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Design, synthesis and in vitro characterization of Glucagon-Like Peptide-1 derivatives for pancreatic beta cell imaging by SPECT

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ABSTRACT

Novel Glucagon-Like Peptide-1 (GLP-1) derivatives containing the metal chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and naturally occurring Indium ($^{113/115}$In) were prepared using solid-phase Fmoc methods. All synthesized peptides contained D-Ala-8, a modification known to improve resistance towards degradation by dipeptidyl peptidase-IV. The effect of increased distance between DOTA and the peptide chain was investigated using an (aminoethyl) ethoxy acetyl linker, in order to reduce steric effects imposed by DOTA. Placement of linker and DOTA moieties were also varied within the GLP-1 sequence to test for optimal metal-complex location. The binding affinity of the peptide derivatives was determined in vitro with Chinese hamster ovary cells stably transfected with a human GLP-1 receptor (CHO/GLP-1R) cell line and was shown to be in the nM range. Gamma camera imaging of an insulinoma cell line was carried out using ^{111}In-labeled peptides. Our results suggest that the prepared GLP-1 derivatives are suitable imaging probes for studying pancreatic islet function in vivo.

1. Introduction

Glucagon-Like Peptide-1 (GLP-1) is a peptide hormone produced by intestinal L-cells in response to nutrient ingestion.[1] It is produced through the post-translational processing of its larger precursor, proglucagon, by the enzyme prohormone convertase 1/3 and exists largely as the C-terminally amidated form, GLP-1 (7–36).[2] As GLP-1 binds to its receptor on the pancreatic beta cell, signaling events trigger the release of insulin in a glucose-dependent manner known as the 'incretin effect'.[3] GLP-1 receptor (GLP-1R) signaling events also lead to the enhancement of beta cell survival through stimulation of beta cell growth and differentiation, which plays a role in regeneration of beta cell mass.[4] More importantly the use of GLP-1 based treatments lead to a reduction in blood glucose without body weight gain or hypoglycemia typically observed with other treatments.[5] In some cases, treatments by GLP-1 derivatives have even resulted in weight loss.[6] Therefore, GLP-1R agonists are a viable and powerful therapy in the treatment of Type 2 diabetes.

The structure of the GLP-1R classifies it as part of the Family B (II) Glucagon-Secretin G Protein-Coupled Receptor (GPCR) super family. The structural characteristics of the receptors in this family include a long extracellular N-terminal domain, disulfide bridged cysteine residues in the extracellular domains, and several glycosylation sites.[7,8] Structure-function studies of the GLP-1R have shown that the helical region of GLP-1 interacts with the extracellular N-terminal domain of the GLP-1R, while the N-terminal eight amino acids of GLP-1 interact with residues in the extracellular regions and transmembrane helices of the receptor.[9] More recently, a crystal structure confirming peptide-GLP-1R interactions was reported.[10] Such a complex receptor/ligand interaction demands consideration of only a limited number of sites on the ligand that can be modified for the generation of an imaging probe with GLP-1 as a targeting component.

Since the structural and biochemical characteristics of the interaction between GLP-1 and its receptor have been determined, GLP-1 may be a suitable peptide with which to develop peptide-based imaging probes for the detection and monitoring of pancreatic beta cell mass in vivo. One limiting factor is the short biological half-life of GLP-1 (1–2 min) due to rapid enzymatic degradation,[11,12] as the alanine residue at position 8 is a cleavage site for the plasma protease dipeptidyl peptidase-IV (DPP-IV). Thus, in order to develop

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GLP-1-based diagnostics, it is important to increase the biological half-life of the parent peptide, which can be done through modification of ^{8}Ala to ^{8}D-Ala.[13,14] Alternatively, acylation with myristic acid can be employed to increase plasma stability to 13 h for therapeutic applications.[5] An alternate approach can be the use of exendin-4, a 39-amino acid peptide originally isolated from *Heloderma suspectum*,[15] which is also a GLP-1R agonist with a plasma half-life of approximately 26 min, largely due to the substitution of a glycine residue for alanine at position 8.

Until now, radioiodination has been the only method reported for GLP-1 radiolabeling. However, iodinated peptides, such as [^{123}I]GLP-1 for detection of insulinomas, have been reported to be unsuitable as a result of the instability of the radioiodine label in vivo.[16] Radiometal conjugates, on the other hand, have been shown to exhibit higher in vivo stabilities when compared to their radioiodinated analogues.[16,17] In this study, the choice of using a metal chelator over radioiodination of GLP-1 analogues was established based on the structural stability of reported compounds.

One class of chelators commonly used for the radiolabeling of peptides are cyclen derivatives, such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA),[18–20] which are inert and thermodynamically stable, making them good candidates for radiolabeling of peptides and antibodies.[20,21] Examples of such studies include DOTA-labeled somatostatin analogues (eg., DOTA-NOC (1-Nal3-octreotide),[22] and RGD peptides.[23] A review by De León-Rodríguez and Kovacs was recently published on DOTA-peptide conjugates and their applications.[20] To date, DOTA has not been employed as a chelator for the radiolabeling of GLP-1. The conjugation of DOTA to a peptide using solid-phase chemistry, the most suitable synthetic approach for peptides, requires the employed ligands to be compatible with the organic solvents commonly used in solid-phase peptide synthesis (SPPS). The commercially available tris-*t*Bu-DOTA (Fig. 1), satisfies these requirements and can be conjugated, via an unprotected carboxylic acid, to free amino groups using tetramethyluronium coupling agents such as HBTU (*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate) or HATU (2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium). This, however, requires the use of the proper orthogonal protecting groups for all amine containing amino acids in the peptide.

The metals gallium and indium have several medically useful radioisotopes that can be used for diagnostic imaging, the most widely used of which are Ga-67 and In-111. Recently, the use of Ga-68 for positron diagnostic imaging has also gained significant interest.[24] While both of these metals are able to undergo stable coordination to cyclen chelators such as DOTA, in this study we utilized In-111 as a suitable and widely available radioisotope for non-invasive imaging. Optimal In-labeling is achieved at slightly acidic pH levels (pH 4–5) when it exists as a hexaaquo ion, while it undergoes extensive hydrolysis below pH 3.4, and forms soluble hydroxides under basic conditions.[25]

Considering that GLP-1 receptors are expressed on beta cells in high density, we propose that the use of radiolabeled GLP-1, in conjunction with the non-invasive imaging technique single photon emission computed tomography (SPECT), can provide a method of studying beta cell mass in vivo. This hypothesis is supported by a recent report on the generation of the probe [^{40}Lys(Ahx-DTPA-^{111}In)NH$_2$]exendin-4, which was shown to target GLP-1R for the molecular imaging of insulinomas in transgenic Rip1Tag2 mice.[17,26,27] Here, we report the development of new GLP-1 based metal-conjugated peptides, with increased structural stability and optimized binding affinity for beta cell imaging.

2. Results and discussion

2.1. Design of GLP-1 analogues

The main objective of this study was the development of novel GLP-1 analogues with enhanced structural stability and strong GLP-1R binding for the imaging of pancreatic islets in vivo. The primary difficulty in attaching DOTA to GLP-1 is interference with peptide-receptor binding caused by the addition of a metal-complex to the peptide structure. Therefore, an integral part of this research project was the determination of optimal attachment sites for the metal chelator in order to circumvent negative impacts on the binding affinity of the peptide. Figure 2 illustrates one of the peptide derivatives before labeling. Improvement in structural stability of the developed GLP-1 analogues was achieved by substitution of L-Ala at position 8 with D-Ala, which is a modification previously reported to increase the resistance of this peptide to degradation by DPP-IV.[13] Other substitutions at this position, namely ^{8}Aib, ^{8}Gly, ^{8}Ser, and ^{8}Thr, have also been reported to increase the structural stability of GLP-1; however, the latter three substitutions have a negative impact on the binding affinity and potency of the peptide as an endocrine hormone.[13]

A report by Lee et al. showed that covalent coupling of PEG (polyethylene glycol) to specific sites on GLP-1 may improve the overall therapeutic effect of the peptide while maintaining its biological activity.[28] For instance, PEGylated GLP-1 conjugates, PEG$_{2K}$-N^{ter}-GLP-1 and PEG$_{2K}$-$^{26/34}$Lys-GLP-1, were prepared and analyzed in vitro. The latter was a mixture of derivatized peptide at positions 26 and 34 which could not be separated. The pharmacokinetic profile of each peptide was studied in vivo, with the finding that while PEGylation at ^{34}Lys did not increase the biological half-life of the peptide, it did increase its potency in vivo.[28] Another report by Madsen et al. explored the structure–activity relationship of liraglutide analogues, coupled to fatty acids, with respect to potency on cloned human GLP-1R. The authors noted that in most cases, long and bulky fatty acids decreased the potency of the peptide.[29] Knudsen et al. reported that GLP-1 can be derivatized at the C-terminal part of the peptide, with both short/long fatty acids and amino acid derived spacers, and still maintain its potency.[30] There have also been reports on albumin conjugated GLP-1 analogues with enhanced stability towards enzymatic degradation.[31]

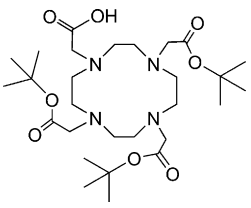


Figure 1. Structure of tris-*t*Bu-DOTA.

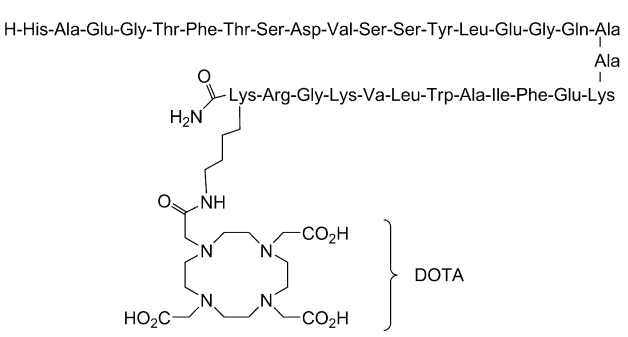


Figure 2. Structure of ^{37}Lys-DOTA-GLP-1.

In order to reduce any steric effects imposed by the macrocyclic DOTA ring on the binding site of the peptide derivatives, a short polyethyleneglycol chain, 2-[2-(2-aminoethyl)ethoxy]acetic acid (AEEA), was employed as a spacer between the main peptide chain and DOTA. Considering that this spacer was to be used at varying sites on the peptide chain, a relatively short PEG was selected in order to minimize any negative impacts on the potency or binding affinity of the peptide as shown by previously published reports discussed above. The AEEA spacer was synthesized in our laboratory according to a published procedure.[32]

There have been a number of reports that have identified residues within the sequence of GLP-1 that are critical for optimal binding and proper biological functionality. Deletion of the N-terminal eight amino acids, or substitution of residues within the N-terminal 10 amino acids, resulted in a dramatic reduction in receptor affinity.[9,33] Alanine scanning experiments have shown that amino acids in positions 7, 10, 12, 13 and 15 were directly involved in receptor binding and activation, whereas those in positions 28 and 29 maintained the secondary structure of the peptide necessary for receptor recognition.[34] These studies therefore highlight the importance of the N-terminal region of GLP-1 in binding to and activating its receptor. Leger et al. synthesized a number of GLP-1 HSA (human serum albumin) analogues and subsequently tested the stabilizing effect of bioconjugation in the presence of DPP-IV, as well as receptor binding and activation.[35] From this study, they concluded that the C-terminus is the best point of modification both in terms of stability, especially with D-Ala at position 8, and biological activity.[35] Figure 3 summarizes the results that have been reported on structure–activity relationships of GLP-1.[36]

According to the reported Ala scans, structural modification at positions 14, 16–18, 20, 22, 27 and 30–35 may be possible without having a major impact on the binding affinity of the peptide. The HSA study showed that the only site suitable for side group modification with bulky ligands is position 37 (C-terminus).[35] However, a report on GLP-1 PEGylation,[28] indicated that positions 26 and 34 could also be functionalized with bulky ligands without a major impact on binding affinity of the peptide. Therefore, based on the structure–activity relationships reported to date along with the reported modifications discussed above, we selected positions 22, 26, 34 and 37 as sites for the attachment of DOTA to GLP-1. To the best of our knowledge, substitution of large ligands at position 22 has not been previously reported. The modification of the peptide at positions 22 and 37 required substitution of Gly (originally at this site), with Lys, whereas those at positions 26 and 34 already contained a Lys residue.

2.2. Synthesis of Indium-GLP-1 analogues

GLP-1 analogues were synthesized using standard Fmoc-SPPS methods with an automated multi-well synthesizer. A representative synthetic route is described in Scheme 1, illustrating the preparation of ^{37}Lys-DOTA-GLP-1(7–37) and the related analogue containing a spacer between the peptide and the DOTA, ^{37}Lys-AEEA-DOTA-GLP-1(7–37). *N*-Fmoc removal was achieved using 20% v/v piperidine in DMF with the Kaiser test being used to identify the presence of free primary amino groups, hence determining the proper duration for Fmoc deprotection. Amino acids with reactive side chains were protected with acid labile orthogonal protecting groups such as O*t*Bu, *t*Bu, Trt and Pbf. The site of DOTA attachment, the ε-amine of a lysine side chain positioned at amino acid 22, 26, 34, or 37, was protected with the temporary protecting group 4-methyltrityl (Mtt), which was later removed using a solution of 2% trifluoroacetic acid (TFA) in DCM (dichloromethane) with triisopropylsilane as a scavenger. Other lysine residues in the peptide main chain were protected with Boc groups, which are much less acid labile. Small samples of the peptide in preparation were taken at varying intervals and, after full deprotection, were subsequently analyzed using ESI-MS and RP HPLC. Typical yields obtained for the purified GLP-1 analogues were 10–15%. The purity of the peptides along with their respective ESI-MS characterization are given in Table 1. While purification of most products resulted in purities in excess of 90%, due to the difficult nature of preparing these lengthy sequences and the very similar HPLC retention times of peptide byproducts formed during syntheses, compound **5** was obtained with only an 85% purity.

Initial labeling experiments were carried out using naturally occurring Indium-113/115 (Scheme 2), in order to obtain IC_{50} values, synthesize HPLC standards, and determine radiolabeling conditions while preventing unnecessary exposure to radiation. High temperatures are required for this reaction in order to speed up the formation of the product. In addition, a sodium acetate buffer was employed in order to maintain the pH at the desired level, thus preventing the formation of insoluble indium oxides.[37] Products were purified using preparative HPLC and subsequently characterized using ESI-MS, which in all instances indicated complete labeling of peptides. Final compounds were analyzed by RP-HPLC for purity determination prior to biological evaluation. In total, eight novel GLP-1 analogues containing indium were prepared, with four positional variations (22, 26, 34, 37), each position being prepared with and without the AEEA spacer.

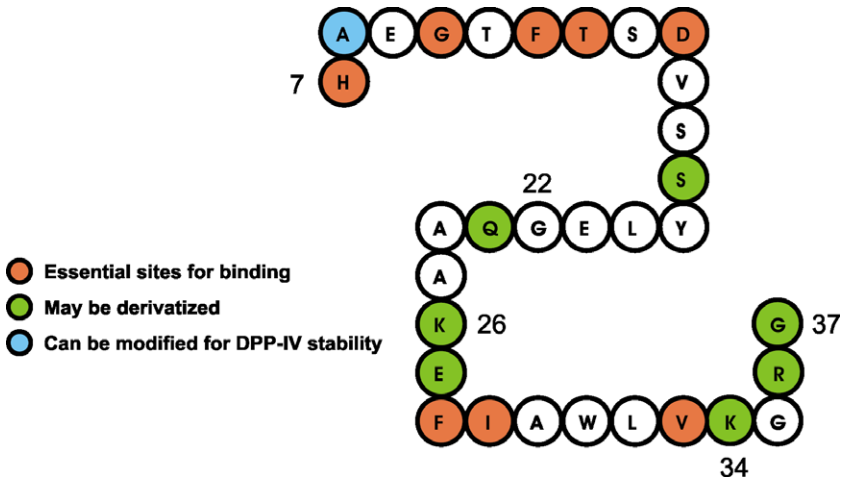
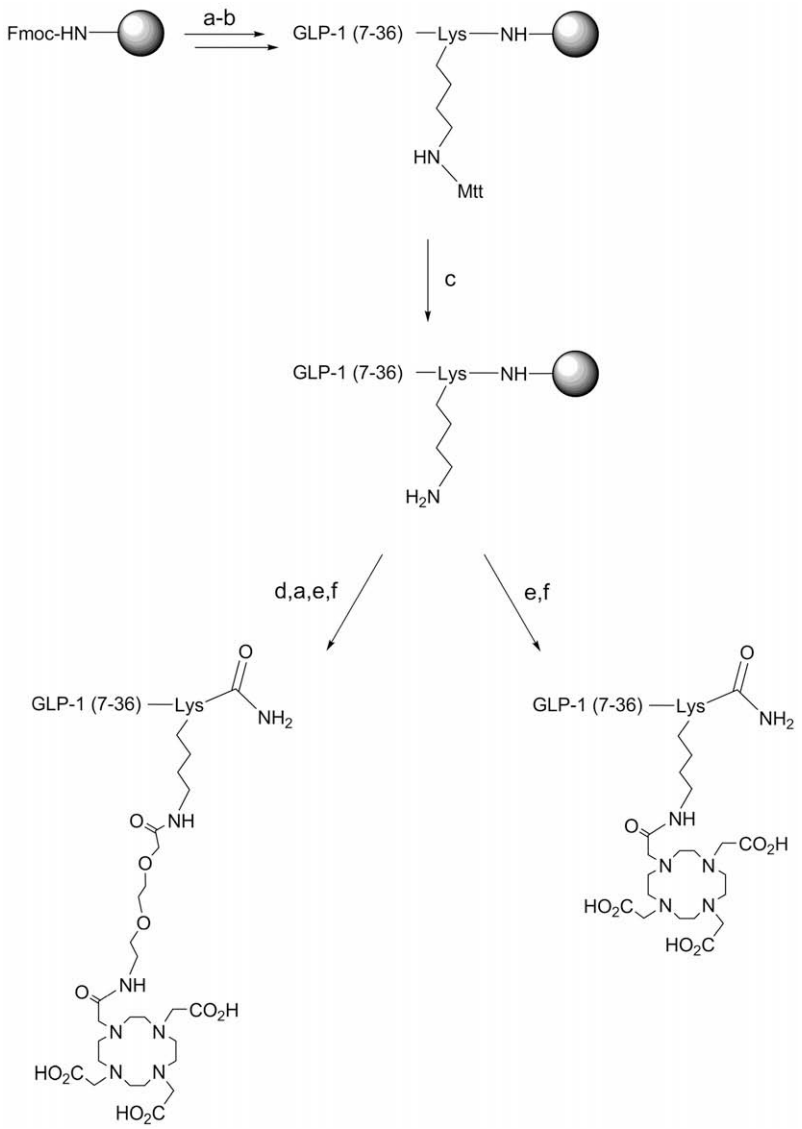


Figure 3. A schematic of the structure activity relationship of GLP-1.[36]



Scheme 1. Employed synthetic pathway for the preparation of ^{37}Lys-DOTA-GLP-1 and ^{37}Lys-AEEA-DOTA-GLP-1(7–37). Reagents: (a) 20% pip/DMF; (b) Fmoc amino acid, HBTU, DIPEA; (c) 2% TFA, triisopropylsilane; (d) Fmoc-AEEA-OH, HBTU, DIPEA; (e) DOTA(tBu)$_3$, HBTU, DIPEA; (f) 88% TFA + scavengers.

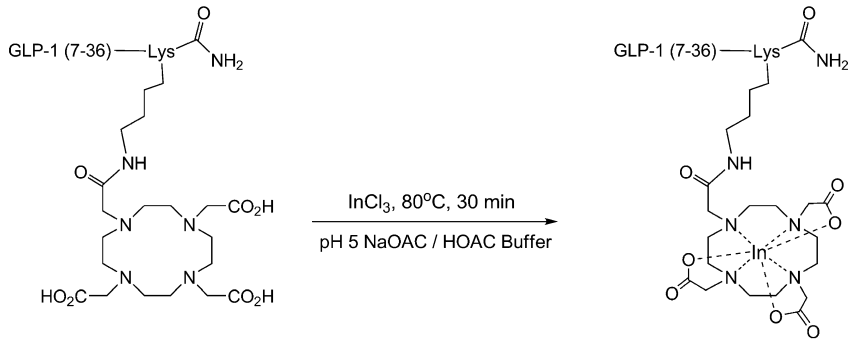
Table 1
Analysis of synthesized indium ($^{113/115}$In) containing GLP-1 analogues by ESI-MS and RP HLPC

Compd	Peptide	Purity (%)	Calculated m/z	Observed m/z
1	^{22}Lys-In-DOTA-GLP-1 (7–36)	90.0	1290.2 $[M+3H]^{3+}$	1290.3 $[M+3H]^{3+}$
2	^{22}Lys-AEEA-In-DOTA-GLP-1 (7–36)	98.0	1339.4 $[M+3H]^{3+}$	1340.3 $[M+3H]^{3+}$
3	^{26}Lys-In-DOTA-GLP-1 (7–36)	95.0	1266.6 $[M+3H]^{3+}$	1265.8 $[M+3H]^{3+}$
4	^{26}Lys-AEEA-In-DOTA-GLP-1 (7–36)	90.0	1314.9 $[M+3H]^{3+}$	1314.6 $[M+3H]^{3+}$
5	^{34}Lys-In-DOTA-GLP-1 (7–36)	85.3	1266.6 $[M+3H]^{3+}$	1266.3 $[M+3H]^{3+}$
6	^{34}Lys-AEEA-In-DOTA-GLP-1 (7–36)	97.0	1314.9 $[M+3H]^{3+}$	1314.6 $[M+3H]^{3+}$
7	^{37}Lys-In-DOTA-GLP-1 (7–37)	98.0	1309.3 $[M+3H]^{3+}$	1309.5 $[M+3H]^{3+}$
8	^{37}Lys-AEEA-In-DOTA-GLP-1 (7–37)	95.3	1357.6 $[M+3H]^{3+}$	1357.4 $[M+3H]^{3+}$

2.3. In vitro studies

A GLP-1 competitive receptor binding assay was used to evaluate the binding affinity of the indium labeled analogues. In these studies the competitive binding of [^{125}I]exendin-4 with the indium coordinated peptide, on CHO cells stably expressing the human GLP-1R (CHO-GLP-1R cells),[33] was used as a measure of the respective IC_{50} value. Figure 4 shows typical plots obtained from competitive studies, in this instance for compounds **2**, **7** and **8**. Table 2 shows the obtained IC_{50} values of all eight peptides.

The best results for GLP-1R binding were obtained for GLP-1 derivatives modified at positions 22 and 37. For the peptide with DOTA conjugated at position 37 (**7**), a favorable IC_{50} of 63 nM was determined, while the presence of the AEEA spacer at position 37 (**8**) decreased slightly the binding affinity of the peptide to 89 nM. This was an unexpected outcome as the C-terminus of the



Scheme 2. Indium labeling of DOTA-GLP-1 derivatives.

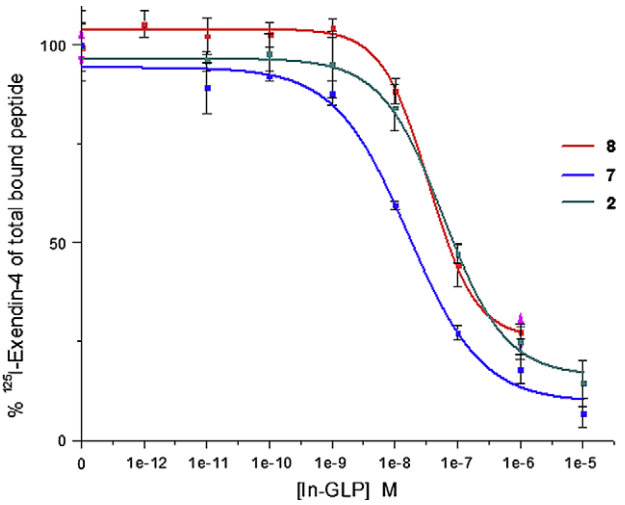


Figure 4. Competitive binding of compounds **2**, **7** and **8** versus ^{125}I-exendin-4 on CHO/GLP-1R cells.

Table 2
Receptor binding and cAMP measurements for $^{113/115}$In-GLP-1 peptide analogues

Compd	IC_{50} (nM)	EC_{50} (nM)
GLP-1	5.0 ± 0.1	0.12 ± 0.01
1	598 ± 45	–
2	93 ± 7	0.74 ± 0.07
3	970 ± 73	–
4	2660 ± 129	–
5	268 ± 16	–
6	573 ± 39	–
7	63 ± 4	0.78 ± 0.07
8	89 ± 3	0.93 ± 0.05

The first column shows the IC_{50} values obtained from in vitro competitive binding studies. Peptides **2**, **7** and **8** were selected for cAMP studies and EC_{50} values are shown in the second column.

peptide is the least sterically hindered position along the peptide chain. Although side chain modification at position 22 has not been recommended in the past literature, our results indicate that, when coupled with the proper spacer, modification at this position has minimal negative effect on the binding affinity of the peptide, as indicated by **2** with an IC_{50} of 93 nM. Considering that the original residue at this position, ^{22}Gly, is predicted to disrupt the helical structure of GLP-1,[38–40] it is possible that its replacement with ^{22}Lys may decrease the helical distortion, thereby allowing the addition of the metal-complex with only minimal impact on its binding affinity to GLP-1R. The use of the AEEA spacer was also observed to have a negative impact on the binding affinity of the peptide when it was coupled to the Lys side chain at positions 26 and 34. This could have been caused by destabilization of the helical conformation of the GLP-1 derivatives as a result of steric hindrance or strain caused by the long AEEA chain.

2.4. cAMP studies

The production of intracellular cAMP is a measure of GLP-1R activiation.[41] As GLP-1 binds to its receptor on the beta cell, intracellular Gαs is activated, resulting in the stimulation of transmembrane adenylyl cyclases (tmACs) and elevation of cAMP levels.[42] This in turn promotes glucose-dependent insulin secretion. In this study, the ability of peptides **2**, **7**, and **8** to activate GLP-1R was assessed by measuring cAMP levels in response to 0.1 nM to 1 μM of peptide using a previously published method.[34] All three peptides increased cytoplasmic cAMP concentrations in CHO/GLP-1R cells with sub-nanomolar EC_{50} values (Table 2). These experiments therefore demonstrate that these analogues can act as GLP-1R agonists.

2.5. Plasma stability studies

In order to examine the protease stability of the developed probes, compound **7** was selected for plasma stability studies and compared with native GLP-1 (7–36). After incubation of the peptide in rat plasma at varying time intervals, the products were examined using RP-HPLC and identified using ESI-MS. The calculated half-life of native GLP-1 was 15 min while that of compound **7** was 13 h. These results, which are in agreement with published plasma stability data for native GLP-1 and [Ser^8]-GLP-1 (7–36) amide,[43] indicate enhanced protease stability for the ^{8}D-Ala modified peptides developed in this study.

3. ^{111}In-labeling

Based on the accumulated biological data for these novel GLP-1 analogues, compound **8** was selected for radiolabelling with In-111 and subsequent evaluation in insulinoma cells. Radiolabeling was carried out in pH 5 NaOAC/HOAc buffer over 30 min at 70 °C. The decay corrected radiochemical yield for [^{111}In]-**8** was 60% and the radiopurity was 98%. Monitoring of the reaction progress and characterization of the ^{111}In-labeled peptide were carried out using RP-HPLC with $^{113/115}$In-labeled peptide **8** used as a non-radioactive standard for chromatographic comparison of retention time. Figure 5 shows the UV and radiochromatograms corresponding to this reaction. To further demonstrate the ability to radiolabel these DOTA-GLP-1 derivatives, the radiolabeling of **2** was also carried out under the same conditions as mentioned above. The radiochemical yield and purity in this case were 84% and 97%, respectively. Figure

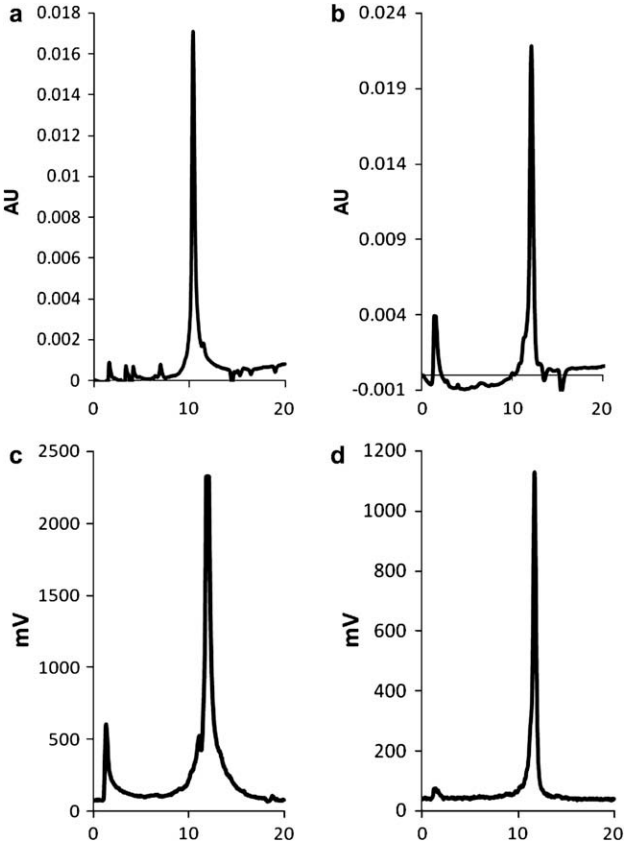


Figure 5. The HPLC (a) UV chromatogram of the standard unlabeled **8**; (b) UV chromatogram of [$^{113/115}$In]-**8**; (c) radiochromatogram of [^{111}In]-**8** before purification; and (d) radiochromatogram of [^{111}In]-**8** after Sep-Pak purification.

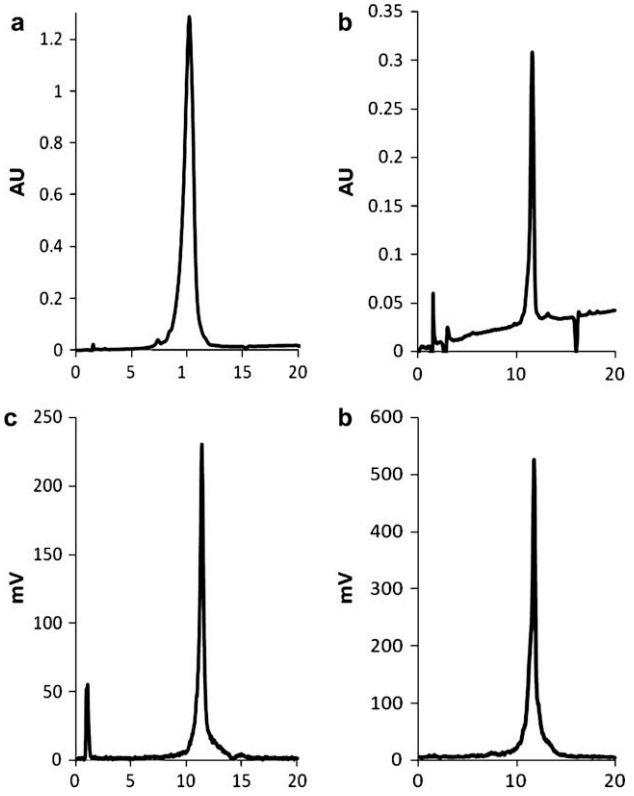


Figure 6. The HPLC (a) UV chromatogram of the standard unlabeled **2**; (b) UV chromatogram of [$^{113/115}$In]-**2**; (c) radiochromatogram of [^{111}In]-**2** before purification; and (d) the radiochromatogram of [^{111}In]-**2** after Sep-Pak purification.

6 shows the UV and radiochromatograms corresponding to the preparation of [^{111}In]-**2**.

To demonstrate that our [^{111}In]-labeled peptides can be used for imaging, an in vitro imaging study was carried out using [^{111}In]-**8** and the clonal insulin-producing beta cell line INS-1 832/13, which expresses GLP-1R. The planar gamma camera image in Figure 7 demonstrated specific binding of the GLP-1 analogue, as it was displaced by exendin-4. In this image, wells 1 and 2, containing the binding buffer and INS-1 832/13 cells, were used as a control to measure the background noise. Wells 3 and 4, containing INS-1 832/13 cells and [^{111}In]-**8**, indicated binding of the radiolabeled peptide. The image of wells 5 and 6 clearly indicated displacement of the radiolabeled peptide by exendin-4 thus further proving that the GLP-1 derivative was bound to GLP-1R on the surface of the INS-1 832/13 cells. This imaging study proved [^{111}In]-**8** to be a potential SPECT imaging agent for monitoring pancreatic beta cell mass in vivo.

4. Conclusion

The objective of this study was to develop novel GLP-1 analogues for the targeting of GLP-1R on pancreatic beta cells. We have developed eight GLP-1 analogues, all shown to have affinity for GLP-1R. Three of the developed derivatives, namely compounds **2**, **7** and **8**, showed optimal IC_{50} values indicating that positions 22 and 37 of GLP-1 are the most suitable for indium-DOTA conjugation. While the presence of the AEEA spacer was beneficial at position 22, it had a negative impact on the binding affinity of the peptide analogues at positions 26, 34, and 37. The SPECT images obtained from the competitive study between [^{111}In]-**8** and exendin-4 in INS-1 832/13 cells further demonstrated the potential of the developed probes to monitor beta cell mass. Future studies will employ the most promising peptides reported here, compounds **2**, **7** and **8**, as candidates for pancreatic beta cell imaging in vivo.

4.1. Experimental procedures

Common solvents and reagents were purchased from VWR, Fisher Scientific, or Sigma–Aldrich and used as received, unless stated otherwise. Sterile, deionized water was used in all aqueous procedures. All Fmoc protected amino acids, except Fmoc-Lys(mtt)-OH (Nova Biochem), and HBTU were obtained from Peptides International. Fmoc-Rink amide MBHA resin (4-(2′,4′-dimethoxyphenyl-(9-fluorenylmethoxycarbonyl)-aminomethyl)-phenoxy-acetamido-norleucyl-4-methyl benzhydrylamine resin), and Boc-His(Boc)-OH·DCHA (dicyclohexylamine) were obtained from Nova Biochem. The hydrophilic linker 2-[2-(2-aminoethoxy)ethoxy]acetic acid (AEEA) was synthesized in our laboratory.[29] DOTA-tris(*t*Bu)-ester was obtained from CheMatech. Indium trichloride tetrahydrate (99.99%) was obtained from Strem Chemicals. Indium-111 and [^{125}I]-exendin were obtained from MDS Nordion and PerkinElmer Inc., respectively. RP-C18 Sep-Pak SPE cartridges were obtained from Waters. CHO/GLP-1R and INS-1 832/13 cells were provided by Dr. Michael Wheeler from University of Toronto and Dr. Christopher Newgard from Duke University, respectively.

4.2. Peptide syntheses

Fmoc-based solid-phase peptide synthesis was carried out using an APEX 396 autosynthesizer (AAPPTEC) with 0.05 mequiv of 0.27 mmol/g Fmoc-Rink amide mBHA resin and a threefold excess of the protected amino acids. Fmoc removal, carried out with 20% piperidine in DMF (*N*,*N*-dimethylformamide) over two

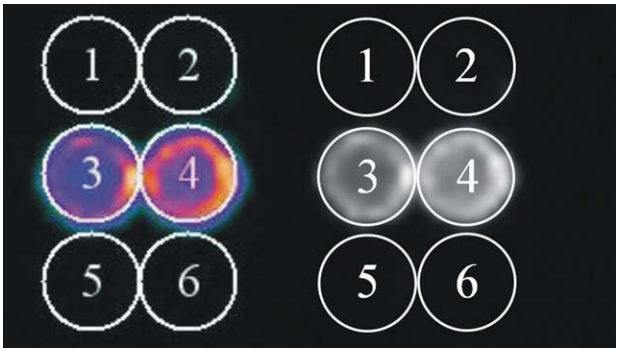


Figure 7. In vitro gamma camera imaging study with INS-1 832/13 cells. Wells 1 and 2 = background control (no probe added); 3 and 4 = [^{111}In]-**8**; 5 and 6 = [^{111}In]-**8** + 10^{-6} M unlabeled exendin-4 (cold exendin-4 block). Left image, color contoured; right image, grey-scale.

cycles (10 and 20 min), was followed by amino acid activation with three equivalents HBTU and six equivalents DIPEA (*N,N*-diisopropylethylamine) (10 min) and subsequent coupling over 30 and 120 min cycles.

Methyl trityl deprotection was carried out in a glass peptide reaction vessel using 5% triisopropylsilane (v/v) + 2% TFA (v/v) in CH_2Cl_2 over 3 min and repeated 10 times. Coupling of DOTA/AEEA to this site, or the coupling of DOTA to AEEA, followed the same methodology used in amino acid coupling, however the coupling times were increased to 18 and 24 h for AEEA and DOTA, respectively. Microcleaved samples were used to monitor reaction progress and peptide purity via HPLC.

Full deprotection of synthesized peptides was accomplished using a solution of 88% TFA (v/v) + 5% H_2O (v/v) + 5% phenol (m/v) + 2% triisopropylsilane (v/v) over 6 h. The cleaved peptides were then precipitated using *tert*-butyl methyl ether (TBME) and centrifuged (2200 rpm for 15 min). After removing the resulting supernatant, the peptide pellet was rinsed with TBME, vortexed and centrifuged again (2200 rpm for 15 min). The supernatant was removed, then the peptide pellet was dissolved in water, frozen at 78 °C and lyophilized.

4.3. Purification by RP-HPLC/ESI-MS

Peptides were analyzed using an analytical reverse-phase HPLC column (RP-HPLC) (Grace Vydac Protein/Peptide RP-C18 column 4.6 × 250 mm, 5 μm). This system was equipped with a Waters 600 controller, Waters Prep degasser, and Waters MassLynx software (version 4.1). Employed mobile phases were 0.1% CF_3CO_2H in water (eluent A) and 0.1% CF_3CO_2H in CH_3CN (eluent B). The linear gradient used was 30–50% of B with a flow rate of 1.5 mL min^{-1} over 20 min. The column eluate was monitored using a Waters 2998 Photodiode array detector set at 220 and 254 nm.

Peptides were purified using a preparative RP-HPLC column (Grace Vydac Protein/Peptide RP-C18 column 22.0 × 250 mm, 10 μm) on the same system mentioned above. The detection method along with eluents and gradients were the same as those stated above, with the exception of the flow rate being set at 20 mL min^{-1}. The collected fraction was then lyophilized to a solid and subsequently analyzed by ESI-MS (electrospray ionization mass spectrometry) (Waters Micromass Quattro Micro™ API). Purity of final products was determined by analytical RP-HPLC (reverse-phase high performance liquid chromatography).

4.4. $^{113/115}$In-labeling

In a typical reaction, 5 mg of the GLP-1 analogue, 3 mL of pH 5 NaOAc/HOAc buffer, and 7 mg of $InCl_3{\cdot}4H_2O$ (0.02 mmol) were placed in a 25 mL glass round bottom flask. The reactants were dissolved by sonication at 25 °C. The reaction was then carried out at 70 °C for 30 min. The resulting reaction mixture was allowed to cool before purification by a C18 RP sep-pak© (conditioned with 5 mL of ethanol and 15 mL of H_2O). After passing the reaction mixture through the sep-pak©, 10 mL of water was used as eluent in order to wash out residual unreacted $InCl_3{\cdot}4H_2O$. A 10 mL aliquot of 0.1% TFA in CH_3CN was used to wash out the labeled product. The resulting solution was then mixed with 10 mL of H_2O (to lower the percentage component of acetonitrile), frozen at −78 °C and subsequently lyophilized.

4.5. Radiolabeling

To a clean conical glass vial was added 20 μL of the prepared GLP-1 analogue (1 mg/mL of pH 5 NaOAc/HOAc buffer). This aliquot was dissolved in 200 μL of the buffer (10-fold dilution to obtain micromolar concentrations), to which 5.63 mCi of $^{111}InCl_3$ was added. The reaction mixture was then heated at 70 °C for 30 min, before purification by a RP-C18 SPE sep-pak© cartridge (conditioned with 5 mL of ethanol and 15 mL of water). After passing the reaction mixture through the sep-pak©, 10 mL of water was used as eluent in order to wash out residual unreacted $InCl_3$. A 5 mL aliquot of EtOH was used to wash out the radiolabeled product. The sep-pak eluate was evaporated on a rotary evaporator prior to in vitro studies. The reaction progress and product purity was analyzed using analytical RP-HPLC (Waters Symmetry, 4.6 × 150 mm, 5 Å, C-18 column) coupled to a gamma detector. This system employed a Waters 1525 Binary HPLC pump, Waters 2487 dual λ absorbance detector, Waters In-Line degasser and Breeze software (version 3.30).

4.6. Binding assays

All GLP-1 receptor binding studies were conducted using Chinese hamster ovary cells stably transfected with the human GLP-1 receptor (CHO/GLP-1R), generously donated by Dr. Michael Wheeler (University of Toronto). Receptor binding was measured by the competitive binding of [^{125}I]-exendin-4 with increasing concentrations of the synthesized $^{113/115}$Indium labeled GLP-1 analogues, using a previously published method.[33] Briefly, CHO/GLP-1R cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (Invitrogen). On the day of the experiment, cells were rinsed 2× with warm Hank's buffered saline solution (HBSS) and dissociated in HBSS containing 2 mM EDTA. Approximately one million cells were incubated for 60 min at 37 °C in binding buffer (DMEM containing 0.1% Trasylol (Bayer) and 0.1% bovine serum albumin (Sigma), pH 7.4), 0.77 μmol [^{125}I]exendin-4 (Amersham) and variable concentrations of each GLP-1 analogue (10^{-5} to 10^{-11} M). After incubation, cells were centrifuged at 2800 rpm for 15 min. After removal of the supernatants, the cell pellets were washed with 200 μL of cold binding buffer and re-centrifuged before the final pellet was counted in a gamma counter. Binding curves and IC_{50} (half maximal inhibitory concentration) values were generated using MS Excel, Sigma Plot, and Origin Lab 8. This study was repeated three times per peptide.

4.7. cAMP studies

Activation of the GLP-1R by the peptide analogues was assessed by stimulation of cAMP using a previously published method.[33] Briefly, CHO/GLP-1R cells were plated at a density of 1×10^5 cells/well in a 24-well plate 48 h prior to the day of the experiment. Cells were incubated for 30 min at 37 °C in DMEM

containing 1 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma) and 10 nM of the peptide analogues. Media were removed, cells were rinsed twice with cold HBSS and scraped in 200 μL of 80% ethanol. cAMP levels were measured using a cAMP radioimmunoassay kit (Perkin Elmer, Shelton CT) as per the manufacturer's instructions. EC_{50} values were calculated using Matlab.

4.8. Plasma stability studies

Plasma stability studies were carried out according to previously published procedures.[43] Briefly, GLP-1 (7–36) (BaChem) and compound **7** (1 μg/μl peptide in acetate buffer pH 6.2) were incubated with rat plasma (final sample volume 1 ml; final peptide concentration 20 μM) over increasing time-points at 37 °C. The incubation period ended with the addition of 40 μL aqueous 10% TFA. Samples were then applied to C18 reverse-phase cartridges (Light C18 sep-pak©), washed with 4 ml aqueous 0.1% TFA, and eluted with 2 ml 60% acetonitrile in aqueous 0.1% TFA. After lyophilization, samples were analyzed using analytical RP-HPLC (Grace Vydac Protein/Peptide RP-C18 column 4.6 × 250 mm, 5 μm). This system was equipped with a Waters 600 controller, Waters Prep degasser, and Waters MassLynx software (version 4.1). Employed mobile phases were 0.1% CF_3CO_2H in water (eluent A) and 0.1% CF_3CO_2H in CH_3CN (eluent B). The linear gradient used was 30–50% of B with a flow rate of 1.5 mL min^{-1} over 20 min. The column eluate was monitored using a Waters 2998 Photodiode array detector set at 220 and 254 nm. Products were identified using ESI-MS. The half-life of the peptides was determined using GraphPad Prism 5.

4.9. In vitro imaging

INS-1 832/13 cells were plated in 6-well plates at a density of one million cells per well. Two wells did not receive any radiolabeled peptide; two wells were incubated with 50 μCi [^{111}In]-8 for 30 min at 37 °C; and two wells were incubated with 50 μCi [^{111}In]-8 and 1 μM cold exendin-4. After the incubation period, media were removed, cells were rinsed twice with cold HBSS and covered with 500 μL culture media. Cells were imaged for 30 min with a GE Millenium II gamma camera using a medium-energy general purpose collimator. Images were acquired and analyzed using GE Xeleris software.

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Supplementary data

Supplementary data (MS spectra and HPLC chromatograms for compounds **1**–**8**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.032.

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