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Exploration of structure–activity relationships at the two C-terminal residues of potent 11mer Glucagon-Like Peptide-1 receptor agonist peptides via parallel synthesis

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ABSTRACT

We report the identification of potent agonists of the Glucagon-Like Peptide-1 receptor (GLP-1R) via evaluation of two positional scanning libraries and a two-dimensional focused library, synthesized in part on SynPhase™ Lanterns. These compounds are 11 amino acid peptides containing several unnatural amino acids, including (in particular) analogs of biphenylalanine (Bip) at the two C-terminal positions. Typical activities of the most potent peptides in this class are in the picomolar range in an *in vitro* functional assay using human GLP-1 receptor.

1. Introduction

Type 2 diabetes is the most prevalent form of diabetes, accounting for 90–95% of the estimated 20.8 million people with diabetes in the United States alone [5]. Patients with type 2 diabetes initially develop resistance to the effects of insulin, resulting in an elevation of blood glucose levels. The patient's production of insulin by pancreatic β-cells eventually decreases, leading to an even greater elevation of glucose levels in the blood.

Glucagon-Like Peptide-1 (GLP-1) was first identified in 1983 [3,4], and has become an important subject of interest for the treatment of type 2 diabetes [14]. GLP-1 is an incretin hormone that is normally secreted in response to food intake and regulates insulin release in a glucose-dependent manner. GLP-1 has been implicated in promoting satiety after meals, beta-cell growth, delayed gastric emptying, and decreased glucagon and gluconeogenesis [9]. The broad, beneficial effects of GLP-1 make it an attractive therapeutic target [6,10,15]. However, the enzyme dipeptidyl peptidase IV (DPP-IV) readily cleaves GLP-1 at the 2-position alanine residue, inactivating the agonist activity of GLP-1. Consequently, the short plasma half-life of GLP-1 *in vivo* (less than 5 min) precludes the parent peptide from being a useful treatment for type 2 diabetes.

GLP-1 binds to and activates the GLP-1 receptor (GLP-1R). GLP-1R is a member of the class B family of G-protein-coupled receptors (GPCRs) [8]. Receptors in this family typically consist of two domains; an N-terminal extracellular binding domain (N-domain), and a C-terminal domain of 7 α-helices that span the membrane (juxtamembrane domain, or J-domain). It is believed that high affinity binding of ligands to the receptor occurs at the N-domain, inducing a conformational change that allows the N-terminal domain of the ligand to bind to the J-domain to activate the receptor and intracellular G protein. With few exceptions, the class B GPCRs have proven to be difficult targets for development of small molecule agonist therapeutics; nearly all agonists reported to date consist of peptides of moderate length (30–60 amino acids) [8].

Most agonists reported for GLP-1R are closely related in both sequence and size to the endogenous peptide GLP-1(7–37), the

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GLP-1:
His[7]-Ala-Glu-Gly[10]-Thr-Phe-Thr-Ser-Asp[15]-Val-Ser-Ser-Tyr-Leu[20]-Glu-Gly-Gln-Ala-Ala[25]-Lys-Glu-Phe-Ile-Ala[30]-Trp-Leu-Val-Lys-Gly-Arg-Gly[37]

Exendin-4:
His[1]-Gly-Glu-Gly-Thr[5]-Phe-Thr-Ser-Asp-Leu[10]-Ser-Lys-Gln-Met-Glu[15]-Glu-Glu-Ala-Val-Arg[20]-Glu-Phe-Ile-Glu-Trp[25]-Leu-Lys-Asn-Gly-Gly[30]-Pro-Ser-Ser-Gly-Ala[35]-Pro-Pro-Pro-Ser[39]

Fig. 1. Sequences of GLP-1(7–37) agonist peptide (top), and Exendin-4 (bottom).

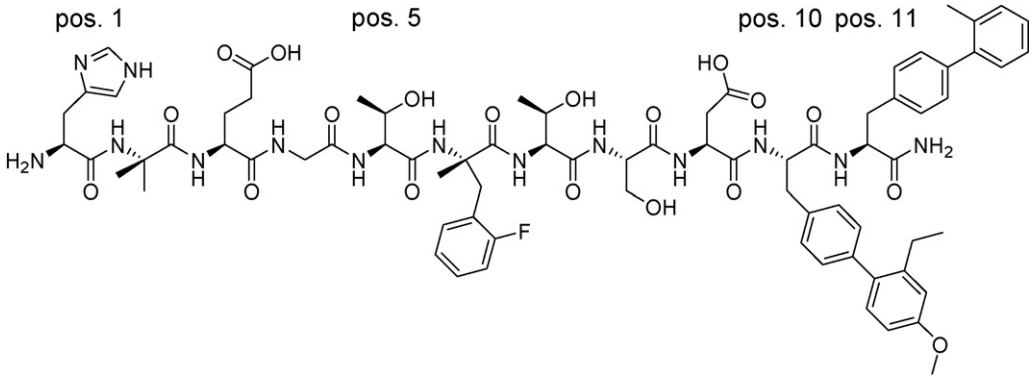


Fig. 2. Lead peptide **1**, with sequence His[1]-Aib-Glu-Gly-Thr[5]-[α-MePhe(2-F)]-Thr-Ser-Asp-Bip(2-Et,4-OMe)[10]-Bip(2-Me)[11]-NH_2.

sequence of which is shown in Fig. 1. A number of organizations are working to develop drugs which act as metabolically stabilized GLP-1R agonists [13]. One example of a potent and relatively stable GLP-1 agonist, recently approved for use with type 2 diabetes by the US Food and Drug Administration, is exenatide (marketed under the name Byetta™). Exenatide is a synthetic version of the naturally occurring peptide Exendin-4, which is found in Gila Monster lizard saliva (Fig. 1). Another peptide reported to be in clinical development by Novo Nordisk is Liraglutide (NN2211), which also has a sequence closely related to that of GLP-1 (Liraglutide = γ-L-gutamoyl(*N*-α-hexadecanoyl)-Lys[26], Arg[34]-GLP-1(7–37)) [1]. These larger peptide agonists have high binding affinities for the GLP-1 receptor (typically in the picomolar range). The primary focus of our research has also focused on finding compounds with a longer plasma half-life than GLP-1, while retaining or improving upon GLP-1's potent functional activity.

We have reported the identification of 11 amino acid peptides that are potent GLP-1 agonists when tested against human and mouse GLP-1 receptors (**1**, Fig. 2) [11]. These peptides contain four non-proteinogenic amino acids, display GLP-1-like functional agonist activity in the low picomolar range, and have greatly improved plasma half-lives over that of GLP-1 itself. The exact binding mode of these peptides to GLP-1R is currently unknown. The first nine amino acids (positions 1–9 in our series, as shown in Fig. 2) are closely related to those found in the N-terminal region of GLP-1. The two C-terminal biphenyl amino acids (positions 10 and 11 in our series) essentially replace the 22 Val[16]-Gly[37] amino acids of GLP-1. We sought to identify novel position 10 and position 11 biphenylalanine (Bip) amino acids as part of our efforts to clarify structure–activity relationships around lead peptide **1**.

We elected to examine a variety of substituted Bip amino acids at either position 10 or 11, while holding the rest of the 11mer peptide sequence constant. The "parent sequence" used for the constant part of the peptides was taken from **1**. SynPhase™ Lanterns (Mimotopes, Melbourne, Australia) were selected for use as a solid support to facilitate the generation of the libraries. The SynPhase-PS™ Lantern consists of an inert polymer base onto which a polystyrene surface has been grafted. The polystyrene surface is functionalized with a chemical linker to allow organic synthesis to occur. Each lantern used in this project incorporated a radiofrequency tag. This tag accommodated the application of deterministic "split and mix" synthesis techniques with identification and isolation of individual final products. Lanterns functionalized with the Rink amide linker, a strong acid labile linker, were selected to allow for the deprotection of the *tert*-butyloxycarbonyl (Boc) protecting group under mildly acidic conditions without cleaving the amino acid itself from the support. (Several early attempts to accomplish the Suzuki reaction with Fmoc-protected 4-iodophenylalanine on the Lantern solid support failed to yield the desired product, and resulted in loss of the Fmoc group from the amino acid.)

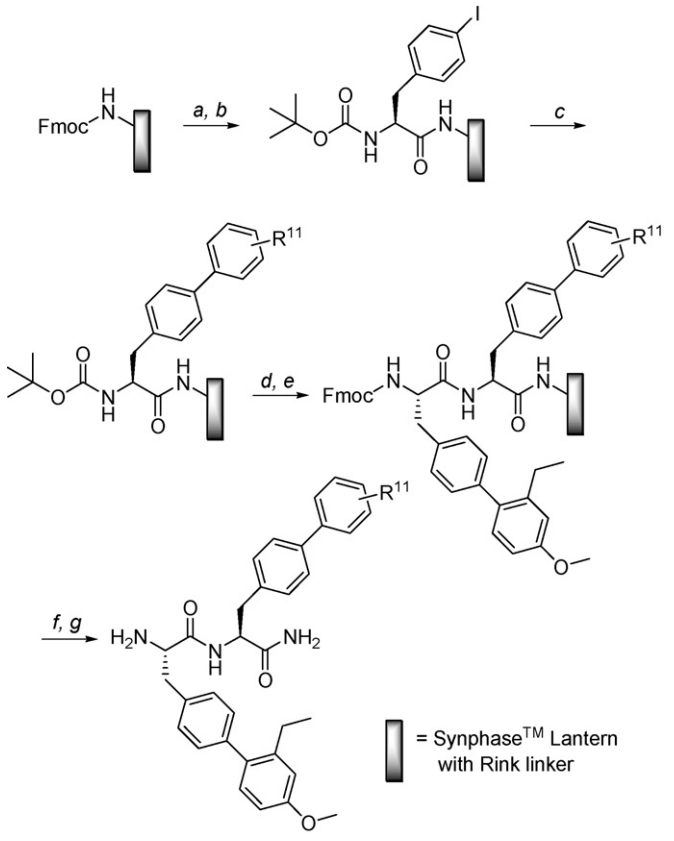
2. Methods

2.1. Abbreviations

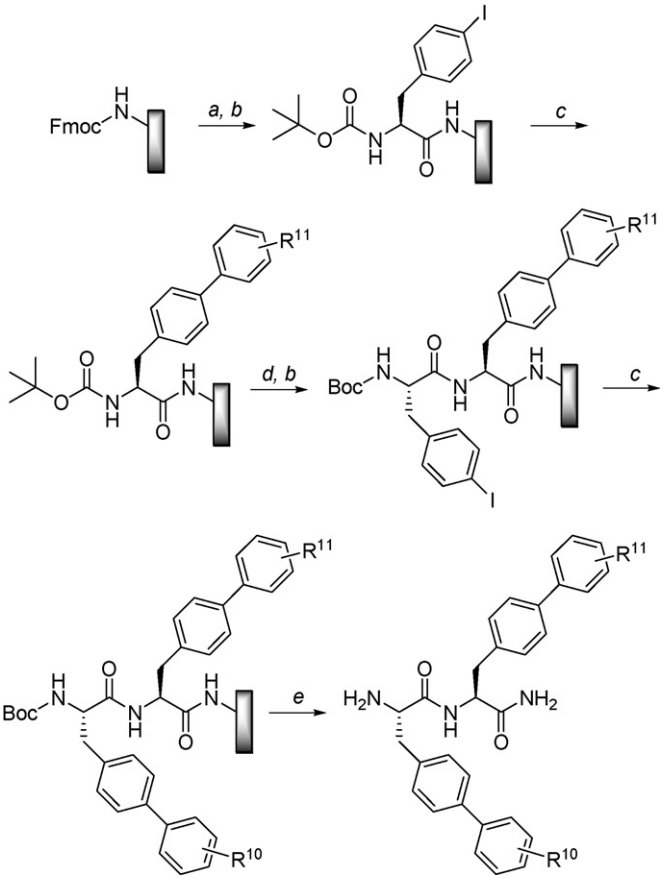
Bip = Biphenylalanine; Boc = *tert*-butoxycarbonyl; cAMP = cyclic adenosine monophosphate; DIC = diisopropylcarbodiimide; DIEA = *N*,*N*-diisopropylethylamine; DMA=*N*,*N*-dimethylacetamide; DMF= *N*,*N*-dimethylformamide; Fmoc = fluorenylmethoxycarbonyl; HOBt = 1-hydroxybenzotriazole; HPLC = high pressure liquid chromatography; K_3PO_4 = potassium phosphate; LC–MS = liquid chromatography/mass spectroscopy; MeOH = methanol; PyBop = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; $[Pd(PPh_3)_4]$ = tetrakistriphenylphosphine palladium(0); SAR = structure–activity relationship; SPA = scintillation proximity assay; TFA = trifluoroacetic acid; THF = tetrahydrofuran.

2.2. Solid phase synthesis of position 10 and 11 biphenyl amino acid dipeptides

Synthesis of diverse position 11 Bip analogues was accomplished as shown in Scheme 1. Access to a diverse set of position 10 Bips was achieved in an analogous method after first coupling the completed position 11 Bip amino acid (synthesized in bulk using standard solution-phase methods) to the Lantern. Generation of the "positional scanning" sets was accomplished as shown in Scheme 3,



Scheme 1. Reagents and conditions: (a) DMF/piperidine (8:2, excess), 1 h. (b) Boc-L-Phe(4-I)-OH (2 equiv.), DIC (2.1 equiv.), HOBt (2.1 equiv.), DIEA (4 equiv.), DMF/CH_2Cl_2 (1:1), 18 h. (c) Arylboronic acid (4 equiv.), aq. K_3PO_4 (2 M solution, 8 equiv.), $Pd[P(Ph)_3]_4$ (0.1 equiv.), DMA, 80 °C, 16 h. (d) 1N HCl in 1,4-dioxane (10 equiv.), 1 h. (e) Fmoc-Bip(2′-Et,4′-OMe)-OH (1.3 equiv.), PyBOP (1.4 equiv.), HOBt (1.4 equiv.), DIEA (4 equiv.), DMF/CH_2Cl_2 (2:1) 24–48 h. (f) DMF/piperidine (8:2), 1 h. (g) 1:1 TFA/CH_2Cl_2 (1:1), 2 h.

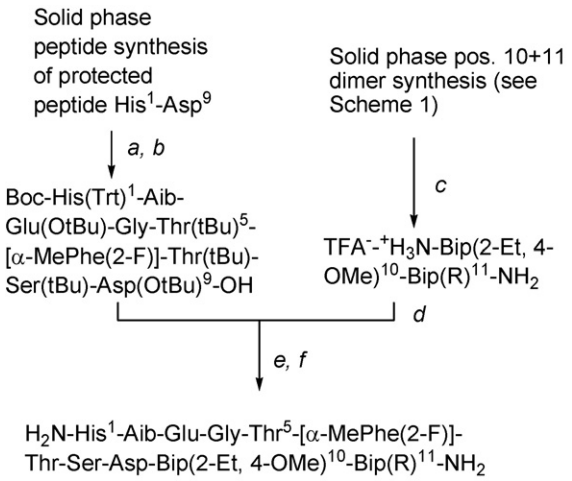


Scheme 2. Reagents and conditions: (a) DMF/piperidine (8:2, excess), 1 h. (b) Boc-L-Phe(4-I)-OH (2 equiv.), DIC (2.1 equiv.), HOBt (2.1 equiv.), DIEA (4 equiv.), DMF/CH_2Cl_2 (1:1) 18 h. (c) Arylboronic acid (4 equiv.), aq. K_3PO_4 (2 M solution, 8 equiv.), $Pd[P(Ph)_3]_4$ (0.1 equiv.), DMA, 80 °C, 16 h. (d) 1N HCl in 1,4-dioxane (10 equiv.), 1 h. (e) TFA/CH_2Cl_2 (1:1) 2 h, repeat.

using a convergent synthesis to take advantage of the fact that positions 1–9 were held constant throughout the peptides described.

The solid-phase synthetic route allowing us to access substituted Bips at positions 10 and 11 was developed based on Suzuki-Miyaura coupling chemistry [12]. For example, for position 11 Bips, Boc-L-4-iodophenylalanine was loaded onto SynPhase-PS™ Lanterns with Rink amide linker via activation with 1,3-diisopropylcarbodiimide in the presence of 1-hydroxybenzotriazole and *N*,*N*-diisopropylethylamine. The palladium-catalyzed Suzuki cross-coupling was achieved using $[Pd(PPh_3)_4]$ as catalyst, K_3PO_4 as base, at least 4 equiv. of arylboronic acid, and heating in *N*,*N*-dimethylacetamide at 80 °C overnight. We determined that the Boc-carbamate can be deprotected without significant cleavage of amino acid from the Rink linker using 1N HCl in 1,4-dioxane for 60 min [16]. The Lanterns were washed with DMF, MeOH, and CH_2Cl_2 between reactions and after deprotections (3 washes per solvent, 3–5 min per wash), as well as washing with dilute base (typically 10% Et_3N in DMF) after removal of the Boc protecting group.

The synthesis of the C-terminal dimers for the 2D combinatorial library is described in Scheme 2. The variations for both positions 10 and 11 were introduced on the SynPhase™ Lanterns via Pd-catalyzed coupling of arylboronic acids to support-bound Boc-L-4-iodophenylalanine. After cleavage of the dimers from the Lanterns, the peptides were completed via the solution-phase fragment coupling shown in Scheme 3.



Scheme 3. Convergent synthesis of 11mer peptides. Reagents and conditions: (a) standard solid-phase Fmoc-based peptide synthesis on 2-chlorotrityl chloride resin. (b) dichloromethane/acetic acid/2,2,2-trifluoroethane (8:1:1), 1 h. (c) 5:5:0.25 TFA:CH_2Cl_2:triisopropylsilane, 2 h (excess, repeat). (d) macroporous carbonate resin (3 equiv.) in THF, 2 h. (e) DIC (1.0 equiv.), HOBt (1.1 equiv.), 9:1 $CHCl_3$:DMF, 16 h. (f) 95:2.5:2.5 TFA/H_2O/triisopropylsilane (excess), 2 h.

2.3. Solution-phase 9 + 2 fragment coupling to generate desired 11mer peptides

A convergent approach to the synthesis of the full peptides was adopted, where the positions 1–9 segment of the peptide (free C-terminal carboxylic acid, side chains and N-terminal amine protected) was coupled to the positions 10 + 11 dimer in solution [2] (Scheme 3). Both the side chain-protected N-terminal 9mer and the C-terminal dimer were synthesized on solid supports (resin and Lanterns, respectively), then cleaved and purified prior to the solution-phase fragment condensation reaction. Product 11mer peptides then underwent acidic side chain deprotection and preparative purification via HPLC.

2.4. Characterization of product peptides

The products were characterized by analytical LC–MS chromatography and observation of the $M+H^+$ ion via electrospray mass spectroscopy.

Conditions for analytical LC–MS chromatography: 10–20 μL of a 1 mg/mL solution of product in 1:1 CH_3CN/H_2O was injected onto a Shimadzu/Waters LC–MS system equipped with a Phenomenex Luna C-18 4.6 mm × 50 mm column, and running a 10–90% aq. MeOH + 0.1% trifluoroacetic acid gradient over 4 min. Product purity was determined via UV detection of peaks at 220 nm, and identity was confirmed by observation of the (M+2H+)/2 ion in the mass trace.

Conditions for electrospray mass spectroscopy of product peptides: Product peptide was dissolved at 1 mg/mL in 1:1 CH_3CN/H_2O and injected onto a Finnigan SSQ7000 mass spectrometer equipped with an electrospray ionization source. Both ES^+ and ES^- spectra were obtained to confirm identity of product peptides.

2.5. GLP-1 agonist functional assay

The compounds were assayed for GLP-1 agonist functional activity as follows: a Chinese Hamster Ovary (CHO) cell line expressing human GLP-1R was utilized in primary cell based assays that measure the intracellular cAMP generated by GLP-1 analog stimulation. This scintillation proximity assay (SPA) was performed in a 96-well adherent cell format using a radioimmunoassay (RIA) based kit. Upon treatment with peptidyl agonists, the non-radioactive cAMP synthesized in the cell lysate competed with the ^{125}I-labeled cAMP tracer for the limited amount of anti-cAMP antibody, which binds to SPA beads that are covalently coated with anti-rabbit antibody, causing the light signal emitted by the SPA beads to decrease. The decrease of signal is proportional to the amount of intracellular cAMP generated by GLP-1 analog stimulation.

CHO cells stably expressing human GLP-1R were plated at 2×10^4 cells/well in sterile 96-well white clear-bottom plates and incubated overnight before assaying. On the assay day, after aspirating the growth media, the cells were treated with 50 μL of compound at varying concentrations, or buffer control in phosphate-buffered saline (PBS) without $MgCl_2$ and $CaCl_2$, with 0.1 mM 3-isobutyl-1-methylxanthine and 0.05% bovine serum albumin for 20 min at room temperature. The solution was then aspirated, and 50 μL lysis buffer was added immediately, followed by addition of 70 μL of assay buffer containing ^{125}I-labeled cAMP tracer, rabbit anti-cAMP antibody and SPA beads that were covalently coated with anti-rabbit antibody (provided in the Amersham cAMP SPA assay kit). The plates were incubated at room temperature for 12 h before counting on a TriLux Microbeta reader. The cAMP standard curve with 12 concentrations was established independently using known amounts of non-radioactive cAMP. The amount of cAMP from treated cells was converted to picomoles (pmol) of cAMP by interpolating from the cAMP standard curve. The data were normalized and plotted as the percentage of the response induced by 10 nM GLP-1. The EC_{50} value of compounds was defined as the concentration of compound that stimulated 50% of maximal cAMP synthesis by 10 nM GLP-1 in CHO cells as the positive control. The values shown in the tables are the means of four experiments, with assay-to-assay variability typically within ±2-fold based on results with a control compound.

Table 1
In vitro GLP-1 agonist functional activity for human receptor, for peptides **1**–**47** containing reported 11mer sequence with Aib in position 2 and Bip(2′-Me) at position 11. All position 10 amino acids were of the L-configuration.

Compound	Position 10 second aryl group	Human GLP-1 cAMP EC_{50}, pM[a]
1	2-Ethyl-4-methoxyphenyl	146
2	2-Methoxypyridin-3-yl	135
3	2-Ethylphenyl	157
4	Quinolin-5-yl	184
5	2-Methyl-4-methoxyphenyl	281
6	3,5-Dimethylisoxazol-4-yl	303
7	*o*-Tolyl	336
8	2,3-Dimethylphenyl	338
9	2-Methyl-4-fluorophenyl	351
10	Isoquinolin-4-yl	388
11	3,5-Dimethoxyphenyl	388
12	2-Chlorophenyl	392
13	Quinolin-6-yl	410
14	2-Methyl-5-fluorophenyl	475
15	2,3-Dihydrobenzofuran-5-yl	553
16	2-Methyl-4-chlorophenyl	560
17	2-Fluoro-3-methoxyphenyl	589
18	2-Methoxy-5-isopropylphenyl	624
19	3-Fluoro-4-methoxyphenyl	631
20	Naphthalen-1-yl	676
21	Pyridin-4-yl	689
22	*m*-Tolyl	729
23	2-Fluoro-6-methoxyphenyl	801
24	2,4-Dichlorophenyl	876
25	3-Isopropylphenyl	1090
26	6-Methoxynaphthalen-2-yl	1190
27	4-Methoxymethylphenyl	1230
28	3-Methoxyphenyl	1270
29	4-Methoxypyridin-3-yl	1300
30	4-Phenoxyphenyl	1330
31	Benzo[*d*][1,3]dioxol-5-yl	1360
32	6-Ethoxynaphthalen-2-yl	1420
33	3,4-Dimethylphenyl	1440
34	Quinolin-3-yl	1500
35	4-Chlorophenyl	1510
36	3,5-Dimethylphenyl	1880
37	3-Methyl-4-methoxyphenyl	1890
38	3-Chlorophenyl	2100
39	4-Methylnaphthalen-1-yl	2120
40	2,3-Difluorophenyl	2530
41	4-Chloro-4-fluorophenyl	3840
42	4-Isopropylphenyl	3970
43	3,5-Difluorophenyl	4190
44	3-Methyl-4-chlorophenyl	4610
45	3,5-Dichlorophenyl	11,700
46	2-Phenoxyphenyl	15,300
47	Quinolin-8-yl	30,400

[a] Values are means of four experiments, with assay-to-assay variability typically within ±2-fold based on results with a control compound.

2.6. Pharmacokinetics in dogs

The pharmacokinetic parameters of peptide **49** were determined in male beagle dogs. Following an overnight fast, each animal received peptide **49** by subcutaneous injection given near the shoulder blades (67 μg/kg). The dosing vehicle was propylene glycol:phosphate buffer (50:50). Serial blood samples were collected in EDTA-containing microcentrifuge tubes at predose, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 24, and 30 h post-dose after subcutaneous administration. Approximately 0.3 mL of blood was collected at

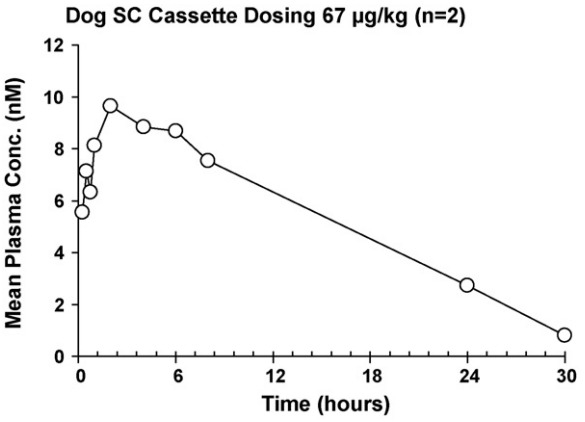


Fig. 3. Plasma concentration vs. time profiles of peptide **49** in the dog.

each time point. Blood samples were immediately centrifuged at 4 °C. The obtained plasma was frozen with dry ice and stored at −20 °C. Plasma drug levels were determined using the LC–MS/MS assay.

3. Results

Previous research within our group suggested that Bip(2′-Et,4′-OMe) (from lead peptide **1**) was a preferred residue at position 10 [11]. We wished to exhaustively examine the SAR at position 10 and explore this hypothesis. Therefore, a wide variety of arylboronic acids were used in this first "positional scanning" library. The first set of compounds explored a variety of Bip analogs at position 10, while holding positions 1–9 constant (as shown in Fig. 2), and position 11 constant as Bip(2′-Me). The results for these peptides in an agonist assay using human GLP-1 receptor are shown in Table 1.

We next proceeded to explore position 11. This position was moderately explored in previous research within our group [7,11], so we selected a limited set of arylboronic acids to complete our SAR analysis. Functional activity for the resulting 15 peptides is shown in Table 2. Both these results and those obtained previously suggest that position 11 is considerably more "permissive" than position 10 regarding substitution on the distal ring of the Bip.

Table 2
In vitro GLP-1 agonist functional activity for human receptor, for peptides **48**–**62** containing reported 11mer sequence with Aib in position 2 and Bip(2′-Et,4′-OMe) at position 10. All position 11 amino acids were of the L-configuration.

Compound	Position 11 second aryl group	Human GLP-1 cAMP EC_{50}, pM[a]
48	3,5-Dimethylphenyl	78
49	2-Chlorophenyl	88
50	Quinolin-5-yl	89
51	3,4-Dimethoxyphenyl	100
52	3-Chlorophenyl	103
53	3-Methoxyphenyl	108
54	Pyridin-3-yl	118
55	3-Chloro-4-fluorophenyl	123
56	4-Methoxypryidin-3-yl	144
57	Quinolin-6-yl	160
58	Quinolin-8-yl	220
59	3-Isopropylphenyl	228
60	4-Isopropylphenyl	241
61	Quinolin-3-yl	244
62	2,4-Dichlorophenyl	249

[a] Values are means of four experiments, with assay-to-assay variability typically within ±2-fold based on results with a control compound.

Table 3
Pharmacokinetic profile of peptide **49**.

Species	SC dose	T_{max} (h)	C_{max} (nM)	AUC (nM h)	SC $t_{1/2}$ (h)
Dog	67 µg/kg	5	11	168	8

The pharmacokinetic parameters of peptide **49** in male beagle dogs ($n = 2$), following subcutaneous (SC) administration are summarized in Table 3. The plasma concentration–time profiles are presented in Fig. 3. The time to reach peak concentrations (T_{max}) after a subcutaneous dose of 67 µg/kg occurred at 5 h. The maximum plasma concentration (C_{max}) after subcutaneous administration was 11 nM. The estimated apparent elimination half-life was 8 h. These results encouraged us to pursue our two-dimensional (2D) matrix library, which is described below.

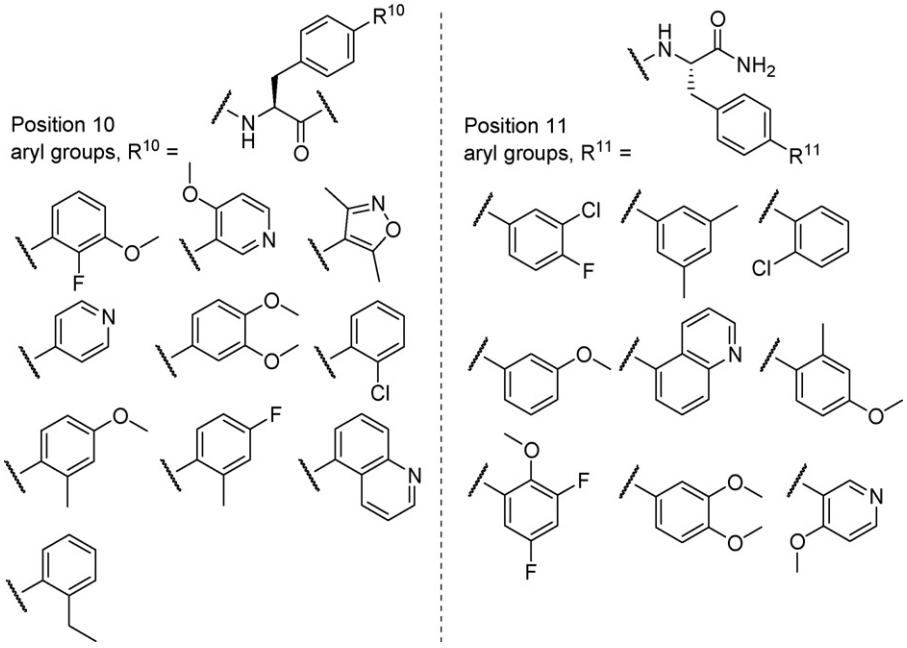


Fig. 4. Aryl groups used at positions 10 and 11 in the 10 × 9 matrix library.

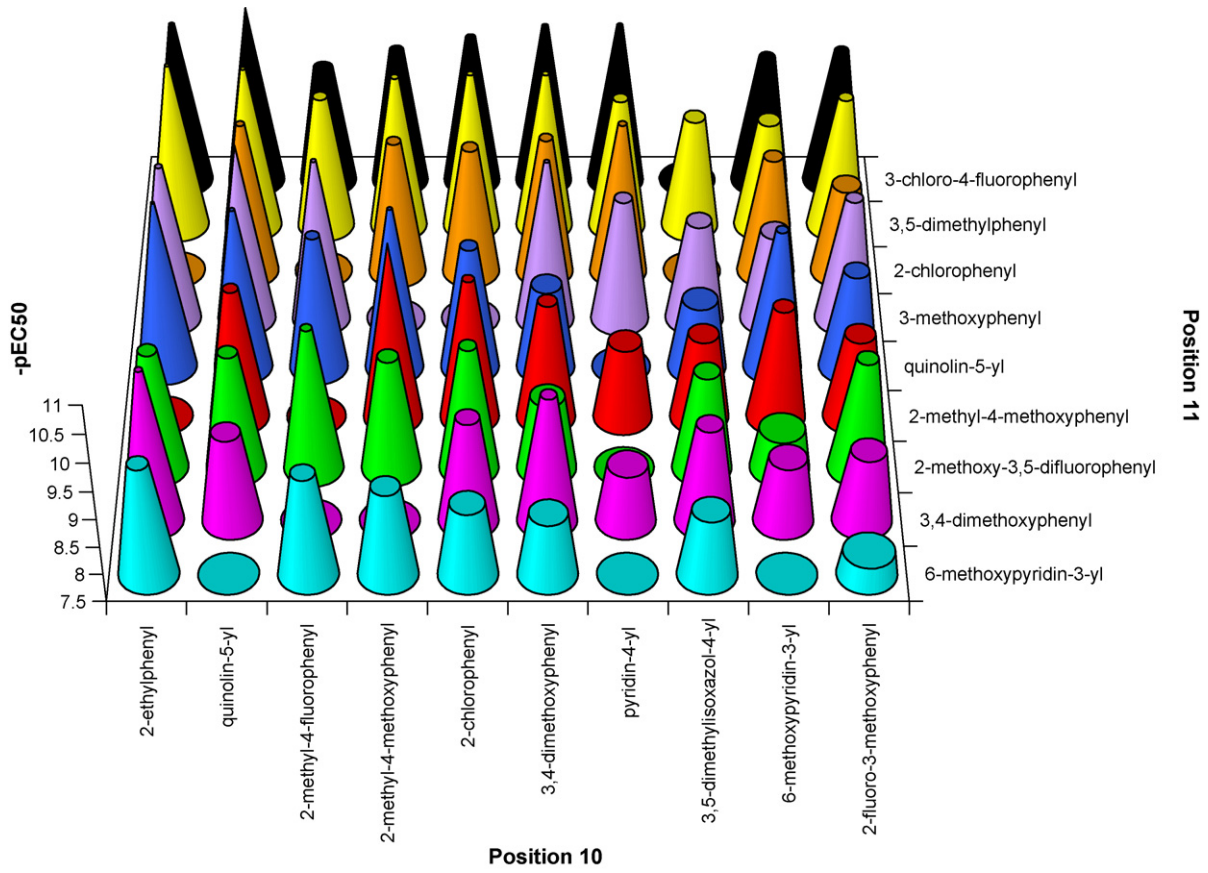


Fig. 5. Graph of matrix results, displaying – pEC_{50} activity on the *z*-axis (i.e. taller cones indicate more potent GLP-1 agonists), position 10 amino acid on the *x*-axis, and position 11 amino acid on the *y*-axis. "Not obtained" values are at the base of the graph.

The aryl groups for the 90 compound 2D library were chosen based either on the GLP-1R agonist potency displayed in the positional scan libraries, or on similarity to the corresponding side chain from the lead compound **1**. An effort was made to include side chains with a range of properties, e.g. electron-withdrawing substituents, electron-donating substituents, heteroaromatic, etc. The Bip side chains ultimately incorporated into the library are shown in Fig. 4.

Upon completion of the synthesis and purification of the 2D library, we were able to obtain 75 out of 90 anticipated peptides in sufficient yield and purity for inclusion in our GLP-1 agonist *in vitro* assay. Results for the peptides in the GLP-1 functional assay against human receptor are shown in Table 4 , and are depicted graphically in Fig. 5 in Section 4.

4. Discussion

From the positional scanning library exploring position 10 it's perhaps not surprising that compounds **3** and **5**, containing Bips that are close analogs to Bip(2′-Et,4′-OMe) (from lead compound **1**), display good GLP-1 agonist activity. Several interesting and unexpected results were also observed; the good activities of peptides **4**, **6**, **10**, and **13** (products of quinoline or isoquinoline boronic acids) suggested the possibility that larger Bip analogs at position 10 could promote binding to the GLP-1 receptor. The position 11 scanning library was less definitive, with a number of side chains displaying good agonist potency.

One of the more potent compounds from the second library, **49**, was examined via cassette dosing in dogs to evaluate the pharmacokinetic properties of this series (Table 3). We were pleased to observe that the peptide had a half-life of 8 h after SC administration, much longer than the less than 5 min typically seen with the native GLP-1 peptide. The long apparent half-life of the 11mer suggests that potent agonists in this class could have potential as practical therapeutic agents for the treatment of type 2 diabetes.

We ultimately wished to identify new, more potent peptides with novel Bip analogs at positions 10 and 11. Much of our previous research, including the two initial positional scanning libraries detailed in Tables 1 and 2, focused on modifying a single amino acid while holding the rest of the peptide constant [7,11]. While this approach is ideal for side chains that bind to the receptor independently, it does not allow for the exploration of possible cooperative effects. We elected to synthesize a 90 compound two-dimensional (10×9) library focusing on positions 10 and 11 with two key purposes in mind: (1) identification of any cooperative effects resulting in highly potent agonists; (2) production of a number of potent agonists that could be subsequently pursued in more advanced (e.g. *in vivo*) studies.

Several trends in activity are apparent after examination of the data in Table 4 and Fig. 5 (higher cones equal more potent agonists). At position 10, a substituent at the *ortho* position of the second aromatic ring of the biphenyl system promotes agonist activity, as seen with the 2-ethylphenyl, 2-methyl-4-fluorophenyl, 2-methyl-4-methoxyphenyl, and 2-chlorophenyl Bips. The activity of the quinolin-5-yl substituent at position 10 observed with **4** in the first positional scan library was validated by the potent activities of compounds **135**–**139**, all of which also contain the quinolin-5-yl substituent. The potency of the 3,4-dimethoxyphenyl substituent at position 10 was likewise affirmed by the activity of compounds **99**, **100**, and **102**. Overall, side chains bearing close

Table 4
In vitro GLP-1 agonist functional activity for human receptor, for peptides **63**–**152** obtained from 10 × 9 matrix library, containing variation at positions 10 and 11.

Compound	Position 10 second aryl group	Position 11 second aryl group	Human GLP-1 cAMP EC_{50}, pM[a]
63	2-Fluoro-3-methoxyphenyl	3-Chloro-4-fluorophenyl	148
64	2-Fluoro-3-methoxyphenyl	3,5-Dimethylphenyl	159
65	2-Fluoro-3-methoxyphenyl	2-Chlorophenyl	851
66	2-Fluoro-3-methoxyphenyl	3-Methoxyphenyl	214
67	2-Fluoro-3-methoxyphenyl	Quinolin-5-yl	574
68	2-Fluoro-3-methoxyphenyl	2-Methyl-4-methoxyphenyl	994
69	2-Fluoro-3-methoxyphenyl	2-Methoxy-3,5-difluorophenyl	331
70	2-Fluoro-3-methoxyphenyl	3,4-Dimethoxyphenyl	1670
71	2-Fluoro-3-methoxyphenyl	6-Methoxypyridin-3-yl	11,700
72	6-Methoxypyridin-3-yl	3-Chloro-4-fluorophenyl	190
73	6-Methoxypyridin-3-yl	3,5-Dimethylphenyl	402
74	6-Methoxypyridin-3-yl	2-Chlorophenyl	270
75	6-Methoxypyridin-3-yl	3-Methoxyphenyl	827
76	6-Methoxypyridin-3-yl	Quinolin-5-yl	110
77	6-Methoxypyridin-3-yl	2-Methyl-4-methoxyphenyl	322
78	6-Methoxypyridin-3-yl	2-Methoxy-3,5-difluorophenyl	6020
79	6-Methoxypyridin-3-yl	3,4-Dimethoxyphenyl	2240
80	6-Methoxypyridin-3-yl	6-Methoxypyridin-3-yl	Not obtained
81	3,5-Dimethylisoxazol-4-yl	3-Chloro-4-fluorophenyl	Not obtained
82	3,5-Dimethylisoxazol-4-yl	3,5-Dimethylphenyl	352
83	3,5-Dimethylisoxazol-4-yl	2-Chlorophenyl	Not obtained
84	3,5-Dimethylisoxazol-4-yl	3-Methoxyphenyl	542
85	3,5-Dimethylisoxazol-4-yl	Quinolin-5-yl	1870
86	3,5-Dimethylisoxazol-4-yl	2-Methyl-4-methoxyphenyl	991
87	3,5-Dimethylisoxazol-4-yl	2-Methoxy-3,5-difluorophenyl	557
88	3,5-Dimethylisoxazol-4-yl	3,4-Dimethoxyphenyl	562
89	3,5-Dimethylisoxazol-4-yl	6-Methoxypyridin-3-yl	2070
90	Pyridin-4-yl	3-Chloro-4-fluorophenyl	54
91	Pyridin-4-yl	3,5-Dimethylphenyl	166
92	Pyridin-4-yl	2-Chlorophenyl	52
93	Pyridin-4-yl	3-Methoxyphenyl	216
94	Pyridin-4-yl	Quinolin-5-yl	Not obtained
95	Pyridin-4-yl	2-Methyl-4-methoxyphenyl	1350
96	Pyridin-4-yl	2-Methoxy-3,5-difluorophenyl	Not obtained
97	Pyridin-4-yl	3,4-Dimethoxyphenyl	2990
98	Pyridin-4-yl	6-Methoxypyridin-3-yl	Not obtained
99	3,4-Dimethoxyphenyl	3-Chloro-4-fluorophenyl	55
100	3,4-Dimethoxyphenyl	3,5-Dimethylphenyl	67
101	3,4-Dimethoxyphenyl	2-Chlorophenyl	128
102	3,4-Dimethoxyphenyl	3-Methoxyphenyl	50
103	3,4-Dimethoxyphenyl	Quinolin-5-yl	1000
104	3,4-Dimethoxyphenyl	2-Methyl-4-methoxyphenyl	259
105	3,4-Dimethoxyphenyl	2-Methoxy-3,5-difluorophenyl	1550
106	3,4-Dimethoxyphenyl	3,4-Dimethoxyphenyl	170
107	3,4-Dimethoxyphenyl	6-Methoxypyridin-3-yl	2360
108	2-Chlorophenyl	3-Chloro-4-fluorophenyl	86
109	2-Chlorophenyl	3,5-Dimethylphenyl	62
110	2-Chlorophenyl	2-Chlorophenyl	187
111	2-Chlorophenyl	3-Methoxyphenyl	Not obtained
112	2-Chlorophenyl	Quinolin-5-yl	208
113	2-Chlorophenyl	2-Methyl-4-methoxyphenyl	106
114	2-Chlorophenyl	2-Methoxy-3,5-difluorophenyl	198
115	2-Chlorophenyl	3,4-Dimethoxyphenyl	427
116	2-Chlorophenyl	6-Methoxypyridin-3-yl	1570
117	2-Methyl-4-methoxyphenyl	3-Chloro-4-fluorophenyl	143
118	2-Methyl-4-methoxyphenyl	3,5-Dimethylphenyl	97
119	2-Methyl-4-methoxyphenyl	2-Chlorophenyl	146
120	2-Methyl-4-methoxyphenyl	3-Methoxyphenyl	Not obtained
121	2-Methyl-4-methoxyphenyl	Quinolin-5-yl	48
122	2-Methyl-4-methoxyphenyl	2-Methyl-4-methoxyphenyl	63
123	2-Methyl-4-methoxyphenyl	2-Methoxy-3,5-difluorophenyl	304
124	2-Methyl-4-methoxyphenyl	3,4-Dimethoxyphenyl	Not obtained
125	2-Methyl-4-methoxyphenyl	6-Methoxypyridin-3-yl	446
126	2-Methyl-4-fluorophenyl	3-Chloro-4-fluorophenyl	294
127	2-Methyl-4-fluorophenyl	3,5-Dimethylphenyl	154
128	2-Methyl-4-fluorophenyl	2-Chlorophenyl	Not obtained
129	2-Methyl-4-fluorophenyl	3-Methoxyphenyl	49
130	2-Methyl-4-fluorophenyl	Quinolin-5-yl	138
131	2-Methyl-4-fluorophenyl	2-Methyl-4-methoxyphenyl	Not obtained
132	2-Methyl-4-fluorophenyl	2-Methoxy-3,5-difluorophenyl	97
133	2-Methyl-4-fluorophenyl	3,4-Dimethoxyphenyl	167
134	2-Methyl-4-fluorophenyl	6-Methoxypyridin-3-yl	446
135	Quinolin-5-yl	3-Chloro-4-fluorophenyl	28
136	Quinolin-5-yl	3,5-Dimethylphenyl	72
137	Quinolin-5-yl	2-Chlorophenyl	73

Table 4 (*Continued*)

Compound	Position 10 second aryl group	Position 11 second aryl group	Human GLP-1 cAMP EC_{50}, pM[a]
138	Quinolin-5-yl	3-Methoxyphenyl	27
139	Quinolin-5-yl	Quinolin-5-yl	50
140	Quinolin-5-yl	2-Methyl-4-methoxyphenyl	157
141	Quinolin-5-yl	2-Methoxy-3,5-difluorophenyl	260
142	Quinolin-5-yl	3,4-Dimethoxyphenyl	785
143	Quinolin-5-yl	6-Methoxypyridin-3-yl	Not obtained
144	2-Ethylphenyl	3-Chloro-4-fluorophenyl	52
145	2-Ethylphenyl	3,5-Dimethylphenyl	45
146	2-Ethylphenyl	2-Chlorophenyl	Not obtained
147	2-Ethylphenyl	3-Methoxyphenyl	61
148	2-Ethylphenyl	Quinolin-5-yl	38
149	2-Ethylphenyl	2-Methyl-4-methoxyphenyl	Not obtained
150	2-Ethylphenyl	2-Methoxy-3,5-difluorophenyl	240
151	2-Ethylphenyl	3,4-Dimethoxyphenyl	63
152	2-Ethylphenyl	6-Methoxypyridin-3-yl	305

[a] Values are means of four experiments, with assay-to-assay variability typically within ±2-fold based on results with a control compound.

resemblance to the parent position 10 side chain in **1** are potent, and the activity of several more novel position 10 side chains was confirmed.

A wider variety of side chains at position 11 displayed high levels of activity, as expected from our previous results. Substitution is tolerated at nearly every position on the second aryl ring, though less so at the *para* position: peptides with the 2-methyl-4-methoxyphenyl and 3,4-dimethoxyphenyl side chains at position 11 tend towards lower activity versus peptides with substituted side chains at position 11. Given the *ortho*-substituted position 11 side chain in **1**, we were interested to observe that many of the more potent side chains from the 2D library lacked an *ortho*-substituent at this position (e.g. representative peptides **99**, **102**, **109**, **135**, **138**, and **145**).

Several side chains included in the 2D library are generally unfavorable in terms of conferring agonist activity. These include 3,5-dimethylisoxazol-4-yl, 5-methoxypyridin-3-yl, and 2-fluoro-3-methoxyphenyl at position 10, and 3,4-dimethoxyphenyl and 6-methoxypyridin-3-yl at position 11.

5. Conclusions

In conclusion, a series of short (11 amino acid) GLP-1 receptor agonist peptides were optimized for agonist potency against the human GLP-1 receptor using iterative parallel synthesis. Through the use of two positional scanning libraries, followed by a two-dimensional library focused primarily on highly active side chains, we were able to identify several new position 10 and position 11 Bip analogues which result in peptides with high agonist potency. One analog displayed an apparent half-life of 8 h following subcutaneous administration in a dog PK study. The final, 2D (10×9) library that was focused on optimization of compound potency was also highly successful, with 22 compounds (29% of 76 compounds tested) having agonist activity of less than 100 pM. By extensively exploring SAR at the two C-terminal positions via positional scanning libraries, then using that data to design a final 2D library, we were able to rapidly explore SAR, identify new biphenylalanine analogs at both positions 10 and 11, and generate a number of highly active agonists for potential use in follow-up *in vitro* and *in vivo* studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2010.04.013.

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