglutide and **2** where the fatty acid was increased from C16 to C18 and **4** with **5** where the fatty acid was changed from C16 diacid to C18 diacid showed in both cases a significant reduction in potency (Table 1).

It therefore became an ambitious design challenge to demonstrate that it was possible to design an analogue that was efficacious at a low dose while still being reversibly bound to albumin with an affinity sufficient to protract the systemic clearance.

A series of Aib[8], Arg[34] GLP-1 (7−37) analogues derivatized at Lys[26] were prepared, and the *in vitro* potency and receptor binding was compared with those of liraglutide. The first campaign was aimed at investigating how much the potency and receptor binding could be affected by modification of the γGlu linker motif between the peptide and the C16 fatty acid in the side-chain of liraglutide (Table 2). The results showed that the linker can have significant impact on potency and receptor binding. It is interesting to observe that the largest difference in binding affinity was in the presence of 2% albumin where there was 23-fold difference between **8** with a γGlu linker and **12** with γGlu-3xOEG linker. The difference in functional potency was only four- to five-fold for this pair. There was a general tendency for improved binding affinity in the presence of albumin with increasing length of linker (**8** < **10** = **11** < **12**). The ratio between binding at low and high albumin affinity varied from two- to 42-fold, and the effect of adding albumin to the assay was highest for **8**, which had the shortest linker. The effect of the Ala to Aib modification in position 8 (liraglutide and **8**) had no significant effect on binding affinity, whereas it seemed to impact the potency slightly in a negative direction. This observation is opposite to what has been described for nonderivatized GLP-1 where the Aib modification has a slightly positive effect on receptor binding.[25]

In order to increase albumin binding beyond that of liraglutide, the C16 fatty acid was exchanged with other

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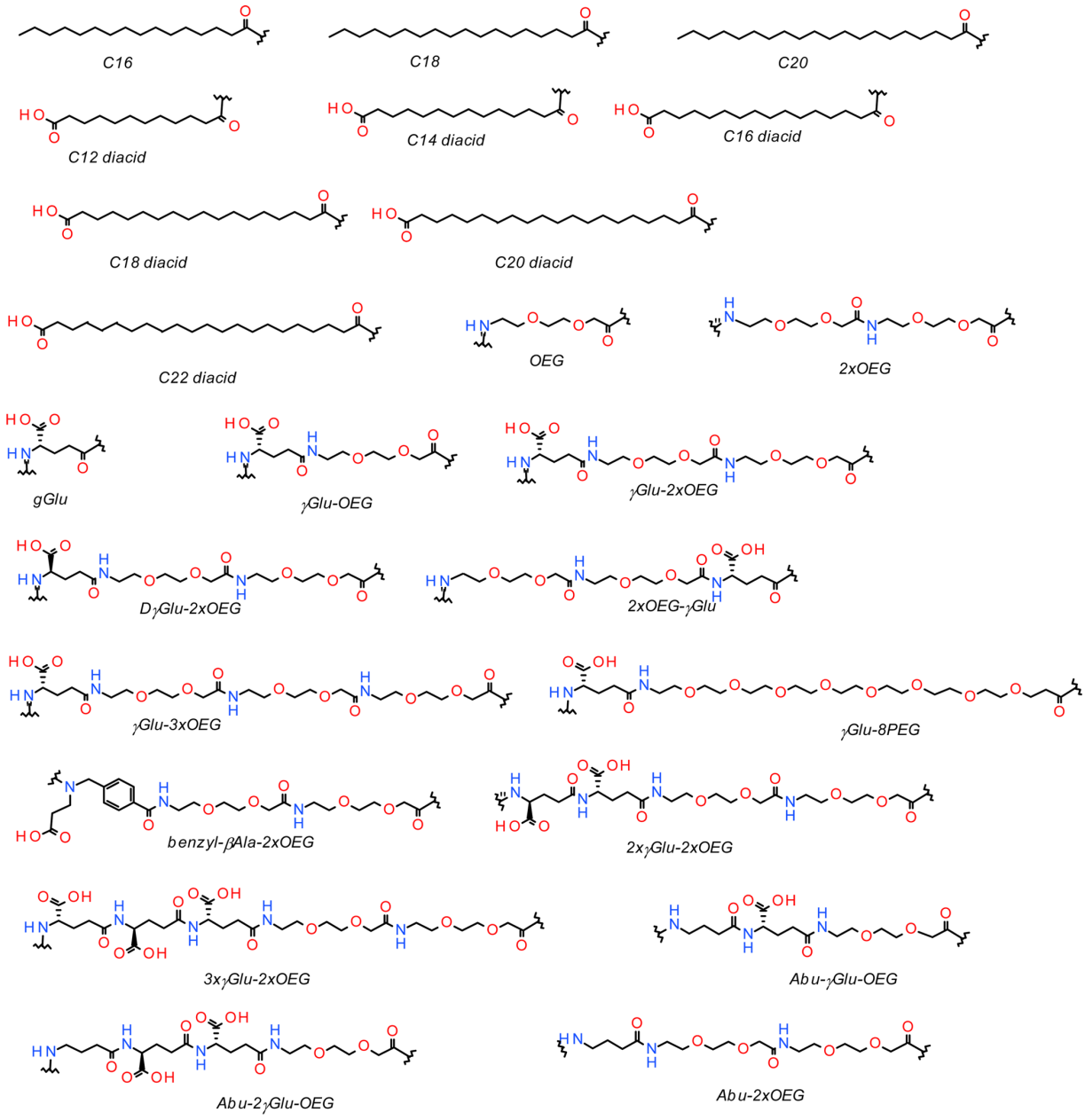

Figure 1. Abbreviations of linkers and protractors used to derivatize GLP-1 analogues in Tables 1−4.

lipophilic moieties in a series of derivatives with potential for increased albumin affinity. Based on the knowledge about the interaction of fatty acids with albumin, it was expected that increased lipophilicity, as well as the location of the acidic group, could have an impact on albumin affinity.[26−28] A series of derivatives were prepared where the properties of the albumin binding moiety were investigated (Table 2). Increasing the fatty acid length from C16 to C18 had a slightly negative impact on both potency and binding in the absence of albumin using the Arg[34] GLP-1 peptide (compared **2** with liraglutide). The results are in accordance with earlier reported data,[8] which concluded that lengthening the fatty acid chain had a negative impact on receptor affinity. However, it was observed that the binding in the presence of 2% albumin was improved by going from C16 to C18. An important observation was that in the Aib[8], Arg[34] GLP-1 series, the binding affinity with and without albumin was significantly improved by going from C16 to C18 fatty acid combined with the γGlu-2xOEG linker (compare **11** and **13**). This result gave the first insight toward the importance of the linker. A series of analogues with increasing fatty acid chain length (C18 and C20) using γGlu-2xOEG and γGlu-3xOEG was prepared (Table 2). Interestingly, the potency that was lost in the C18 analogue **2** was regained by introducing the γGlu-2xOEG linker in **13** which is even more potent than liraglutide. Even the C20 analogue **14** had improved potency compared to liraglutide.

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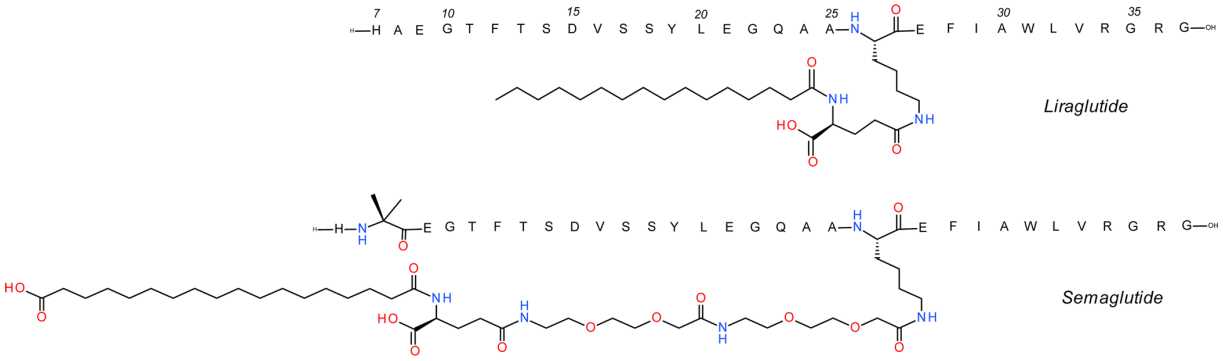

Figure 2. Structures of liraglutide and semaglutide.

Table 2. Derivatives of Aib[8], Arg[34] GLP-1 (7–37) with C16 to C20 Acids and C12 to C16 Diacids and Attached to Lys[26] Using γGlu and OEG Based Linkers[a]

analogue	sequence modification	protractor	linkage	acylation position	GLP-1R binding (IC_{50}, nM) (SEM) 0% HSA	GLP-1R binding (IC_{50}, nM) (SEM) 2% HSA	ratio 2%/0% HSA	GLP-1R potency (EC_{50}, pM) (SEM) 0% HSA
7	Aib[8], Arg[34]	none	none		0.17 (0.01)	0.08 (0.01)	0.5	6.2 (0.2)
8	Aib[8], Arg[34]	C16	γGlu	Lys[26]	0.12 (0.05)	5.05 (1.52)	42	19.2 (2.3)
9	Aib[8], Arg[34]	C16	2xOEG	Lys[26]	0.32 (0.01)	0.75 (0.10)	2.3	11.1 (0.5)
10	Aib[8], Arg[34]	C16	γGlu-OEG	Lys[26]	0.16 (0.03)	3.40 (0.86)	21	14.7 (1.6)
11	Aib[8], Arg[34]	C16	γGlu-2xOEG	Lys[26]	0.19 (0.07)	3.78 (1.05)	20	2.7 (0.3)
12	Aib[8], Arg[34]	C16	γGlu-3xOEG	Lys[26]	0.03 (0.01)	0.22 (0.14)	8.0	4.3 (0.2)
13	Aib[8], Arg[34]	C18	γGlu-2xOEG	Lys[26]	0.04 (0.01)	1.99 (0.59)	50	3.2 (0.7)
14	Aib[8], Arg[34]	C20	γGlu-2xOEG	Lys[26]	0.18 (0.02)	0.88 (0.08)	4.9	4.8 (0.6)
15	Aib[8], Arg[34]	C12 diacid	γGlu-2xOEG	Lys[26]	5.16 (1.16)	4.75 (0.80)	0.9	42.6 (7.0)
16	Aib[8], Arg[34]	C14 diacid	γGlu-2xOEG	Lys[26]	2.65 (0.45)	8.87 (1.90)	3.3	15.7 (0.8)
17	Aib[8], Arg[34]	C16 diacid	2xOEG	Lys[26]	3.25 (0.11)	12.8 (1.4)	3.9	166 (61)
18	Aib[8], Arg[34]	C16 diacid	γGlu-2xOEG	Lys[26]	0.94 (0.14)	20.5 (2.3)	22	8.6 (0.9)

[a]GLP-1 receptor binding at 0% and 2% albumin (HSA) using BHK cells expressing the human GLP-1R and in vitro potency measured in BHK cells that express both the human GLP-1R and a luciferase reporter system.

In the original design of liraglutide, the γGlu linker was introduced to compensate for the loss of the acidic group of palmitate used for amide linkage. In order to investigate the impact of the position of the acidic group and its importance for the binding to basic residues of albumin, a series of derivatives comprising a terminal acid on the albumin binding side chain was next prepared and tested.[29−31] The first two analogues were made using C16 diacids in combination with different linkers to the peptide backbone. As seen from Table 2, the addition of a terminal acid had a large negative impact on potency and binding (compare **17** with **9** and **18** with **11**). The effect was quite dramatic with about 10-fold decreased affinity for **18** compared to **11**. The small series of C16 diacid analogues in Tables 1 and 2 also confirmed that the linker had significant impact on potency. The impact of the Aib[8] substitution was not considered to be responsible for this huge difference, as there was no difference between the two nonderivatized analogues **7** and **11** (Tables 1 and 2).

The GLP-1 receptor affinity in the absence of albumin was used to get a measure of the true receptor affinity without competition with albumin affinity in the assay. Several attempts were made to measure the direct binding of the analogues to albumin in a screening mode, but due to the high unspecific adhesion of several of the analogues to various surfaces it has so far not been possible to obtain a reliable assay for albumin binding affinities. An alternative but indirect method to identify derivatives with high albumin affinity was to pick those analogues that had a right shift of the binding dose−response curve when high concentrations of albumin were in the assay compared to binding at low albumin concentration. Thus, we introduced the IC_{50} (high albumin)/IC_{50} (low albumin) ratio (BR ratio) as a surrogate of the albumin affinity with awareness of the pitfalls of this approach. This kind of data filter would of course not pick those derivatives that may have high affinity to albumin and the receptor simultaneously, but as we did not have a robust direct albumin affinity assay, this was a pragmatic screening plan to select analogues for animal pharmacokinetics studies.

It was clear that there was a remarkable effect on the BR ratio by modifying the sequence, the acylation site, and the linker. The BR ratio varied from around 0.9 for **15** to 940 for semaglutide among the peptides in this study. Also the *in vitro* receptor binding affinities and the potencies were remarkably different with a potency span of more than 2000-fold including the outlier **31** and greater than 100-fold for the main population of the series.

Based on the experience of modifying the fatty acids and the linker, it was concluded that the linker could have a pronounced impact on the *in vitro* properties. It was decided to focus more on this topic, and we hoped that extension of the

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Table 3. Derivatives of Aib[8], Arg[34] GLP-1 (7−37) with C18 to C22 Diacid Protractors Attached to Lys[26] Using γGlu, OEG, Abu, and Benzyl Containing Linkers[a]

analogue	sequence modification	protractor	linkage	acylation position	GLP-1R binding (IC_{50}, nM) (SEM) 0% HSA	GLP-1R binding (IC_{50}, nM) (SEM) 2% HSA	ratio 2%/0% HSA	GLP-1R potency (EC_{50}, pM) (SEM) 0% HSA
19	Aib[8], Arg[34]	C18 diacid	none	Lys[26]	1.86 (0.27)	27.0 (7.5)	14	269 (19)
20	Aib[8], Arg[34]	C18 diacid	γGlu	Lys[26]	0.21 (0.01)	112 (8)	541	9.9 (0.7)
21	Aib[8], Arg[34]	C18 diacid	γGlu-OEG	Lys[26]	0.17 (0.01)	79.7 (10.7)	477	4.8 (0.2)
semaglutide	Aib[8], Arg[34]	C18 diacid	γGlu-2xOEG	Lys[26]	0.38 (0.06)	357 (98)	940	6.2 (0.6)
22	Aib[8], Arg[34]	C18 diacid	DγGlu-2xOEG	Lys[26]	0.13 (0.01)	30.0 (3.0)	230	7.1 (0.4)
23[b]	Aib[8], Arg[34]	C18 diacid	γGlu-2xOEG	Lys[26]	0.80 (0.36)	12.9 (6.8)	16	5.6 (1.4)
24	Aib[8], Arg[34]	C18 diacid	γGlu-3xOEG	Lys[26]	0.71 (0.04)	6.17 (0.13)	8.6	27.7 (2.1)
25	Aib[8], Arg[34]	C18 diacid	γGlu-dPEG8	Lys[26]	5.31 (0.92)	11.4 (1.6)	2.1	47.3 (8.7)
26	Aib[8], Arg[34]	C18 diacid	2xγGlu-2xOEG	Lys[26]	0.25 (0.04)	19.5 (8.1)	77	27.8 (4.0)
27	Aib[8], Arg[34]	C18 diacid	3xγGlu-2xOEG	Lys[26]	0.36 (0.04)	15.2 (0.8)	43	67.0 (21.1)
28	Aib[8], Arg[34]	C18 diacid	Abu-2xγGlu-OEG	Lys[26]	1.77 (0.29)	16.8 (2.5)	9.5	70.3 (7.1)
29	Aib[8], Arg[34]	C18 diacid	Abu-2xOEG	Lys[26]	0.20 (0.08)	8.80 (4.86)	44	21.2 (2.1)
30	Aib[8], Arg[34]	C18 diacid	Abu−γGlu-OEG	Lys[26]	0.58 (0.11)	20.7 (3.3)	36	10.7 (1.6)
31	Aib[8], Arg[34]	C18 diacid	Benzyl-βAla-2xOEG	Lys[26]	9.19 (1.73)	51.7 (23.0)	5.6	5990 (1040)
32	Aib[8], Arg[34]	C20 diacid	γGlu-2xOEG	Lys[26]	0.10 (0.02)	8.43 (2.22)	85	11.5 (1.3)
33	Aib[8], Arg[34]	C22 diacid	γGlu-2xOEG	Lys[26]	0.21 (0.01)	24.8 (2.2)	116	24.4 (2.9)

[a]GLP-1 receptor binding at 0% and 2% albumin (HSA) using BHK cells expressing the human GLP-1R and *in vitro* potency measured in BHK cells that express both the human GLP-1R and a luciferase reporter system. [b]Analogue **23** has a C-terminal amide.

linker would impact the albumin affinity and PK in a similar manner as earlier reported for monoacid derivatives.[8,10,24] After the positive results with the C16 diacids, a campaign was designed using C18 diacid combined with a larger set of linkers and varying the acylation position.

A series of analogues with the Aib[8], Arg[34] GLP-1 (7−37) backbone was derivatized at Lys[26] with C18 diacids via various linkers (Table 3). The *in vitro* potency varied from 5990 pM for **31** with the bulky benzyl-containing linker (Benzyl-βAla) to 4−6 pM with the most promising linkers. It was still not clear how the structural features of the linker affects the potency, but it was observed that simple linkers composed of OEG and/or γGlu gave the highest potencies with EC_{50} values below 10 pM, as seen for semaglutide, **20**, **21**, and **22**. The receptor binding was also affected by the choice of linker with a span of 70-fold from around 9 nM for **31** to 0.1−0.2 nM for **21** and **22** in the absence of albumin. When 2% albumin was present in the assay the right-shift of the binding dose−response (BR ratio) varied a lot, from around two-fold for **25** up to 940-fold for semaglutide, due to different binding affinities for albumin.

There was a dramatic effect on the BR ratio by changing from C16 diacid in **18** to C18 diacid in semaglutide, which was hypothesized to be due to an increased albumin affinity. An attempt was made to increase this BR ratio further by expanding the fatty acid length to C20 diacid in **32** and C22 diacid in **33** but the BR ratio actually dropped due to improved receptor affinity at 2% HSA for these analogues compared to semaglutide. One surprising observation of the series of C12 diacid to C22 diacid (**15**, **16**, **18**, semaglutide, **32**, and **33**) is that the *in vitro* potency is gradually improved from C12 diacid in **15** (42.5 pM) to C18 diacid in semaglutide (6.2 pM) but is again attenuated for **32** with C20 diacid (11.5 pM) and for **33** with C22 diacid (24.4 pM), thus concluding that C18 diacids is the optimal choice with respect to *in vitro* potency.

An earlier observation was that it seemed possible to acylate GLP-1 in several positions without losing receptor affinity,[8,10] and also in this study we decided to investigate how the position of acylation could affect the *in vitro* properties. A relatively complex approach was tried where we permutated not only the linker but also the attachment point and a few mutations of the peptide backbone. In order to be able to draw structural conclusions, the C18 diacid was kept constant throughout (Table 4). Among this set of GLP-1 derivatives, four peptides had EC_{50} potency values below 10 nM (**35**, **36**, **38**, and **43**) combined with high receptor affinity below 0.5 nM. However, the BR ratio was relatively low, and only three derivatives had a BR ratio above 100 (**34**, **38** and **43**).

Pharmacokinetic studies are more resource demanding, hence, pharmacokinetics were only investigated for a limited number of the analogues. The *in vivo* extension of half-life has previously been shown to be greatly impacted by the albumin binding moiety for a series of non-DPP-4 stabilized analogues (including liraglutide).[8] In the present work, we hypothesized that for DPP-4 stabilized analogues the increase in albumin binder lipophilicity would also increase the exposure (increase systemic half-life). This was evaluated using a series of analogues with the Aib[8], Arg[34] GLP-1 (7−37) backbone derivatized at Lys[26] linked to various chain length (C12 to C20) diacids using a γGlu-2xOEG linker (Figure 3).

As depicted in Figure 3 the trend was quite clear; there is an increased exposure *in vivo* with increased chain length and relative lipophilicity of the albumin binder going from C12 to C14, C16, C18, and C20 diacid moiety. However, backbone substitution and modifying the linkers gave rise to a much less clear picture and unpredictable impact on the pharmacokinetic properties. The semaglutide analogue exhibited the highest BR ratio (940), but there was no apparent correlation between the indirect measure of albumin affinity (BR ratio) and the observed protraction (terminal systemic half-life) across the analogues tested. This is exemplified by the analogue **32**, which had a BR ratio of 85 but a protraction quite similar to semaglutide.

Native GLP-1 is rapidly metabolized by DPP-4, and liraglutide also has been shown to be metabolized to a minor degree by

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Table 4. Derivatives of GLP-1 (7−37) Peptides Attached with C18 Diacids via γGlu and OEG Containing Linkers and to 16, 22, 25, 26, 27, 36, 37, and 38 Positions of the Peptides[a]

analogue	sequence modification	protractor	linkage	acylation position	GLP-1R binding (IC_{50}, nM) (SEM) 0% HSA	GLP-1R binding (IC_{50}, nM) (SEM) 2% HSA	ratio 2%/0% HSA	GLP-1R potency (EC_{50}, pM) (SEM) 0% HSA
34	26Arg^{26}, 34Arg^{34},Lys^{36}	C18 diacid	2xOEG	Lys^{36}	0.11 (0.01)	14.5 (0.8)	127	7.0 (0.3)
35	Aib^{8}, Lys^{27}	C18 diacid	γGlu-2xOEG	Lys^{27}	0.06 (0.01)	5.39 (1.39)	98	4.6 (0.4)
36	Aib^{8}, Lys^{16}	C18 diacid	γGlu-2xOEG	Lys^{16}	0.50 (0.13)	25.5 (4.9)	51	9.9 (1.4)
37	Aib^{8}, Aib^{22}, Aib^{35}, Lys^{37}	C18 diacid	OEG	Lys^{37}	0.77 (0.06)	19.3 (0.7)	25	40.9 (0.4)
38	Aib^{8}, Lys^{22}	C18 diacid	γGlu-2xOEG	Lys^{22}	0.06 (0.01)	17.7 (2.8)	280	2.7 (0.1)
39	Aib^{8}, Lys^{25}	C18 diacid	γGlu-2xOEG	Lys^{25}	1.13 (0.29)	41.6 (10.5)	37	41.8 (4.1)
40	Aib^{8}, Arg^{26}, Arg^{34}, Lys^{36}	C18 diacid	none	Lys^{36}	0.52 (0.04)	23.0 (2.4)	45	159 (21)
41	Aib^{8}, Arg^{26}, Arg^{34}, Lys^{38}	C18 diacid	none	Lys^{38}	0.31 (0.13)	7.55 (1.44)	24	87.3 (15.4)
42	Aib^{8}, Arg^{26}, Arg^{34}, Lys^{38}	C18 diacid	2xOEG	Lys^{38}	0.11 (0.01)	3.99 (0.58)	36	13.2 (1.4)
43	Aib^{8}, Arg^{26}, Arg^{34}, Lys^{38}	C18 diacid	γGlu-2xOEG	Lys^{38}	0.09 (0.01)	30.1 (1.1)	334	1.5 (0.1)
44	Aib^{8}, Arg^{34}, Lys^{38}	C18 diacid	2xOEG	Lys^{38}	0.27 (0.05)	6.73 (3.65)	25	67.5 (0.9)
45	Gly ^{8}, Arg^{34}	C18 diacid	2xOEG	Lys^{26}	0.10 (0.03)	6.5 (1.5)	64	26.0 (0.5)

[a]GLP-1 receptor binding at 0% and 2% albumin (HSA) using BHK cells expressing the human GLP-1R and *in vitro* potency measured in BHK cells that express both the human GLP-1R and a luciferase reporter system.

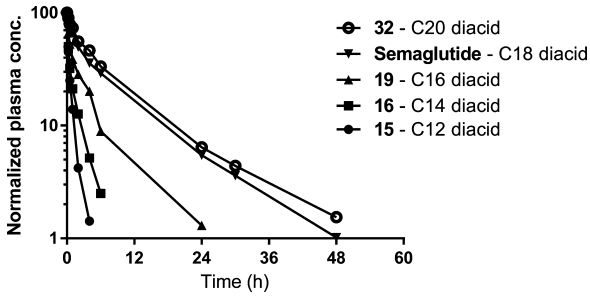

Figure 3. *In vivo* protraction in rats following i.v. administration of **32** (5.5 nmol/kg), semaglutide (4.2 nmol/kg), **19** (3.3 nmol/kg), **16** (5.5 nmol/kg), and **15** (5.3 nmol/kg).

DPP-4 in the Ala^{8}-Glu^{9} position of the N terminus.[32,33] The impact on *in vivo* protraction of the lack of DPP-4 protection is illustrated in Figure 4.

Figure 4 depicts that semaglutide with Aib^{8} has a longer systemic half-life compared to the analogue **6** with Ala^{8} and

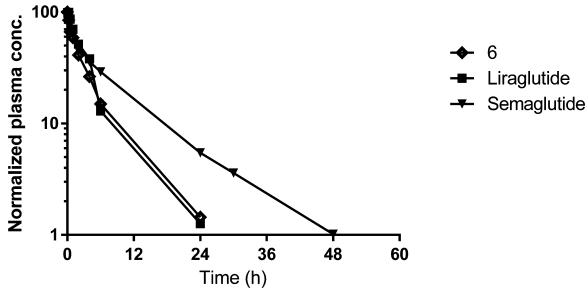

Figure 4. *In vivo* protraction in rats following i.v. administration of **6** (5.8 nmol/kg), liraglutide (5.8 nmol/kg), and semaglutide (4.2 nmol/kg). Compound **6** is the Ala^{8} version of semaglutide and susceptible to enzymatic degradation.

otherwise similar backbone structure, linker, and protractor moiety. The susceptibility to enzymatic degradation *in vivo* of **6** was verified by identification of circulating metabolites (data not shown). In fact, **6** has quite similar *in vivo* protraction as liraglutide indicating the importance of not only the stronger albumin binding of semaglutide compared to liraglutide but potentially also the extra enzymatic stabilization in order to obtain long systemic half-life.

As mentioned earlier, in order to limit the risk of immunogenicity responses in patients, we aimed to select a peptide structurally similar to liraglutide and native GLP-1. Thus, acylation in position 26 and as few mutations as possible were prioritized features. Among the possible candidates, it was quite encouraging that semaglutide fulfilled these structural priorities and had an attractive pharmacokinetic profile combined with acceptable receptor potency.

***In Vivo* Characterization Studies.** The *in vivo* characterization of the analogues in the screening program included evaluation of the pharmacokinetic properties in pigs (Göttingen mini-pigs) and *in vivo* efficacy in *db/db* mice.

In Table 5 the pharmacokinetic parameters of liraglutide and semaglutide are presented. The volume of distribution of

Table 5. Pharmacokinetic Evaluation in Göttingen Mini-Pigs Following Administration of Semaglutide (2 nmol/kg i.v. or 2 nmol/kg s.c.) and liraglutide (0.5 nmol/kg i.v. or 1.0 nmol/kg s.c.)[a]

	i.v. administration			s.c. administration		
	Cl (L/h/kg)	Vz (L/kg)	$T_{1/2}$ (h)	T_{max} (h)	MRT (h)	F (%)
semaglutide	0.0016	0.1019	46.1	12	63.6	94%
liraglutide	0.0038	0.0674	12.4	7	23.0	66%

[a]The key pharmacokinetic parameters (Cl, Vz, T_{max}, $T_{1/2}$, MRT and F) of semaglutide and liraglutide as reference in Göttingen mini-pigs.

liraglutide after i.v. administration was 0.067 L/kg (67 mL/kg). This is very close to the blood volume in mini-pigs (65 mL/kg) indicating either a limited distribution outside the circulation or a fast equilibrium of the liraglutide concentration between the circulation and the peripheral tissue. Body clearance of liraglutide after i.v. administration was estimated to 0.0038 L/h/kg (0.063 mL/min/kg), and together with the observed volume of distribution it leads to a half-life of 12.4 h after i.v. administration.

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The volume of distribution of semaglutide was 0.102 L/kg (102 mL/kg). This is 1.5-fold higher than for liraglutide, indicating lower concentrations of semaglutide measured in blood, which is consistent with a higher degree of albumin binding. The body clearance after i.v. administration was also two-fold lower for semaglutide compared to liraglutide, suggesting that the albumin binding might reduce the clearance. This leads to a half-life of semaglutide after i.v. administration of 46.1 h, which is more than three-fold longer than observed for liraglutide. The observed mean residence time (MRT) following s.c. administration was approximately three-fold longer for semaglutide compared to liraglutide. An almost complete subcutaneous bioavailability was observed for semaglutide (94%), whereas the subcutaneous bioavailability for liraglutide was 66%. It was speculated that an increased albumin binding and strong protection toward DPP-4 degradation could facilitate increased bioavailability, but further studies have to be made to conclude.

The db/db mouse, a hyperglycaemic, hyperinsulinaemic, and obese model of type 2 diabetes, was used during the initial *in vivo* screening studies of GLP-1 analogues to investigate the antihyperglycaemic and body weight lowering efficacy. A dose–response study (0.3 to 100 nmol/kg) showed that semaglutide had a dose-dependent efficacy and duration of action of up to 48 h (Figure 5A). A similar study with liraglutide (Figure 5B)

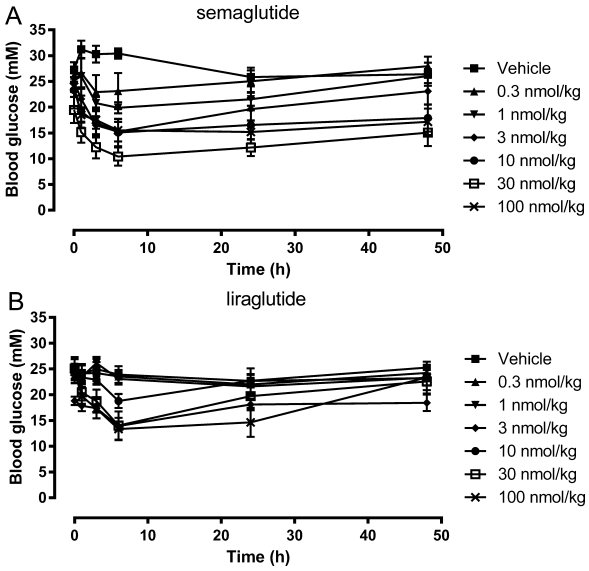

Figure 5. Dose response for blood glucose lowering of semaglutide (A) and liraglutide (B) in diabetic db/db mice after s.c. dosing of 0.3 to 100 nmol/kg.

clearly showed a shorter duration of action and less potency for liraglutide compared to semaglutide. Among the tested GLP-1 analogues, semaglutide was the most potent with an EC_{50} of <2 nmol/kg (calculated as the delta AUC blood glucose during 0–48 h after dosing).

HSA Affinity Assessed by Analytical Ultracentrifugation. We did not obtain measurements of the absolute binding constant between the ligands and albumin. Instead, the albumin binding of a subset of analogues was evaluated *in vitro* by analytical ultracentrifugation[34] to obtain measures of relative albumin affinities. Figure 6 depicts the fraction of bound analogue relative to that of liraglutide. As expected the native

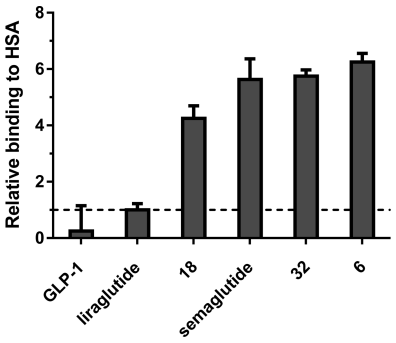

Figure 6. Relative binding affinities of GLP-1 analogues to HSA measured by analytical ultracentrifugation. Fraction of analogue bound to HSA relative to liraglutide assessed by analytical ultracentrifugation. The relative fractions of bound vs free analogue were determined in solutions of 100 μM analogue and 10 μM HSA in PBS-buffer, pH 7.4.

peptide displayed negligible binding to albumin. Among those analogues that are acylated in position 26 there is a clear trend that with increasing length of the carbon chain (**18**, C16 diacid; semaglutide, C18 diacid; and **32**, C20 diacid), the analogues display a successively increased affinity to albumin binding relative to liraglutide (4.3, 5.6, and 5.8 times, respectively). This trend relates to the longer plasma half-life observed with increasing length of the fatty diacids and correlates much better than the BR ratio. However, this correlation does not hold for other modifications. As an example, the absence of the Aib^8 in analogue **6** appeared favorable for HSA binding to the same extent as for semaglutide, but unfortunately, this analogue has a much shorter half-life in rats compared to semaglutide (Figure 6). It could be speculated that the DPP-4 cleavage for the unprotected analogue **6** is the main reason for this lack of correlation.

Crystal Structure of the Semaglutide Peptide Backbone in Complex with the GLP-1 Receptor Extracellular Domain. Crystallization trials of semaglutide in complex with the GLP-1 receptor extracellular domain resulted in poorly diffracting crystals. The unacylated semaglutide backbone, however, gave nicely diffracting crystals resulting in a high resolution structure (1.8 Å). Not surprisingly, due to high sequence identity, the overall structure was almost identical to native GLP-1(7–37)-OH.[35] In both structures, Lys^{26} interacted with Glu^{128} of GLP-1R, but receptor mutagenesis suggested previously that this interaction was not very important and indeed acylation of Lys^{26} had only minor impact on receptor affinity. The native Lys^{34} of GLP-1(7–37)-OH appeared highly flexible in complex with the GLP-1R extracellular domain, whereas Arg^{34} of semaglutide adopts a more well-defined conformation oriented toward Glu^{27} (Figure 7A). A water molecule was coordinated by the guanidine group of Arg^{34}, the backbone carbonyl, and carboxyl group of Glu^{27}. The water molecule appeared to mediate an electrostatic interaction between Arg^{34} and Glu^{27}. Arg^{34} was originally introduced in liraglutide to enable site-specific acylation of Lys^{26} and had no apparent effect on binding to the GLP-1R.

In the previous crystal structure of native GLP-1(7–37)-OH, it was not possible to build a representative model of Arg^{36}-Gly^{37}-OH.[35] The electron density of the semaglutide backbone C-terminus was better defined, suggesting a specific conformation upon binding to GLP-1R, although the density of the C-terminal carboxyl group was still rather weak. The backbone nitrogen of Gly^{35} and Arg^{36} formed a hydrogen bond with the backbone carbonyl oxygen of Leu^{32} and Trp^{31}, respectively

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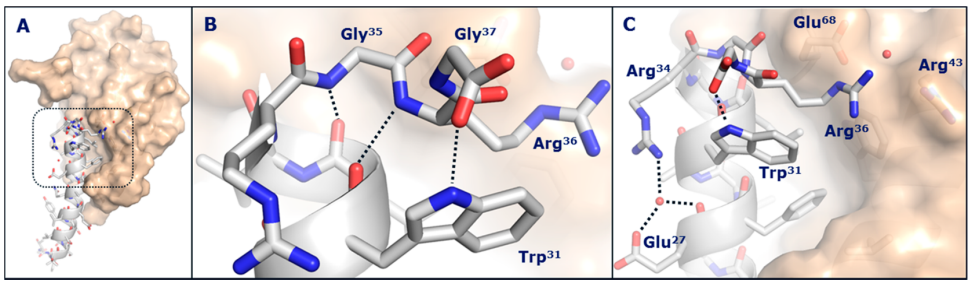

Figure 7. Crystal structure of the semaglutide peptide backbone (gray) in complex with the GLP-1 receptor extracellular domain (golden surface). Individual residues are shown in sticks where nitrogen and oxygen atoms are colored blue and red, respectively. (A) Overall structure with boxed area magnified in B and C. (B) The structure of the C-terminus of the semaglutide peptide backbone. Hydrogen bond interactions are illustrated as dotted lines. (C) Arg^{36} closes the hydrophobic ligand−receptor interface by aligning with Trp^{31} and Glu^{68}. A water molecule is coordinated by Glu^{27} and Arg^{34}.

(Figure 7B). The C-terminal carboxyl group may form a hydrogen bond with the ring nitrogen of Trp^{31}. The side-chain of Arg^{36} aligned with and filled the gap between Glu^{68} of GLP-1R and Trp^{31} of GLP-1, putting a lid on the hydrophobic ligand−receptor interface (Figure 7C). The methyl groups of Arg^{36} on GLP-1 formed hydrophobic interactions with Trp^{31} of the GLP-1R on one side, and Glu^{68} of GLP-1R on the other side. Exendin-4 occupied the same gap using Gly^{30}-Pro^{31}. The guanidine group of Arg^{36} interacted with the carboxyl group of Glu^{68} and a water molecule between Glu^{68} and Arg^{43} of GLP-1R. C-terminal truncation of GLP-1 reduced the GLP-1R affinity supporting a role of Arg^{36} in receptor binding as also suggested by the structural observation shown here (unpublished binding data). The role of Glu^{68} in ligand binding was previously investigated by receptor mutagenesis.[36,37]

■ CONCLUSION

In summary, we have described the structure−activity relationship of a series of GLP-1 analogues derivatized with various fatty acids on the epsilon amine of lysines with the aim to obtain a potent GLP-1 analogue with a plasma half-life suitable for once-weekly administration and with low peak to trough ratio of the plasma concentrations at steady state. The starting point was the once daily GLP-1 analogue liraglutide with a half-life of approximately 12 h in pigs as well as in humans. We were able to obtain analogues with terminal half-lives of 46.1 h in mini pigs after i.v. dosing combined with GLP-1 receptor binding affinities similar to liraglutide. A dose response study of semaglutide in db/db mice demonstrated a duration of action of at least 48 h at a dose of 10 nmol/kg. The selection of semaglutide for further *in vivo* studies and as the lead candidate for further development was, in addition to the preclinical data, also based on its structural resemblance to liraglutide in order not to introduce unnecessary risks into the clinical program. Semaglutide is currently in phase 3 clinical development.

■ EXPERIMENTAL SECTION

Synthesis of Peptides. All GLP-1 analogues were prepared by standard solid phase peptide synthesis using the Fmoc strategy. Resins used were Rink-amide or preloaded Wang resins with a loading of 0.25−0.6 mmol/g.

For analogues with a side chain, the lysine to be modified was incorporated as Fmoc-Lys(Mtt)-OH and the N-terminal amino acid as Boc-His(Trt)-OH. After completion of the synthesis, the Mtt group was removed by a 30 min treatment with hexafluoroisopropanol-dichloromethane (3:1), and the side chain built by the Fmoc strategy. Finally, the peptide was cleaved from the resin by a 2 h treatment with TFA/water/triisopropylsilane (95:2.5:2.5) followed by ether precipitation and ether wash.

The analogues were purified by RP-HPLC on a C18-column in acetonitrile/TFA, and purity and identity of the product established by UPLC and LCMS.

The purity of the peptides was analyzed on UPLC and LCMS and confirmed to be of ≥95% purity for all key analogues.

***In Vitro* Binding.** Homogeneous binding competition of ^{125}I-GLP-1 was performed on plasma membranes prepared from BHK cells expressing the hGLP-1R (clone FCW467-12A). Membranes were suspended to a concentration of 0.06 mg/mL in a 20 mM HEPES buffer (pH 7.4) containing 10 mM EDTA and kept on ice until used. Compounds to be tested were diluted in an assay buffer consisting of 50 mM HEPES (pH 7.4) containing 5 mM EGTA, 5 mM $MgCl_2$, and 0.005% Tween-20 over the concentration range of $1 \times 10^{-14} - 1 \times 10^{-7}$ M. Wheat germ agglutinin SPA beads (PerkinElmer RPNQ0001) were suspended in assay buffer at 10 mg/mL just prior to addition. Incubations were started for the low HSA (0.005% HSA) assays by adding 50 μL assay buffer to each well in a 96-well assay plate (PerkinElmer OptiPlate-96 6005290). For the high HSA (2%) assays 50 μL of 8% HSA stock in assay was added to each well in the assay plate. For all assays a 25 μL aliquot of the compound dilutions were added to the assay plate followed by a 50 μL of the cell membrane preparation and 50 μL of the SPA bead suspension. A 25 μL of ^{125}I-GLP-1 (7−36)-NH_2 (480 pM, 6651 Bq) was added, and the plates were incubated for 2 h at 30 °C. The assay was terminated by centrifugation of the assay plate for 10 min at 1500 rpm. The plate was read in a TopCount NXT instrument. Data was imported into GraphPad Prism version 6.02 and IC_{50} values were determined using the following nonlinear regression equation: log(inhibitor) vs response (four parameters).

***In Vitro* Functional Assay.** Frozen aliquots of BHK cells that express both the hGLP-1R and CRE firefly luciferase (clone FCW467-12A/KZ10-1) were thawed, washed twice in PBS, and suspended in assay buffer (DMEM w/o phenol red, 10 mM HEPES, 1× GlutaMAX (Gibco 35050-038), 1% ovalbumin, and 0.1% Pluronic F-68). Cells were plated out into 96-well plates at 5000 cells/well in a volume of 50 μL. Compounds to be tested were diluted in assay buffer and a 50 μL aliquot transferred to the plate containing the cells to reach final assay concentrations of $1 \times 10^{-14} - 1 \times 10^{-7}$ M. The plate was incubated for 3h at 5% CO_2 at 37 °C. The plate was allowed to stand at room temperature for 15 min prior to adding 100 μL of steadylite plus reagent (PerkinElmer 6016757). The plate was covered to protect it from light and shaken at room temperature for 30 min. The plate was read in a TopCount NXT instrument. Data was imported into GraphPad Prism version 6.02, and EC_{50} values were determined using the following nonlinear regression equation: log(agonist) vs response (three parameters).

Crystal Structure. The GLP-1R extracellular domain and the complex with the semaglutide peptide backbone were prepared as described previously.[35] The crystals were grown in the presence of 0.24 M sodium malonate pH 7.0, 20% (w/v) PEG3350 (JCSG screen QIAGEN), and 10 mM hexaethylene glycol monooctyl ether (Detergent Screen HT

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Hampton Research). Diffraction data was collected at our in-house X-ray source (Rigaku MicroMax-007 HF rotating anode and Rayonix165 CCD detector) and processed using XDS. The structure was solved and refined in Phenix, molecular replacement using Phaser, and refinement using phenix.refine and Coot. Visuals were generated in PyMOL. Coordinates and structure factors are deposited in the Protein Data Bank under accession code 4ZGM.

Biophysics. Materials and Methods AUC, HSA-binding: sedimentation velocity (SV) experiments to study the binding of analogues to HSA were performed in an XL-I Analytical Ultracentrifuge from BeckmanCoulter (Indianapolis, USA). Solutions of 100 μM analogue and 10 μM of purified, monomeric HSA were prepared in PBS-buffer, pH 7.4 and filled into two-sector, sapphire-capped Epon centerpieces of 12 mm path length. After sufficient temperature equilibration to 20 °C, centrifugation was performed at 40 000 rpm and monitored at 250 nm in intensity mode until the majority of all detectable material had sedimented. All analogues had the same extinction coefficient, and the total signal for the different experiments and samples was therefore similar. Data were analyzed with the c(s)-function as implemented in Sedfit (v. 11.8)[34] with an s-grid between 0.5 and 12 S. The resulting sedimentograms were integrated to give the quantities c_{tot} and c_{free} in signal units. The intervals for c_{free} were between 0.5 and 3.5 S, those for c_{tot} between 0.5 and 12 S. The fraction of free analogue Φ_{free} was then calculated according to

$$\Phi_{free} = c_{free}/(c_{tot} - c_{HSA})$$

with c_{HSA} determined independently and assumed to be same for all experiments. Subsequently, the binding relative to that of liraglutide was calculated, relative binding = $((1 - \Phi_{free})/(1 - \Phi_{free,lira}))$. The results from three different experiments were averaged and single standard deviations calculated.

Pharmacokinetics. Pharmacokinetic screening studies were conducted in rats and mini-pigs in accordance with the Protection of Animals Act, the Act on Experiments on Animals, and the Standard Operating Procedures for Experiments on Animals at BioAdvice A/S (Denmark) and Novo Nordisk A/S. The experiments were performed under the supervision and approval of the Danish Government Animal Experiments Inspectorate and the Novo Nordisk Ethical Review Counsel.

Pharmacokinetics in Rats. Male Sprague−Dawley rats (Taconic, Denmark) ($n \geq 3$) of approximately 350−450 g had a central vein catheter (Tygon Microbore Tubing, Cole Parmer) inserted 2−3 cm into right vena jugularis under isoflurane anesthesia and given analgesia during and postsurgery (Anorfin (0.05 mg/kg s.c.) and Rimadyl Vet. (5 mg/kg s.c.)). The rats were allowed to recover for approximately 1 week after surgery prior to study. On study day the rats received a single i.v. bolus over 15sek of 5 nmol/kg (1 mL/kg) of test compound via the catheter and followed by 0.2 mL of sterile saline. Blood samples were collected from vena sublingualis at time points covering the plasma exposure profile with less than 30% extrapolation (12 samples up to 96 h postdosing). Blood samples were collected into ice cooled EDTA tubes and centrifuged for 5 min (4500 G) 20 min at 4 °C. Plasma was transferred and immediately frozen for later measurement of GLP-1 analogue by ELISA or LC/MS.

Pharmacokinetics in Mini-Pigs. Male Göttingen mini-pigs (Ellegaard Göttingen Minipigs A/S, Denmark) of 25−30 kg were used in the pharmacokinetic study. Before the study the pigs were instrumented with central venous catheters. Göttingen mini-pigs ($n \geq 3$) received a single dose of 1−2 nmol/kg of test compound with an injection volume of 0.1 mL/kg. The s.c. injections were given on the right side of the neck, approximately 5−7 cm from the ear and 7−9 cm from the middle of the neck. The injections were given with a stopper on a 21 G needle, allowing 0.5 cm of the needle to be introduced. Individual dose volumes were based on individual body weights. Blood samples were collected from each animal at time points covering the plasma exposure profile with less than 30% extrapolation (12 samples up to 288 h postdosing). Blood samples were collected into ice cooled EDTA tubes and centrifuged for 5 min (4500 G) to 20 min at 4 °C. Plasma was transferred and immediately frozen for later measurement of GLP-1 analogue by ELISA or LC/MS.

Pharmacokinetic Analysis. Plasma concentration−time data from each test compound was analyzed by noncompartmental pharmacokinetics using WinNonlin Professional or Phoenix WinNonlin (Pharsight Corporation/Certera, USA). Calculations of sampling data were performed using individual animal plasma concentration−time values.

Pharmacodynamics. *Test for Effect on Blood Glucose.* Male *db/db* mice (Taconic, Denmark), 10−12 weeks of age and with blood glucose levels over 10 mM, were kept in standard cages and from arrival housed 10 mice per cage, according at ambient controlled conditions. They were given free access to standard chow (Altromin 1324, Brogaarden Aps., Gentofte, Denmark) and acidified (0.4% citric acid) tap water and were kept at a constant room temperature 24 ± 2 °C on a normal day-light cycle with lights on at 6 am. The cages were supplied with wooden wool as nesting material, and the bedding material was aspen chips, changed twice per week as was the bottles of acidified water. After 1 week acclimatization, the nonfasting morning blood glucose was assessed, and 42 mice with blood glucose levels over 10 mM were selected for further experimentation. The mice were allocated to 7 groups ($n = 6$) with matching nonfasting blood glucose levels and thereafter housed 2 mice per cage. The mice were used for 3 times for 3 day experiments whereafter they were euthanized. Principles of laboratory animal care were followed (EU directive no. 86/609) and the type of experiment was approved by the Danish Animal Experiment Inspectorate.

Test Procedure. The peptides were tested subcutaneously at 0.3−100 nmol/kg and were formulated in a phosphate buffer (50 mM phosphate, 0.05% tween 80, pH 8) at concentrations in the range of 0.05 to 17 μM. The mice were dosed at a fixed volume of 6 mL/kg sc. Blood glucose levels were assessed prior to dosing (time 0) 1, 2, 3, 6, 24, and 48 h after dosing. Samples for the measurement of blood glucose were obtained from the tail tip capillary of the conscious mice. Blood, 5 μL, was collected into heparinized capillary tubes and measured using the glucose oxidase method (glucose analyzer BIOSEN 5040, Dreieich, Germany). Samples were measured on the day of sampling. During the experiment, the mice were kept freely moving in their cages. At blood sampling, the mice were slightly constrained using a tail-holder. The mice had been accustomed to this procedure prior to the experiment. The area under the glucose curves were calculated on the actual blood glucose values as well as on delta blood glucose values (subtracted baseline value at time 0). Statistical analysis was made using one-way analysis of variance and Bonferroni's Multiple Comparison Test as post hoc analysis; $P < 0.05$ was regarded as statistically significant. The blood glucose profiles are represented as mean ± SEM versus time.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00726.

Tables of all peptides with analysis as well as data and refinement statistics for the structure (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jela@novonordisk.com.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): Novo Nordisk markets liraglutide for the treatment of diabetes and obesity and has semaglutide in phase 3 clinical development. All authors are or were full time employees of Novo Nordisk, and most hold minor share portions as part of their employment. S.M.K. is a previous employee of NN and

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holds minor share portions as part of her previous employment. J.E.L.A., L.S.C., and K.J.M. hold substantial shares of Novo Nordisk.

■ ABBREVIATIONS USED

BHK, baby hamster kidney; BR ration, IC_{50} (high albumin)/IC_{50} (low albumin); DPP-4, dipeptidyl peptidase 4; GLP-1 RA, glugacon-like peptide-1 receptor agonist; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MRT, mean residence time

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