Enzymatic mono-pegylation of glucagon-like peptide 1 towards long lasting treatment of type 2 diabetes

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ABSTRACT

Human glucagon-like peptide-1 (GLP-1) is a physiological gastrointestinal peptide with glucose-dependent insulinotropic effects which is therefore considered an interesting antidiabetic agent. However, after *in vivo* administration, exogenous GLP-1 does not exert its physiological action due to the combination of rapid proteolytic degradation by ubiquitous dipeptidyldipeptidase IV (DPP IV) enzyme and renal clearance resulting in an extremely short circulating half-life. In this work we describe the conjugation of GLP-1-(7-36)-amide derivatives with polyethylene glycol (PEG) by enzymatic site-specific transglutamination reaction as an approach to reduce both the proteolysis and the renal clearance rates.

The compound GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa monopegylated on the single glutamine residue naturally present in position 23 maintained the ability to activate the GLP-1 receptor expressed in the rat β-cell line RIN-m5F with nanomolar potency along with an increased *in vitro* resistance to DDP IV and a circulating half-life of about 12 h after subcutaneous administration in rats. These properties enabled GLP-(7-36)-amide-Q^{23}-PEG 20 kDa to exert a glucose-stabilizing effect for a period as long as 8 h, as demonstrated by a single subcutaneous injection to diabetic mice concomitantly challenged with an oral glucose load.

The results reported in this work indicate that GLP-(7-36)-amide-Q^{23}-PEG 20 kDa could be a lead compound for the development of long-lasting anti-diabetic agents useful in the treatment of type 2 diabetes affected patients.

1. Introduction

Human glucagon-like peptide-1 (GLP-1), a prototypical incretin hormone that potentiates insulin secretion under elevated glycemic conditions, is a posttranslational cleavage product of proglucagon which is secreted from enteroendocrine L-cells of the intestine after food intake. GLP-1 displays a potent blood glucose-lowering effect through different physiological mechanisms, including the secretion of endogenous insulin in a glucose-dependent manner, the decrease of blood glucagon levels and the reduction of gastric emptying by slowing gastric motility [1]. Besides these properties, GLP-1 stimulates the proliferation and differentiation of new pancreas β-cells leading to increase of β-cell mass [2]. The major form of circulating human GLP-1 is a C-terminal amidated peptide of 30 amino acid residues indicated as GLP-1-(7-36)-amide, while a minor C-terminally glycine extended form of 31 amino acid residues, termed GLP-1(7-37), is also detectable in blood. Both peptides exhibit the same biological activities, are equipotent and exert their effects by binding and activating a specific receptor, named GLP-1 receptor and structurally related to G-protein coupled receptor class 2 family, which is predominantly coupled to stimulation of adenylyl cyclase activity [3]. GLP-1 receptors are expressed in the β-cell of the islets of Langerhans, as well as in gastrointestinal tract and in other tissues including heart, kidney, lung and brain as well as vascular endothelium [4,5].

The insulinotropic action of GLP-1 peptides, that is the stimulation of insulin secretion occurring when plasma glucose levels are above the normal physiological value, makes these compounds potential candidates for the treatment of type 2 diabetes. However, the therapeutic use of GLP-1 peptides is limited by a very short plasma half-life (for example GLP-1-(7-36)-amide has a $t_{1/2} < 1.5$ min after intravenous administration) mainly due to rapid degradation by plasma dipeptidyl peptidase IV (DPP-IV) or CD26, a serine-type protease that cleaves N-terminal dipeptides from polypeptide chains after a proline or alanine residue. The biological activity of naturally circulating GLP-1 peptides is in fact modulated by the N-terminal DPP-IV-mediated

cleavage at the alanine-2 residue to give the inactive metabolite des-His–Ala-GLP-1-(7-36)-amide peptide.

Examples of more stable GLP-1 analogues include exendin-3 and exendin-4, two 39-amino acid peptides originally isolated from the venom of the Gila monster lizard *Heloderma suspectum*, which share approximately 50% sequence identity with GLP-1 itself and are indeed agonists at GLP-1 receptors. A synthetic preparation of exendin-4 (exenatide) has been approved in both USA and Europe as adjunctive therapy to for the treatment of type 2 diabetes based on two daily subcutaneous injections [6]. Exenatide, which differs from GLP-1 in N-terminal position 2, is DPP-IV resistant and is therefore essentially eliminated by glomerular filtration. However, when injected intravenously, it displays a plasma half-life of about 30 min.

The rapid inactivation and/or the clearance of GLP-1 peptides and analogues raises the need of developing degradation-resistant GLP-1 receptor agonists capable of exhibiting prolonged duration of action with respect to natural GLP-1 peptides after *in vivo* administration.

In the present work we generated long-lasting insulinotropic peptides through the conjugation of GLP-1 peptides and analogues to polyethylene glycol (PEG) by enzymatic site-specific transglutamination reaction. Our results indicate that these compounds could find therapeutical applications in type 2 diabetes in combination with suitable pharmaceutical formulations and/or slow release delivery systems.

2. Materials

GLP-1-(7-36)-amide and GLP-1-(7-36)-amide mutants, prepared according to the fluorenylmethyl chloroformate chemistry and with a purity > 90%, were custom synthetized by Pepscan (Lelystad, Netherlands). Linear methoxy-polyethylene glycol-amine MW 5,000 and 20,000 Da were purchased from IRIS Biotech (Marktredwitz,Germany). Branched methoxy-polyethylene glycol-amine MW 50,000 Da was obtained by NOF Corporation (Tokyo, Japan).

Dipeptidyl peptidase IV from porcine kidney (10 U/mg) and exenatide were purchased from Sigma-Aldrich (St. Louis, MO, USA).

$[\alpha\text{-}^{32}P]$ATP (30–40 Ci/mmol) and $[2,8\text{-}^3H]$cyclic AMP (25 Ci/mmol) were obtained from Perkin–Elmer (Boston, MA, USA).

Unless otherwise specified, all other chemicals and reagents were of analytical grade from Sigma-Aldrich and Fluka (Milan, Italy).

Macrocap SP chromatographic resin was from GE Healthcare (Uppsala, Sweden).

3. Methods

3.1. Analytical assays

Approximately 3 μg of non-reduced sample was separated by SDS-PAGE in 15% polyacrylamide with Tris–glycine buffer [7]. Resolved protein bands were fixed with glutaraldheyde and detected by Coomassie Blue staining. Biorad protein markers with mass range from 6.6 to 203.3 kDa were used as molecular weight reference.

RP-HPLC analysis of pegylated GLP-1-peptides was performed on a C18 Supelco Discovery Bio Wide Pore column, 4.6 × 250 mm, 5 μm particle size, (Bellefonte, PA, USA) at +45 °C and UV detection at 215 nm; elutions were carried out at 0.75 ml/min starting from the mobile phases A (0.1% v/v trifluoroacetic acid in water) and B (0.1% v/v trifluoroacetic acid in acetonitrile) with the following gradient: 15% B for 2 min; 15–34% B from 2 to 10 min; 34–56% B from 10 to 20 min and 56–90% B from 20 to 27 min; the column was finally washed with 90% B for 5 min.

Transglutaminase enzymatic activity was measured at 37 °C according to a colorimetric method based on the chromogenic hydroxamate procedure using N-α-carbobenzoxy-L-glutaminyl-glycine as substrate [8]. The calibration curve was prepared using L-glutamic acid-γ-monohydroxamate and one unit (U) of enzyme activity was

defined as the amount of enzyme that catalyzed the formation of 1 μmol of L-glutamyl mono- hydroxamic acid per minute.

Transglutaminase mass determination was performed by RP-HPLC analysis on a C4 Vydac 214TP52 column at +40 °C and UV detection at 215 nm; elutions were carried out at 0.2 ml/min starting from the mobile phases A (0.1% v/v trifluoroacetic acid in water) and B (0.1% v/v trifluoroacetic acid in acetonitrile) with the following gradient: 30–59% B from 0 to 13 min; 59–85% B from 13 to 20 min. Being not available a certified transglutaminase reference standard, transglutaminase was quantified by peak areas comparison of standard bovine albumin (BSA) preparations separated in the same conditions.

3.2. Enzymatic pegylation

3.2.1. Purification of microbial transglutaminase

Microbial transglutaminase (EC. 2.3.2.13) from *Streptoverticillium mobaraensis* (Activa WM, 81–135 U/g) was obtained from Ajinomoto (Tokyo, Japan) and purified by cation exchange chromatography. Briefly, a filtered enzyme solution in 50 mM sodium acetate–50 mM sodium chloride buffer (pH 5.5) was loaded on Macrocap SP chromatography column equilibrated with the same buffer and eluted with 50 mM sodium acetate–50 mM sodium chloride buffer (pH 5.8). The transglutaminase pooled fractions displayed a protein concentration of 0.368 mg/ml and a specific activity of 26.7 U/mg determined by combining RP-HPLC mass determination and colorimetric assay of enzymatic activity.

3.2.2. Pegylation reactions

GLP-1-(7-36)-amide dissolved in 20 mM potassium dihydrogen phosphate buffer (pH 7.4) at 0.5 mg peptide/ml was mixed with linear monomethoxy-polyethylene glycol-amine (mPEG-NH$_2$) of 5 or 20 kDa or with branched 50 kDa mPEG-NH$_2$ to achieve a 20:1 mPEG-NH$_2$:GLP-1 molar ratio and with 0.25 U/ml of partially purified microbial tranglutaminase. The resulting solution was maintained under mild agitation for 16 h at room temperature to obtain GLP-1(7-36)-amide monopegylated at glutamine 23.

The double mutant GLP-1-(7-36)-(Q23N–A30Q)-amide was reacted in the same conditions with linear 20 kDa mPEG-NH$_2$ to obtain the corresponding monopegylated derivative at glutamine 30.

The double mutant GLP-1-(7-36)-(T11Q–Q23N)-amide at a concentration of 0.5 mg/ml in 20 mM phosphate buffer pH 7.4 was reacted for 16 h at room temperature with linear 5 kDa mPEG-NH$_2$ (40:1 PEG:GLP-1 molar ratio) and with 0.25 U/ml of partially purified microbial transglutaminase to obtain the corresponding monopegylated derivative at glutamine 11.

All monopegylated GLP-1-(7-36)-amide derivatives were purified by cation exchange chromatography after dilution of reaction mixtures with 20 mM sodium acetate buffer (pH 4). The solutions were loaded on a Macrocap SP chromatography column and eluted with 10 column volumes of a NaCl linear gradient from 0 to 500 mM. The fractions containing the mono-pegylated GLP-1-(7-36) amides were concentrated by ultrafiltration and analyzed by RP-HPLC and SDS-PAGE.

3.3. In vitro stability of pegylated GLP-1(7-36)-amides towards DPP-IV digestion

GLP-1-(7-36)-amide and purified mono-pegylated GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa, GLP-1(7-36)-amide-Q^{23}-PEG 20 kDa, GLP-1-(7-36)-amide-Q^{23}-branched PEG 50 kDa, GLP-1-(7-36)-(Q23N–A30Q)-amide-Q^{30}-PEG 20 kDa and GLP-1(7-36)-(A11Q–Q23N)-amide-Q^{11}-PEG 5 kDa were dissolved in phosphate buffered saline (PBS) at a concentration of 0.1 mg/ml (calculated as peptide content) and were incubated at 37 °C with porcine DPP-IV (0.05 U/ml of peptide solution). At different incubation times, 50 μl was removed from

the reaction mixtures, and treated with 2.5 µl of 10% (v/v) trifluoroacetic acid to block the enzymatic reaction. The extent of degradation was evaluated by the increase of N-terminal dipeptide His–Ala measured by RP-HPLC on a C18 Supelco Discovery Bio Wide Pore column (4.6 mm × 250 mm, 5 µm particle size) at room temperature and UV detection at 215 nm. Elutions were carried out at 0.75 ml/min starting from the mobile phases A (0.1% v/v trifluoroacetic acid in water) and B (0.1% v/v trifluoroacetic acid in acetonitrile) with the following gradient: 100% A for 6 min; from 0% to 36% B from 6 to 15 min; from 36% to 55% B from 15 to 31 min and from 55% to 95% B from 31 to 32 min; the column was finally washed with 95% B for 5 min.

3.4. Determination of in vitro biological activity of GLP-1(7-36)-amide and its pegylated derivatives.

In vitro biological studies were performed on cell line RIN-m5F derived from a radiation-induced transplantable rat insulinoma, that not only expresses glucagon-like peptide-1 receptors in a sufficient number but also serves as a model cell line for the beta-cells [9].

3.4.1. Cell membrane preparation

Rat RIN-m5F insulinoma cells were from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI 1640 medium supplemented with 11 mM glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin from Invitrogen (Carlsbad, CA, USA), 9 mM NaHCO$_3$, 2 mM CH$_3$COCOONa and 10 mM HEPES and 10% (v/v) foetal calf serum (FCS) (Euroclone, Milano, Italy). Cells were maintained in sterile 75 cm^2 Corning tissue culture flasks (NewYork, NY, USA) at 37 °C in a humidified atmosphere of 5% CO$_2$/95% air using a Heraeus HERA cell 150 incubator (Hanau, Germany). Cells were grown in 100 mm plastic Petri dishes to ~80–90% confluency, the culture medium was removed and the cells were washed with ice-cold phosphate buffered saline (pH 7.4). Thereafter, the cells were scraped into an ice-cold buffer containing 10 mM HEPES/NaOH (pH 7.4), 1 mM EGTA and 1 mM MgCl$_2$ and lysed with a Dounce tissue grinder. The cell lysate was centrifuged at 1000 g for 5 min at 4 °C to remove unbroken cells and nuclei. The supernatant was collected and centrifuged at 32,000 g for 20 min at 4 °C. The pellet was resuspended in the homogenization buffer at a protein concentration of 2.0–2.5 mg/ml measured with the method of [10], using BSA as a standard. The membrane preparations were either used immediately or stored at −80 °C.

3.4.2. Adenylyl cyclase assay

The adenylyl cyclase activity of RINm5F cell membranes was assayed in a reaction mixture (final volume 100 µl) containing 50 mM HEPES/NaOH (pH 7.4), 2.3 mM MgCl$_2$, 0.3 mM EGTA, 0.2 mM [α-^{32}P]ATP (50 cpm/pmol), 0.5 mM [^{3}H]cyclic AMP (80 cpm/nmol), 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 U/ml of creatine phosphokinase, 10 µM GTP, 50 µg of BSA, 10 µg of bacitracin and 10 kallikrein inhibitor units of aprotinin. Preliminary experiments indicated that the stimulation of adenylyl cyclase activity by GLP-1 (7-36)-amide was maximal at 10 µM GTP. Peptide dilutions were freshly prepared in 0.1% BSA just before the experiments, constantly kept on ice and used only once. The reaction was started by the addition of the tissue preparation (30–35 µg of protein) and was carried out at 30 °C for 10 min. The reaction was stopped by the addition of 200 µl of a solution containing 2% (w/v) SDS, 45 mM ATP, 1.3 mM cyclic AMP (pH 7.5). Cyclic AMP was isolated by sequential chromatography on Dowex and alumina columns as described [11]. The recovery of [^{32}P]cyclic AMP from each sample was calculated on the basis of the recovery of [^{3}H]cyclic AMP. Three separate membrane preparations were used. Assays were carried out in duplicate.

3.4.3. In vitro insulinotropic action

The insulinotropic activity of GLP-1 amide and its derivates was evaluated by static incubation of RIN-m5F cells [12,13]. Cells were seeded in 12-multiwell plates at a density of 3 × 10^5 cells/well, and grown to 60–70% confluency. On the third day the growth medium was gently removed and replaced 24 h before the trial.

Acute tests for insulin release were preceded by 2 h pre-incubation at 37 °C/5% CO$_2$ in fresh RPMI 1640.

Test incubation was performed in the presence of 2 mM glucose and GLP-1-(7-36)-amide or its derivatives dissolved in RPMI 1640 without FCS at the final concentrations ranging from 10^{-12} to 10^{-6} M. After 30 min incubation the supernatant of each well was collected and centrifuged at 1000 g in a Eppendorf centrifuge equipped with F45-24-11 rotor for 4 min at 10 °C. The amount of insulin content was detected forthwith by using a rat insulin ELISA kit from Mercodia AB (Uppsala, Sweden).

3.5. In vivo biological activity of GLP-1(7-36)-amide and its pegylated derivatives.

All animal experiments were performed in accordance with the provisions of the European Economic Community Council Directive 86/209 concerning the protection of animals used for experimental and other scientific purposes, recognized and adopted by the Italian Government with the approval decree D.M. no. 230/95-B.

3.5.1. Glucose stabilizing effect in diabetic mice

The in vivo glucose-stabilizing effect of GLP-1(7-36)-amide and its pegylated derivatives was studied in 7- to 11-week-old diabetic db/db mice obtained from Charles River (Calco, Italy). The mice were distributed in three groups of seven animals each. Animals were food-deprived for 18 h before and 3 h after oral administration of a glucose solution (1.5 g/kg body weight). Thirty minutes before glucose administration, animals were treated subcutaneously with a solution containing either GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa (40 µg/ml) or GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa (40 µg/ml) or saline control solution in a volume of 2.5 ml/kg body weight to give a final dosage of 100 µg/kg. Blood samples were collected from the tail vein of conscious mice and glucose concentration was measured by a Gluco tester Ascensia Elite from Bayer (Milano, Italy) before food withdrawing, immediately prior to injections and 15, 30, 60, 120, 180, 240, 300, 1440, 1500 and 1620 min post injection.

3.5.2. Pharmacokinetic studies

Pharmacokinetic studies were performed in two groups of four adult Sprague-Dawley male rats weighing about 400 g obtained from Charles River (Calco, Italy). The animals were treated by subcutaneous injection of 1 mg/kg of GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa and GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa dissolved at a concentration of 0.5 mg/ml in 20 mM acetate–0.14 M NaCl buffer (pH 4.0). Blood samples (200 µl) were collected from tail vein at time 0 and 3, 6, 9, 24, 32, 48, 72 and 96 h after products administration into heparinized tubes and plasma was separated by centrifugation. Plasma GLP-1-(7-36)-amide concentration was determined by an ELISA method performed in 96-well polystyrene microtiter plates coated overnight at 4 °C with 100 µl/well of mouse monoclonal antibody specific for the amidated C terminus of GLP-1(7-36)-amide obtained from AntibodyShop (Gentofte, Denmark). The following day the plate was washed once with washing solution (PBS containing 0.1% v/v Tween 20) and blocked by incubation for 1 h with 200 µl/well saturating solution (PBS containing 5% w/v BSA and 0.1% v/v Tween 20). Wells were then washed four times, incubated for 1 h with 100 µl/well of standard and plasma samples and washed four times. Hundred microliter of biotinylated mouse monoclonal antibody specific for mid-molecular epitope of GLP-1 (AntibodyShop) was added to each well. After 1 h of incubation, the plate was washed four times, incubated for another hour

with 100 μl/well Streptavidin-Horseradish Peroxidase Conjugate obtained from Vector (Burlingame, CA, USA) and washed five times. Finally, the plate was developed by incubation for 10 min in the dark with 100 μl/well of TMB peroxidase substrate from Sigma and the reaction was stopped by the addition of 100 μl 1 N H_2SO_4 per well. Absorbances were measured at 450 nm on a Biorad microplate reader (Milan, Italy).

3.6. Statistical analysis

Results are reported as mean ± SEM. Statistical analysis of the difference between means was performed by Student's *t* test.

Concentration–response curves were analyzed by using a non-linear curve fitting computer program (GraphPad Prism, San Diego, CA, USA), which yielded EC_{50} (concentration producing half-maximal response) and E_{max} (maximal effect) values. The relative potency was calculated according to the formula: $1/(EC_{50}d/EC_{50}r)$, in which $EC_{50}d$ and $EC_{50}r$ are the EC_{50} values of the GLP-1 analog and GLP-1(7-36)-amide, respectively. The relative efficacy was calculated as percent of the GLP-1(7-36)-amide E_{max} value.

The pharmacokinetics parameters were estimated using a non-compartmental model. The half-life ($t_{1/2}$) was calculated on the elimination phase of the log transformed pK curve. The maximal concentration (C_{max}) and time of maximal concentration (T_{max}) are the experimental data point corresponding to the maximum value of GLP-1 plasma concentration. The area under the curve (AUC) was calculated by using the linear trapezoidal rule.

4. Results

4.1. Pegylation of GLP-1-(7-36)- amide with bacterial transglutaminase

The single glutamine residue naturally present in position 23 of GLP-1(7-36)-amide easily reacts with linear 5 kDa and 20 kDa alkylamino-monometoxy-PEG and a catalytic amount of bacterial transglutaminase to give the GLP-1(7-36)-amide-Q^{23}-monopegylated derivative with yields of about 60%. However, the same enzymatic pegylation either does not occur or occurs with lower yields when a single glutamine residue substituted a native residue in other positions of the peptidic chain of GLP-1(7-36)- Q23N-amide mutant, as shown in Table 1.

Monopegylated derivatives of natural GLP-1(7-36)-amide as well as of GLP-1(7-36)-Q23N-amide mutants were purified by cation-exchange chromatography with yields around 80% to obtain homogenous products as demonstrated by analytical RP-HPLC (Fig. 1). As expected [14], the pegylation increased the apparent size of the conjugated peptides; for example, GLP-1(7-36)-amide-Q^{23}-PEG 20 kDa, which has a calculated molecular weight of around 23.3 kDa, migrated in SDS-PAGE with an apparent molecular mass of 44–46 kDa (Fig. 2).

4.2. Stability towards DPP-IV digestion and biological activity in vitro

GLP-1-(7-36)-amide, its mutants and the derivatives mono-conjugated with 5, 20 or 50 kDa PEG on Gln11, Gln23 and Gln30 were subjected to *in vitro* proteolysis by incubation with porcine DPP-IV. The results demonstrated a high degradation rate for both native GLP-1-(7-36)-amide and its Q23N–A30Q double mutant, which showed 50% hydrolysis of N-terminal dipeptide after a few minutes and about 30 min respectively. Conversely, GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa and GLP-1-(7-36)-amide-Q^{23}-PEG 50 kDa as well as GLP-1-(7-36)-(Q23N–A30Q)-amide-Q^{30}-PEG 20 kDa, exhibited a similar slower degradation rate reaching about 50% hydrolysis after 8 h incubation. GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa showed a lower stability, reaching 50% degradation after about 4 h incubation, most likely due to a minor shielding effect of the shorter PEG chain. However, both
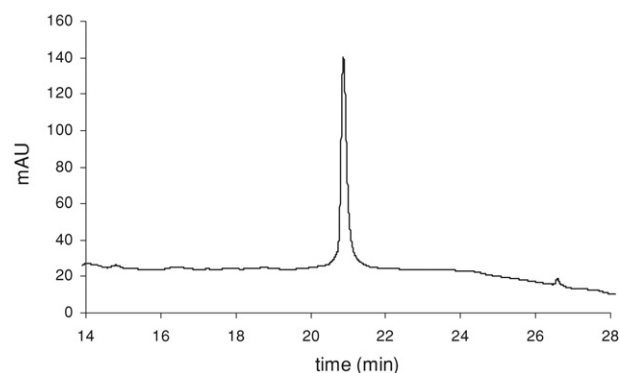

Fig. 1. RP-HPLC analysis of purified GLP-1-(7-36)-amide-Q^{23}-PEG 20kDa performed on a C18 Supelco Discovery Bio Wide Pore column (4.6 × 250mm) at 45 °C and UV detection at 215nm. Gradient elution was carried out at 0.75ml/min starting from the mobile phases A (0.1% v/v trifluoroacetic acid in water) and B (0.1% v/v trifluoroacetic acid in acetonitrile).
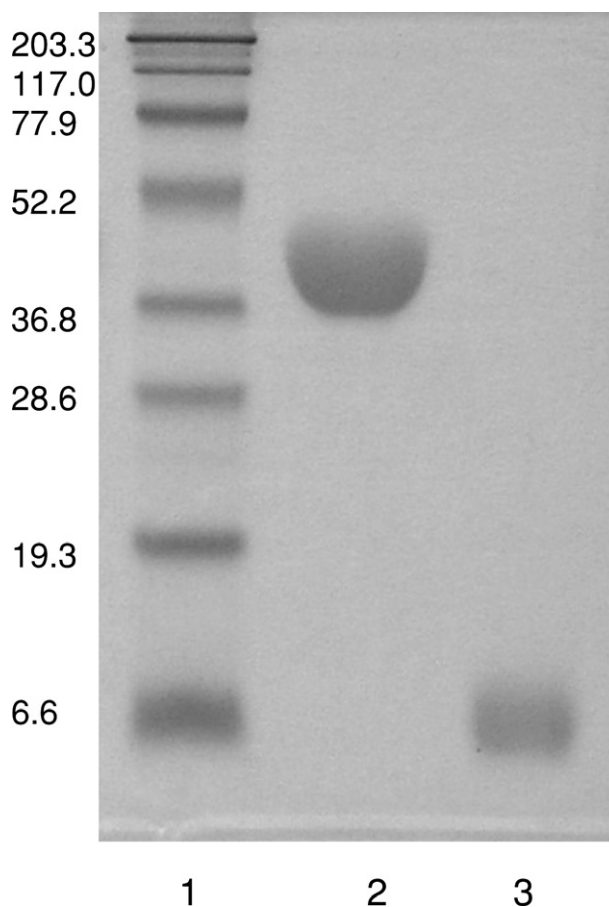

Fig. 2. SDS-PAGE of GLP-1-(7-36)-amide-Q^{23}-PEG 20kDa and GLP-1-(7-36)-amide-Q^{23}-PEG 5kDa carried out on 15% running gel and Coomassie Blue stained after glutaraldehyde fixation. Lane 1: standard protein markers; lane 2: GLP-1-(7-36)-amide-Q^{23}-PEG 20kDa; lane 3: GLP-1-(7-36)-amide-Q^{23}-PEG 5kDa.

PEG chain length and conjugation site were important determinants of proteolytic resistance, as demonstrated by the high stability of GLP-1-(7-36)-(T11Q–Q23N)-amide-Q^{11}-PEG 5 kDa (15% degradation after 8 h incubation), where the 5 kDa PEG chain is attached to a site closer to the N-terminus, which is cleaved by DPP-IV (Fig. 3).

The ability of the pegylated GLP-1 derivatives to activate the GLP-1 receptor was evaluated by measuring the stimulation of adenylyl cyclase in RIN-mf5 cell membranes. GLP-1-(7-36)- amide and the GLP-1 mimetic peptide exenatide stimulated cyclic AMP formation in

Table 1
Summary of enzymatic pegylations of GLP-1-(7 - 36)-amide and of its Q23N mutants, performed in phosphate buffer pH 7.4 for 16 h at room temperature.

Monopegylated GLP-1-(7 - 36)-amide derivatives	Peptide/mPEG-NH$_2$ (Molar ratio)	MTGase (unit/ml)	Pegylation yields (%)
GLP-1-(7 - 36)-amide-Q^{23}-PEG 5kDa	1:20	0.25	60
GLP-1-(7 - 36)-amide-Q^{23}-PEG 20kDa	1:20	0.25	60
GLP-1-(7 - 36)-amide-Q^{23}-PEG 50kDa*	1:20	0.25	25
GLP-1-(7 - 36)-(T11Q – Q23N)-amide-Q^{11}-PEG 5kDa	1:40	0.25	40
GLP-1-(7 - 36)-(Q23N – A30Q)-amide-Q^{30}-PEG 20kDa	1:20	0.25	60
GLP-1-(7 - 36)-(A8Q – Q23N)-amide-Q^{8}-PEG 20kDa	1:20	0.25	No pegylation
GLP-1-(7 - 36)-(S17Q – Q23N)-amide-Q^{17}-PEG 20kDa	1:20	0.25	No pegylation
GLP-1-(7 - 36)-(S18Q – Q23N)-amide-Q^{18}PEG 20kDa	1:20	0.25	No pegylation

* Reaction performed with branched 50kDa PEG-NH$_2$.

Table 2
Potencies and relative efficacies of GLP-1-(7 - 36)-amide, exenatide, GLP-1 mutants and pegylated GLP-1 peptides in stimulating adenylyl cyclase activity and insulin production in RINm5F cells. Values are the mean ± SEM of three experiments.

| Samples	*In vitro* cAMP stimulation in plasma membranes of rat β-cell line RIN-m5F			*In vitro* insulin secretion in rat β-cell line RIN-m5F		
	EC_{50} (nM)	Relative potency	Relative efficacy	EC_{50} (nM)	Relative potency	Relative efficacy
GLP-1(7 - 36)amide	0.43 ± 0.14	1	100	0.6 ± 0.2	1	100
Exenatide	0.52 ± 0.10	0.827	100	0.3 ± 0.1	2	92
GLP-1 -(7- 36)-(Q23N – A30Q)- amide				1.4 ± 0.3*	0.42	84.5
GLP-1-(7 - 36)- (T11Q – Q23N)-amide	29.9 ± 1.0*	0.014	80	28.8 ± 0.7*	0.02	100
GLP-1-(7 - 36)-amide-Q^{23}-PEG 5 kDa	9.3 ± 0.03*	0.046	100	2.7 ± 0.7*	0.22	98
GLP-1-(7 - 36)-amide-Q^{23}-PEG 20 kDa	27.0 ± 0.5*	0.016	100	34.4 ± 0.8*	0.017	80
GLP-1-(7 - 36)-amide-Q^{23}-PEG 50 kDa				1870 ± 1.0*	0.0003	94.7
GLP-1-(7 - 36)-amide-Q^{11}-PEG 5 kDa	153 ± 3*	0.0028	83	106.3 ± 0.5*	0.005	93
GLP-1-(7 - 36)-amide-Q^{30}-PEG 20 kDa				35 ± 3.0*	0.017	95

* $p < 0.05$ by Student's *t* test.
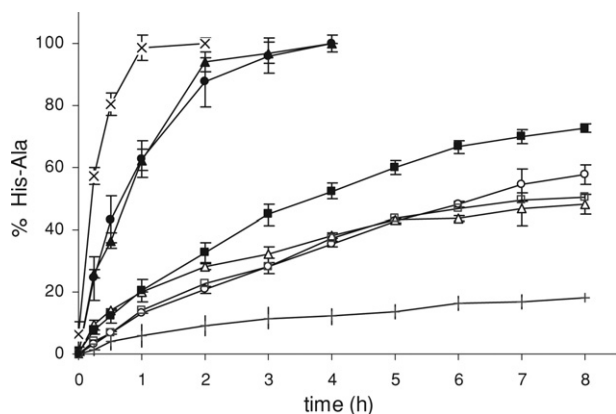
Fig. 3. *In vitro* enzymatic degradation of GLP-1-(7-36) amide (x), GLP-1-(7-36)-amide-Q^{23}-PEG 20KDa (○), GLP-1-(7-36)-(Q23N/A30Q)-amide-Q^{30}-PEG 20 kDa (△), GLP-1-(7- 36)-(Q23N–A30Q)-amide (▲), GLP-1-(7-36)-amide-Q^{23}-PEG 5KDa (■), GLP-1-(7-36)-amide-Q^{23}-PEG 50KDa (□), GLP-1-(7-36)-(T11Q–Q23N)-amide-Q^{11}-PEG 5 kDa (+), GLP-1-(7-36)-(T11Q–Q23N)-amide (●), towards porcine dipeptidyldipeptidase IV after 8 h incubation at 37 °C. Time course cleavage of N-terminal dipeptide Hys–Ala was measured by RP-HPLC carried out on a C18 Supelco Discovery Bio Wide Pore column (4.6mm × 250mm), at room temperature and UV detection at 215nm, by gradient elutions at a flow rate of 0.75ml/min starting from the mobile phases A (0.1% v/v trifluoroacetic acid in water) and B (0.1% v/v trifluoroacetic acid in acetonitrile).

a concentration-dependent manner with E_{max} values corresponding to 360 ± 22 and 358 ± 26% increase of basal value ($p < 0.001$, $n = 4$) and EC_{50} values of 0.43 and 0.52 nM, respectively (Table 2). All the pegylated derivatives displayed E_{max} values equal to that of GLP-1-(7-36)-amide, conversely, important differences were observed in the EC_{50} values, as pegylation significantly reduced the potency in stimulating GLP-1 receptor signaling. It is noteworthy that the relative potency of GLP-1-(7-36)-amide pegylated on Gln23 with 5 kDa PEG was higher than the corresponding derivative pegylated with 20 kDa

PEG, indicating a lower impact of the shorter PEG chain on the agonist activity.

Additional experiments were conducted to characterize the ability of GLP-1-(7-36)-amide, GLP-1(7-36)-amide mutants and the corresponding pegylated derivatives to induce insulin secretion from RIN-mf5 cells in the presence of 2 mM glucose (Table 2). All peptides and pegylated derivatives stimulated insulin release in a concentration-dependent manner within the range of 10^{-12}–10^{-6} M. As observed in the adenylyl cyclase assay, the major differences were observed in the EC_{50} values, with a loss of potency occurring for GLP-1 mutants and pegylated derivatives in comparison with native GLP-1(7-36)-amide.

4.3. In vivo activity of pegylated GLP-1-(7-36)-amide derivatives

Time-course of glucose stabilizing capabilities of GLP-1-(7-36)-amide, GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa and GLP-1-(7-36)-amide-Q^{23}-PEG-20 kDa were assessed in diabetic mice challenged with an oral glucose load administered 30 min after a subcutaneous injection of 100 μg/kg of pegylated GLP-1 derivatives or saline. As shown in Fig. 4, the glucose-lowering effect of GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa was maintained for about 8 h while the effect of GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa lasted not more than 3.5 h.

4.4. Pharmacokinetic properties

The pharmacokinetic profiles of GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa and GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa administered by subcutaneous injections of 1 mg/kg were studied in adult rats (Fig. 5). GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa and GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa plasma concentrations peaked respectively at 3 and 9 h, with C_{max} values of 160 and 640 ng/ml. GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa disappeared from plasma with a $t_{\frac{1}{2}}$ value of 1.7 h, while the circulating concentration of GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa decreased at a slower rate with a $t_{\frac{1}{2}}$ value of 17.1 h and after 96 h

Table 3

Main pharmacokinetic parameters calculated after subcutaneous administration of GLP-1-(7 - 36)-amide-Q^{23}-PEG 5kDa and GLP-1-(7 - 36)-amide-Q^{23}-PEG 20kDa.

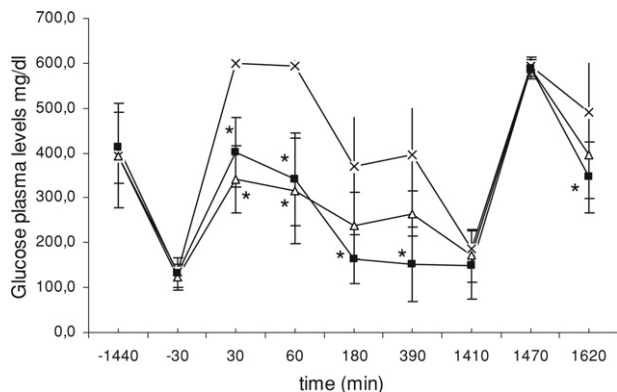| Sample	t_{max} (h)	C_{max} (ng/ml)	AUC* (ng/ml × h)	$t_{1/2}$ (h)
GLP1-(7 - 36)-amide-Q^{23}-PEG 20kDa	9	684.4	16,799.8	12.1
GLP1-(7 - 36)-amide-Q^{23}-PEG 5kDa	3	160.4	1408.3	1.7

* AUC_{96h}.

Fig. 4. Blood glucose levels in overnight fasted db/db diabetic mice during an oral glucose tolerance test (1.5g/Kg) performed 30min after a s.c. injection of vehicle (x) or of 100μg/kg GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa (△) and GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa (■). * $p < 0.05$ by Student's test
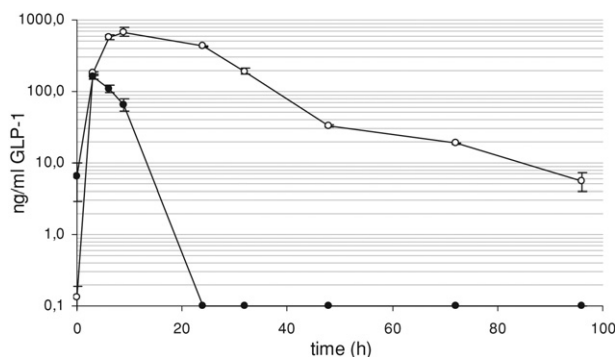

Fig. 5. Plasma concentration of GLP-1(7-36)-amide following subcutaneous administration. with 1mg/kg of GLP-1-(7-36)-amide-Q^{23}-PEG 5kDa (•) and GLP-1-(7-36)-amide-Q^{23}-PEG 20kDa (○) to rats. Blood samples for determination of GLP-1-(7-36)-amide concentration were collected from tail vein. Each point represents the average of four animals.

was still around 5 ng/ml. On the basis of their pharmacokinetic profiles, the calculated average $AUC_{(0 \to 96 h)}$ for subcutaneous administration of GLP-1-(7=36)-amide-Q^{23}-PEG 5 kDa and GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa was respectively 1408 and 16,800 ng/ml × h (Table 3).

The significant contribution of 20 kDa pegylation to the increase of circulating half-life of GLP-1 is clearly demonstrated by literature data of GLP-1-(7-36)-amide administered subcutaneously to rats displaying half-lives between 90 and 216 min [15].

5. Discussion

In this paper, we report for the first time the enzymatic pegylation of GLP-1 peptides as an approach to obtain modified incretin mimetic peptides displaying maintenance of therapeutically useful biological activity, resistance to endogenous peptidase degradation and longer circulating half-life.

It is well-known that the conjugation of peptides or proteins to linear or branched high molecular weight PEG modifies the physicochemical properties of the conjugated complexes maintaining, totally or in part, the biological function of the original non-conjugated molecule, such as the capability of receptor recognition and activation. Concomitantly, the conjugated PEG chain may hamper physical contacts between the protein and both specific and non-specific proteolytic enzymes, thus preventing or reducing the enzymatic proteolysis [16].

Pegylated therapeutic proteins, as any other drug, should ideally be homogeneous products with well defined structural and functional characteristics. However, conventional chemical pegylation of proteins is based on non-specific reactions with nucleophilic residues (most commonly the ε-amino group of surface lysine residues or the side-chain carboxylic groups) and frequently produces different extent of conjugation and/or mixtures of pegylated positional isomers. Each of these isomers could display variations in biological properties relevant to clinical applications, including pharmacological activity and side-effects.

It has been reported that polypeptidic chains can be pegylated by exploiting the preferential reactivity, at acidic pH, of the alpha-amino group of N-terminal residue with aldehyde-functionalized monometoxy-PEG chains followed by reductive alkylation [17]. Unfortunately, the N-terminal selective chemical pegylation of GLP-1 peptides results in a monoconjugated derivative with negligible *in vivo* activity due to the fact that the N-terminal histidine residue is crucial for effective receptor recognition and maintenance of biological action [18,19].

The selective chemical pegylation of one of the two lysine residues of GLP-1-peptides to give biologically active monopegylated derivatives either on Lys^{26} or on Lys^{34} residue was only possible through a low-yield, complex and multi-step method involving chemical protection of lysine ε-amino groups, chromatographic separation of Lys^{26} and Lys^{34} protected GLP-1 isomers, conventional independent chemical pegylation of each isomer followed by removal of protecting group from the non-reacted lysine residue [19].

In addition, therapeutical administration of human peptides bearing amino acid substitutions and/or additions as well as of peptides of non-human origin, such as exenatide, may induce undesired immune response that may decrease the drug efficacy or cause adverse events in patients. Indeed, clinical studies have shown that 40–50% of patients treated with exenatide for 30 weeks developed anti-exenatide antibodies, which, at least in some cases, impaired treatment efficacy [20].

To overcome these limitations, we developed mono-pegylated derivatives of GLP-1-(7-36)-amide and mutants by using the transglutaminase of *Streptoverticillium mobaraensis*, a monomeric enzyme with a molecular mass of about 38 kDa and a Ca^{2+} independent catalytic activity. This enzyme is able to catalyze an acyl transfer reaction between a γ-carboxyamido group of peptide-bound glutamine residues and monomethoxy-PEG chains functionalized with primary alkylamines [21].

In this work we exploited this reaction to pegylate the single glutamine residue naturally present on the incretin mimetic peptide GLP-1-(7-36)-amide or the single glutamine residue introduced into the GLP-1-(7-36)-(Q23N)-amide mutants in substitution of other native residues. Although no specific consensus site has been identified

around the glutamine residues modified by bacterial transglutaminase catalyzed reaction, it is commonly believed that the enzyme is able to recognize as substrate glutamine residues located on solvent accessible, flexible and locally unfolded region of the protein chain encompassing the glutamine residues [22].

Considering the properties of bacterial tranglutaminase and the fact that GLP-1-(7-36)-amide has no defined structure in aqueous solution [23], one may expect that any single glutamine residue introduced into the peptidic chain of the mutant GLP-1-(7-36)-(Q23N)-amide would provide an alternative substrate for the transglutaminase catalyzed monoconjugation reaction with mPEG-NH$_2$. However, when a number of Q23N-GLP-1 glutamine-containing mutants, including derivatives with a single glutamine substitution in positions 8, 11, 17, 18 and 30 were reacted with amino functionalized mPEG in the presence of bacterial transglutaminase it was surprisingly found that only two mutants were substrates for transglutaminase. In fact, with the exception of GLP-1-(7-36)-(Q23N–A30Q)-amide and GLP-1-(7-36)-(A11Q–Q23N)-amide monopegylated on the single glutamine residue in positions 30 and 11, respectively, the other mutants did not give the expected monopegylated derivatives (Table 1).

These results restricted the number of potential GLP-1-(7-36)-amide mutants as candidates for enzymatic monopegylation and this number was further reduced by excluding the residues involved in GLP-1-receptor binding, that is residues in positions 7, 10, 12, 13, 15, 19, 21, 29 and 32 [24,9].

On the basis of these considerations as well as of described experimental results, we chose to investigate native GLP-1-(7-36)-amide pegylated on the single Gln23 residue as potential candidate for treating type 2 diabetes. In fact, this derivative showed: (a) simple preparation with acceptable yields, (b) maintenance of sufficiently high level of agonist activity at the GLP-1 receptors, (c) increased proteolytic stability, (d) long circulating half-life and (e) improved glucose-stabilizing capability.

Monopegylated GLP-1-(7-36)-amide peptides are easily prepared by an enzyme catalyzed reaction of aqueous buffer solutions of peptides in the presence of 20 M excess of mPEG-NH$_2$ and of preparation of S. mobaraensis transglutaminase. The enzymatic pegylations were carried out at room temperature and the yields were generally not higher than 60% due to the transglutaminase which causes an inter-molecular crosslinking of GLP-1 by formation of an isopeptide bond involving the side chains of Gln and Lys residues, as shown for example in Fig. 6 which refers to the preparation of GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa. One step purification of pegylated GLP-1 peptides was then performed by loading the reaction mixture on a cation-exchange chromatography column and eluting with a saline gradient. It is worth noting that the enzymatic pegylation of GLP-1-(7-36)-amide peptides is only catalyzed by bacterial tranglutaminases but not by mammalian tranglutaminases. Indeed, we did not obtain any pegylated GLP-1 peptide when the reactions were carried out in the presence of purified guinea-pig liver transglutaminase (data not shown), confirming the broader substrate specificity of bacterial enzyme already reported in literature [21].

The biological activities of a small peptide as the 30 residue GLP-1-(7-36)-amide, whose molecular mass is only 3298 Da, can be greatly influenced by the chain length of conjugated PEG, as exemplified by the behavior of GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa. In the case of this product, the conjugation of a 5 kDa PEG chain reduced the potency in stimulating cyclic AMP formation and insulin release by 21- and 5-fold, respectively, as compared with the non-pegylated GLP-1-(7-36)-amide, whereas it protected the peptide from in vitro proteolysis by DPP-IV, as 50% degradation required 4 h and less than 10 min for the pegylated and non-pegylated peptide, respectively. However, the increase of hydrodinamic size conferred by a single 5 kDa chain was not sufficient to reduce the renal clearance of GLP-1-(7-36)-amide-Q^{23}-PEG 5 kDa to a great extent, as demonstrated by the $t_{1/2}$ value of 1.7 h found in the rat pharmacokinetic study. On the other hand,
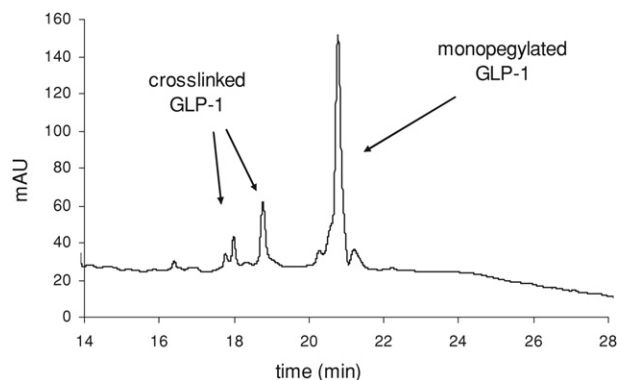

Fig. 6. RP-HPLC analysis of pegylation mixture after 16 h reaction at room temperature of GLP-1-(7-36) amide (0.5mg/ml) with mPEG-NH$_2$ 20kDa in 20mM phosphate buffer pH 7.3 in the presence of 0.25unit/ml of bacterial transglutaminase. Reaction mixture was applied to a C18 Supelco Discovery Bio Wide Pore column (4.6 × 250 mm^2) RP-HPLC and analysis was carried out by gradient elution at +45 °C and UV detection at 215nm with a flow rate of 0.75ml/min starting from the mobile phases A (0.1% v/v trifluoroacetic acid in water) and B (0.1% v/v trifluoroacetic acid in acetonitrile).

the GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa, conjugated with a larger 20 kDa PEG chain, showed a significant circulating half-life increase with a $t_{1/2}$ value of 12.1 h as well as a better resistance to in vitro DPP-IV proteolytic degradation. It is worth noting that the mutant GLP-1-(7-36)-(Q23N–A30Q)-amide monopegylated on Gln30 with a PEG 20 kDa gave exactly the same degradation rate and that no further improvement of resistance to DPP-IV degradation was obtained when GLP-1-(7-36)-amide was pegylated with a much bigger 50 kDa branched PEG chain.

5.1. Conclusions

Finally, even if the agonist potency of GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa was reduced by 50–60 fold with respect to the non-pegylated GLP-1-(7-36)-amide, the combination of resistance towards DDP-IV and reduced renal clearance enabled GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa to display a glucose-stabilizing action lasting up to 8 h in diabetic mice. Although the pharmacokinetic parameters of biodrugs derived from animals may not be predictive in humans, it is reasonable to estimate that the therapeutic application of GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa for the treatment of diabetes will require more than once-a-day injections.

Studies are therefore in progress to investigate whether the incorporation of GLP-1-(7-36)-amide-Q^{23}-PEG 20 kDa in sustained release formulations will be able to control hyperglycemia in type 2 diabetic patients on the basis of once a week or twice a month administration.

References

[1] Holst J.J., Deacon C.F., Vilsbøll T., Krarup T., Madsbad S. Glucagon-like peptide-1, glucose homeostasis and diabetes. Trends in Molecular Medicine. 2008;14:161–168.

[2] Holst J.J. The physiology of glucagon-like peptide 1. Physiological Reviews. 2007;87:1409–1439.

[3] Mayo K.E., Miller L.J., Bataille D., Dalle S., Goke B., Thorens B. et al. Glucagon receptor family. Pharmacological Reviews. 2003;55:167–194.

[4] Arhèn B. Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. Nature Reviews Drug Discovery. 2009;8:369–385.

[5] Ban K., Hossein Noyan-Ashraf M., Hoefer J., Bolz S., Drucker D.J., Husain M. Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. Circulation. 2008;117:2340–2350.

[6] Davidson M.B., Bate G., Kirkpatrick P. Exenatide. Nature Reviews Drug Discovery. 2005;4:713–714.

[7] Laemmli U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature. 1970;227:680–685.

[8] Folk J., Cole P.W. Transglutaminase: mechanicistic features of the active site as determined by kinetic and inhibitor studies. Biochimica et Biophysica Acta.

1966;122:244–264.

[9] Gallwitz B., Witt M., Paetzold G., Morys-Wortmann C., Zimmermann B., Eckart K. et al. Structure/activity characterization of glucagon-like peptide-1. European Journal of Biochemistry. 1994;225:1151–1156.

[10] Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976;72:248–254.

[11] Salomon Y., Londos C., Rodbell M. A highly sensitive adenylyl cyclase assay. Analytical Biochemistry. 1974;58:541–548.

[12] Green B.D., Gault V.A., Mooney M.H., Irwin N., Bailey C.J., Harriot P. et al. Novel dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-(7-36)-amide have preserved biological activities in vitro conferring improved glucose-lowering action in vivo. Journal of Molecular Endocrinology. 2003;31:529–540.

[13] Pan C.Q., Buxton J.M., Yung S.L., Tom I., Yang L., Chen H. et al. Design of a long acting peptide functioning as both a glucagon-like peptide-1 receptor agonist and a glucagon receptor antagonist. Journal of Biological Chemistry. 2006;18:12506–12515.

[14] Pasut G., Veronese F.M. PEGylation for improving the effectiveness of therapeutic biomolecules. Drugs Today (Barc). 2009;45:687–695.

[15] Parkes D., Joda C., Smith P., Nayak S., Rinehart L., Gingerich R. et al. Pharmacokinetic actions of exendin-4 in the rat: comparison with glucagon-like peptide-1. Drug Development Research. 2001;53:260–267.

[16] Veronese F.M., Pasut G. PEGylation, successful approach to drug delivery. Drug Discovery Today. 2005;10:1451–1458.

[17] Kinstler O.B., Gabriel N.E., Farrar C.E., DePrince R.B. N-terminally chemically modified protein composition and methods. US Pat. no. 5,985,265; 1999.

[18] Hareter A., Hoffmann E., Bode H-P., Goke B., Goke R. The positive charge of the imidazole side chain of histidine[7] is crucial for GLP-1 action. Endocrine Journal. 1997;44:701–705.

[19] Youn Y.S., Chae S.Y., Lee S., Jeon J.E., Shin H.G. Evaluation of therapeutic potentials of site-specific PEGylated glucagon-like peptide-1 isomers as type 2 anti-diabetic treatment. Insulinotropic activity, glucose-stabilizing capability and proteolytic stability. Biochemical Pharmacology. 2007;73:84–93.

[20] Drucker D.J., Nauk M.A. The incretin system: glucagon-like peptide-1 receptor agonist and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet. 2006;368:1696–1705.

[21] Sato H. Enzymatic procedure for site-specific pegylation of proteins. Advanced Drug Delivery Reviews. 2002;54:487–504.

[22] Fontana A., Spolaore B., Mero A., Veronese F.M. Site-specific modification and PEGylation of pharmaceutical proteins mediated by transglutaminase. Advanced Drug Delivery Reviews. 2008;60:13–28.

[23] Thornton K., Gorenstein D.G. Structure of glucagon-like peptide (7-36) amide in dodecylphosphocholine micelle as determined by 2D NMR. Biochemistry. 1994;33:3532–3539.

[24] Adelhorst K., Hedegaard B.B., Knudsen L.B., Kirks O. Structure-activity studies of glucagon-like peptide-1. Journal of Biological Chemistry. 1994;269:6275–6278.