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Evaluation of therapeutic potentials of site-specific PEGylated glucagon-like peptide-1 isomers as a type 2 anti-diabetic treatment: Insulinotropic activity, glucose-stabilizing capability, and proteolytic stability

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ABSTRACT

PEGylation has been considered to be a good biotechnique for improving the therapeutic value of glucagon-like peptide-1 (GLP-1) analogs for the treatment of type 2 diabetes. Despite the attractive anti-diabetic potentials, GLP-1 does not exert its full biological action because of its extremely short life-time *in vivo* due to rapid proteolytic degradation. Here, the enzyme-resistant mono-PEGylated GLP-1 isomers substituted at Lys^{26}- or Lys^{34}-amine were prepared through a newly devised site-specific PEGylation process using a maleic anhydride-protection/deprotection method. The therapeutic potentials of these site-specific PEGylated GLP-1 isomers (Lys^{26}- or Lys^{34}-PEG-GLP-1) along with His^{7}-(N-terminus) PEG-GLP-1 were evaluated by examining their insulinotropic activity, glucose-stabilizing capability, and proteolytic stability. Lys^{34}-PEG-GLP-1 was found to have the well-preserved insulinotropic activity (93% efficacy versus GLP-1) in isolated rat pancreatic islets. Furthermore, Lys^{34}-PEG-GLP-1 showed the most prominent glucose-stabilizing capability, evaluated via an oral glucose tolerance test in db/db mice by considering the following three crucial factors: (i) maximum blood glucose level (BGL), (ii) required time to lower the BGL below 100 mg/dl, and (iii) total hypoglycemic degree. Additionally, Lys^{34}-PEG-GLP-1 had longer half-lives than the other PEGylated GLP-1s in the dipeptidyl peptidase IV (DPP IV) inhibitor-treated liver or kidney homogenate, and its stability against DPP IV was also comparable to that of Lys^{26}-PEG-GLP-1. Taken together, Lys^{34}-PEG-GLP-1 displayed the promising characteristics in all evaluations versus His^{7}- or Lys^{26}-PEG-GLP-1. This site-specific PEGylated GLP-1 analog would have therapeutic usefulness for treating type 2 diabetes on account of the well-preserved insulinotropic activity, the increased proteolytic stability, and thereby the improved glucose-stabilizing capability.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is a proglucagon-derived hormone secreted from enteroendocrine L-cells of the intestine in response to orally ingested foods. GLP-1 is attracting increasing interest on account of its prominent benefits in type 2 diabetes [1–3]. GLP-1 acts as a potent incretin to lower the postprandial elevated blood glucose level by stimulating insulin

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secretion and inhibiting glucagon secretion in a glucose-dependent manner [4]. GLP-1 also produces anorectic effects associated with diminished appetite and reduced energy consumption [5,6], and stimulates β-cell proliferation [7]. All these properties have made GLP-1 an attractive treatment.

However, upon release into the circulation, GLP-1 is subjected to severe proteolytic attack by a few dominant enzymes. Therefore, its biological half-life in plasma is only a few minutes (~2 min) [8]. GLP-1 is rapidly inactivated by dipeptidyl peptidase IV (DPP IV), which cleaves GLP-1 at the penultimate N-terminal site of Ala^8 [3]. DPP IV is a ubiquitous enzyme that is particularly abundant in the kidney and liver, and is also present as a soluble form in the plasma [8]. GLP-1 is also a good substrate for neutral endopeptidase (NEP) 24.11 [9,10]. NEP 24.11 has a widespread tissue distribution and is found in particularly high levels in the kidney. NEP 24.11 has been shown to hydrolyze the carboxyl sides of GLP-1 at Asp^{15}, Ser^{18}, Tyr^{19}, Glu^{27}, Phe^{28}, and Trp^{31} [1,9,10]. The proteolysis of GLP-1 means that under physiological conditions, native GLP-1 cannot survive long enough to maintain an effective therapeutic level. For this reason, it has been suggested that the enzyme-resistant GLP-1 forms may be feasible as a promising type 2 anti-diabetic agent.

As with other bioactive peptides, GLP-1 has distinct amino acids involved in the biological action or receptor binding, and metabolism. Therefore, a site-mutation or chemical modification at the specific sites of GLP-1 might result in chimeric GLP-1 analogs with altered bioactivity and proteolytic stability. His^7 replacement by aromatic Tyr or Phe decreased the level of receptor (GLP-1R) binding and activation of GLP-1. His^7 covalent modification with the acyl chain (e.g. acetyl or hexanoyl) was shown to have comparable or increased insulinotropic activity *in vivo* despite the decreased GLP-1R binding *in vitro* [10]. In particular, [D-Ala^2]GLP-1 exerted markedly enhanced hypoglycemic activity in diabetic db/db mice by abolishing the DPP IV sensitivity without a loss of bioactivity *in vitro* [10]. A strong GLP-1R agonist, exendin-4, which is clearly resistant to DPP IV and NEP 24.11 enzymes, showed markedly greater hypoglycemic efficacy than GLP-1 as a result of its extended biological half-life (~3–4 h) *in vivo* and preserved insulinotropic activity *in vitro* [1,12–14]. Therefore, in order to optimize glucose-stabilizing efficacy *in vivo*, it is important to strike a balance between the bioactivity and proteolytic stability of GLP-1. In this respect, a site-specific modification presents a means of maximizing the therapeutic potential of GLP-1 by controlling this balance.

The chemical modification of therapeutic peptides and proteins with polyethylene glycol (PEG), which is known as PEGylation, can increase the therapeutic potential by enhancing the proteolytic resistance, diminishing glomerular filtration, thereby prolonging the biological life-time [15,16]. In particular, PEGylation is associated with an enhanced resistance to various peptidases. PEGylated peptides and proteins have a capability of elevating the proteolytic resistance by shielding the metabolic sites due to increased streric hindrance [17,18]. PEGylation of the other DPP IV-sensitive peptides have been shown to increase their resistance using this mechanism [19–22]. Furthermore, the site-specificity in PEGylating peptides can render them therapeutically attractive by optimizing the proteolytic stability relative to the bioactivity *in vitro*.

Previous studies have demonstrated that PEGylation greatly contributes to improving the poor pharmacokinetic/pharmacodynamic profile of GLP-1 [23-24]. However, in those studies, the PEGylated GLP-1s appeared to exert a restricted therapeutic efficacy because they were prepared using a conventional non-specific PEGylation method. In this study, three different mono-PEGylated GLP-1 isomers were prepared independently through a newly devised site-specific PEGylation process. The therapeutic potentials of these site-specific PEGylated GLP-1 isomers were further evaluated by considering insulinotropic activity, glucose-stabilizing capability, and proteolytic stability.

2. Materials and methods

2.1. Materials

Glucagon-like peptide-1 (7–36)amide (GLP-1) was purchased from Bachem (Torrance, CA, USA). Monomethoxy polyethylene glycol succinimidyl propionate (mPEG-SPA, MW 2000) and monomethoxy polyethylene glycol propionaldehyde (mPEG-PALD, MW 2000) were obtained from Nektar Therapeutics (Huntsvile, AL, USA) and Sunbio (Orinda, CA, USA), respectively. Insulin enzyme immunoassay (EIA) kits were acquired from Mercodia (Uppsala, Sweden). All other reagents used including maleic anhydride (MA) and formic acid, unless otherwise specified, were obtained from Sigma (St. Louis, MO, USA). Type 2 diabetic C57BL/6 db/db mice (male, 7–8 weeks old) were purchased from the Korea Research Institute of Bioscience and Biotechnology, Daejon, Korea).

2.2. Site-specific preparation of PEGylated GLP-1s

Site-specific PEGylation of GLP-1 was achieved separately using two different methods based on the amine types, i.e. N-terminus- or Lys-amine, as shown in Fig. 1. First, the N-terminus (His^7)-amine specific PEGylation was performed using a PEG-aldehyde derivative, which is known to specifically attach to the alpha amine of peptides, according to the procedure described previously [23]. A portion (2.4 mg) of $mPEG_{2\,K}$-PALD was added to 1 ml of GLP-1 (2 mg/ml) in a 50 mM acetate buffer solution (pH 5.5) containing 20 mM sodium cyanoborohydride ($NaCNBH_3$) (molar ratio of GLP-1 to PEG = 1–2). The reaction was allowed to continue at 4 °C for 2 h, and was quenched by adjusting the pH to ~2.2 with 1% TFA/deionized water (DW). Second, the Lys (Lys^{26} or Lys^{34})-amine specific PEGylation was accomplished through an amine-protection/deprotection method using a reversible amine-protective reagent, maleic anhydride (MA), as previously described [25,26]. To achieve this, two series of MA-protected GLP-1s of His^7, Lys^{34}-di-MA-GLP-1 and His^7, Lys^{26}-di-MA-GLP-1 were prepared for the Lys^{26}- and Lys^{34}-amine specific PEGylations, respectively. Briefly, GLP-1 (1 mg/ml) in a 50 mM phosphate buffer solution (PB, pH 7.0) was reacted with a 6-time molar excess of MA at room temperature for 10 min. The reaction mixture was then subjected to reversed-phase high-performance liquid chromatography (RP-HPLC) on a CAPCELL PAK C_{18} column (250 μm × 4.6 μm, 5 μm, Shiseido Co. Ltd., Japan) at ambient temperature. Gradient elution was carried out at a flow-rate of 1.0 m/min with solvent A (0.1% TFA in DW) and solvent B (0.1% TFA in acetonitrile), using a 36–46% B linear

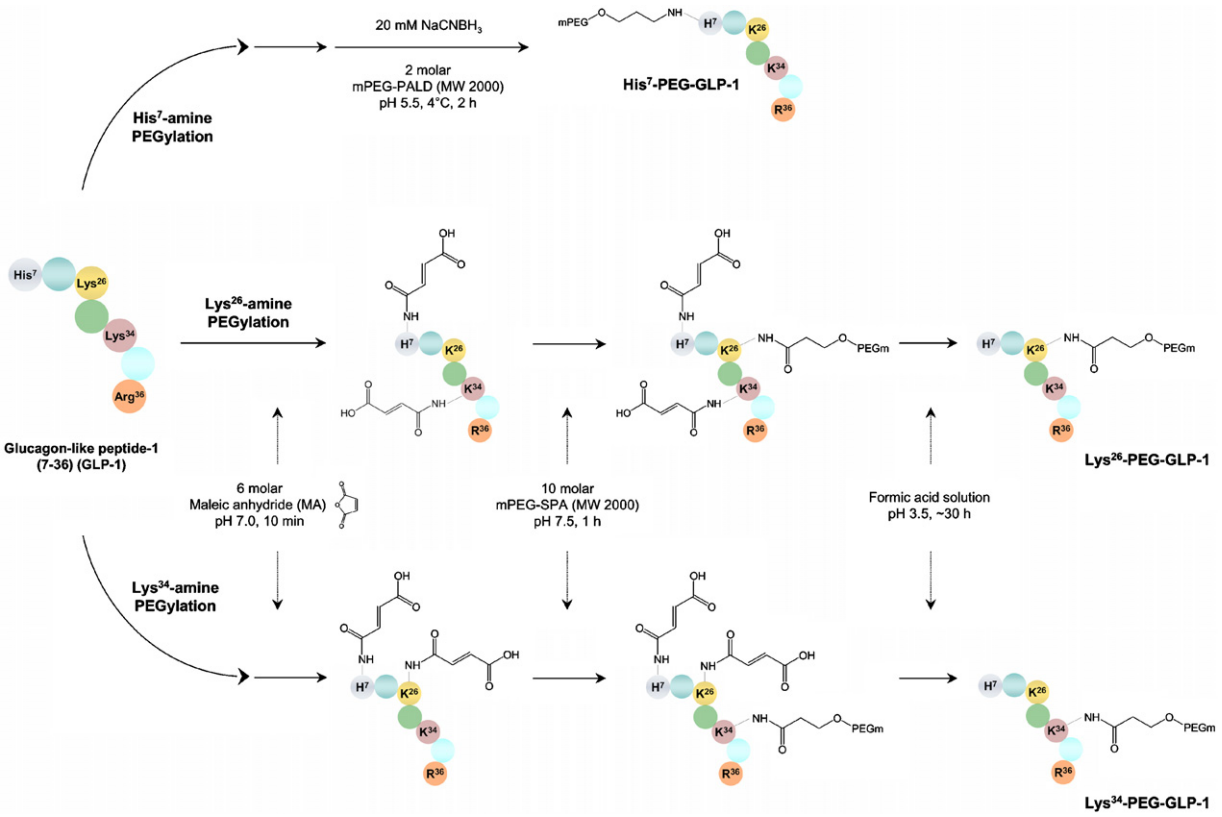

Fig. 1 – Site-specific PEGylation strategy for GLP-1 according to its target amines. His^{7}-PEG-GLP-1 was prepared using an N-terminus specific PEGylation method, and Lys^{26}- and Lys^{34}-PEG-GLP-1 were prepared using a MA-protection/deprotection strategy.

gradient over 15 min. Eluates were monitored at 215 nm. Fractions were collected separately, dried under nitrogen, and stored in PB (100 mM, pH 7.5) at 4 °C until needed. The Lys-specific PEGylation process consisted of two independent reaction steps (Fig. 1): the first reaction step involved the conjugation of MA-protected GLP-1s with mPEG-SPA, and the second involved the deprotection of the MA groups from the PEGylated MA-protected GLP-1s. PEGylation itself was carried out using a conventional method. A 10-time molar excess of $mPEG_{2\ K}$-SPA was added to aliquots (1 ml) of MA-protected GLP-1s in 100 mM PB (pH 7.5, 1 mg/ml), and the reaction was allowed to continue at ambient temperature for 1 h. The PEGylated MA-protected GLP-1s were purified using the afore-mentioned RP-HPLC procedure, and was then dialyzed with a formic acid solution (pH 3.5) using a dialysis kit (MW cutoff = 3500, Gene Bio-Application Ltd., Israel). MA-deprotection was conducted to incubate the dialyzed solutions containing PEGylated MA-protected GLP-1s at 37 °C. After approximately 30 h, the resulting mixture was subjected again to the same RP-HPLC procedure, and the MA-deprotected PEGylated GLP-1 fractions were collected separately, dried under nitrogen, and stored in a 10 mM phosphate buffer saline solution (PBS, pH 7.4) at 4 °C until needed.

2.3. *Characterization of GLP-1 derivatives*

GLP-1 and its various derivatives (Table 1), along with their conjugation sites were identified using a slight modification of a Lys-C enzyme digestion method followed by MALDI-TOF MS (Voyager-RP Biospectrometry Workstation, PerSeptive Biosystems, Cambridge, MA) analysis described elsewhere [23,27].

2.4. *Insulinotropic activity in isolated rat pancreatic islets*

Insulinotropic activities of GLP-1 and PEGylated GLP-1s in rat pancreatic islets were assessed using a method described previously [23,24]. Briefly, male Sprague–Dawley rats weighting 240–280 g were anesthetized by an intraperitoneal (i.p.) injection of a mixture of ketamine and xylazine (90/10 mg/kg). Rat pancreas was inflated by injecting a cold Hank's balanced buffered salt solution (HBSS, pH 7.4, Sigma) containing 1.5 mg/ml type V collagenase (Sigma). The isolated islets were purified by centrifugation with a stepwise Ficoll (Amersham Biosciences AB, Uppsala, Sweden) gradient at 2400 rpm for 25 min. Purified islets were cultured with a RPMI 1640 culture medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA) and 1% penicillin-streptomycin (Gibco) at 37 °C in a humidified 95% air/5% CO_2 atmosphere. After a 2-day maintenance period, the islets were washed and incubated in a Krebs–Ringer bicarbonate (KRB, Sigma) buffer solution. Twenty islets were incubated for 2 h in 2 ml of KRB–HEPES in the same atmosphere at 37 °C. The concentration of insulin released was determined using an EIA kit (Mercodia).

Table 1 – Characterization of GLP-1 (7–36) derivatives and their conjugation sites

GLP-1 (7–36) derivatives	Conjugated moieties	Calculated total masses (*m*/z[a])	Possible Lys-C enzyme-digested fragments	
			Calculated masses (*m*/z)	Observed masses (*m*/z)
His^7, Lys^{26}-di-MA-GLP-1	Maleic anhydride	3494.8	His^7-MA–Lys^{26}-MA–Lys^{34}: 3281.5; Gly^{35}–Arg^{36}: 231.2	3281.1 (Peak a)
His^7, Lys^{34}-di-MA-GLP-1			His^7-MA–Lys^{26}: 2196.3; Glu^{27}–Lys^{34}-MA–Arg^{36}:1316.6	1316.3 and 2196.7 (Peak b)
His^7-PEG-GLP-1	$mPEG_{2\ K}$-PALD	5355 (Mn[b])	His^7-$PEG_{2\ K}$-Lys^{26}: 4154 (Mn); Glu^{27}-Lys^{34}:1005.2; Gly^{35}-Arg^{36}: 231.2	1006.1 and 4138 (Mn)
Lys^{26}-PEG-GLP-1	$mPEG_{2\ K}$-SPA	5550 (Mn[b])	His^7-Lys^{26}-$PEG_{2\ K}$-Lys^{34}: 5340 (Mn); Gly^{35}-Arg^{36}: 231.2	5305 (Mn)
Lys^{34}-PEG-GLP-1			His^7-Lys^{26}: 2098.2; Glu^{27}-Lys^{34}-$PEG_{2\ K}$-Arg^{36}: 3474 (Mn)	2100.2 and 3422 (Mn)

GLP-1 derivative samples treated with a Lys-C enzyme solution were subjected to MALDI-TOF mass spectrometry. Their conjugation sites were confirmed by comparing the observed masses with the calculated masses.

[a] Mass/charge.

[b] Number-averaged molecular mass.

2.5. *Glucose-stabilizing capability in type 2 diabetic db/db mice*

Glucose-stabilizing capabilities of GLP-1 and PEGylated GLP-1s in db/db mice were assessed using a method described elsewhere [24]. At 30 min before administrating glucose orally, diabetic mice, which had been fasted for 18 h, received an i.p. injection of saline, GLP-1, and PEGylated GLP-1s (5 nmol/kg). At 0 min, a 1.0 g/kg dose of glucose in DW was administered orally to each mouse ($n = 7–11$). At predetermined times, a drop of blood was drawn from the tail vein, and the blood glucose levels were determined by using a one-touch blood glucose meter (ACCU-CHEK® Sensor, Roche Diagnostics Corp., USA). The glucose-stabilizing capability was evaluated by considering the following three factors: (i) maximum blood glucose level (BGL_{max}), (ii) required time to lower the blood glucose level below 100 (±5) mg/dl ($T_{BGL < 100\ mg/dl}$), and (iii) total hypoglycemic degree ($HGD\%_{total}$). The total hypoglycemic degree (% versus saline group) was calculated as follows: $[(AUC_{saline,\ 0–180\ min} - AUC_{test,\ 0–180\ min})/AUC_{saline,\ 0–180\ min}] \times 100$.

2.6. *Proteolytic stability evaluation of site-specific PEGylated GLP-1 isomers*

Proteolytic stabilities of GLP-1 and PEGylated GLP-1s were evaluated in dipeptidyl peptidase IV (DPP IV) enzyme, rat liver and kidney homogenate solutions containing 10% DPP IV inhibitor (v/v, Linco Research Inc., St. Charles, MO, USA) using a slight modification of a method reported elsewhere [17,23]. DPP IV enzyme solution was prepared to a concentration (100 mU/ml) in a PBS solution (pH 7.4). DPP IV inhibitor-treated rat liver or kidney homogenate solution was prepared from the supernatants of their original homogenized tissue organs [24], and each protein concentration was adjusted to 40 mg (BSA)/ml. A portion (25 μl) of either GLP-1 or PEGylated GLP-1s solution (200 μg/ml) was mixed with the same volume of the enzyme solutions (pre-incubated at 37 °C for 15 min), and further allowed to incubate at 37 °C. At predetermined times, the incubations were quenched by adding 200 μl of 1% TFA/DW and ice-cold methanol for the DPP IV enzyme and the DPP IV inhibitor-treated tissue homogenates, respectively. The resulting mixtures were centrifuged at 12,000 rpm for 5 min. Supernatants were then subjected to RP-HPLC on a LiChrospher 100 RP-18 (250 μm × 4.0 μm, 5 μm, Merck, Germany) at ambient temperature. The following linear gradient profile was used: 36–48% B over 20 min. Eluates were monitored at 215 nm. The peak areas were measured, and the degradation half-lives obtained from time versus residual amount curves were calculated by assuming first-order kinetics.

2.7. *Data analysis*

Data are presented as means ± S.D.s. Significances were determined using the Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. *Preparation and characterization of MA-protected GLP-1s*

The conjugation of GLP-1 with MA resulted in four main peaks in the RP-HPLC chromatogram, as shown in Fig. 2. These fractions collected were subjected to MALDI-TOF MS, and their identities depending on the number of attached MA were assigned by determining the molecular mass. Among these, the second and third peaks (peak a and b, respectively) corresponded to di-MA-GLP-1s, and these fractions were further subjected to Lys-C enzyme digestion followed by MALDI-TOF MS analysis. As summarized in Table 1, the molecular masses of the Lys-C digested peaks a and b fragments revealed their own identities depending on the sites of attached MA, compared to the calculated masses. Subsequently, peaks a and b were assigned as the His^7, Lys^{26}-di-MA-GLP-1 and His^7, Lys^{34}-di-MA-GLP-1, respectively. In this way, two di-MA-GLP-1 conjugates were prepared, which remained open at the target amines (Lys^{26} or Lys^{34}) for site-specific PEGylation and protected the GLP-1 at the other amines.

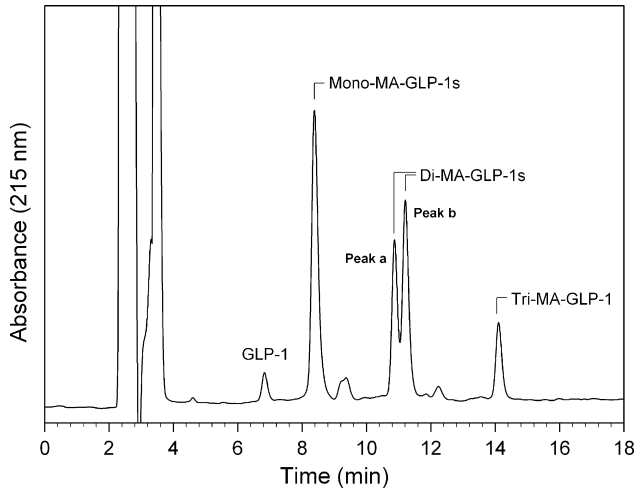

Fig. 2 – RP-HPLC chromatogram of the maleic anhydride (MA) conjugated GLP-1 mixture. Reaction mixture was applied to a C_{18} analytical column at ambient temperature. Gradient elution was carried out at a flow-rate of 1.0 ml/min using with solvent A (0.1% TFA in deionized water) and solvent B (0.1% TFA in acetonitrile), using a 36–46% B linear gradient over 15 min.

3.2. *Site-specific preparation of PEGylated GLP-1 isomers*

The His^7-amine specific PEGylation of GLP-1 was performed using an N-terminus specific PEGylation method, as previously described [23,24]. The molecular masses of His^7-amine specific PEGylated GLP-1 (His^7-PEG-GLP-1) and its Lys-C digested fragments confirmed that this isomer has only one PEG molecule at the His^7-amine site (Table 1). Separately, Lys (Lys^{26} or Lys^{34})-amine specific PEGylations of GLP-1 were performed using the His^7, Lys^{34}-di-MA-GLP-1 and His^7, Lys^{26}-di-MA-GLP-1 derivatives, respectively. Since these have only one amine available for PEGylation at Lys^{26} or Lys^{34}, an excess of PEG could be added to increase the reaction yield, without site-competition. The reaction yields at this step were approximately >90% in both cases. The MA groups of the PEGylated di-MA-GLP-1s were further detached by incubating them with a formic acid solution (pH 3.5) at 37 °C. The total and Lys-C digested masses of the Lys^{26}- and Lys^{34}-amine specific PEGylated GLP-1s (Lys^{26}- and Lys^{34}-PEG-GLP-1s) showed appropriate identities according to the number and site of PEGylation (Fig. 3 and Table 1).

3.3. *In vitro insulinotropic activity*

The effects of GLP-1 and PEGylated GLP-1s on glucose-dependent insulin release were evaluated in the isolated rat pancreatic islets to determine their bioactivities. At a low glucose concentration of 5.5 mM, the islets did not show any significant release of insulin in response to GLP-1 or PEGylated GLP-1s: insulin levels were in the range of 3.5 ± 1.4 to 5.1 ± 1.0 pM/islet/h in all groups including the saline. However, at a high glucose concentration of 16.8 mM, a significant dose-response release of insulin was observed in the islets after stimulation with GLP-1 and PEGylated GLP-1s. As shown

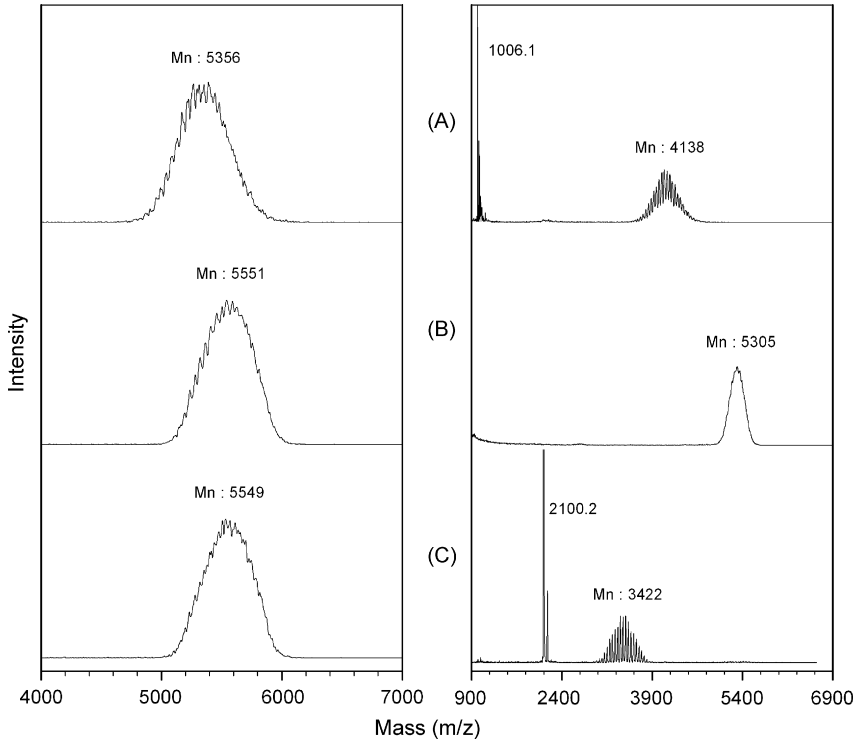

Fig. 3 – MALDI-TOF mass spectra of the PEGylated GLP-1s (left panel) and their Lys-C digested fragments (right panel): (A) His^7-PEG-GLP-1; (B) Lys^{26}-PEG-GLP-1; and (C) Lys^{34}-PEG-GLP-1. A Lys-C enzyme solution (10 μg/ml in 50 mM Tris–HCl, pH 8.5) was mixed with the GLP-1 derivative sample solutions (100 μg/ml), and the digestion was allowed to continue at 37 °C for 30 min. The resulting samples were subjected to MALDI-TOF MS.

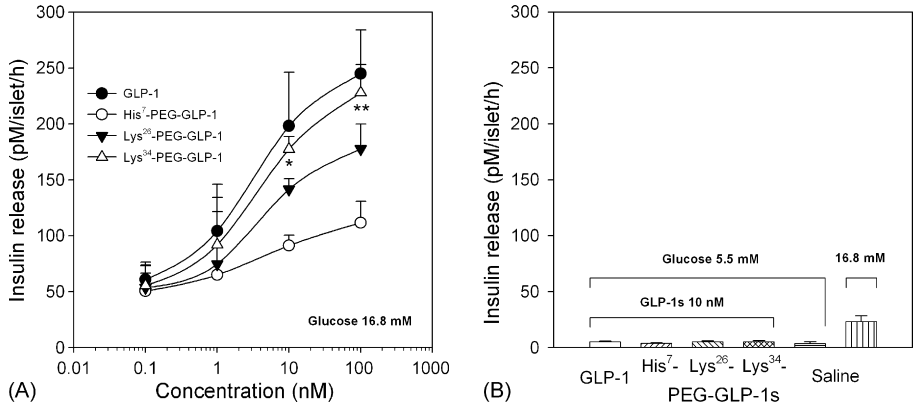

Fig. 4 – Insulinotropic activities of GLP-1 and PEGylated GLP-1s in isolated rat pancreatic islets: (A) dose-response curves of each GLP-1 species at a glucose concentration of 16.8 mM; (B) insulin releases by each GLP-1 species (10 nM) and saline at 5.5 mM or by saline at 16.8 mM. Data are presented as means ± S.D.s of four to six determinations. $^{*}P > 0.49$ over GLP-1, $P < 0.04$ over Lys^{26}-PEG-GLP-1, and $P < 0.0001$ over His^{7}-PEG-GLP-1; $^{**}P > 0.57$ over GLP-1, $P < 0.07$ over Lys^{26}-PEG-GLP-1, and $P < 0.001$ over His^{7}-PEG-GLP-1.

in Fig. 4 and Table 2, the potency (EC_{50}, nM) of GLP-1 and PEGylated GLP-1s (His^{7}-, Lys^{26}-, and Lys^{34}-PEG-GLP-1s) was not significantly altered. In contrast, their efficacy (E_{max}: insulin release, pM/islet/h) increased clearly and differed from the GLP-1 species at a peptide concentration of 100 nM (111.5 ± 19.2 to 244.8 ± 39.2 nM, respectively; Table 2). In particular, Lys^{34}-PEG-GLP-1 was as effective in stimulating the islets to release insulin at this concentration as the native GLP-1 ($P > 0.57$), and was more efficacious than His^{7}- and Lys^{26}-PEG-GLP-1s ($P < 0.001$ and $P < 0.07$, respectively). The glucose-dependency score, represented as the increased fold of the efficacy response at 16.8 mM glucose relative to that at 5.5 mM (10 nM peptide), of Lys^{34}-PEG-GLP-1 was comparable to that of native GLP-1 and clearly higher than those of the other isomers (Table 2).

3.4. *In vivo glucose-stabilizing capability*

In order to evaluate the glucose-stabilizing capability of GLP-1 and PEGylated GLP-1s, an oral glucose tolerance test (OGTT) was performed after an i.p. GLP-1 species administration (−30 min) in type 2 diabetic db/db mice (Fig. 5A). The glucose-stabilizing capability was evaluated comprehensively by considering three factors, (i) BGL_{max}, (ii) $T_{BGL < 100\ mg/dl}$, and (iii) $HGD\%_{total}$. Lys^{34}-PEG-GLP-1 was found to have the most significant glucose-stabilizing capability in all factors evaluated. The BGL_{max} value of Lys^{34}-PEG-GLP-1 (241.0 ± 54.6 mg/dl) was clearly lower than those of saline, GLP-1, and His^{7}-PEG-GLP-1 (446.0 ± 63.8, 383.5 ± 25.3, and 364.7 ± 40.2 mg/dl, respectively), and similar to that of Lys^{26}-PEG-GLP-1 (243.7 ± 25.6 mg/dl). As shown in Fig. 5A and Table 2, Lys^{34}-PEG-GLP-1 rapidly lowered the blood glucose level (BGL) <100 mg/dl within 45 min after administration. In contrast, the $T_{BGL < 100\ mg/dl}$ values of the saline and GLP-1 groups, and of the His^{7}-PEG-GLP-1 and Lys^{26}-PEG-GLP-1 groups were delayed to 180 and 120 min, respectively. As a result, Lys^{34}-PEG-GLP-1 showed the greatest total hypoglycemic degree, as shown in Fig. 5B and Table 2. In particular, the hypoglycemic degree of Lys^{34}-PEG-GLP-1 was significantly higher than that of Lys^{26}-PEG-GLP-1 ($P < 0.00005$).

Table 2 – Therapeutic efficacy scores of GLP-1 and PEGylated GLP-1s: *in vitro* insulinotropic activity and *in vivo* glucose-stabilizing capability

Groups/ properties	Insulinotropic activity			Glucose-stabilizing capability		
	Potency: EC_{50} (nM)	Efficacy: E_{max} (pM/islet/h) (retention%)	Glucose-dependency[a]: fold increased	BGL_{max}[b] (mg/dl)	$T_{BGL < 100\ mg/dl}$[c] (min)	$HGD\%_{total}$[d] (vs. saline)
Saline	–	–	–	446.0 ± 63.8	180	–
GLP-1	3.37 ± 0.75	244.8 ± 39.2 (100.0%)	40.5	383.5 ± 25.3	180	21.9 ± 11.5
His^{7}-PEG-GLP-1	5.18 ± 1.23	111.5 ± 19.2 (45.4%)	24.5	364.7 ± 40.2	120	34.1 ± 12.4
Lys^{26}-PEG-GLP-1	4.59 ± 1.01	177.5 ± 22.3 (72.5%)	28.9	243.7 ± 25.6	120	46.8 ± 5.8
Lys^{34}-PEG-GLP-1	4.22 ± 1.00	227.7 ± 25.1 (93.0%)	35.0	241.0 ± 54.6	45	61.7 ± 3.7

Insulinotropic activity was determined using isolated rat pancreatic islet cell ($n = 6$), and glucose-stabilizing capability was evaluated using an oral glucose tolerance test (OGTT) in type 2 diabetic db/db mice ($n = 7–11$).

[a] Increased fold of the efficacy response at 16.8 mM glucose relative to that at 5.5 mM (10 nM peptide).

[b] Maximum blood glucose level.

[c] Required time to lower the blood glucose level below 100 (±5) mg/dl.

[d] Total hypoglycemic degree. Significances were shown in each figure.

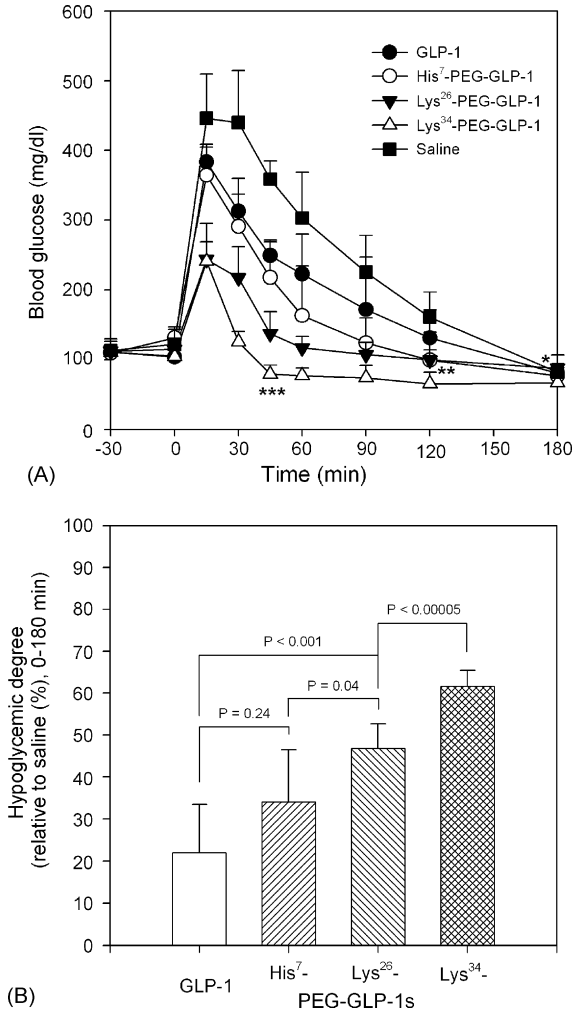

Fig. 5 – (A) Glucose-stabilizing profiles of GLP-1 and PEGylated GLP-1s in type 2 diabetic db/db mice fasted for 18 h, after the intraperitoneal administration of the drugs (−30 min, 5 nmol/kg) and the oral administration of glucose (0 min, 1.0 g/kg). (B) Hypoglycemic degrees of each GLP-1 species relative to the saline. Data are presented as means ± S.D.s of seven to eleven determinations. Asterisks (*) represent the required time for the saline and GLP-1 to lower the glucose concentration below 100 (± 5) mg/dl; double asterisk (**) for His^7-PEG-GLP-1 and Lys^{26}-PEG-GLP-1; and triple asterisk (***) for Lys^{34}-PEG-GLP-1.

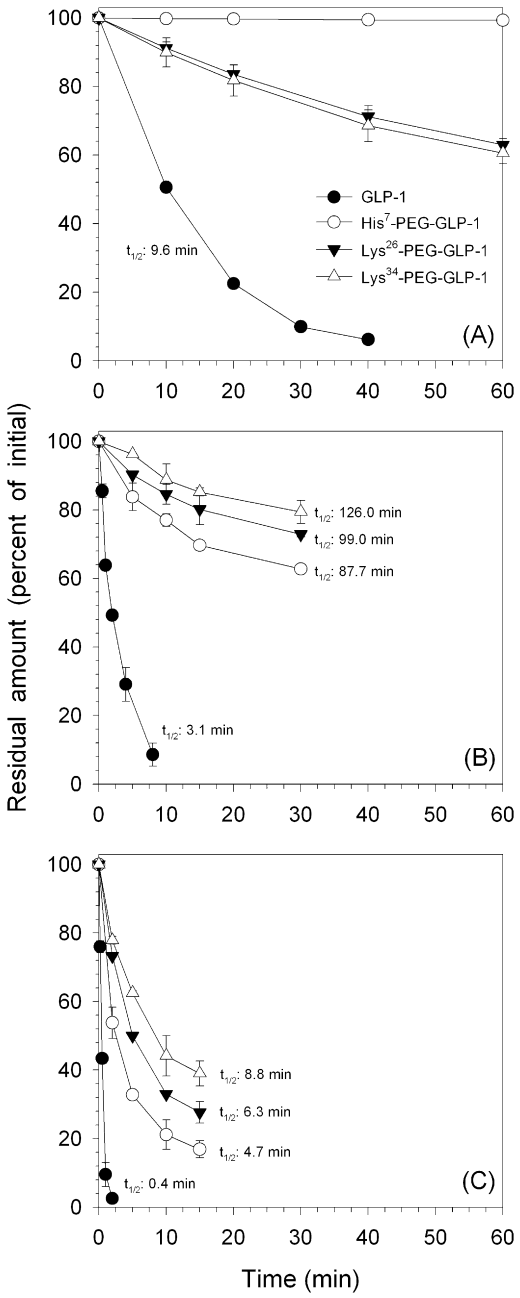

Fig. 6 – Degradation profiles of GLP-1 and PEGylated GLP-1s incubated with (A) isolated DPP IV enzyme, (B) liver and (C) kidney homogenates containing 10% (v/v) DPP IV inhibitor. Residual amount of the samples were quantified by RP-HPLC. Data are presented as means ± S.D.s for three determinations.

3.5. *Proteolytic stability*

The proteolytic stabilities of the PEGylated GLP-1 isomers were observed in an isolated DPP IV enzyme and a DPP IV inhibitor-treated liver or kidney homogenate. Consistent with previous studies [23,24], GLP-1 was rapidly degraded in all these enzyme systems, with half-lives of 9.6, 3.1, and 0.4 min, respectively (Fig. 6A). In contrast, the PEGylated GLP-1s showed much higher resistance to these enzymes. The DPP IV enzyme barely degraded His^7-PEG-GLP-1 because of its N-terminus modification. However, it had significantly shorter half-lives than those of Lys^{26}- and Lys^{34}-PEG-GLP-1s in both DPP IV inhibitor-treated tissue homogenates (Fig. 6B and C). In particular, Lys^{34}-PEG-GLP-1 had longer half-lives (126.0 and 8.8 min) than those of both His^7-PEG-GLP-1 and Lys^{26}-PEG-GLP-1 (99.0 and 6.3 min; 87.7 and 4.7 min, respectively) in the DPP IV inhibitor-treated liver and kidney homogenates, respectively. In addition, the stability of Lys^{34}-PEG-GLP-1 against DPP IV was comparable to that of Lys^{26}-PEG-GLP-1 ($t_{1/2}$: 88.1 versus 95.7 min) (Fig. 6C).

4. Discussion

Conventional non-specific PEGylation generates a mixture consisting of heterogeneous isomers according to the number and site of PEG-attachment. Some of these PEGylated isomers may be biologically inactive or metabolically unnecessary because the target functional groups (e.g. amino-, thiol-, and carboxyl-groups, etc.) of therapeutic peptides are randomly distributed and may sometimes be either proximal to an active or distal to a metabolic site [28]. Therefore, site-specific PEGylation has been viewed as a way of circumventing this pharmacological ineffectiveness [22,29–31].

The amine-directed mono-PEGylation of GLP-1 results in the three different isomers of His^7-, Lys^{26}-, and Lys^{34}-PEG-GLP-1. Initially, an attempt was made to prepare the isomers by isolating from a PEGylated mixture using a modification of the RP-HPLC methods described previously [18,27]. However, isomer separation was not simply achieved in this case: the respective peaks overlapped one another due to their very similar hydrophobicities even under optimized RP-HPLC conditions. For this reason, previous studies [23,24] primarily used two series of mono-PEGylated GLP-1s, (i) His^7-PEG-GLP-1 prepared using an N-terminus specific PEG reagent or (ii) Lys (Lys^{26} or Lys^{34})-PEG-GLP-1 mixture prepared by pH control. In particular, a homogenous PEGylated GLP-1 at only one target Lys-amine could not be obtained, although Lys^{34}-PEG-GLP-1 was a more predominant form than Lys^{26}-PEG-GLP-1. Therefore, the precisely controlled site-specific preparation for these isomers was a prerequisite to accomplish the further therapeutic evaluations. Accordingly, a unique PEGylation method using a MA-protection/deprotection strategy was devised.

His^7, Lys^{34}-di-MA-GLP-1 and His^7, Lys^{26}-di-MA-GLP-1 derivatives were introduced in order to prepare the two Lys^{26}- and Lys^{34}-PEG-GLP-1s independently. MA groups in these two derivatives were conjugated to their respective aimed amines of GLP-1 (Table 1), and this protection provided a much greater opportunity for the Lys-amine specific PEGylation (Fig. 1). Based on these derivatives, PEGylation was carried out by simply adding a molar excess of PEG without having any negative influence on the overall process. The MA groups of the PEGylated MA-protected GLP-1 intermediates were then clearly removed by a simple deprotection process (Fig. 1). Through this successive process (MA-protection, PEG-attachment, and MA-deprotection), both Lys^{26}- and Lys^{34}-PEG-GLP-1s could be successfully prepared. Separately, His^7-PEG-GLP-1 was simply prepared via the N-terminus specific PEGylation process in high reaction yield >85%, as described in previous studies [23,24]. As shown in Fig. 3, the MALDI-TOF mass spectra of the PEGylated GLP-1 isomers and their Lys-C enzyme digested fragments showed that all these isomers contained only one PEG molecule at their own target amine sites.

Biological evaluation of the site-specific PEGylated GLP-1 isomers was performed *in vitro*. Since His^7-PEG-GLP-1 was shown to have little bioactivity *in vitro* [22,23], more emphasis was placed on comparing the bioactivities of the Lys^{26}- and Lys^{34}-amine PEGylated GLP-1s. As shown in Fig. 4, Lys^{34}-PEG-GLP-1 was as effective in exerting an insulinotropic action as native GLP-1 ($P > 0.57$). Interestingly, Lys^{34}-PEG-GLP-1 had a higher insulinotropic activity than Lys^{26}-PEG-GLP-1: both the efficacy and potency in Lys^{34}-PEG-GLP-1 were higher than Lys^{26}-PEG-GLP-1. It is well-known that the imidazole ring structure of the His^7 of GLP-1 is essential for its biological action [11,32]. In contrast, both the Lys^{26}- and Lys^{34}-amines of GLP-1 were weakly associated with its biological action [33]. Therefore, Lys^{34}-amine PEGylation may have a lower hindrance to the N-terminus of GLP-1 than Lys^{26}-amine PEGylation due to likely spatial distance. These results are consistent with those reported in a previous study. Maleimido derivatized GLP-1-albumin conjugate at Lys^{34}-amine was found to have a significantly stronger GLP-1R binding and cAMP stimulation than those of the conjugates at Lys^{26}- or His^7-amine [34]. Therefore, compared with Lys^{26}-amine, CJC-1131 appeared to be constructed to have a maleimido derivative at an additional Lys^{37}-amine on the GLP-1 frame, which is distal to the N-terminus [34]. Subsequently, it was assumed that the spatial distance, to some extent, from the N-terminus was associated with maintaining the GLP-1 bioactivity. Separately, the glucose-dependency in insulin release, which is an important biological property of GLP-1 to prevent hypoglycemia, was also maintained after PEGylation, as shown in Fig. 4B. This suggests that the PEGylated GLP-1s can be used as a potential replacement for the native GLP-1 (Table 2).

Glucose-stabilizing capabilities of GLP-1 and PEGylated GLP-1s were evaluated in terms of BGL_{max}, $T_{BGL < 100\ mg/dl}$, and $HGD\%_{total}$ via an OGTT in type 2 diabetic db/db mice. As shown in Fig. 4, GLP-1 did not stabilize effectively the blood glucose level against the oral glucose challenge because of its short biological life-time as reported previously [1]. Also, the level was not quite stabilized until 180 min by an i.p. His^7-PEG-GLP-1 administration owing to the greatly reduced insulinotropic activity *in vitro* (Fig. 3 and Table 2). In contrast, Lys^{34}-PEG-GLP-1 displayed the prominent glucose-stabilizing efficacy *in vivo*. In particular, Lys^{34}-PEG-GLP-1 was found to be superior to GLP-1 and the other isomers in all those considerations (BGL_{max}, $T_{BGL < 100\ mg/dl}$, and $HGD\%_{total}$). Lys^{34}-PEG-GLP-1 had the lowest peak glucose level (BGL_{max}; 241.0 ± 54.6 mg/dl) and showed the fastest stabilization ($T_{BGL < 100\ mg/dl}$; 45 min) in the blood glucose level, following an oral glucose administration. Consequently, it was shown to exert the greater hypoglycemic efficacy ($HGD\%_{total}$; 61.7 ± 3.7%) than the other isomers. This improved glucose-stabilizing capability of Lys^{34}-PEG-GLP-1 is primarily associated with its well-preserved *in vitro* insulinotropic activity due to its higher receptor binding or activation. Another crucial reason for this finding appeared to be associated with its increased proteolytic stability.

As with other peptides, GLP-1 has been reported to be cleared by two dominant elimination pathways, proteolytic degradation and glomerular filtration [1]. However, in this study, the latter factor was excluded because PEGylated GLP-1s are likely to have similar molecular sizes due to their consistent mono-PEGylation presence, indicating the same glomerular filtration rate. Therefore, the main focus was on the proteolytic stability in the well-known two dominant enzymes of DPP IV and NEP 24.11. The isolated DPP IV enzyme, and DPP IV inhibitor-treated liver or kidney homogenate were used for this set of stability experiments. DPP IV and NEP 24.11 are mainly present in the kidney and liver. Therefore, a DPP IV inhibitor-treated kidney or liver homogenate was presumed to

be a suitable NEP 24.11 enzyme pool in this system. As shown in Fig. 6A, the DPP IV resistance of Lys^{34}-PEG-GLP-1 was comparable to that of Lys^{26}-PEG-GLP-1, indicating a similar steric hindrance to the Ala^2 site. Furthermore, Lys^{34}-PEG-GLP-1 was found to have superior resistance to His^7-PEG-GLP-1 as well as Lys^{26}-PEG-GLP-1 in the DPP IV inhibitor-treated tissue homogenates, indicating the effectiveness of Lys^{34}-amine specific PEGylation in shielding the proteolysis from various enzymes including NEP 24.11. Thus, the increased stability of Lys^{34}-PEG-GLP-1 against these two predominant enzymes must be another main reason for its improved biological efficacy *in vivo*.

Taken together, the site-specific Lys^{34}-PEG-GLP-1 was found to have significantly improved *in vivo* glucose-stabilizing efficacy than the other PEGylated GLP-1 isomers (His^7- or Lys^{26}-PEG-GLP1). The current study shows that this observation is clearly due to the dual advantages by the Lys^{34}-amine specific PEGylation of GLP-1 in terms of well-preserved insulinotropic activity and increased proteolytic stability. Therefore, an optimized balance between these two factors is essential for preparing and selecting site-specific PEGylated peptide isomers with promising therapeutic potential. Moreover, we are undertaking its additional desirable therapeutic effects of GLP-1, including an examination of the anorectic properties *in vivo*. In addition, the devised site-specific PEGylation technique using a unique MA-protection/deprotection method has great therapeutic usefulness because of its ability to selectively produce the most attractive PEGylated form of therapeutic peptides.

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