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Bioorganic & Medicinal Chemistry Letters 14 (2004) 4395–4398
Bioorganic & Medicinal Chemistry Letters

Identification of CJC-1131-albumin bioconjugate as a stable and bioactive GLP-1(7–36) analog

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Received 11 May 2004; revised 21 June 2004; accepted 21 June 2004

Abstract—A series of analogs of GLP-1(7–36) amide containing a *N*ε-(2-{2-[2-(3-maleimidopropylamido)ethoxy]ethoxy}acetyl)lysine has been synthesized and the resulting derivatives were bioconjugated to Cys34 of human serum albumin (HSA). The GLP-1-HSA bioconjugates were analyzed in vitro to assess the stabilizing effect of bioconjugation in the presence of DPP-IV as well as GLP-1 receptor binding and activation. Compound **9** (CJC-1131) having the point of attachment to albumin at the *C*-terminal of GLP-1 and a D-alanine substitution at position 8 was identified as having the best combination of stability and bioactivity.

GLP-1(7–36) (**1**, Fig. 1) is a well known and well studied insulinotropic endocrine hormone with potential as a treatment for type 2 diabetes.[1] However, it suffers from a short half-life in plasma due primarily to dipeptidyl-peptidase IV (DPP-IV) degradation.[2] Several strategies have been explored to circumvent this problem such as (1) modifications at the *N*-terminus or the residue at position 8 to stabilize the molecule,[3] (2) attachment of a lipophilic chain to a lysine residue on GLP-1,[4] or (3) DPP-IV inhibitors to improve plasma half-life of endogenous GLP-1.[5]

The plasma half-life of a bioactive peptide can be improved through bioconjugation to serum albumin resulting in protection against enzyme degradation and decreased elimination through the kidney.[6] In this communication, we report the stabilizing effect of the position of attachment of GLP-1(7–36) to Cys34 of human serum albumin (HSA) in the presence of DPP-IV as well as the in vitro binding to and activation of the GLP-1 receptor.

The GLP-1(7–36) peptide conformation has mostly been studied in solution with α-helical structure inducing solvent systems.[7] The recent publications of the solution structure of Exendin-4,[8] structure–activity relationship (SAR) studies of insertion of a lipophilic chain at positions 26[4a] and 34[4b] on GLP-1(7–36) as well as recent reports on the interaction of GLP-1(7–36) and Exendin-4 with the GLP-1 receptor[9] were more in agreement with our SAR study. The α-helix portion of **1** is thought to start near Asp15 and extends through to Arg36.[9b] From this, the eight derivatives shown in Figure 1 were prepared. Compound **2** had the maleimido group attached to His7 at the *N*-terminus. Free His7 was expected to be essential for conferring the peptide's bioactivity. Compounds **3** and **4** had the maleimido attachment on the outside portions of the α-helical segment of the peptide thus exposing the residues important for binding. The maleimido was also attached to a lysine already present in the peptide in **5** and **7**. Furthermore, **8** and **9** had an added lysine at the *C*-terminus to accommodate the maleimido group. In **6**, Phe28 was replaced with a lysine to support the maleimide. Phe28 resides on the side of the α-helical structure important for activity, so **6** was included as a negative control. Compound **9** had Ala8 replaced by a D-alanine to confer proteolytic stability.

The syntheses of the compounds shown in Figure 1 were accomplished using a solid phase synthesis strategy. A general protocol as exemplified by the syntheses of **2** and **3** is shown in Scheme 1.

Fmoc-AEEA (*N*-Fmoc-2-[2-(2-aminoethoxy)ethoxy]acetic acid) was introduced into the sequence in the same

Keywords: GLP-1; Albumin conjugate; DPP-IV.
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doi:10.1016/j.bmcl.2004.06.066

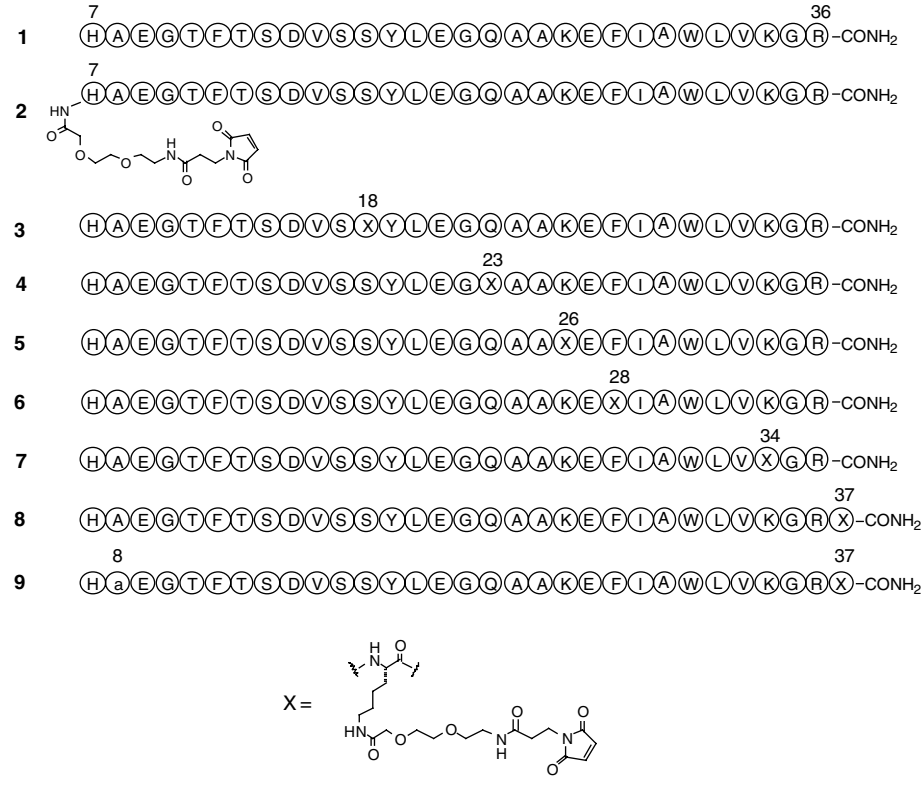
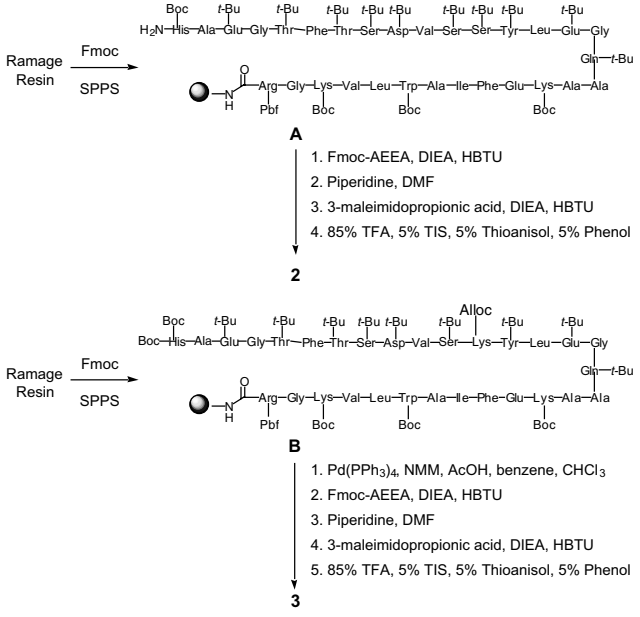


Figure 1. Human GLP-1(7–36), and 8 positional maleimido derivatives.



Scheme 1. General solid phases synthesis protocol in the preparation of maleimido GLP-1(7–36) derivatives.

fashion as an amino acid residue followed by MPA (maleimidopropionic acid) to give compound **2** after cleavage, isolation, and purification. The other syntheses were done in the same manner as compound **3** where an Alloc protecting group was introduced at the *N*ε position of the lysine at the desired anchoring site. The selective removal of Alloc was accomplished using palladium(0)[10] followed by the protocol described above with Fmoc-AEEA and MPA. The yields and analytical data are displayed in Table 1.

Table 1. Compounds obtained by solid phase synthesis

Compound	Yield[a] (%)	M_r[b]		Purity (HPLC)
		Expected	Measured[c]	
2	14	3591.8	3593.4	98.3
3	12	3632.8	3634.4	92.4
4	9	3591.8	3593.3	97.2
5	11	3591.8	3593.5	90.1
6	4	3572.8	3574.6	91.7
7	20	3591.8	3593.6	90.6
8	17	3719.9	3721.6	90.5
9	19	3719.5	3721.9	97.0

[a] TFA salt.
[b] M_r of free base.
[c] Average value from multiple charged species by ES/MS.

The conjugates were prepared by incubation of peptides **2**–**9** in the presence of HSA[11] followed by purification using hydrophobic chromatography.[12] The conjugates were analyzed by LC/(ES)MS and found to be free of unreacted starting material and other albumin species such as mercaptalbumin and cysteinylated albumin at the Cys34 position. The MS results and the yields (with respect to the amount of maleimide) following conjugation and purification are shown in Table 2.

DPP-IV is a ubiquitous multifunctional plasma enzyme responsible for the metabolism and deactivation of GLP-1. It recognizes alanine and proline residues at

Table 2. Analysis of HSA bioconjugates

Conjugate	M_r		Yield (%)[b]
	Predicted[a]	Measured	
2-HSA	70,037	70,029	63
3-HSA	70,079	70,072	62
4-HSA	70,038	70,033	58
5-HSA	70,038	70,033	61
6-HSA	70,001	70,009	41
7-HSA	70,037	70,026	57
8-HSA	70,165	70,157	56
9-HSA	70,165	70,160	74

[a] Using the measured molecular weight of an albumin standard analyzed by LC/MS prior to conjugate sample analysis.
[b] Yield (with respect to maleimide) following conjugation and purification.

the P1 site and in the case of GLP-1 causes the release of the His-Ala dipeptide fragment. GLP-1(9–36) does not activate the GLP-1 receptor.

The conjugates described in Table 2 were assessed for their resistance toward DPP-IV degradation. The enzyme stability assay was done by placing the conjugates in the presence of DPP-IV (porcine) and reporting the abundance signal at the 24 h time point relative to a control solution that had no enzyme.[13] The results are displayed in Table 3.

2- and **3**-HSA conjugates were stable in the presence of the DPP-IV enzyme. When the point of attachment on the GLP-1 molecule was incrementally moved further away from the *N*-terminal, a directly proportional loss of stability was observed. The least stable derivative was **8**-HSA with attachment at the *C*-terminal of GLP-1. Replacement of the L-alanine (**8**-HSA) at position 2 with a D-alanine residue (**9**-HSA) restored stability. It is noteworthy that the *N*-terminal of HSA (Asp-Ala) is not cleaved in the presence of DPP-IV in our assay.

Table 3. In vitro stability and bioactivity of GLP-1 albumin bioconjugates compared to native GLP-1(7–36)

Compound	DPP-IV stability Amount remaining after 24 h	Receptor binding[a] IC_{50} (nM)	cAMP production[b]	
			EC_{50} (nM)	E_{max}[c] (%)
1	0%	14 ± 1	15.4[d]	100
1[e]		0.14 ± 0.00	0.4 ± 0.1	100
2-HSA	>99%	5354[d]	>1000[d]	<10[d]
3-HSA	>99%	713 ± 50	28 ± 21	88 ± 5
4-HSA	68%	454 ± 4	37 ± 9	88 ± 4
5-HSA	49%	804 ± 124	72 ± 11	80 ± 13
6-HSA	53%	>10,000[d]	>1000[d]	<10[d]
7-HSA	55%	296 ± 3	49 ± 11	79 ± 16
8-HSA	36%	87 ± 8	24 ± 15	84 ± 22
9-HSA	98%	108 ± 42	15 ± 1	94 ± 6

Binding and cAMP production experiments were done in duplicate and repeated twice ($n = 2$).
[a] Human GLP-1 receptor expressing CHO cell membrane homogenates.
[b] Human GLP-1 receptor expressing CHO cells.
[c] E_{max} is the maximum elevation of cAMP relative to **1** as 100%.
[d] $n = 1$.
[e] Contains 1% DMSO.

The binding[14] and activation[15] of the GLP-1 receptor by the GLP-1(7–36)-HSA conjugates were evaluated in vitro. The reference GLP-1 was tested with and without DMSO and a difference was observed. Binding and activation concentrations of native GLP-1 were lower in the presence of DMSO while the DMSO did not affect the bioconjugate binding and activation concentrations (data not shown). We mention this because the apparent loss of activity is greater when comparing conjugate results with those of the native peptide in the presence of DMSO. DMSO was reported to cause a synergistic effect on GLP-1 mediated insulin secretion in vitro,[16] although in our study, it is not clear if the results are due to enhanced solubility, conformational or synergistic effects on the native peptide. The fact that the conjugate is not affected by the presence of DMSO reduces the likelihood of the synergistic effect. This DMSO effect on the native peptide in vitro merits further investigation.

The results in Table 3 show that there was reduction in binding and activation of the GLP-1 receptor when the GLP-1(7–36) peptides were attached to the larger HSA molecule. However, there was variability based on the position of attachment. The importance of the *N*-terminal amino acid was demonstrated with **2**-HSA where this conjugate lost a great deal of its binding ability and did not activate the GLP-1 receptor.

Moving the point of attachment away from the *N*-terminus resulted in better binding and activation of the GLP-1 receptor except for **6**-HSA where the face of the peptide exposing the important residues was presumably unavailable for binding. The result for **6**-HSA is in agreement with the approximated structure of GLP-1[17] and the data reported from an alanine scan across the peptide.[18] Compound **8**-HSA with the point of attachment at the *C*-terminus had the best binding in this assay but this conjugate was less stable as discussed above.

Clearly, the compound with the best combination of binding, bioactivity, and DPP-IV stability was **9**-HSA conjugate. The precursor compound **9** is also known as CJC-1131 and has been demonstrated to be efficacious in a db/db mouse model.[6b] Further pre-clinical and clinical evaluations of this compound are currently ongoing.

Acknowledgements

We would like to thank Peter Bakis for his skilled technical help in peptide synthesis.

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11. Each maleimido GLP-1(7–36) derivative was solubilized in nanopure water at a concentration of 10 mM then diluted to 1 mM into a solution of HSA (25%, Cortex-Biochem, San Leandro, CA). The samples were then incubated at 37 °C for 30 min. Prior to their purification, each conjugate solution was diluted to 5% HSA in 20 mM sodium phosphate buffer (pH 7) composed of 5 mM sodium octanoate and 750 mM $(NH_4)_2SO_4$.
12. Using an ÄKTA purifier (Amersham Biosciences, Uppsala, Sweden), each conjugate was loaded at a flow rate of 2.5 mL/min onto a 50 mL column of butyl sepharose 4 fast flow resin (Amersham Biosciences, Uppsala, Sweden) equilibrated in 20 mM sodium phosphate buffer (pH 7) composed of 5 mM sodium octanoate and 750 mM $(NH_4)_2SO_4$. Under these conditions, all GLP-1(7–36)-HSA conjugates adsorbed onto the hydrophobic resin whereas essentially all nonconjugated (unreacted) HSA eluted within the void volume of the column. Each conjugate was further purified from any free (unreacted) maleimido GLP-1(7–36) derivatives by applying a linear gradient of decreasing $(NH_4)_2SO_4$ concentration (750–0 mM) over four column volumes. Each purified conjugate was then desalted and concentrated using Amicon® ultra centrifugal (30 kDa) filter devices (Millipore Corporation, Bedford, MA). Finally, each conjugate solution was immersed into liquid nitrogen, lyophilized, and stored at −80 °C.
13. The conjugate (10 mg) was solubilized in 1X PBS® pH 7.1 (250 μL) and added directly to a vial of DPP-IV enzyme (5 mU, porcine, Calbiochem, San Leandro, California). The mixture was incubated at 37 °C under mixing conditions and aliquots were taken at the indicated time and immediately frozen at −80 °C. Sample were thawed just prior to LC/(ES)MS analysis.
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