In vitro functional quality characterization of NOTA-modified somatropins

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Abstract

Chemical modifications on protein biopharmaceuticals introduce extra variability in addition to their inherent complexity, hence require more comprehensive analytical and functional characterization during their discovery, development and manufacturing. Somatropin (*i.e.* recombinant human growth hormone, rhGH) modified with the chelating agent S-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) allows the incorporation of radiometals for research and possible theranostic purposes. We previously demonstrated that this conjugation leads to multiple substitution degrees and positional isomers within the product. *In vitro* techniques at molecular and cellular level were now applied to assess their functional quality: (i) size exclusion chromatography (SEC) demonstrated functional complexation with human growth hormone binding protein (hGHBp) to the different NOTA-modified somatropins, as well as to gallium chelated NOTA-functionalities (Ga-10:1 NOTA:somatropin); (ii) native MS offered in-depth information: a substitution degree up to four NOTAs was still functional; (iii) circular dichroism (CD) analysis confirmed the complexation of unmodified and NOTA-modified somatropin to hGHBp; and (iv) a hGHR bioassay demonstrated initiation of the signal transduction cascade, after binding of all investigated products to the receptor presented on cells with a similar potency (pEC_{50} value between 9.53 and 9.78) and efficacy (E_{max} values between 130 and 160%). We conclude that the NOTA-modified somatropins do not possess a significantly different in vitro functionality profile compared to unmodified somatropin. Techniques such as SEC, MS and CD, traditionally used in the physicochemical characterization of proteins, have a demonstrated potential use in the functionality evaluation not only in drug discovery and development but also in quality control settings.

K**eywords:** NOTA-modified somatropin, growth hormone, cancer, native mass spectrometry, size exclusion chromatography, growth hormone receptor bioassay, circular dichroism, ligand binding assays.

1. INTRODUCTION

Protein biopharmaceuticals are an established class within the medicinal product landscape and their members include complex molecules such as hormones, cytokines, monoclonal antibodies (mAbs), fusion proteins and therapeutic enzymes [1-3]. Compared to small molecules, the protein biopharmaceuticals form a more complex product class inherent to (i) their size, *e.g.* mAbs are approximately 150 kDa consisting of different chains, and (ii) their chemical and/or enzymatic modifications/truncations originating from expression, manufacturing and/or storage [4,5]. One cloned recombinant protein can thus lead to many variants that each contribute to the overall quality, safety and efficacy of the product. In comparison to the unmodified protein biopharmaceuticals, chemical modifications such as polyethylene glycol (PEG), bifunctional chelating agents (*e.g.* 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA)) and drug conjugations, render the protein additional functionalities. These modifications can, for example, influence the protein efficacy and create opportunities to deliver a payload such as a radionuclide or cytotoxic drug [6]. These chemical modifications typically target different reactive amino acid residues throughout the protein sequence such as lysine and cysteine residues, leading to different substitution degrees and positional isomers within the product, hence introducing more heterogeneity to the product [7,8]. Control of a specific conjugation site is usually impossible and conjugation at the target-binding interface can lead to unwanted or abolished interactions, as well as an altered pharmacokinetic profile [9,10].

The development of a heterogeneous active pharmaceutical ingredient (API) into a medicinal product requires more regulatory effort and a thorough chemical and functional characterization during development and manufacturing prior to clinical or commercial release. These requirements come along with the need for more advanced analytical tools and further investment in their development. Chromatographic, electrophoretic and mass

spectrometric approaches are traditionally applied for the physicochemical characterization (*e.g.* substitution degree, position isomers, oligomerization, molecular weight, etc.) [11-13], while classical biochemical and cellular assays [14] as well as emerging techniques such as biosensors [15,16] have been applied in the functional characterization. Nowadays, a combined approach within analytical techniques is made from solely physicochemical to a combined functional characterization.

Growth hormone (GH) is a key anabolic hormone, known to stimulate lipolysis during fasting [17,18], to regulate skeletal muscle metabolism [18,19] and to have an important role in bone metabolism throughout life [20]. The last decades, the interest in the actions of GH and the GH receptor (GHR) in cancer progression is growing [21-29]. For example, a higher GHR expression was observed in prostate carcinoma [30] and breast cancer tissue [31], as well as a higher GHR and GH expression in prostate cancer cell lines [32] and in large cell neuroendocrine carcinoma tissue [33]. The pleiotropic effects of GH are mediated after binding to a predimerized receptor presented at the cell surface of target cells, thereby forming a functional 2:1 GHR:GH complex and initiating the signal transduction cascade [19,29,34].

Modification of biological substances with chelating agents (*e.g.* NOTA) allow the incorporation of radiometals for single-photon emission computed tomography/positron emission tomography (SPECT/PET)-research, diagnostic or therapeutic purposes. Such NOTA-somatropins have been previously developed and chemically characterized [8]. In this study, we evaluated the *in vitro* functional quality by means of traditional physicochemical techniques and cellular techniques.

2. MATERIALS AND METHODS

2.1 Chemicals and materials

The S-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) was purchased from Macrocyclics Inc. (Dallas, TX, USA). Zomacton® 4 mg, (Ferring, somatropin Ph.Eur.) was obtained from the Ghent University Hospital (Ghent, Belgium) and human growth hormone binding protein (hGHBp) from MyBiosource (San Diego, USA). The purity quality evaluation, using SDS-PAGE, is represented in Supplementary information **Figure S1**. PD-10 sephadex G-25M columns were acquired from GE healthcare (Diegem, Belgium). Water was purified in-house using an Arium Pro VF TOC purification system (Sartorius, Göttingen, Germany), yielding 18.2 MΩ*cm and ≤ 5 ppb TOC quality water. Other chemicals and solvents were purchased from Merck (Overijse, Belgium), Sigma Aldrich (Diegem, Belgium), Biosolve (Valkenswaard, The Netherlands) or Fischer Scientific (Erembodegem, Belgium), all high quality (>98% purity) and/or HPLC/MS grade. For size exclusion chromatography (SEC) a Waters Alliance 2695 HPLC, equipped with a photodiode array detector 2996 and Waters multi λ fluorescence detector 2475 was used. Native mass spectrometry (MS) studies were conducted on a Waters Synapt G2-Si high-resolution quadrupole time of flight mass spectrometer (Waltham, MA) equipped with a LockSpray dual electrospray ion source. The PathHunter Cytosolic Tyrosine Kinase (CTK) assay with JAK2 target was from DiscoverX (Fremont, USA). CD analysis was performed on a Jasco J-815 CD spectrometer (Tokyo, Japan).

2.2 Synthesis of NOTA-modified somatropin and complexation with gallium

The synthesis procedure of 1:1 NOTA:somatropin, 3:1 NOTA:somatropin and 10:1 NOTA:somatropin was previously detailed by Bracke *et al.*[8] and are the resulting products of respectively equimolar, three times and ten times molar excess of p-SCN-Bn-NOTA over

somatropin during synthesis. For the gallium labeling of 10:1 NOTA:somatropin, approximately 40.5 nmol lyophilized protein was dissolved in 0.1 M ammonium acetate solution pH 5.5. A GaCl3 solution was made as follows: 285 µL of a 4 mM GaCl3 bulk solution in 0.1 M HCl was added to 570 µL 0.05 M NaOH and 1140 µL ammonium acetate solution pH 5.5, supplemented with 0.2 mM acetylacetone. 1500 µL of this solution was transferred into a 2 mL Protein LoBind Eppendorf tube, containing 450 µL of the 10:1 NOTA:somatropin solution (40.5 nmol). The solution was mixed and incubated for 1 h at 37°C in the dark, while mixing at 750 rpm. The sample solution (1.950 mL) was loaded onto a PD-10 column (previously rinsed using 25 mL of PBS, pH 7.4). Before elution, the column was washed with 550 µL of PBS and then gallium labeled NOTA-somatropin was eluted using 2 mL of PBS, pH 7.4, into a 15 mL tube. Chemical quality control (QC) of the NOTA-conjugation and gallium chelation using trypsin and chymotrypsin peptide mapping was performed as previously described with analogous results[8].

2.3 Analytical size-exclusion chromatography

Analytical SEC was performed with two columns, both equipped with suitable guard columns: (1) BioSep-SEC-S 2000, 7.8 (i.d.) x 300 mm (5 µm) (Phenomenex, Utrecht, The Netherlands); (2) a YMC-Pack Diol-120, 4.6 (i.d.) x 150 mm (3 µm) (Achrom, Machelen, Belgium). The analysis used 0.2 M anhydrous potassium phosphate monobasic (KH_2PO_4) and 0.2 M anhydrous potassium phosphate dibasic (K_2HPO_4), pH 6.7-7.2 mobile phase at a flow rate of 1 mL/min and 0.5 mL/min, respectively. The injected sample volume was 20 µL (column 1) and 10 µL (column 2). Chromatography was performed at 22°C ± 3°C. Fluorescence detection was used with excitation and emission wavelengths of 280 nm and 340 nm, respectively. Complex standard (hGHBp + somatropin) and complex samples (hGHBp + NOTA-modified somatropins) were prepared at equal volumes of 50 µL using a

fixed concentration of hGHBp (130 nM) and addition of somatropin, 1:1 NOTA:somatropin, 3:1 NOTA:somatropin, 10:1 NOTA:somatropin or Ga-10:1 NOTA:somatropin at 29.5, 59, 118 or 237 nM (final concentration). The single proteins were evaluated under the same conditions. The protein stocks and dilutions were made in a 0.01% m/v polysorbate 20 solution in mobile phase. The relative affinities of the NOTA-modified somatropins were calculated according to Roswall *et al.* [35], assuming no changes in response factor after gallium chelation and NOTA-conjugation of somatropin:

$$Relative\ hGHBp\ binding\ affinity = \frac{Peak\ area\ 2{:}1\ complex_{complex\ sample}}{Peak\ area\ 2{:}1\ complex_{complex\ standard}} \times \frac{Peak\ area\ somatropin_{single\ protein\ standard}}{Peak\ area\ NOTA{:}somatropin_{single\ protein\ sample}} \quad (1)$$

2.4 Native MS analysis

A Waters Synapt G2-Si high-resolution quadrupole time of flight mass spectrometer (Waltham. MA) equipped with a LockSpray dual electrospray ion source was used to acquire non-denaturing MS data of standard (*i.e.* somatropin) and samples (*i.e.* NOTA-modified somatropins) in positive mode (ESI^+). A leucine enkephalin solution (200 pg/µL leucine enkephalin in 50/50 V/V ACN/H_2O + 0.1% m/v formic acid) was used as the lock mass during the experiment, generating a reference ion for positive ion mode ($[M + H]^+$ = 556.2771). Standards and samples were injected (10 µL) and directly infused (*i.e.* without chromatographic separation) at a flow rate of 10 µL/min. The optimized conditions of analysis were as follows: the source temperature was set at 150°C, desolvation gas temperature was 300°C, cone gas flow was 150 l/h, desolvation gas flow was 800 l/h, capillary voltage was 2.5 kV and sampling cone voltage was 50.0 V. Data were acquired between m/z 500 and 5000 Da. The concentration of standard/sample and hGHBp during analysis were fixed at 666 nM and 365 nM, respectively. All compounds were dissolved in a 25 mM ammonium acetate solution pH 6.8-7.0.

The data between m/z 3000-4000 of the complex sample and complex standard were further analysed using a Python program. Data were smoothed using a moving average filter (301 points). The baseline was fitted in regions without protein signal with a quadratic function. Peak deconvolution was performed on offset data (*i.e.* baseline subtracted) using the sum of exponential Gaussian peak algorithm, using the least-squares fitting technique.

2.5 Circular dichroism analysis

CD analysis was performed on a nitrogen-flushed Jasco J-815 spectrometer. Multiscanning was required for high data precision. At a scan speed of 50 nm/min, 3 continuous scans were recorded in the 185–360 nm regions. CD spectra were reported in millidegrees (m^o). The spectrometer was calibrated with (1S)-(+)-10-camphorsulfonic acid according to European Pharmacopeia. The study was conducted in duplicate by adding two consecutive volume of 10 µl of 13.3µM analyte stock solution (somatropin and 10:1 NOTA:somatropin respectively) directly into the Hellma Analytics cuvette of 1 mm path length, containing an initial volume of 200 µl of 640 nM hGHBp in 12 mM phosphate buffer (pH 7.4).

2.6 *In vitro* GHR bio-assay

The PathHunter Cytosolic Tyrosine Kinase (CTK) Functional Assay (JAK2 target) was used for the profiling of NOTA-modified somatropins in agonist and antagonist format. The cells (U2OS cell background) were seeded in a total volume of 20 µL Cell Plating Reagent (*i.e.* 1% Charcoal/Dextran-treated Fetal Bovine Serum) into 384-well microplates. For agonist determination, cells were incubated with sample to induce a response. An intermediate dilution of sample stocks was prepared to generate 5X sample in assay buffer (0.1% BSA in PBS). Five microliter of 5X sample was added to each well and incubated for three hours. In antagonist mode, cells were pre-incubated with sample for 60 min at 37°C, followed by hGH

agonist incubation for three hours (EC80 challenge; 0.012 µg/mL hGH). Assay signal was generated through a single addition of 12.5 or 15.0 µL (50% V/V) of PathHunter Detection reagent cocktail for agonist and antagonist assays respectively, followed by a one hour incubation at room temperature. The microplates were read and signals were generated with a Perkin Elmer Envision™ instrument for chemiluminescent signal detection in relative luminescence units (RLU). Human GH and INCB018424, *i.e.* a potent, selective and orally bioavailable inhibitor of JAK1 and JAK2 [36,37], were used as positive controls in agonist and antagonist format, respectively. All compounds under investigation were analyzed using a concentration range between 0.5 pM and 0.01 µM, in a logdose-spaced manner. Four individual independent replicates were performed, yielding four curves and obtaining four EC_{50} for potency (expressed as pEC_{50} values) and four E_{max} values for efficacy.

For agonist mode assays, percentage activity was calculated using the following formula:

$$\%\ \text{Activity} = 100\% \times \frac{(\text{mean RLU of test sample} - \text{mean RLU of blank})}{(\text{mean MAX RLU control} - \text{mean RLU of blank})} \quad (2)$$

For antagonist mode assays, percentage inhibition was calculated using the following:

$$\%\ \text{Inhibition} = 100\% \times \frac{1-(\text{mean RLU of test sample} - \text{mean RLU of blank})}{(\text{RLU EC80 control} - \text{mean RLU of blank})} \quad (3)$$

Data was further analyzed using non-linear regression (least square regression) to calculate the RC_{50} (*i.e.* EC_{50} or IC_{50}) and E_{max} values (Prism 5 software, Graphpad, La Jolla, USA):

$$E = Effect = E_{min} + \frac{E_{max} - E_{min}}{1+10^{([\log(RC_{50}) - X] \times hill\ slope)}} \quad (4)$$

The pEC_{50} (=logRC_{50} in the above four parameters logistic sigmoid function) and E_{max} values of the different compounds were compared using ANOVA (SPSS Statistics 24 software, New York, USA).

3. RESULTS

3.1 Analytical size-exclusion chromatography

Analytical size exclusion chromatography (SEC) allows to evaluate the *in vitro* binding capacity of NOTA-modified somatropins to the soluble form and extracellular part of the human GHR, *i.e.* human growth hormone binding protein (hGHBp). The suitability of this method, known as the High Performance Receptor Binding Chromatography (HPRBC) method [35], was confirmed using the unmodified somatropin standard and hGHBp on two SEC columns. **Figures 1A** and **1B** show the stoichiometric analysis of hGHBp and somatropin, *i.e.* the concentration dependent dimerization mechanism with 2:1 hGHBp:somatropin complexes in excess hGHBp conditions and increasing 1:1 complexes in excess somatropin (analyte) conditions. The 2:1 hGHBp:somatropin complex elutes first, followed by the 1:1 complex, somatropin and hGHBp. This ligand binding complexation is in agreement with the observations made in prior work [35,38]. The hGHBp single protein chromatogram has several peaks indicating that the applied hGHBp consisted of a mixture of hGHBp-isoforms and impurities, including hGHBp oligomers (Supplementary information **Figure S2**). Indeed, analysis on SDS-PAGE confirmed the presence of higher molecular weight substances (Supplementary information **Figure S1**).

All NOTA-modified somatropins were in a 2:1 complex when approximately 2-4.5 times molar excess of hGHBp was used (**Figure 1C** and **1D**), which demonstrated the binding-functionality and dimerization capacity of these NOTA-modified somatropins, as well as of the gallium chelated NOTA-modified somatropin towards hGHBp (**Figure 1D**). The relative affinity of the samples increased upon NOTA-modification (1.21, 1.35, 1.42 and 1.81 for 1:1 NOTA:somatropin, 3:1 NOTA:somatropin, 10:1 NOTA:somatropin and Ga-10:1 NOTA:somatropin, respectively). The formation of a 1:1 hGHBp:analyte complex was

also observed when the NOTA-modified somatropin samples were in excess relative to hGHBp (**Figure S3**).

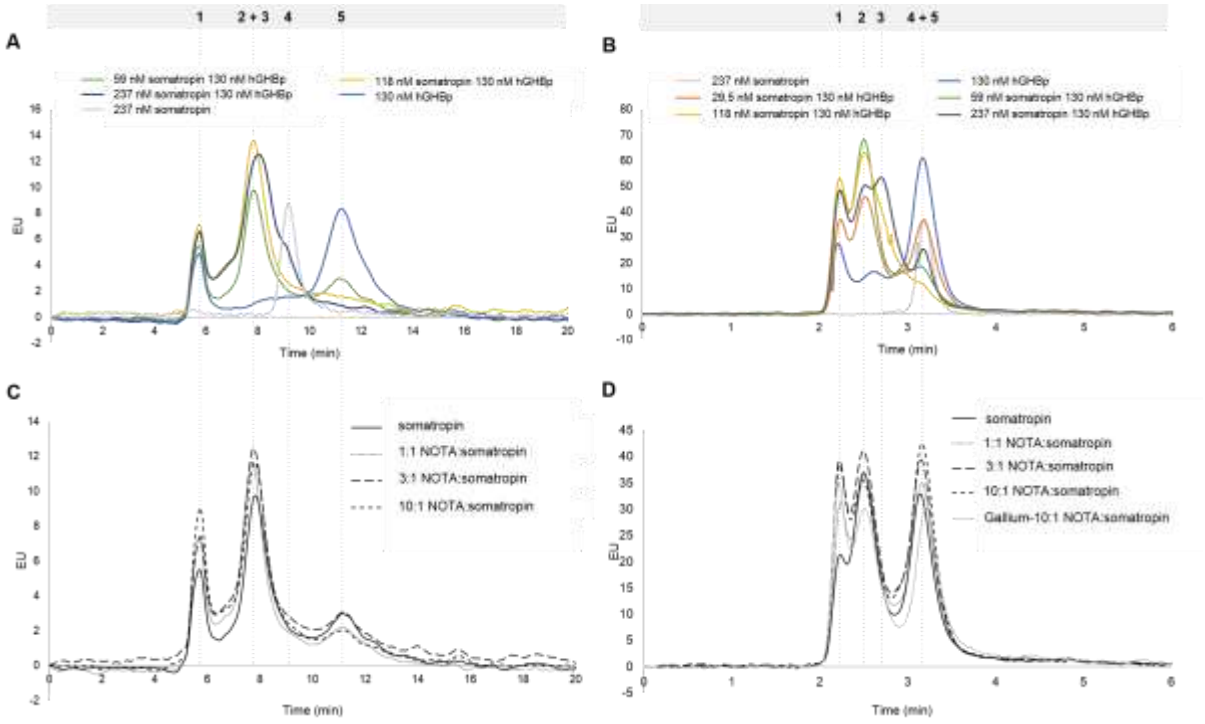

Figure 1: Analytical SEC analysis of (NOTA-modified) somatropin and hGHBp. (A) Overlay chromatograms of the stoichiometric analysis of somatropin standard and hGHBp on column 1 and (B) column 2. (C) Functional activity evaluation of NOTA-modified somatropin samples with a two times molar excess of hGHBp on column 1, and (D) a 4.5 times molar excess on column 2. (1): Impurity from the hGHBp sample, (2): 2:1 hGHBp: somatropin, (3): 1:1 hGHBp:somatropin, (4): somatropin and (5): hGHBp.

A comparison of both silica based SEC columns is enclosed in **Table S1** (supplementary information). They both have a different molecular weight range, but overlap in the region of interest (22 – 78 kDa). The single proteins were also analysed (**Figure S3**). Each NOTA-conjugation increases the molecular weight with 449.5 Da and, as previously observed, the substitution degree increases in the samples from 1:1 to 10:1 NOTA:somatropin [8]. We observed a longer elution time for the NOTA-modified somatopins. The increased polarity (*i.a.* the addition of three negative charges and removal of one positive charge) as a result of

the NOTA-substitution, likely creates more interactions with the hydrophilic deactivated stationary phases, hence resulting in an increased elution volume. The hGHBp (Mw: 29 kDa) and somatropin (Mw: 22 kDa) single protein standards elute at different elution times on column 1, thereby complementing the information provided by column 2 and simplifying the identification of excess somatropin or hGHBp in the different complex samples. The different elution times of hGHBp compared to somatropin is likely due to physicochemical interactions with the column stationary phases.

3.2 Native MS analysis of protein complexes

Confirmation and more in depth exploration of the SEC results, *i.e.* interrogation of the different product species (somatropins containing one, two or more NOTA-substitutions) for hGHBp-binding, was done by native MS. Native MS, also referred to as non-denaturing MS, allows the detection of the different substitution species-hGHBp-complexes in a single mass spectrum under non-denaturing conditions (*i.e.* typically volatile solutions such as ammonium acetate with reduced formation of adducts in the gas phase [39]).

In general, the observed percentage substitution degree within the NOTA-modified somatropin samples (**Figure 2E** and **Figure S4**) are in agreement with previously reported data [8]. The spectra of the standard and sample complex solutions are given in **Figure 2**. In the standard complex, ions corresponding to 1:1 (**Figure 2A**) and 2:1 hGHBp:somatropin (**Figure S5**) complexes are detected. However, the increasing species complexity within the 1:1 to the 10:1 NOTA:somatropin samples leads to a lower concentration of a single substitution degree species, and hence, a lower signal of the different hGHBp-complex species. Moreover, using a ~2 times molar excess of analyte relative to hGHBp creates a large fraction of 1:1 hGHBp:analyte complex, as observed in our analytical SEC data. Analyte with a substitution degree up to four NOTAs, was still able to form a complex with hGHBp. The

semi-quantitative substitution degree species distribution in the single protein analyte samples (**Figure 2E**) suggests a similar distribution within the hGHBp:analyte complexes (**Figure 2F**). There were no free hGHBp ions detected in the complex samples, indicating the hGHBp-binding functionality of the different species within the samples.

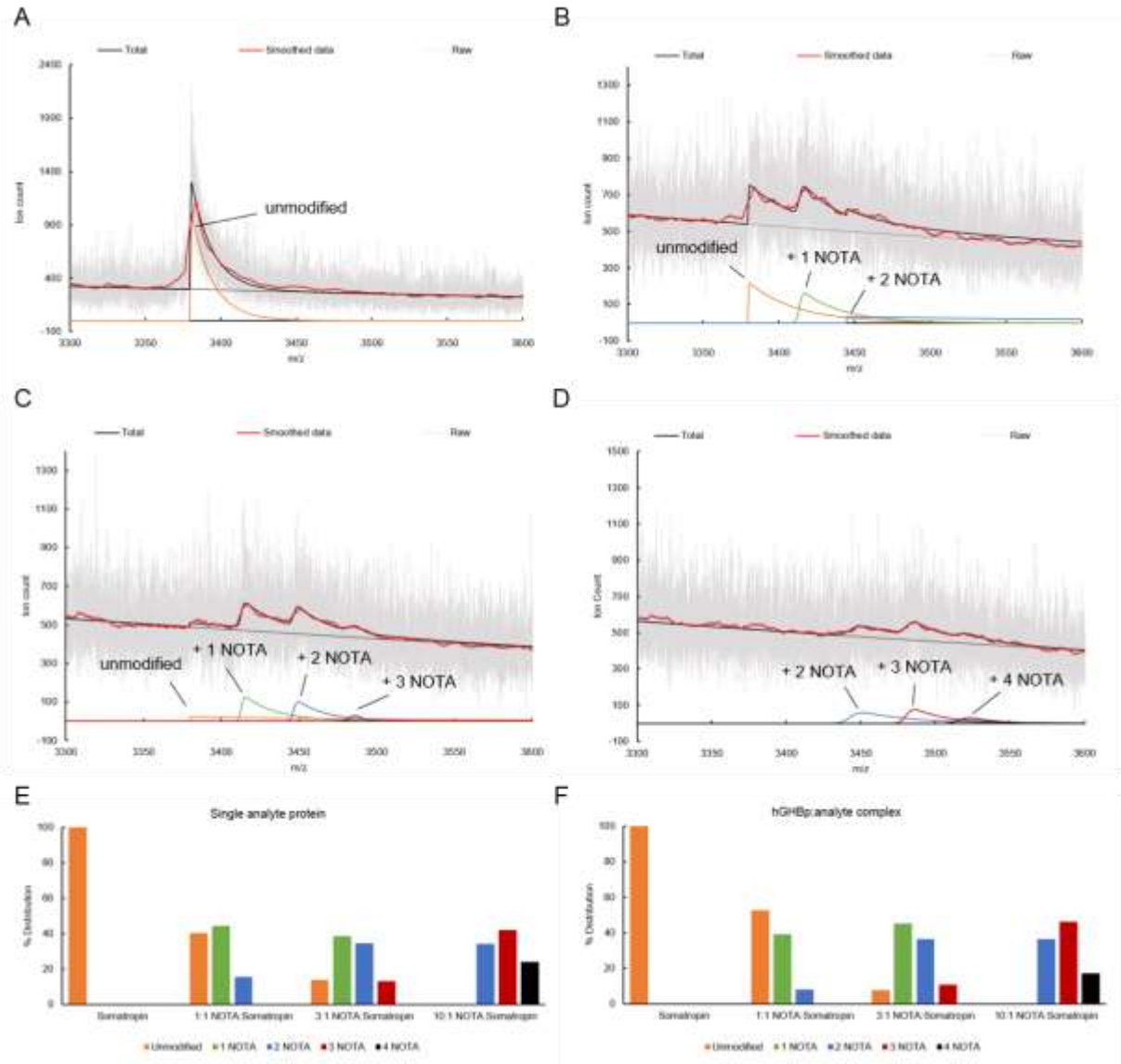

Figure 2: Native MS spectra analysis of the complex samples. A-D: Deconvolution analysis of the 1:1 complex standard/samples, analyte:hGHBp ions (z=15) are shown. The upper part of each curve shows the raw data in gray, the smoothed data in red and the total sum of the peak deconvolution in black. At the bottom of each curve is the baseline subtracted peak deconvolution shown, with orange: unmodified somatropin:hGHBp complex, green: 1 NOTA-somatropin:hGHBp complex, blue: 2 NOTA-somatropin:hGHBp complex, dark red: 3 NOTA-

somatropin:hGHBp complex and black: 4 NOTA-somatropin:hGHBp complex. (A) Analyte: somatropin standard. (B) Analyte: 1:1 NOTA:somatropin. (C) Analyte: 3:1 NOTA:somatropin. (D) Analyte: 10:1 NOTA:somatropin. (E) Species distribution analysis of the single protein standard and samples (z=10). (F) Species distribution analysis within the analyte:hGHBp complex (z=15); analytes are shown on the x-axis.

3.3 Circular dichroism analysis

CD was used to additionally confirm the (modified) somotropin-hGHBp complex formation. For proteins, the experimentally measured difference in left- and right-handed circularly polarized light in the far UV (below 250nm) is mainly due to the peptide bone (amide chromophore), reflecting the secondary structure of the protein. Ligand-binding interactions can be measured by CD due to the change in the secondary structure of one or both ligands upon interactions and hence a difference in CD spectrum. Hence, due to the additive properties of the UV-CD response[40-41], the comparison between the sum of the 2 individual spectra (*i.e.* the calculated, addition spectrum) with the experimentally obtained complex-analytes will demonstrate an interaction: if the experimentally observed and simulated-sum spectra are identical, there is no detectable interaction, whereas if the spectra are different, a binding interaction unambiguously took place. In **Figure 3A** and **3C**, the result confirms our hypothesis, where the difference in 185-250nm range was observed indicating the interaction between unmodified somatropin and hGHBp. Similar spectral differences were found for the modified 10:1 NOTA:somatropin (**Figures 3B** and **3D**), indicating that both molecules show similar secondary structure change upon binding to hGHBp.

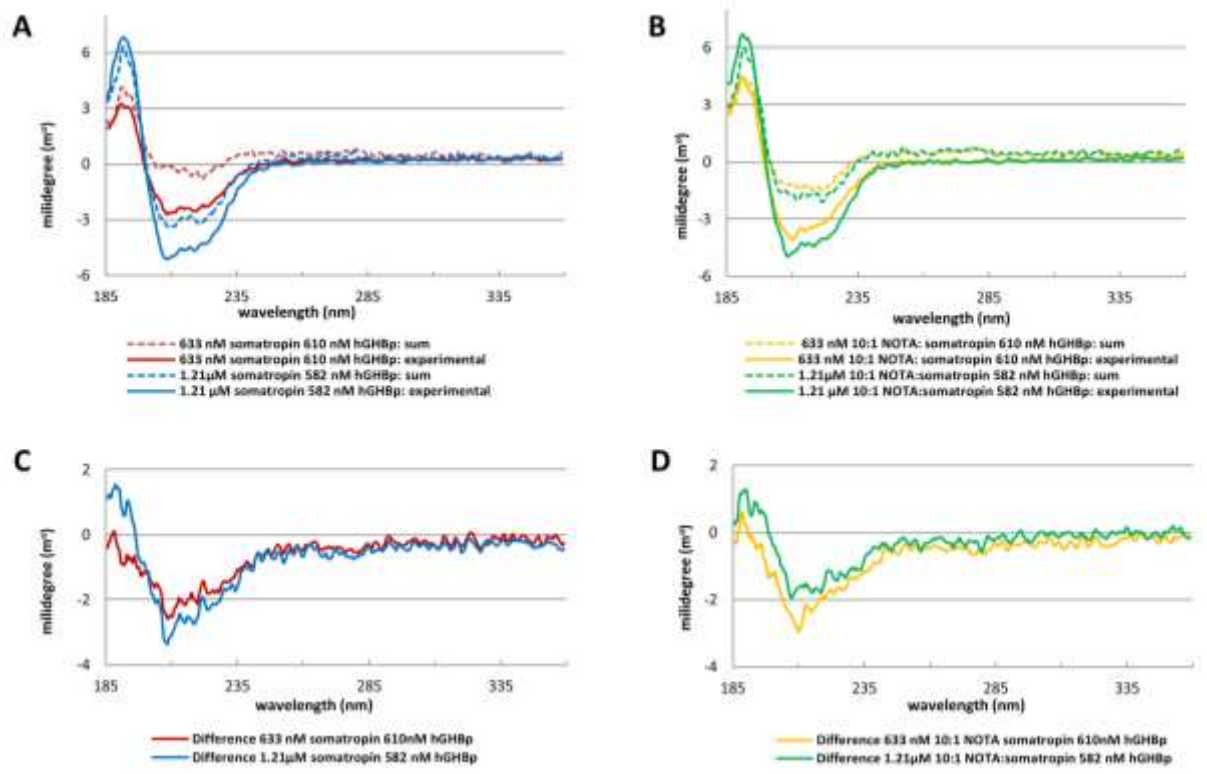

Figure 3. CD spectra of (NOTA-modified) somatropin and hGHBp (n=2). (A) spectra of somatropin as analyte; (B) spectra of 10:1 NOTA:somatropin as analyte; (C) difference spectra with somatropin as analyte; (D) difference spectra with 10:1 NOTA:somatropin as analyte.

3.4 *In vitro* GHR bio-assay

The functional quality of NOTA-modified somatropin for binding to the full length human GHR and activation/inhibition of the signal transduction cascade was evaluated using a PathHunter Cytosolic Tyrosine Kinase (CTK) functional GHR bioassay. This bioassay involves the intracellular JAK2 target, because this JAK2 pathway is the classical route of GHR signaling [42].

An overview of the results is given in **Figure 4** and **Table 1**. All NOTA-modified somatropins were able to bind to the hGHR in agonist mode. No antagonistic effects were found at concentrations up to 10 nM, not even for the 10:1 NOTA:somatropin sample in which no unmodified somatropins were detected [8]. The efficacy of all samples in the agonist format (*i.e.* the E_{max} values) did not statistically differ (ANOVA, $p>0.05$), as well as the

potency (*i.e.* expressed as pEC_{50} values) of investigated somatropins (pEC_{50} values ranged between 9.53 and 9.78).

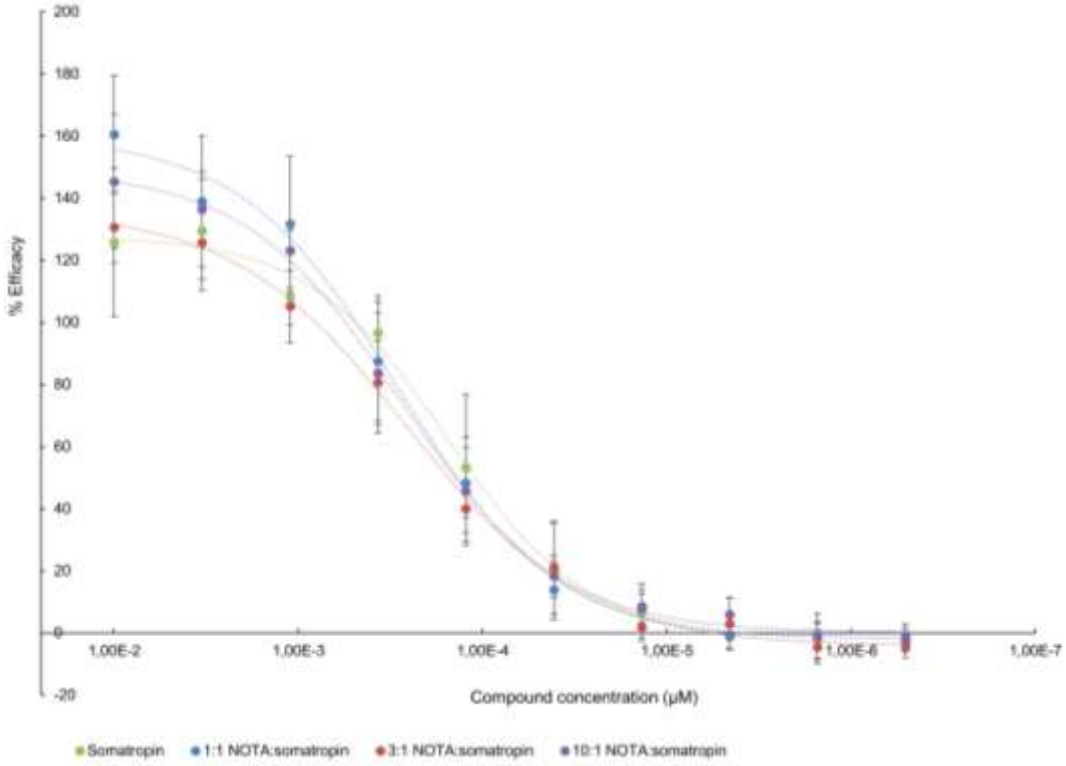

Figure 4: Dose response curves of the hGHR bioassay in agonist format, error bars represent the standard deviation (n = 4).

Table 1: Overview of the hGHR bioassay overall results with their standard deviation (n=4)

Compound Name	pEC_{50}	E_{max}(%)
Somatropin Ph. Eur.	9.78 ± 0.10	130 ± 13
1:1 NOTA:somatropin	9.54 ± 0.22	160 ± 21
1:3 NOTA:somatropin	9.56 ± 0.26	139 ± 18
1:10 NOTA:somatropin	9.53 ± 0.07	151 ± 17

4. DISCUSSION

The thorough regulatory requirements for biopharmaceuticals have shifted the use of techniques and methods that were previously solely used for physicochemical characterization

to a combined functionality characterization. For example, in the European Pharmacopoeia, SEC is generally used in the test for related substances of higher molecular mass in the protein monographs (*e.g.* Ph. Eur.8.8: 01/2008:0951 [43]). Also MS is generally used for peptide mapping purposes or direct analysis of proteins, allowing identification of the substitution degree of the conjugate/post-translational modification, as well as identification of the modification hot spot [8,11]. By the inclusion of a receptor or target within the sample, the functional quality characterization of the product can be established: complexation, indicative for a functional product, shifts the elution volume to the left (HPRBC/SEC), shifts the m/z values to higher values (native MS) and shifts the UV-CD signals.

During the HPRBC/SEC analysis, we observed mainly the 2:1 hGHBp:somatropin in conditions with molar excess of hGHBp, while in conditions with molar excess of somatropin, also the 1:1 hGHBp:somatropin complex is detected. Somatropin contains two receptor binding sites, a high affinity site I and a low affinity site II [44,45], which are mechanistically important in the concentration dependent binding that we observe in the SEC/HPRBC. After binding of receptor 1 to the high affinity site I, receptor 2 makes contacts on somatropin and on the somatropin-bound-receptor 1, yielding a 2:1 complex. Especially in a biological context, only after the formation of a functional 2:1 complex, the receptor undergoes a conformational change, leading to a signal transduction cascade.

Native MS is nowadays an established technique during the drug discovery phases, such as during the chemical characterization of modified proteins [46], the characterization of protein-metal complexes destined for either diagnostic or therapeutic purposes [47], and, during the investigation of interactions between protein drugs with their therapeutic targets/physiological partners, including unmodified proteins and their modified versions [48-51]. In this study, the different substitution-degree species within the protein product were functionally evaluated for hGHBp binding, thereby complementing the information provided by SEC/HPRBC.

Confirmation was obtained by the conformation-sensitive UV-CD result under physiological conditions.

In addition to these traditional techniques, also new emerging tools such as biosensors are making an evolution from lab bench equipment to work horses in the industrial settings. For example, we demonstrated that the binding kinetics of these NOTA-modified somatropins were not significantly different from somatropin using a SAW biosensor technique with an antibody ligand (K_D between 15 and 19 nM) [52].

Finally, we used a classical cell assay with full length receptor. We observed a similar potency and efficacy of all investigated somatropin products, meaning that the NOTA-modified somatropins are functional in a biological context and thus confirm our previous results from HPRBC/SEC, native MS and CD. The NOTA-conjugation hotspot within somatropin is K70, followed by K158, and with a lower reactivity K140 and K172 [8]. Based on the crystal structure of hGHBp$_2$:hGH (**Figure 5**), K172 is pointed to the receptor binding site I and has the potential to sterically hinder the receptor after modification with NOTA. Using mutational studies, Cunningham *et al.* demonstrated that K172 is a binding hotspot within site I [44]. A reduced affinity for GHR was also observed after conjugation of K172 with PEG5000 [53]. Compared to PEG with an average molecular weight of 5000 Da, the NOTA addition here is much smaller (449.5 Da). The other identified lysine residues prone to conjugation show enough conformational freedom to have no direct interference with receptor binding. Our results show that all investigated products, including species with a substitution degree of four NOTAs, can bind to the receptor and hence display receptor binding-functionality.

Thus, we have investigated the *in vitro* functionality of the NOTA-modified somatropins on two levels: (i) on molecular level using hGHBp (HPRBC/SEC, native MS and CD), with emphasis on the switch from classical physicochemical characterization techniques to more

functionality characterization, and (ii) on cellular level by the detection of activation/inhibition of the GHR-signal transduction cascade in a biological context.

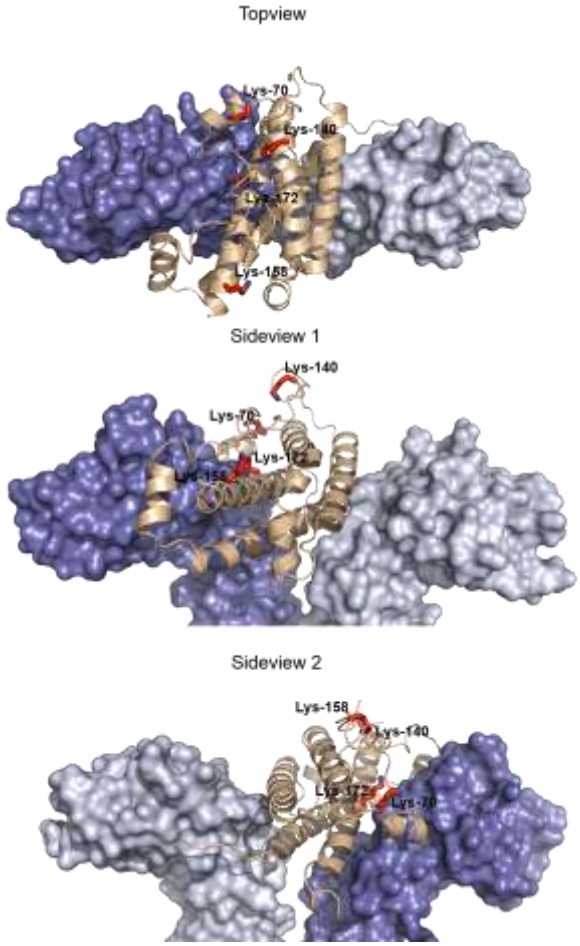

Figure 5: Position of the conjugation hotspots (Lys-70, Lys-158, Lys-140 and Lys-172, all in red stick representation) on somatropin (yellow) within the hGHBp$_2$:somatropin complex (PDB: 3HHR).

5. CONCLUSION

HPRBC/SEC and native MS demonstrated that all different NOTA-substituted somatropins, including gallium chelated NOTA-moieties, were able to bind the hGHBp. Native MS showed binding of the products with a substitution degree of 4 NOTAs. The hGHR bioassay using the JAK2 target, demonstrated a similar potency (pEC_{50} values ranged 9.53 between

9.78) and efficacy (E_{max} values ranged 130 between 160) of all investigated compounds (*i.e.* somatropin and the NOTA-modified samples). The use of molecular techniques HPRBC/SEC, native MS and CD, as well as a cell assay complemented the obtained results to evaluate the functional quality of the NOTA-modified somatropins. We demonstrated that techniques such as SEC, MS and CD, classically used in the physicochemical characterization of proteins, have a potential use not only in the functionality evaluation in drug discovery and development but also in quality control settings.

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