Research Article

Preclinical characterization of eleven new Cys-PEGylated hGH mutants

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ABSTRACT

We synthesized and tested for biological activity eleven new PEGylated hGH derivatives. To this aim we used different strategies. A first group of PEGylable Cys derivatives was prepared by mutating into Cys single specific aminoacid residues located either in the connecting loop between helices 1 and 2 (Ile36 and Phe44) or in the connecting loop between helices 3 and 4 (Ile138 and Phe146). A second group of mutants was synthetized by inserting new Cys close to the aforementioned positions. Other PEGylable mutants were prepared by inserting a Gly$_4$–Cys chain either at the N- or at the C-terminus of the hGH molecule. Finally, a Cys residue of the second hGH disulfide bond (Cys182–Cys189) was made available for PEGylation by mutating its companion Cys to open up the disulfide bridge. All these mutants were tested for their ability to affect Nb2 cell proliferation in vitro and showed different effects. Two mutations (Phe44Cys and +Cys47) severely impaired and two (Ile138Cys and Phe146Cys) increased hGH bioactivity. The mutation Cys189Ser was functionally silent whereas the remaining mutations caused a 15–50% decrease in activity. 20 kDa-pegylation caused a dramatic decrease in bioactivity in all mutants. The r-hGH-(Ile138Cys)–Cys^{138}–PEG derivative showed the highest activity (15% of wild-type unpegylated hGH) and was selected for pharmacockinetic studies in vivo. It showed a fivefold longer half-life and was significantly more effective than wild-type hGH in causing weight gain when given subcutaneously twice a week to hypophysectomized rats.

In conclusion, even when it is directed to residues supposed to have a marginal role in the activity of this hormone, 20 kDa pegylation has detrimental effect on hGH bioactivity. These effects may be counterbalanced by the increase in half-life as it happens in pegylated Ile138Cys–hGH that could represent a promising new long-acting hGH derivative.

Focal points:

- *Bedside*: Current therapy of GH deficiency still has the important limitation of being delivered by daily subcutaneous injections and this reduces the compliance of the small pediatric patients. There is, therefore interest in developing long-acting GH derivative such as the new ones that we present here.
- *Benchside*: By investigating the effect of Cys substitutions at aminoacidic positions that had not been investigated in previous studies, we obtained evidence that Ile138 and Phe146 have a role in GH bioactivity in vitro.
- *Industry*: The mono-pegylated hGH mutant described in this work represents a promising candidate for human clinical pharmacology studies as long-acting derivative of h-GH.
- *Community*: By decreasing the frequency of GH injections, long acting GH could decrease the costs of GH treatment and increase medication adherence.

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1. Introduction

Recombinant DNA technology revolutionized the treatment of dwarfism and starting from mid 80s recombinant human GH hormone represents the standard therapy for short stature due to GH deficit of pituitary origin, Turner disease, chronic kidney disease and Prader–Willi syndrome [1]. More recently the clinical indications for this hormone have been extended to include small for gestational age children [2,3]. Despite the worldwide impressing success of recombinant hGH that sells for about $3 billion a year, little progress in improving the pharmacological properties of this drug has been made since its introduction in the market. More specifically, nowadays as thirty years ago, hGH has

to be administered by daily subcutaneous injections. Although painless needle-free injection systems are now available, the need for daily injections decreases the compliance to the therapy and this can be an important problem in clinical practice considering that the typical patients are small children. Daily injections are needed because of the structural characteristics of the hormone yielding to a short half-life that averages 20 min in humans. The main circulating form of hGH is, indeed, a non-glycosylated polypeptide of 191 amino acids that freely crosses the glomerular filtration membrane to be eliminated in urine [4]. Moreover, hGH is endocyted through its receptors and intracellularly degraded mainly in liver and kidney [5].

Although different strategies can be implemented to prolong the administration interval of short-lived molecules by increasing their half-life or by delaying their absorption [6,7], only one long-acting GH formulation is currently available for use in humans in the European Community and none in the United States [8,9].

In the present paper we report the preclinical characterization of a series of new long-acting hGH derivatives based on protein PEGylation. PEGylation is the covalent binding of polyethylene glycol side (PEG) chains to proteins, yielding to derivatives that are less prone to elimination by kidney filtration than parental proteins because of their larger hydrodynamic volume. Because of the increase in their half-life, PEGylated proteins have long circulation time in the blood and can be administered to patients less frequently than native molecules [10]. Moreover, PEGylated proteins possess reduced immunogenicity and antigenicity as well as enhanced solubility and stability [11]. To achieve protein PEGylation, PEG is functionalized with specific end groups to generate derivatives that can bind to acceptor amino acids that are freely accessible because exposed on protein surface. Among these amino acids, cysteine (Cys) residues are frequently chosen as PEG acceptors because of their reactivity and because they are quite rare in average proteins. This is a critically important point because, by choosing rare PEGylation targets, homogenous final reaction products can be obtained. Conversely, when abundant acceptor sites are used, a heterogeneous population of PEGylated molecules is generated because of the stochastic incorporation of PEG that can be bound to one or more of all its potential acceptor sites. hGH has no free Cys residues because the four Cys in its sequence are all buried inside the molecule to form two disulfide bonds (53–65 and 182–189) [12]. Therefore, in the present work we used specific mutagenesis strategies to make hGH Cys-directed PEGylation possible. Specifically, either we freed Cys from the internal disulfide bonds or we introduced new Cys in the hGH molecule by mutating specific hGH aminoacid residues or by adding one or more Cys as extra amino acids at the carboxy(C)- or at the amino(N)-terminus of the hormone. Eleven new PEGylated hGH derivatives were produced and characterized. One of these compounds proved to be more effective than hGH when injected twice a week and could be promising for further development as long acting hGH form for human therapy.

2. Materials and methods

2.1. r-hGH mutant preparation

Chemically synthesized cDNA coding for mature hGH (NCBI Database accession number J0307), was purchases from BaseClear (Leiden, The Netherlands), cloned into pL161 plasmid to obtain the pL130 plasmid as detailed elsewhere [13], which was used to transform *Escherichia coli* JM109. Mutant hGH cDNAs bearing Cys residue substitutions, addition or insertions were prepared by PCR using a commercial mutagenesis kit (QuickChange®, Stratagene, La Jolla, CA, USA) and the synthetic oligonucleotide primers reported in Table 1.

Because hGH is a secretory protein, its bacterial synthesis by the cytoplasmic route has been classically obtained by introducing a start codon encoding for Met at the N terminus. In this work we used, instead, a proprietary Kex-1 protease-based technique whose details we published in 2008 [13]. Briefly, wild-type and mutant hGH cDNA were cloned into pL161 to obtain constructs encoding for LacZ$_8$–PNP$_{20}$–kex1 peptide-h-GH fusion proteins. These hybrid proteins contain before the NH$_2$ terminal Phe residue of hGH, the first 8 amino acids of the LacZ protein fragment

Table 1

hGH mutants with pegylable Cys residues. The table reports the list of the hGH mutants that we produced to insert free pegylable Cys residues. The mutants were classified in four groups according to the type of mutation that was made: single residue modification, S–S bridge opening, extra Cys insertion or addition of a Cys tag. For each of the mutant the table shows the name of the plasmid and of the bacterial strain that we produced and the sequence of the PCR primers that we use din the mutagenesis protocol.

Type of mutation	Mutation	Plasmid	Recombinant strain	Oligonucleotide primers
Cys substitution of single amino acids	Ile36Cys	pL333	NP506	OL275F: ACTTCTGTTCCTTTGGGCAATAGGCTTCTTCAAACT OL276R: AGTTTGAAGAAGCCTATTGCCCAAAGGAACAGAAGT
	Phe44Cys	pL326	NP497	OL277F: TGGGGGTTCTGCAGGCATGAATACTTCTGTT OL278R: AACAGAAGTATTCATGCCTGCAGAACCCCCA
	Ile138Cys	pL334	NP507	OL279F: AGGTCTGCTTGAAGCACTGCCCAGTCCGGGG OL280R: CCCCGGACTGGGCAGTGCTTCAAGCAGACCT
	Phe146Cys	pL327	NP498	OL281F: TGTGAGTTTGTGTCGCACTTGCTGTAGGTCT OL282R: AGACCTACAGCAAGTGCGACACAAACTCACA
S–S bridge opening	Cys182Ser	pL348	NP527	OL329F: GCCCTCCACAGAGCGGGACTGCACGATGCGCAG OL330R: CTGCGCATCGTGCAGTCCCGCTCTGTGGAGGGC
	Cys189Ser	pL349	NP528	OL331F: AGATCATTAGAAGCCAGAGCTGCCCTCCACAGA OL332R: TCTGTGGAGGGCAGCTCTGGCTTCTAATGATCT
Insertion of an additional Cys	+Cys47	pL351	NP529	GH13F: CATTCCTGCAGTGCAACCCCCAGACCT GH14R: GCATGCCTGCAGGTCGACTCTAGAGTCATTAG
	+Cys146	pL350	NP526	GH12F: CTGGGCAGATCTTCAAGCAGACCTACAGCAAGTGCTTCGACAC GH11R: GTCGACTCTAGAGTCATTAGAAGCC
	+Cys139	pL345	NP521	GH10F: GACTGGGCAGATCTGCTTCAAGCAGACC GH11R: GTCGACTCTAGAGTCATTAGAAGCC
addition of a Cys-tag at the N- or C-terminal	Cys–Gly4 (N-Terminus)	pL330	NP504	GH3F: CGGCCTGTTTAAACGATGCGGTGGCGGTGGCTTCCCAACCATTCCC GH4R: GCTGTAGGTCTGCTTGAAGATCTGCCCAGTCCGG
	Gly4–Cys (C-Terminus)	pL331	NP505	GH1F: AACTGCAGGTCGACTCTAGAGTCATTAGCAGCCACCGCCACCGAAGCCACAG GH2R: CATTCCTGCAGAACCCCCAGACCTCCTCTGTTTC

(LacZ8) (UniProt/SwissProt accession $n°$ Q37953), the first twenty amino acids of purine nucleoside phosphorylase (PNP20) [14] and a short flexible peptide –Glu–Ser–Ser–Met–Ser–Gly–Leu–Phe–Lys–Arg– (kex1 peptide). The last two amino acids of the kex1 peptide, the Lys–Arg dipeptide, are specifically cleaved at C-terminal site of arginine upon exposure to the endoprotease ss–Kex-1-C_{611}. This reaction leads to the release of authentic wt or mutant hGH with Phe at residue 1 in the N terminus [13]. The different plasmids carrying mutant r-hGH cDNAs were used to transform JM109 E. coli competent cells that were selected on LS_{10} medium (5 g/l yeast extract, 10 g/l soytone, 10 g/l sodium chloride) containing 12.5 mg/l tetracycline hydrochloride. A total of 11 different bacterial recombinant strains were obtained expressing different mutant hGH forms (Table 2). The expected plasmid sequence was confirmed in each of these strains by restriction analyses. Bacterial strain cultures were upscaled in 2 l bioreactors operated at a temperature of $37 \pm 1\ °C$, with an oxygen content of about 20% and a pH value of 6.9–7.1. Production culture medium consisted of 20 mM potassium phosphate-pH 7, 0.625 g/l soytone, 1.125 g/l yeast extract, 0.625 g/l glycerol, 1 g/l $MgSO_4$ and 12.5 mg/l tetracycline hydrochloride whereas feed culture medium consisted of 100 g/l soytone, 180 g/l yeast extract, 100 g/l glycerol and 12.5 mg/l tetracycline hydrochloride. The fermentations were terminated when reaching a final OD_{600} value in the range of 40–45 units. Upon completing fermentation, the cell broths were cooled to about $+20\ °C$, harvested and centrifuged to obtain concentrated cell pastes that were stored frozen at $-20\ °C$ until required. Fusion proteins were recovered from cytoplasmic inclusion bodies, renatured, refolded, clarified by centrifugation, ultrafiltrated and dialyzed against 50 mM NaCl, 0.1 M Tris–HCl, 5 mM EDTA-pH 8.0 buffer. Then, enzymatic cleavage of hGH mutant fusion proteins (about 1 g/l) was performed for 18 h at 25 °C after adding 4 mM Ca^{2+} and 0.5 mg/l of purified ss–Kex-1-C_{611} [13]. Released hGH molecules were purified by hydrophobic interaction chromatography on Phenyl Sepharose Fast Flow high substitution resin, followed by anion-exchange chromatography on DEAE Sepharose Fast Flow resin and gel filtration chromatography performed on Sephacryl S-100h resin. Protein purity of the final purification products ranged between 75 and 95%, as assessed by SE-HPLC, RP-HPLC and SDS-PAGE.

2.2. PEGylation of wild-type and mutant r-hGH.

r-hGH mutants (2 mg/ml in 50 mM Tris–HCl buffer pH 7.0) were PEGylated on Cys residues by reacting each of them for 3 h at room temperature with 20 kDa mPEG-maleimide (50 mg/ml in 50 mM Tris–HCl buffer pH 7.0 at a final 1:3 protein/PEG molar ratio). Wild-type hGH (2 mg/ml in 50 mM phosphate buffer, pH 6.5) was PEGylated by N-terminal monoconjugation of its α-amino group. The PEGylation reaction was carried out overnight at room temperature by reductive alkylation using 20 kDa m-PEG-propionaldehyde (50 mg/ml in 50 mM phosphate buffer, pH 6.5 at 1:5 protein/PEG molar ratio) and $NaBH_3CN$ at a final concentration of 3 mM.

MonoPEGylated r-hGH derivatives were purified by anion exchange chromatography performed on a MonoQ chromatography column equilibrated with 20 mM Tris–HCl pH 8.5 and eluted with 10 column volumes of a NaCl linear gradient from 0 to 500 mM concentration. The fractions containing the mono-PEGylated derivatives were concentrated by ultrafiltration and analyzed by reverse phase HPLC (RP-HPLC) and SDS-PAGE. In addition, the aminoacid sequence of mutant r-hGH was determined to confirm the molecular identity of these variants. Total protein concentrations were measured by the bicinchoninic acid assay (BCA) method [15] using a commercial kit (Pierce, Prodotti Gianni, Milan, Italy).

RP-HPLC was performed using a Symmetry 300 C18 column ($2.1 \times 150\ mm^2$, 3.5 μm particle size, Waters Corp., Millford, Massachusetts, USA). Separation was carried out at $+53\ °C$ and at a flow rate of 0.285 ml/min using a gradient of two mobile phases A (0.1% v/v trifluoroacetic acid in water) and B (0.1% v/v trifluoroacetic acid in acetonitrile). Specifically, from 0 to 9 min of the run, the percentage of B was increased from 52.7% up to 57.9%; then, it was increased from 57.9% up to 67.7% in the time interval from 9 to 18 min and from 67.7% up to 90% in the time interval from 18 to 23 min. Absorbance of the eluate was measured at 215 nm using an UV detector.

SDS-PAGE was performed both in reducing and in non-reducing conditions using 12% polyacrylamide gels with tris-glycine buffer. Proteins on the gel were visualized by Coomassie Blue staining.

N-terminal sequence analyses of h-GH, was performed by standard Edman degradation procedure on a Procise 492 Applied Biosystems automatic apparatus (Forster City, CA, USA) according to the manufacturer's instructions.

2.3. In vitro testing of hGH biological activity

The biological activity of wild-type and mutant r-hGH PEGylated derivatives was assessed in vitro by measuring their ability to increase rat lymphoma Nb2-11 cell proliferation as described elsewhere [16]. Briefly, Nb2-11 cells were cultured in Fisher medium supplemented with 0.075% $NaHCO_3$, 10% horse serum, 10% fetal calf serum, 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin at $+37\ °C$ under humidified atmosphere containing 5% CO_2. Cells were starved for 48 h before

Table 2
Pegylation yield attained in wild type and in mutant hGH. As detailed in the methods section hGH mutants bearing free Cys residues were pegylated with m-PEG-maleimide whereas in wild type hGH m-PEG-propionaldehyde was bound to the N terminus using $NaBH_3CN$. Pegylation yield was determined by measuring the amount of pegylated and unmodified hGH by RP-HPLC.

Pegylated hGH derivative	Protein/PEG molar ratio	Pegylation yield (%)
r-hGH-N-term–PEG 20 kDa	1:5	82
r-hGH-(Ile36Cys)–Cys^{36}–PEG 20 kDa	1:3	No pegylation
r-hGH-(Phe44Cys)–Cys^{44}–PEG 20 kDa	1:3	76
r-hGH-(Ile138Cys)–Cys^{138}–PEG 20 kDa	1:3	86
r-hGH-(Phe146Cys)–Cys^{146}–PEG 20 kDa	1:3	66
r-hGH-(Cys182Ser)–Cys^{189}–PEG 20 kDa	1:3	68
r-hGH(Cys189Ser)–Cys^{182}–PEG 20 kDa	1:3	46
r-hGH-(+Cys139)–Cys^{139}–PEG 20 kDa	1.3	60
r-hGH-(+Cys146)–Cys^{146}–PEG 20 kDa	1:3	42
r-hGH-(+Cys47)–Cys^{47}–PEG 20 kDa	1:3	42
Cys^{-5}–Gly$_4$–N-terminal -r-hGH–PEG 20 kDa	1:3	No pegylation
r-hGH–C-terminal Gly$_4$–Cys^{196}–PEG 20 kDa	1:3	22

the proliferation assay. To this aim, 48 h before the assay, they were collected by centrifugation at 350 rpm, washed three times with minimal Fischer medium (without serum) and seeded in the same medium at a density of 2.2×10^4 cells/ml. 24 h later, cells were plated in the same serum-free medium at the density of 4×10^4 cells/well in triplicate in 96 well multiplates and placed in incubator for 22 h. Then, serial dilutions of r-hGH, r-hGH mutants or r-hGH PEGylated derivatives were added to the medium at final concentrations ranging from 0 to 9 ng/ml. Cell proliferation was evaluated after a 24 h incubation with the drugs by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation with a peroxidase-labeled anti-BrdU antibody colorimetric assay using a commercial kit (Biotrak ELISA System, version 2″, GE Healthcare Biosciences, Piscataway, NJ, USA). The data obtained were non-linearly fitted using the GraphPad Prism software (San Diego, CA, USA) to obtain concentration-response curves and to determine the EC_{50} for each tested molecule. The potency of PEGylated r-hGH derivatives was normalized to that of wild-type r-hGH using the formula: $1/(EC_{50}d/EC50r)$, where $EC_{50}d$ and $EC_{50}r$ are the EC_{50} values of mutant and wild-type r-hGH, respectively.

2.4. Studies in vivo

All animal experiments were carried out 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.

2.5. Pharmacokinetic studies

Pharmacokinetic studies were performed in adult Sprague-Dawley male rats weighing about 250 g obtained from Charles River (Calco, Italy). Different groups of four animals each, received a single subcutaneous injection of either wild-type hGH (1 mg/Kg in 20 mM phosphate buffer, 0.14 M NaCl, pH 7.3) or mutant PEGylated r-hGH derivatives (dissolved in the same buffer of wild-type hGH and administered at the dosage of 1 mg/Kg hGH equivalents) and blood samples were sequentially drawn at scalar times from injection to measure plasma hGH concentrations. Specifically, 200 µl blood samples were collected at pre-dose, 5, 15, 30 min, 1, 2, 3, 4, 6, 24 h from injection in the group of animals receiving wild-type hGH, and at pre-dose, 1, 2, 4, 8, 24, 32, 48, 72 and 96 h from injection in those injected with PEGylated derivatives. Plasma hGH concentrations were determined by ELISA using a commercial kit (Active® Human Growth Hormone ELISA kit, Diagnostic Systems Laboratories Inc., Webster, TX, USA). This kit was chosen because in preliminary experiments we found that it recognizes with similar affinity both r-hGH and its PEGylated mutants (data not shown).

Pharmacokinetic analysis was performed using a non-compartmental model and the Microsoft Excel software. Half-life ($t_{1/2}$) of r-hGH and its PEGylated derivatives was calculated on the elimination phase of the plasma concentration to time log transformed curve. Maximal plasma concentration (C_{max}) and time to maximal plasma concentration (T_{max}) were determined as the experimental data points corresponding to the maximum value of plasma concentration to time curves. Area under the plasma concentration curves (AUC) of r-hGH and its PEGylated derivatives were calculated by using the linear trapezoidal rule.

2.6. Pharmacodynamic studies

The bioactivity of r-hGH-(Ile138Cys)-Cys^{138}-PEG was assessed in vivo and compared with that of wild-type GH by measuring the ability to increase body weight in hypophysectomized 4 weeks (body weight 100 g) old Sprague-Dawley male rats that were purchased from Charles River (Calco, Italy). Wild-type and mutant hGH (in 20 mM phosphate buffer, 0.14 M NaCl, pH 7.3) were administered by subcutaneous injection and body weight was measured daily for six days. Four different groups of eight rats each were used in the experiments. The first two groups received two subcutaneous injections at day 1 and 3 of either wild-type r-hGH or r-hGH-(IleCys138)-Cys^{138}-PEG 20 kDa at the dosage of 120 µg r-hGH equivalent/injection/rat (giving a total dose of 240 µg r-hGH equivalent/rat). Animals belonging to the third group received six daily subcutaneous injections of 40 µg r-hGH/rat (giving a total dose of 240 µg r-hGH/rat). The fourth control group was injected with vehicle once a day for six days.

2.7. Statistical analysis

Results are reported as mean $\pm$ SEM. Statistical analysis of the difference between means was performed by ANOVA followed by Bonferroni post-hoc test. The threshold for significance was set at $p < 0.05$.

2.8. Reagents

Restriction enzymes, DNA-modifying enzymes and JM109 K12 E. coli cells were purchased from New England Biolabs (Beverly, MA, USA). Soytone and yeast extracts for the preparation of culture media were from Difco (Voigt Global Distribution, Lawrence, KS, USA). Monomethoxy-PEG-propionaldehyde 20 kDa was obtained from SunBio (Anyang City, South Korea), monometoxy-PEG-maleimide 20 kDa was from Shearwater Corp. (Huntsville, AL, USA). Phenyl Sepharose Fast Flow, DEAE Sepharose Fast Flow and MonoQ resins were from GE Healthcare (Uppsala, Sweden). All other chemicals were purchased from Merck, Sigma-Aldrich or Carlo Erba (Milan, Italy). Nb2-11 cells, Fisher medium, horse serum, fetal calf serum (FCS), penicillin and streptomycin were obtained from Sigma (St. Louis, MO, USA).

3. Results and discussion

3.1. r-h-GH mutant expression and PEGylation

Because there are no free Cys in hGH, we decided to introduce new PEGylable Cys residues in hGH by site directed mutagenesis. We used different approaches to produce eleven different hGH mutants. Specifically, either: i) we substituted a single Cys for another aminoacid in hGH sequence, or ii) we mutated one of the Cys involved in the S^{182}–S^{189} disulfide bridge to leave the other Cys free and available for PEGylation, or we added new Cys residues either iii) as additional new residues inside the hGH sequence mutants, or iv) as a Cys^{-5}–Gly4 or –Gly4–Cys^{196} tag fused to hGH N- or C- terminal residues. Table 2 reports the detailed position of all the mutations that we made categorized according to the aforementioned mutagenesis strategies. As shown in this table, for single aminoacid substitution or insertion we chose residues of the hGH molecule located in the connecting loops between the helices 1 and 2 (Ile36 and Phe44) or 3 and 4 (Ile138 and Phe146) that are not part of the core structure of the hormone and are known not to be involved in the interaction with the hGH receptor [12].

The majority of these mutants easily reacted with linear 20 kDa m-PEG-maleimide to give monoPEGylated derivatives. Reaction yields ranged from 22% for r-hGH-C-terminal Gly4–Cys–Cys^{196}–PEG 20 kDa to 86% for r-hGH–(Ile138Cys)–Cys^{138}–PEG 20 kDa. No PEGylation was observed only in mutants bearing a Cys–Gly4 tag at the N- terminus of r-hGH or a Cys substitution for

Ile in position 36 (Table 2). For in vitro and in vivo studies, PEGylated mutants were purified up to more than 90% as assessed by SDS-PAGE and RP-HPLC (Fig. 1). As reference, a N-terminal derivative of r-h-GH obtained by reductive alkylation with 20 kDa m-PEG-propionaldhyde was also prepared and purified by anion-exchange chromatography.

3.2. In vitro biological activity

The biological activity of the r-hGH mutants both in their unmodified and in their PEGylated forms, was assessed in vitro with the Nb2-11 cell proliferation test as detailed in the methods section and compared with that of unmodified and PEGylated wild-type r-hGH. The results of the proliferation experiments that are reported in Table 3, showed relevant differences in the biological activity in vitro of the different mutants.

The substitution of Cys for Phe44 and the insertion of a Cys in position 47 severely impaired hGH biological activity that was,

Fig. 1. High purity of the preparations of PEGylated and non-PEGylated hGH Cys derivatives. The figure shows the results of two different biochemical analyses that were used to evaluate the purity of the hGH derivatives that we produced: SDS-PAGE in panel A and RP-HPLC in panel B. Only the data obtained with r-hGH-(Ile138Cys) and its PEGylated derivative are shown for reference, but similar results were obtained with all the tested compounds. The SDS-PAGE shown in A was carried out using 12% polyacrylamide. The gel was stained with Coomassie Blue after fixation with glutaraldehyde. Lane 1: standard protein markers; lane 2: r-h-GH-(Ile138Cys); lane 3: r-hGH–(Ile138Cys)–Cys^{138}–PEG 20 kDa; lane 4: r-hGH. In B the RP-HPLC separation of r-h-GH–(Ile138Cys)–Cys^{138}–PEG 20 kDa on a Symmetry 300 C18 column eluted with a water-acetonitrile gradient in 0.1% v/v trifluoroacetic acid is shown. Notice the sharp isolated elution peak consistent with the presence of a single mono-PEGylated hGH species. See Section 2 for further details.

instead increased by 30% when Ile138 or Phe146 were replaced with Cys. The other mutants with single residue substitutions showed a moderate decrease in bioactivity in vitro ranging between 15 and 50% (Table 3). A similar decrease in activity was also observed in +Cys139 and +Cys146 insertion mutants. Biological activity was about 50% lower than in the mutants with a Cys–Gly4 tag on hGH amino- or on the C-terminus than in wild-type hGH. The mutation breaking the second disulfide hGH bond, Cys182Ser and Cys189Ser only marginally affected hGH biological activity in vitro (Table 3).

PEGylation dramatically decreased the biological activity in vitro of native r-hGH and of all its mutants, independently from whether Cys was added or introduced by replacement of another aminoacid. Residual activity expressed as percent of the biological activity of non PEGylated molecule was about 15% in the case of wild-type r-hGH and ranged between 5 and 10% for PEGylated Cys hGH mutants. r-h-GH-(Ile138Cys)–Cys^{138}–PEG 20 kDa was the hGH Cys derivative that showed the highest residual activity and was chosen for further pharmacokinetics and pharmacodynamic studies in vivo.

3.3. Pharmacokinetic properties

Fig. 2 shows the time course of the plasma concentrations of r-hGH, of its N-terminus PEGylated form and of the Cys PEGylated mutant derivatives r-hGH-(Ile138Cys)–Cys^{138}–PEG 20 kDa after a single subcutaneous injection of a dose of 1 mg/kg native r-hGH equivalents. The PEGylated r-hGH derivative showed a longer persistence in circulation than wild-type unmodified r-hGH. Half life was, indeed, 0.8 h in the case of wild-type non-PEGylated r-hGH and longer than 3 h in the PEGylated molecules (Table 4). The highest value was reached with r-h-GH-(Ile138Cys)–Cys^{138}–PEG 20 kDa whose half-life was 4.7 h. PEGylated forms also showed a slower absorption than wild-type r-hGH as indicated by their longer T_{max} and smaller C_{max} values (Table 4). Systemic exposure to the hormone was markedly increased by PEGylation as indicated by the evidence that plasma concentration AUC was about 4 fold higher than after wild-type r-hGH administration when the rhGH-(Ile138Cys)–Cys138–PEG 20 kDa was injected.
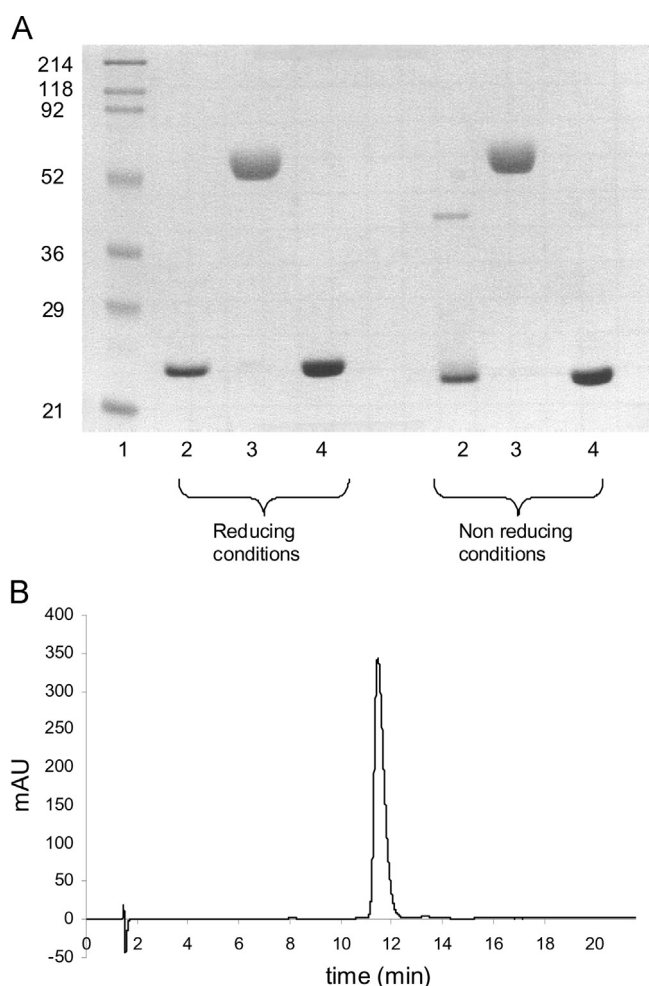
Table 3
In vitro bioactivity of the pegylated and non-pegylated forms of hGH and of its Cys mutants. The table reports the values of the EC$_{50}$ of the concentration-effect curves of Nb2-11 cell proliferation in the presence of progressively higher concentrations of the different forms of hGh under study. The relative potency of these compounds in comparison to wild type unpegylated hGH is also reported. See Section 2 for further details.

| | IN VITRO PROLIFERATIVE ACTIVITY | | | |
| | Before pegylation | | After pegylation | |
	EC$_{50}$ (pg/ml)	Relative potency	EC$_{50}$ (pg/ml)	Relative potency
r-hGH	260 ± 20	100	1724 ± 94*,a	15
r-hGH-(Ile36Cys)	520 ± 45*	50		
r-hGH-(Phe44Cys)	5948 ± 258*	4.4		
r-hGH-(Ile138Cys)	191 ± 29*	136	1752 ± 150*	14.8
r-hGH-(Phe146Cys)	197 ± 53*	132	2553 ± 90*	10
r-hGH-(Cys182Ser)	305 ± 46*	85	2987 ± 140*	8.7
r-hGH(Cys189Ser)	240 ± 29*	108	4270 ± 102*	6
r-hGH-(+Cys139)	551 ± 52*	47		
r-hGH-(+Cys146)	641 ± 65*	40.6	2320 ± 122*	11.2
r-hGH-(+Cys47)	1614 ± 60*	16		
r-hGH-N-terminal – Cys^{-5}–Gly$_4$	543 ± 35*	47.8		
r-hGH-C-terminal Gly$_4$–Cys^{196}	517 ± 78*	50	3951 ± 220*	6.5

a N-terminal pegylated r-h-GH.
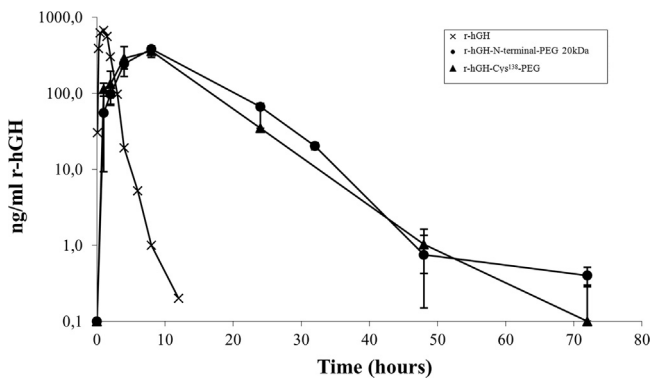* $p < 0.05$ by Student's t test.

Fig. 2. Time course of the plasma concentrations of hGH and of three hGH pegy-lated derivatives after subcutaneous injection in rats. The plot shows the plasma concentrations of r-hGH (×), r-hGH-N-terminal–PEG 20 kDa (•) and r-hGH-(Ile138Cys)–Cys[138]–PEG 20 KDa (▲) measured at different times for 72 h in rats that received at time 0 a single subcutaneous injection of hGH or hGH derivatives at dosage corresponding to 1 mg/kg equivalents. Each point represents the mean ± Std Dev of the values obtained in four animals.

Table 4

Pharmacokinetc parameters of r-hGH and three PEGylated hGH derivatives in rats. The table reports the values of T_{max}, C_{max}, AUC and $t_{1/2}$ obtained in rats after a single subcutaneous administration of either r-hGH, or r-hGH-N-terminal-PEG 20 kDa, or r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa, or r-hGH-(+Cys146)–Cys[146]–PEG 20 kDa at the dosage of 2.5 mg r-hGH equivalent/Kg.

	T_{max} (h)	C_{max} (ng/ml)	AUC* (ng/ml h)	$t_{1/2}$ (h)
r-hGH	1	667.4	1297.4	0.8
r-hGH- N-terminal–PEG 20 kDa	8	382.4	5815.7	3.4
r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa	8	288.4	5285.7	4.7
r-hGH-(+Cys146)–Cys[146]–PEG 20 kDa	24	286.8	10,496.2	3.2

* AUC 72 h.

3.4. In vivo activity of r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa

The biological activity in vivo of r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa was assessed by measuring each day for a week the increase in body weight that it caused when injected to hypo-physectomised rats. For comparison we also evaluated the effect of equivalent doses of unmodified r-hGH. The results of this study showed that a similar increase in body weight was obtained when a cumulative dose 240 μg hGH equivalent/rat was administered by daily injections (40 μg each) of non-PEGylated r-hGH or as two injections (120 μg each) of r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa on day 1 and 3. We also compared the effects of wild-type non-PEGylated r-hGH and of r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa when given with the same administration schedule of two sub-cutaneous injections a week (2 × 120 μ² of r-GH equivalent/rat and day 1 and 3). As shown in Fig. 3, in these experimental conditions our PEGylated mutant hGH was significantly more effective than wild-type unmodified hGH.

3.5. Discussion

In the present paper we reported the preclinical characteriza-tion of eleven new Cys hGH mutants and of their PEGylated derivatives. One of these new PEGylated hGH showed a longer half-life and a higher biological activity than wild type N-terminal PEGylated hGH and could represent a promising new long-acting hGH.

PEGylation is a widely used, well validated, technique that prolongs the half-life of biotechnological drugs by increasing their hydration radius hence preventing glomerular filtration [10]. It also shields molecule surface. While this could be helpful because it prevents receptor-mediated clearance, it can also decrease bio-logical activity by impairing the interaction with the receptors that mediate the pharmacological effects of these drugs. This explains why, especially in the case of small proteins like GH, random PEGylation at multiple sites such as free amino groups usually leads to derivatives with low receptor affinities [17]. Better results can be obtained by directing PEGylation to specific residues that are far from functionally relevant regions of the molecule. Targeted PEGylation can be obtained by introducing new PEGylable resi-dues such as Cys by site directed mutagenenesis. The effectiveness of this approach has been demonstrated already in the case of hGH by Cox et al. [18] who developed a T3C hGH PEGylated derivative that showed an about 100 fold higher in vitro bioactivity than previously characterized amine-PEGylated hGH molecules. The choice of where the PEGylable Cys should be located is absolutely critical because bulky PEG side chains may distort the tridimen-sional structure of the PEGylated molecule and shield biologically relevant sites. hGH is a quite small protein belonging to the family of class I cytokines. As all the members of this family it has a compact tridimensional structure consisting of two couples of α-helices lying on parallel planes [19]. Previous studies dating back to the 90s led to the identification of regions of this hormone that are critical for receptor binding. Two main interaction sites were identified that bind to the two GH-binding sites, site I and site II, of GH receptors. The former includes portions of the central and carboxyl terminal region of helix 4 (residues 167–191), the N-terminal portion of the loop connecting helices 1 and 2 (residues 54–74) and the center of helix 1 (residues 2–19) [20]. The second includes, instead, portions of the N terminus (residues 1–8) and of the juxtaposed hydrophobic faces of helices 1 and 3 (residues 8–21 and 109–128) [20]. None of the positions that we choose to modify belongs to any of these regions and therefore Cys modification of these sites should not unfavorably impact on biological activity of the molecule. A first set of hGH derivatives was obtained, indeed, by substituting with Cys Ile36 and Phe44, two aminoacid residues located in the C-terminal portion of the loop connecting the helices 1 and 2. This portion of the hGH molecule was considered a
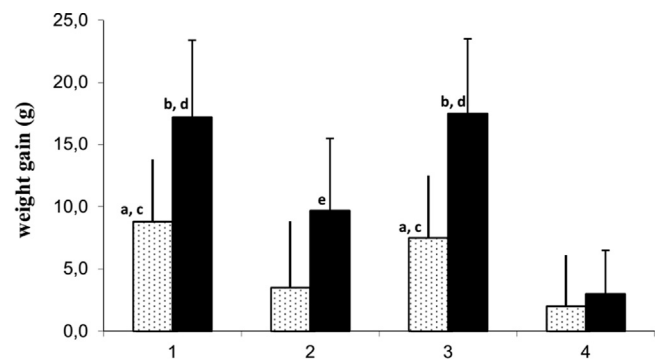

Fig. 3. Biological activity of hGH and of r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa in rats. The bar chart reports the increase in body weight (in grams) that was mea-sured respect to basal values in hypophysectomised rats in response after three

(▦) and six (■) days of treatment with either r-hGH or r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa. Each bar represents the mean ± Std Dev of the values obtained in a group of 8 male Sprague Dawley rats. As indicated in the figure, a first group of rats received daily hGH injections whereas the hormone was injected once every three days in a second group of animals. The group of rats receiving r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa was injected twice a week, once every three days. Rats of the control group received daily vehicle injections. [a]$p < 0.01$ vs control at three days; [b]$p < 0.01$ vs control at six days; [c]$p < 0.05$ vs twice a week r-hGH at three days; [d]$p < 0.05$ vs twice a week r-hGH at six days.

good candidate for Cys mutation also because although one of the two main hGH isoforms, 20 kDa hGH lacks the amino acids from 32 to 46, it has the same growth promoting activity of the higher molecular weight isoform 22 kDa hGH in which these amino acids are present [21]. This suggests that this portion of the hGH molecule is not essential for its biological activity and that mutations in these positions should not cause a decrease in bioactivity. In contrast with this hypothesis, Cys mutation of Phe44 made hGH virtually inactive at the Nb2 proliferation test. This Phe residue that is conserved in GH-related molecules with lactogenic activity, is located in the central portion of mini-helix 1 and hydrophobically interacts with leucine 157 and tyrosines 160 and 164 of the amino terminus of helix 4. Therefore, it can be relevant in maintaining tridimensional configuration of the hormone. Previous mutagenesis studies showed that its substitution with alanine or leucine partially reduced hGH activity at the Nb2 proliferation test whereas its deletion completely abolished the activity of the molecule [22]. Our findings extend these observations by showing that also Cys residue are not as well tolerated as the substitution with small hydrophobic amino acids presumably because prevents the correct folding of the protein. Interestingly, also Cys substitution for Ile36 decreased hGH activity though not so dramatically as when Phe44 was Cys-substituted. Intriguingly, as Phe44, this Ile residue stabilizes hGH tridimensional configuration by interacting with hydrophobic residues in the helices 2 and 4 [12]. Other mutants were prepared by mutating in Cys Ile138 or Phe146, two residues located in the C-terminal portion of the loop joining helices 3 and 4. Both these mutations caused a 30% increase in activity. This result was unexpected because these two residues, do not take part to hormone binding to any of the two receptors and have not been shown to play major structural roles in determining the tridimensional configuration of hGH. The mechanistic bases of this effect that has not been described before, will deserve further investigation. Remarkably, biological activity in vitro did not increase but was lowered by about 50% when instead of introducing Cys by substitution we inserted it as an additional aminoacid.

An additional strategy that we explored to introduce PEGylable Cys residues into hGH was the addition of a short Cys-containing aminoacidic chain at either the amino or carboxy-terminal of the molecule. In both cases we observed a decrease in bioactivity of about 50%. This finding was not unexpected because the amino-terminal of hGH largely overlaps with GHR Site II binding region [20], whereas the COO-terminus is in proximity to the hGH region that binds to GHR site I.

While all the modification strategies that we discussed before were based on the introduction of new Cys residues either by substitution of other amino acids in hGH sequence or by the insertion of new aminoacidic residues, we also explored the possibility to PEGylate one of the four Cys contained in hGH. These Cys that are all evolutionary conserved, are normally inaccessible because involved in two disulfide bonds. The first is located between Cys-53 and Cys-165 and results in a large loop, whereas the second is between Cys-182 and Cys-189, and results in a small loop. To free up one of these Cys and make it available for PEGylation we decided to mutate its companion Cys so that the disulfide bond was broken. Previous work suggested that the disulfilde bond between Cys-53 and Cys-165 could be functionally important because mutations at these residues impair the growth of experimental animals [23] and a Cys53Ser mutation was found in patients with short stature [24]. Conversely, disruption of the second disulfide bond was shown not to significantly affect release and activity of [23,25]. Consistent with these findings we found that substitutions at residues 182 and 189 had a small or negligible effect on biological activity. In contrast with these findings, it has been reported that a byproduct with a thioether link between Cys 182 and Cys 189 that is formed during r-hGH production in bioreactors [26,27], has a significantly decreased receptor affinity and bioactivity in comparison with unmodified hGH [28]. However, this kind of molecular modification is expected to have opposite effects as compared with the bridge opening that we made and to increase the rigidity of the C terminus of GH that is considered one of the most mobile portions of the molecule in normal conditions [27].

Collectively our results showed that Cys modification of hGH could have significant effects on biological activity even though it was directed to residues believed not relevant for normal hGH function. Future structure-activity studies will be needed to clarify this point.

When we performed PEGylation of our hGH Cys derivatives we found a marked decrease in biological activity in vitro that, in the best cases, was only 10–15% of that of wild-type hGH. The most obvious explanation of this result is that our efforts to minimally affect hGH interaction with its receptors by rationally choosing the positions to be PEGylated was thwarted by the steric hindrance of the 20 kDa PEG chain. While our data further emphasize the difficulties in performing a really conservative PEGylation of small proteins, an important finding of our study was that the loss of bioactivity in vitro can be compensated for by the increase in half-life. Indeed, when we tested in vivo r-hGH-(Ile138Cys)–Cys[138]–PEG 20 kDa in rats, our PEGylated mutant with the highest biological activity in vitro, we found that it was more effective than wild-type hGH when administered twice a week in rats although bioactivity in vitro was only about 15% of wild-type hGH. This can be explained by the fivefold increase in half-life that caused a parallel increase in total drug exposure as evaluated by AUC. Therefore, despite the decrease in bioactivity in vitro our Ile138Cys could represent a promising candidate for further development.

3.6. Conclusions

In conclusion, we characterized a number of new hGH Cys mutants and we showed that at least one of their PEGylated derivatives has a pharmacokinetic and efficacy profile suggesting that it could represent an interesting compound for further development as a new long-acting hGH form.

Executive summary table

- Eleven new human growth hormone (h-GH) mutants were produced by introducing free pegylable Cys residues at positions that had not been modified in this way in previous studies.
- These new mutant hGHs were reacted with polyethylene glycol functionalised with a maleimide group to obtain long-acting monopegylated analogs.
- In vitro biological activity of r-h-GH mutants and their Cys-pegylated derivatives was evaluated by proliferation assay using Nb2-11 cell line from rat lymphoma.
- Two mutations (Phe44Cys and +Cys47) severely impaired and two (Ile138Cys and Phe146Cys) increased hGH bioactivity in vitro.
- 20 kDa-pegylation caused a dramatic decrease in bioactivity in all mutants as compared with unpegylated forms.
- The Ile138Cys pegylated derivative had a fivefold longer half-life and was significantly more effective than wild-type hGH in causing weight gain when given subcutaneously twice a week to hypophysectomized rats.

Conflict of interest statement

None declared.

Ethical statement

Not required.

Funding statement

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