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Identification of glycosylated exendin-4 analogue with prolonged blood glucose-lowering activity through glycosylation scanning substitution

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ABSTRACT

Exendin-4, a glucagon-like peptide 1 receptor agonist, is a potent therapeutic xenopeptide hormone for the treatment of type 2 diabetes. In order to further improve in vivo activity, we examined the introduction of sialyl *N*-acetyllactosamine (sialyl LacNAc) to exendin-4. The glycosylated analogue having sialyl LacNAc at position 28 was found to have improved in vivo activity with prolonged glucose-lowering activity.

Exendin-4 is a 39 amino acid peptide originally isolated from the saliva of the Gila monster *Heloderma suspectum*. Exendin-4 has 53% amino acid sequence identity with the incretin hormone glucagon-like peptide 1 (GLP-1) and is a potent GLP-1 receptor agonist.[1] As with GLP-1, exendin-4 stimulates insulin secretion from pancreatic β-cells in response to elevated plasma glucose concentrations. Also, exendin-4 suppresses glucagon secretion, slows gastric emptying, reduces appetite and food intake, and stimulates pancreatic β-cells neogenesis.[2] Though exendin-4 shares certain activities with GLP-1 as seen above, exendin-4 has a longer half-life and greater in vivo activity than GLP-1. Exendin-4 has resistance against proteolytic enzymes, such as dipeptidyl peptidase-IV (DPP-IV) and neutral endopeptidase (NEP) 24.11, while GLP-1 is rapidly degraded by these enzymes.[3] Therefore, synthetic exendin-4 is marketed as a therapeutic for type 2 diabetes. However, exendin-4 still falls short of being a long-term stably acting agent. To reduce the frequency of administration, the development of long-acting agents has been attempted using several formulation technology methods and peptide modification (such as extended-release formulation[4] and PEGylation technology[5]).

As an approach to further improve the pharmacokinetic properties and in vivo activity, we examined the introduction of sialyl *N*-acetyllactosamine (sialyl LacNAc) to exendin-4. In a previous study, we showed that the introduction of simple sialyl LacNAc, a nonreducing terminal trisaccharide of complex type N-linked glycan, is a prospective strategy for improving the pharmacokinetic properties and in vivo activity of the therapeutic peptide,[6] though N-linked carbohydrates are known to improve and prolong the in vivo activity of peptide and protein.[7] Addition of sialyl LacNAc to GLP-1 has been shown to greatly improve its proteolytic stability and in vivo activity.[6] Also, carbohydrates have a marked effect on the solubility, stability, immunogenicity of therapeutic peptide and proteins.[8] These benefits can be expected to improve the therapeutic utility of exendin-4.

Introduction of carbohydrate to N-terminal truncated exendin-4 (9–39) has been done by Meurer et al.[9] The glycosylated exendin-4 (9–39) was reported to effectively antagonize exogenously administered GLP-1 in vivo. However, no actual study on enhancing the therapeutic potential of exendin-4 by glycosylation has been reported. In this Letter, to investigate the effect of glycosylation on improving the in vivo agonistic activity of exendin-4, we synthesized glycosylated exendin-4 analogues, and assessed their cAMP production activity in vitro and blood glucose-lowering activity in diabetic *db*/*db* mice.

Initially, to find glycosylation sites having a small influence on the agonistic activity in vitro, we prepared 40 analogues of exendin-4 with Asn-GlcNAc substitution at each position (1–40) by solid-phase peptide synthesis (SPPS) methodology using fluorenylmethoxycarbonyl (Fmoc) amino acids and Fmoc-Asn(Ac_3GlcNAcβ)-OH as the building blocks (Fig. 1).[10] Their cAMP production activities were assessed using human GLP-1 receptor expressing CHO cells (Fig. 2).[11] To enhance in vivo activity, the carbohydrate

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introduction site needs to be optimized to improve the pharmacokinetic properties while retaining the agonistic activity in vitro. Exendin-4 analogues substituted with Asn-GlcNAc in the C-terminal region (28–40) were found to have activity nearly equal to native exendin-4 (EC_{50}: exendin-4, 0.1 nM). However, substitution with Asn-GlcNAc in the N-terminal region (1–10) led to total loss of agonistic activity. In the central region (11–27), analogues substituted with Asn-GlcNAc at position 12, 13, 16, 17, 20, 21, 24 or 25 retained the agonistic activity, though analogues substituted at other positions did not. Therefore, positions such as 12, 13, 16, 17, 20, 21, 24, 25 and 28–40 can be considered to be potential glycosylation sites having little influence on the agonistic activity. These results are in agreement with the findings of previous structure activity studies of exendin-4. Exendin-4 adopts an α-helical structure in the central region while its N-terminal is significantly frayed, like that of GLP-1.[12] The N-terminal random coil region of exendin-4 is necessary for agonistic activity, and the face of the central helical region composed of amino acid, such as Ser11, Glu15, Ala18, Phe22, Trp25 or Leu26, makes the critical contact with the GLP-1 receptor.[13] On the other hand, the C-terminus of exendin-4 forms a compact folding unit the 'Trp-cage', which surrounds the side chain of Trp25. The Trp-cage plays only a minor role in the interaction with the GLP-1 receptor.[14] Therefore, substitution with Asn-GlcNAc at the opposite side of the receptor binding face in the helical region and C-terminal region resulted in little influence on the receptor binding and agonistic activities.

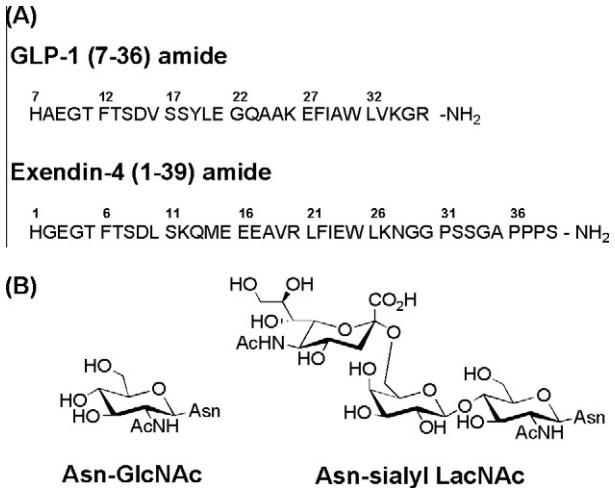

Figure 1. (A) Peptide sequence of GLP-1 and exendin-4. (B) Structure of Asn-GlcNAc and Asn-sialyl LacNAc.

Exendin-4 analogues substituted with Asn-GlcNAc at positions 12, 13, 16, 17, 20, 21, 24, 25 or 28–40, which retained the agonistic activity, were derivatized to analogues having sialyl LacNAc by carbohydrate elongation using glycosyltransferases (Scheme 1).[15] The galactose residue was transferred from uridine-5′-diphosphogalactose (UDP-galactose) to the GlcNAc moiety by β1,4-galactosyltransferase. Sialylation was performed by transfer of sialic acid from cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-NANA) by α2,6-sialyltransferase. All of these glycosylated analogues with sialyl LacNAc were found to retain the agonistic activity, although analogues glycosylated at positions 12, 13, 20, and 25 showed slightly reduced activity compared to the GlcNAc glycosylated analogues (Fig. 2). Introduction of sialyl LacNAc to the positions identified as potential glycosylation sites by Asn-GlcNAc substitution preserved the agonistic activity. On the other hand, alanine substitution scanning could not directly identify the glycosylation sites necessary for retaining the agonistic activity. Alanine substitution scanning of GLP-1 identified positions 9, 11, 14, 16, 17, 20, 32, and 33 as having a minimal effect on the receptor binding and agonistic activity.[16] However, glycosylation at positions 3, 5, 8, 10, 11, 14, 26, and 27 in exendin-4, corresponding to the above positions in GLP-1, led to loss of agonistic activity. Therefore, Asn-GlcNAc scanning substitution is thought to be a prospective strategy for directly identifying glycosylation sites to preserve the receptor binding and agonistic activities.

To investigate the effect of glycosylation on the in vivo activity of exandin-4, the blood glucose-lowering activity of analogues glycosylated with sialyl LacNAc at positions 12, 13, 16, 17, 20, 21, 24, 25 and 28–40 were compared with that of native exendin-4 (non-glycosylated form). After subcutaneous administration of exendin-4 analogues at an equivalent dose (1 nmol/kg) to diabetic *db/db* mice, the blood glucose levels were monitored.[17] Among these glycosylated exendin-4 analogues, the analogue having sialyl LacNAc at position 28 (28N-sialyl LacNAc) was found to show the greatest improvement in in vivo activity with prolonged glucose-lowering activity (Fig. 3). Analogues glycosylated at position 21, 35, and 40 also showed improved in vivo activity, compared to native exendin-4. However, introduction of sialyl LacNAc to position 12, 13, 16, 17, 20, 24, 25, 29–34 and 36–39 did not contribute to improving in vivo activity (data not shown). The pharmacokinetic properties of 28N-sialyl LacNAc were also evaluated in rats by using an immunofluorometric assay. Intravenous administration of 28N-sialyl LacNAc to rats (1 nmol/kg) exhibited a higher plasma concentration as compared to native exendin-4 (data not shown).

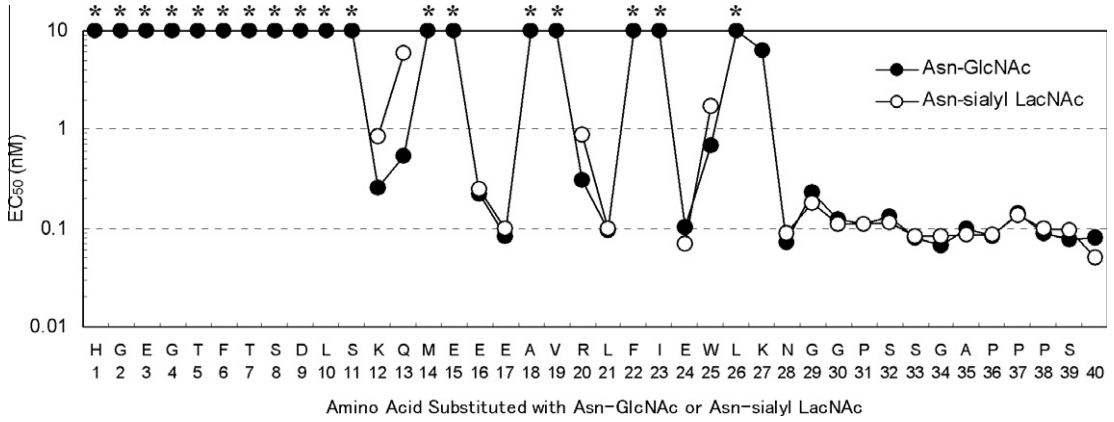

Figure 2. cAMP production activity of Asn-GlcNAc or Asn-sialyl LacNAc substituted analogues. The EC_{50} could not be accurately determined for Asn-GlcNAc substitutions of 1–11, 14, 15, 18, 19, 22, 23, and 26 (designed by *).