International Journal of Pharmaceutics 453 (2013) 533–540

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

N-terminal mono-PEGylation of growth hormone antagonist: Correlation of PEG size and pharmacodynamic behavior

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ARTICLE INFO

Article history:
Received 8 December 2012
Received in revised form 5 May 2013
Accepted 12 June 2013
Available online 21 June 2013

Keywords:
PEGylation
Growth hormone antagonist
N-terminus
Polyethylene glycol
Acromegaly

ABSTRACT

Growth hormone antagonist (GHA), an analog of growth hormone (GH), can inhibit GH action and treat acromegaly. However, GHA suffers from a short plasma half-life of 15–20 min that has limited its clinical application. PEGylation, conjugation with polyethylene glycol (PEG), can increase the plasma half-life of GHA. Single PEG attachment (mono-PEGylation) at N-terminus of GHA has the advantages of product homogeneity and minimization of the bioactivity loss. Conjugation of large PEG molecule may increase the plasma half-life but could potentially decrease the bioactivity of GHA, due to the steric shielding effect of PEG. Thus, N-terminal mono-PEGylation of GHA with 20 kDa and 40 kDa PEG were used to look for a balance of the two competing factors. Sedimentation velocity analysis suggested that 40 kDa PEG was more efficient than 20 kDa PEG to elongate the molecular shape of the conjugate. As reflected by marginal suppression of insulin-like growth factor I (IGF-I), GHA conjugated with 40 kDa PEG was statistically indistinguishable from the saline solution that could not inhibit GH action. In contrast, GHA conjugated with 20 kDa PEG can apparently inhibit GH action, as reflected by IGF-I suppression of 30–43%. Thus, our work demonstrated the effective therapeutic potency of N-terminally mono-PEGylated GHA.

1. Introduction

Acromegaly is a chronic disorder in which a benign pituitary adenoma effects hyper-secretion of growth hormone (GH) (Kopchick et al., 2002; Higham et al., 2009). Acromegaly can be clinically treated with growth hormone antagonist (GHA), an analog of GH that has a molecular weight (M_w) of ~22 kDa with nine amino acid substitutions (Mosharraf et al., 2007; Higham and Trainer, 2008). Human GH has two binding sites for GH receptor (GHR) that is a member of the cytokine receptor superfamily. One GHR binds to site 1 on the GH molecule and then a second molecule of GHR sequentially binds to site 2 on the GH molecule, leading to GHR dimerization (Cunningham et al., 1991). GHA can bind the first GHR with high affinity but abolish the binding with the second GHR (Thorner et al., 1999), thereby preventing the receptor dimerization. Thus, GHA can inhibit GH action by interruption of GHR-linked signal transduction. However, GHA has a short plasma half-life of 15–20 min following subcutaneous injection, which has limited its clinical application (Veldhuis et al., 2002).

PEGylation, chemical conjugation with polyethylene glycol (PEG), is an important and successful approach to increase the serum half-life and to decrease the dosing frequency of therapeutic proteins (Jevsevar et al., 2010; Wang et al., 2010). Thus, PEGylation has been used to improve the therapeutic potentials of GHA. Pegvisomant (Pfizer Inc., USA), an FDA-approved multi-PEGylated GHA, has been developed with a plasma half-life of ~72 h (Bergamaschi et al., 2010; Neggers and van der Lely, 2011). Pegvisomant was prepared by non-specific conjugation of GHA with an average of four-six copies of 5 kDa PEG at 8 lysine residues and N-terminal α-amino group of GHA (Goffin and Touraine, 2002; Sherlock et al., 2011). However, multi-PEGylation may increase the probability of modifying the GHR-binding site of GHA and the resultant product may have different biological properties. Moreover, the multiple sites of PEGylation on GHA cannot be precisely controlled. Refined purification and extensive analysis are thus required to ensure batch-to-batch reproducibility (Veronese, 2001; Porekar et al., 2008).

In recent years, a product with a reproducible and uniform mixture of the PEG isomers is required for regulatory approval. Site-specific mono-PEGylation has received much attention for its pronounced advantages over non-specific multi-PEGylation, such

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http://dx.doi.org/10.1016/j.ijpharm.2013.06.022

as product homogeneity, easy purification and minimization of loss of bioactivity (Doherty et al., 2005; Liu et al., 2012a). In addition, mono-PEGylation at only one site of a protein can provide a good balance between bioactivity and serum half-life. N-terminal mono-PEGylation using aldehyde chemistry is such an approach and has been applied to a variety of proteins and peptides (Nie et al., 2006; Hu et al., 2009, 2011; Liu et al., 2012b). Some products (*e.g.*, PEGylated G-CSF, Neupogen, Amgen Inc., USA) using this approach have been approved by FDA (Wattendorf and Merkle, 2008). PEG aldehydes can preferentially react with α-amino group at N-terminus of a protein under mildly acid conditions, due to the significant difference in p*K*a values between the ε-amino group (p*K*a ~10.5–12.0) and the α-amino group at N-terminus (p*K*a ~7.8) (Wang et al., 2011; Hu et al., 2012). Since the N-terminus of GHA is far from the GHR-binding site, it is possible to develop N-terminally mono-PEGylated GHA for clinical application.

Typically, a PEG mass of 20 kDa or greater has been chosen for mono-PEGylation of a protein to exceed the renal exclusion cut-off (>70 kDa effective M_w) (Veronese and Pasut, 2005). This, in turn, can significantly increase the pharmacokinetic (PK) behavior of the protein. However, the large PEG chain can sterically interfere with the interaction between a protein and its receptor and leads to significant loss of bioactivity of the protein. Recent studies demonstrated that the improved PK behavior can compensate the loss of pharmacodynamic (PD) behavior due to the steric interference of PEG. Although *in vitro* bioactivity of a protein involving receptor interactions is inversely proportional to the attached PEG size, its *in vivo* bioactivity is still not predictive. Thus, the correlation of PEG size and pharmacodynamic behavior of GHA needs to be established.

Our present study is aimed to establish the relationship of the PEG size and the PD behavior of GHA for the development of long-acting therapeutic GHA. Here, 20 kDa and 40 kDa PEG aldehydes were both used for N-terminal mono-PEGylation of GHA. The preparation, structural characterization and *in vivo* PD behavior of the two N-terminally mono-PEGylated GHAs were conducted and compared with each other.

2. Materials and methods

2.1. Preparation of the PEGyalted GHAs

GHA was kindly provided by Pfizer Inc. (USA). GHA was incubated with 20 kDa and 40 kDa PEG propionaldehyde (Jenkem Biotech., China) in 50 mM NaAc–HAc buffer (pH 5.5), respectively. The molar masses for the nominal 20 kDa and 40 kDa PEG propionaldehyde were 20 kDa ± 2 kDa and 40 kDa ± 4 kDa, respectively. The polydispersity of the two PEG reagents was 1.08. The reaction was carried out in the presence of $NaCNBH_3$ at 4 °C for overnight (~16 h). The final GHA concentration was 2.2 mg/ml (*i.e.*, 0.1 mM GHA). The concentrations of PEG reagent and $NaCNBH_3$ were indicated in the Results. Bicinchoninic acid (BCA) protein assay kit (Vigorous Biotechnology, Beijing, China) was used to measure the protein and conjugate concentrations. The determining accuracy of the kit was 0.1–2.0 mg/ml. Since PEG cannot react with BCA, the concentration of conjugate species was measured in terms of GHA equivalents.

2.2. Purification of the PEGylated GHAs

The two reaction mixtures were loaded onto a Superdex 200 column (1.6 cm × 60 cm, GE Healthcare, USA). The column was equilibrated and eluted with 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1.0 ml/min. The effluent was detected at the absorbance of 280 nm. The fractions corresponding to the mono-PEGylated GHA with 20 kDa PEG (M-P20K-GHA) and with 40 kDa PEG (M-P40K-GHA) were pooled as the arrows indicated in the chromatogram, respectively. The fractions were concentrated using an Amicon filtration system (Millipore, USA) with a 10 kDa cutoff membrane and stored at −80 °C.

2.3. Characterization of the PEGylated GHAs

The GHA samples were loaded on a Superdex 200 column (1 cm × 30 cm, GE Healthcare, USA). The column was equilibrated and eluted with 20 mM sodium phosphate buffer (pH 7.0). The effluent was detected at the absorbance of 280 nm. SDS-PAGE analysis of the GHA samples was conducted using 14% polyacrylamide gel (Hu et al., 2005). Afterwards, proteins were identified by Coomassie blue staining.

2.4. Characterization of the sites of PEGylation

Tryptic digestion of the GHA samples was performed in 50 mM NH_4HCO_3 containing 2 M urea (pH 8.3) at an enzyme-to-substrate ratio of 1:100 (w/w) at 37 °C for 10 h. The resultant peptides were analyzed by a Vydac C4 column (4.6 mm × 250 mm). The column was equilibrated and eluted with 5% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). Then, the column was eluted with a linear gradient of 5–50% ACN containing 0.1% TFA for 100 min. The flow rate was 0.5 ml/min and the effluent was monitored at 214 nm.

2.5. Circular dichroism

The GHA samples were analyzed by far-UV circular dichroism (CD) at 25 °C, using a JASCO J-810 spectropolarimeter (JASCO, Tokyo, Japan) with a 0.1 cm light path cuvette (Hu et al., 2007). All the GHA samples were at a GHA concentration of 0.3 mg/ml in 20 mM sodium phosphate buffer (pH 7.0). Molar ellipticity ([θ]) was expressed in deg cm^2/dmol based on a mean residue weight of 191.

2.6. Intrinsic fluorescence

The intrinsic fluorescence spectra of the GHA samples were measured by a Hitachi F-4500 fluorescence spectrometer (Hitachi, Japan) at room temperature (Li et al., 2012). The emission spectra were recorded from 300 to 400 nm using an excitation wavelength of 280 nm. Excitation and emission slit widths were 2.5 nm. All the samples were at a GHA concentration of 0.3 mg/ml in 20 mM sodium phosphate buffer (pH 7.0). A cuvette with 1 cm path-length was used.

2.7. Dynamic light scattering

Molecular radii of the GHA samples were measured by dynamic light scattering (DLS) on a DynaPro Titan TC instrument (Wyatt, USA) at 25 °C. The GHA samples were at a GHA concentration of 1.0 mg/ml in 20 mM sodium phosphate buffer (pH 7.0). The GHA samples were centrifuged at 12,000 × *g* for 10 min before analysis.

2.8. Analytical ultracentrifugation

Sedimentation velocity measurements were performed by analytical ultracentrifugation on a ProteomeLab XL-1 (Beckman, USA) equipped with an An-60Ti rotor. The GHA samples with nominal concentration (A_{280} = 0.65) were centrifuged at 60,000 rpm in 20 mM sodium phosphate buffer (pH 7.0) at 20 °C. The absorption optics were used to detect boundary movement at 280 nm. The sedimentation coefficient (S) and the ratio of frictional coefficient (f/f_0) were determined. These values were normalized to standard conditions.