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Development of a long acting human growth hormone analog suitable for once a week dosing

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ABSTRACT

Human growth hormone was conjugated to a carrier aldolase antibody, using a novel linker by connecting a disulphide bond in growth hormone to a lysine-94 amine located on the Fab arm of the antibody. The resulting CovX body showed reduced affinity towards human growth hormone receptor, reduced cell-based activity, but improved pharmacodynamic properties. We have demonstrated that this CovX-body, given once a week, showed comparable activity as growth hormone given daily in an in vivo hypophysectomized rat model.

Somatotroph cells located in the anterior pituitary gland intermittently releases growth hormone (hGH), which has many physiological functions including growth promotion, protein synthesis, lipolysis and regulation of metabolic processes.[1] Although growth hormone has many biological functions, its major target organs are bone and muscle, promoting the growth of the bone and increasing the mass of the muscles.[2] GH deficiency in children results in a condition called pituitary dwarfism, which is characterized by slowed long bone growth, younger facial features and sometimes a chubby body build. Puberty may come late or not at all in older children. It is estimated that 10,000–15,000 children in the United States have growth failure due to growth hormone deficiency.[3]

Recombinant human growth hormone (Genotropin®), a 191 amino acid polypeptide chain, is widely used to treat diseases due to growth hormone deficiency both in children and adults.[4] GH is given as a daily subcutaneous injection due to its short half-life of 20–30 min. Rapid proteolysis and ligand–receptor internalization result in a short half-life. A long acting growth hormone receptor (GHR) agonist with sufficient stability to be dosed weekly is therefore a highly desirable alternative especially for young children. A long acting N-terminally PEGylated growth hormone was advanced to phase II clinical trials but subsequently the studies were terminated due to injection site lipoatrophy.[5,6]

CovX-Bodies represent a novel class of biotherapeutic agents created by the fusion of a therapeutic with an antibody, which serves as a carrier protein scaffold. The fused payload determines the targeting property of a CovX-Body whereas the antibody scaffold provides an antibody like pharmacokinetic and distribution profile which prevents it from crossing the blood–brain barrier. CovX-Bodies are formed by the conjugation of a pharmacophore functionalized with an azetidinone (AZD) linker with a specific lysine residue in the Fab region of a humanized antibody that serves as the carrier scaffold. We have successfully applied this technology to peptide based therapeutic agents and we envisioned the application to the protein therapeutic space such as GH.[7–9] We developed a long-acting hGH analogue conjugated to an aldolase carrier antibody engineered in such a way that human growth hormone was irreversibly linked to one of the Fab arms. The resulting conjugated molecule, known as GH-CovX-Body, has the pharmacological properties of GH with the benefit of extended therapeutic half-life.[10] To the best of our knowledge, this is the first demonstration that half-life of human growth hormone can be extended by conjugating to a carrier antibody that has no biological function.

Growth hormone has two binding sites (site 1 and 2) and binds to two growth hormone receptors in 1:2 complex and initiate intracellular signaling event through receptor homo-dimerization, which is critical for signaling.[11,12] After the signaling event, the entire receptor–ligand complex is internalized resulting in signal termination.[13] By preventing the internalization one could keep the GH in circulation longer. PEGylated growth hormone, which showed reduced affinity and improved half-life, was suitable for once a week dosing.[14] However the development of PEGylated growth hormone was discontinued due to injection site lipoatrophy. Our approach for developing long-acting growth hormone was to reduce the affinity in a way that maintains the required

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potency but likely reduces the internalization rate (or efficiency) and then use such a modified molecule with CovX-Body conjugation to give the desired pharmacokinetic profile. We reasoned that by lowering the affinity of GH towards GHr one could keep the GH in circulation longer since the lower affinity could result in reduced internalization. Several mutants of growth hormone have been shown to have reduced affinity towards GHr, which have the potential to stay in the circulation longer.[15] These studies showed that the affinity towards the receptor can be reduced by as much as 30-fold without affecting the cellular EC_{50}. Our internal studies have shown that by modulating the affinity towards its receptor, hGH can affect the internalization without affecting cellular activity. The traditional approach to modulate the protein affinity towards its receptor is to mutate appropriate amino acids on the surface of the protein. One of the mutated amino acids is then used to attach a linker which could be connected to an antibody. We decided to work with wt-GH for generating an agonist with reduced affinity to reduce the risk of formation of an antibody to the drug molecule.

The crystal structure studies have shown that GH is a four helix bundle with two disulphide bonds which are located towards the C-terminal end of GH (Fig. 1).[16] Several residues of the α-helix on the C-terminal side located in site 1 of GH interact with GH receptor.[17] Our approach to develop a growth hormone with lower affinity was to introduce small conformational changes towards the C-terminal end which would disrupt some of the interactions with the receptor. Initially we examined reaction of linkers containing

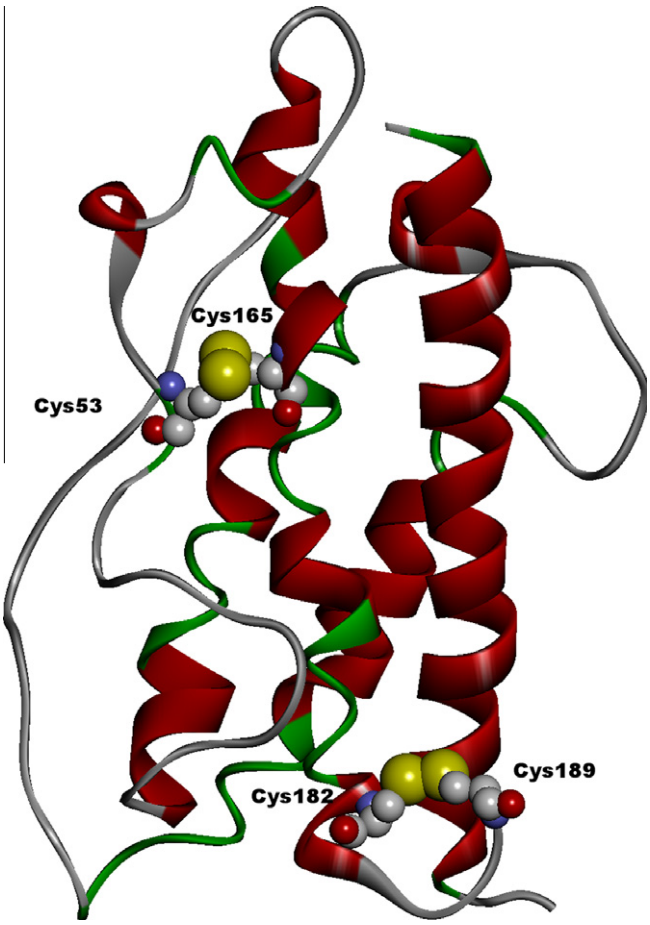
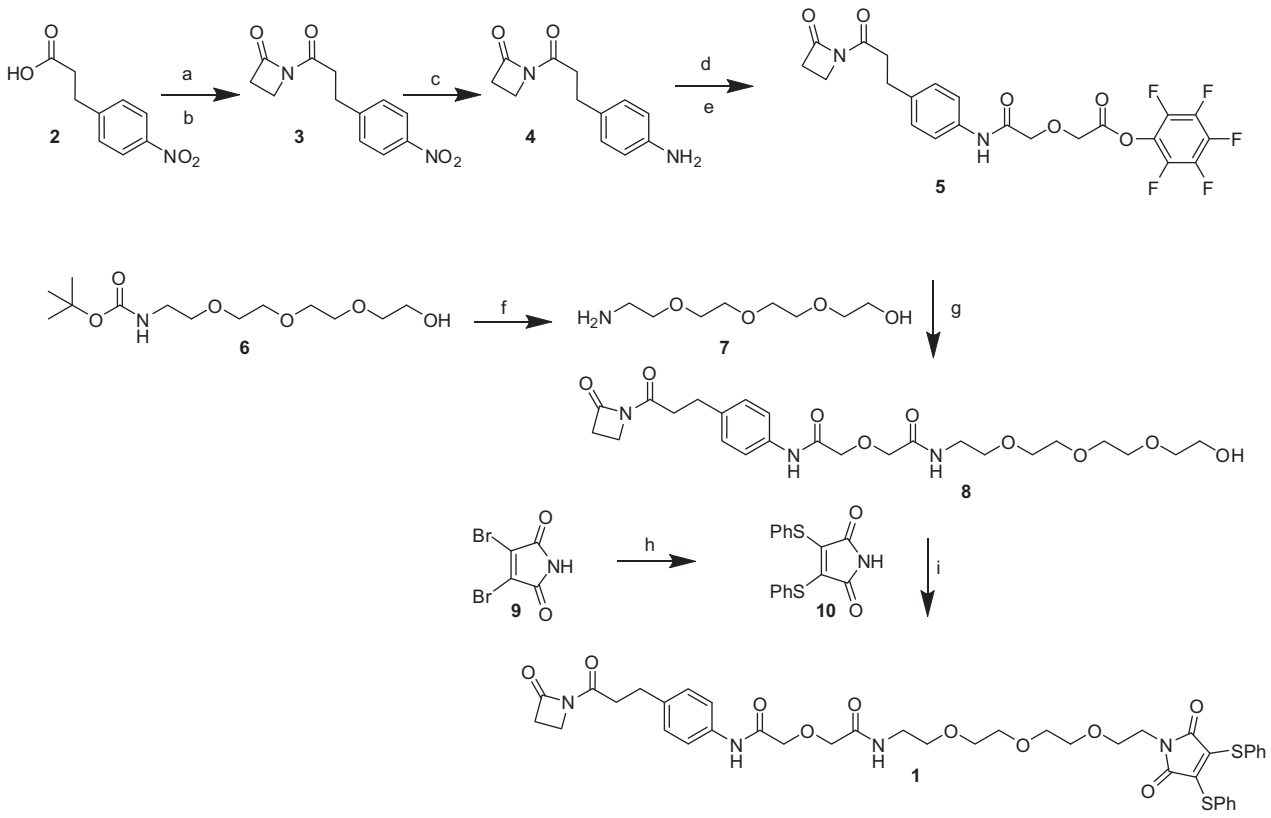


Figure 1. X-ray crystal structure of hGH (PDB ID: 1hgu). Both disulphide bonds are towards the C-terminal end. The disulphide bond between Cys182–Cys189 is more easily accessible for the introduction of a linker. The crystal structure of wild-type growth hormone at 2.5 Å resolution (PDB 1D: 1hgu).

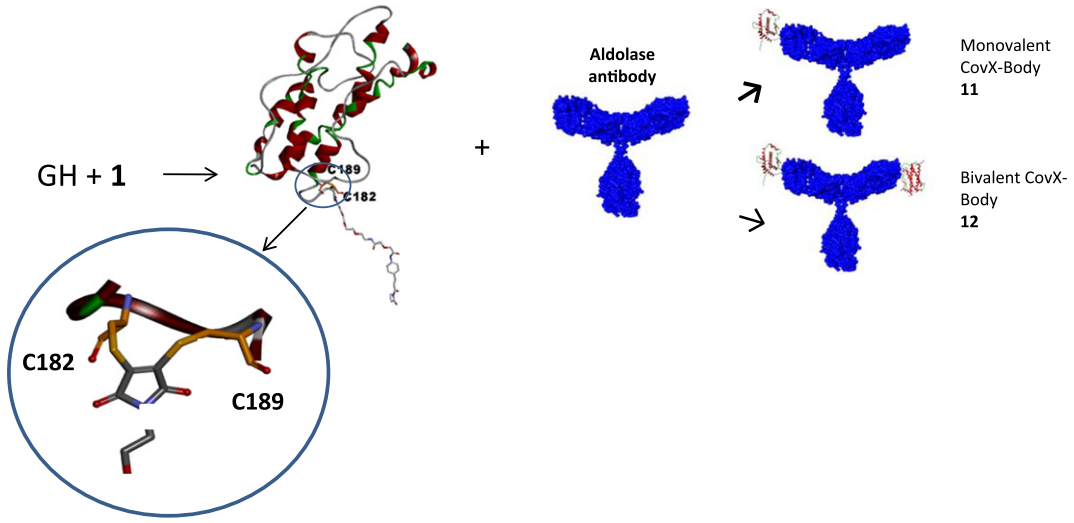
N-hydroxysuccinamide[18] with an amine of a lysine for linker connection. However, this approach resulted in a mixture of products due to natural abundance of lysines on the surface of GH. Our next approach involved inserting a maleimide based linker in between a C-terminal disulphide bond (Cys182–Cys189) and connect GH to the aldolase antibody. This approach offered several advantages. The maleimide group was small and could be inserted between a disulphide bond with high selectivity and efficiency in buffered solutions at room temperature.[19] The examination of crystal structure revealed that both cysteine residues are buried inside and not easily accessible. Hence we reasoned that even if the linker is broken, the cysteine disulphide modification should not elicite any meaningful immunogenic response. The growth hormone by itself has a very short half-life and it is removed from the circulation through receptor internalization if it is cleaved. By introducing a linker at the above location, we anticipated a change in conformation of the C-terminal end which would lower the affinity of GH towards its receptor. It was anticipated that these conformational changes would allow the GH analog to bind to the receptor long enough to trigger the signaling event but not long enough to internalize immediately. We also anticipated by connecting the GH to the aldolase antibody, the GH was protected from proteolysis and stays in circulation longer. On one end of the linker we introduced a diphenylthiomaleimide group which reacts with a thiol group of the protein and on the other end an electrophile to connect to the antibody. The aldolase antibody has a lysine at position 93 of the heavy chain and located deep in the hydrophobic binding pocket on each of the two Fab arms.[20] We have developed phenylpropanoylazetidin-2-one to react selectively with Lys-93 located deep in the hydrophobic pocket as described earlier.[21,22] We proposed linker **1** which would be useful for conjugating either peptides or proteins to the aldolase antibody, provided the peptide or protein has at least one disulphide bond.

The synthesis of a disulphide linker **1** is shown in Scheme 1. 3-(4-Nitrophenyl)propionyl chloride was prepared from **2** as described.[23] The carboxylic acid group in **2** was converted to propionyl acid chloride using thionyl chloride. An amide proton in azetidin-2-one was deprotonated with n-butyl lithium in tetrahydrofuran and reacted with the propionyl chloride to give **3**. Hydrogenation over palladium on carbon gave **4** that was treated with 1,4-dioxane-2,6-dione to give an intermediate acid and the acid group was converted to pentafluorophenyl ester **5** by treating with diisopropylcarbodiimide and pentafluorophenol. The treatment of pentafluorophenyl ester **5** with 2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethanol (**7**) resulted in compound **8** that was converted to **1** as described.[24]

The preparation of the GH-aldolase antibody conjugate CovX-Body) is shown in Scheme 2. Wt-hGH (10 mg/mL in 5 mM sodium phosphate and 34 mM glycine at pH 7.5) was treated with Tris[2-carboxyethyl] phosphine (TCEP, 0.287 mg in 36 μL of 0.1 M sodium phosphate at pH 7.0) for 20 min at room temperature. Compound **1** (1.8 mg in 150 μL of dimethylsulfoxide) was added and shaken very gently overnight at room temperature. The protein was purified using a PD-10 column (PBS buffer, pH 7.4, Mediatech Cat. No. 21-040-CM) and the excess linker and TCEP were removed. Accurate measurement of the intact mass of GH + compound **1** using a QTof mass spectrometer confirmed the addition of one linker onto the GH protein. About 95% of the total GH protein was observed containing 1 linker, with only trace levels of underivatized protein or protein with two (2) or greater linkers attached.[25] Further analysis by MALDI-TOF In-Source Decay mass spectroscopy methodology was used to establish that the linker was attached selectively between the disulphide bond of Cys-182 and Cys-189 in GH.[25] GH + 1 protein was desalted and mixed 2:1 with the matrix 'super' dihydroxybenzoic acid (sDHB, Bruker Daltonics) and spotted on a stainless steel target. In-Source Decay MS generates protein fragment ions sequentially from the N- and C-termini



Scheme 1. Reagents and conditions: (a) $SOCl_2$, toluene, rt; (b) azetidin-2-one, *n*-BuLi, THF, −78 °C to rt, 57%; (c) H_2, 1 N HCl, Pd/C, methanol, 35 °C, 65%; (d) 1,4-dioxane-2,6-dione, *N*,*N*-diisopropylethylamine, dichloromethane, rt, 88%; (e) diisopropylcarbodiimide, pentafluorophenol, THF, rt, 81%; (f) trifluoroacetic acid, dichloromethane, rt, 88%; (g) 7, DMF, rt, 77%; (h) thiophenol, sodium bicarbonate, methanol, rt, 99%; (i) diisopropyl azodicarboxylate, triphenyl phosphine, neopentyl alcohol, dichloromethane, THF, −78 °C to rt, 69%.



Scheme 2. Preparation of GH-CovX body.

of proteins. After linker derivatization of GH, extensive terminal sequence coverage was observed, which terminated only at residue 53 on the N-terminus and residue 165 on the C-terminus, which suggests the presence of an intact disulfide bridge between these residues. It was further determined that fragment ions containing Cys residues 182 and 189 were modified with a mass corresponding to the linker **1**.[25] The protein with the linker was added to the antibody (20 mg/mL, 10 mM Histidine, 130 mM Glycine, 130 mM Sucrose, pH 6.5, 1:1 molar ratio) and very gently shaken over night at room temperature. The reaction mixture was purified using size exclusion column (CM column, buffer A: 20 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.0; buffer B: 20 mM 2-(*N*-morpholino)ethanesulfonic acid, 1 M sodium chloride, pH 6.0) to isolate **11** (34%) and **12** (32%). Compounds **11** and **12** were isolated in >95% pure based on mass spectral analysis. Compound **11** has one GH per antibody and compound **12** has two GHs per antibody.

These two compounds were evaluated using NB2-11 cells which proliferate in response to lactogenic hormones such as GH. These cells were maintained in Fisher's Medium supplemented with 10% fetal bovine serum and 10% Lactogen deficient horse serum. Prior to GH exposure, cells were serum starved overnight in Lactogen deficient media (Fisher's Medium, 1% Fetal Bovine Serum, 10% Horse Serum, 0.05 mM 2-mercaptoethanol). Culture plates were prepared with starved NB2 cells and stimulated with GH for 48 h. Following GH stimulation, the amount of NB2-11 cell proliferation was determined and found to show identical biological activity in in vitro assays (Table 1).[26] Since there is no advantage for having more than one growth hormone molecule per antibody, we have chosen **11** to study further.[27]

Compound **11** was evaluated in vivo in hypophysectomized rats. PEGylated growth hormone (PEG-hGH) was included in this study.[28] Pituitary glands in young female Sprague Dawley rats were surgically removed and the rats were placed randomly in four different groups. Three groups of rats were subcutaneously dosed once a week with compound **11** at three different doses (1.5, 5, 50 mg/kg/week). Two control groups (hypophysectomized rats and non- hypophysectomized (normal) rats) were given weekly s.c. doses of PBS. Compound **11**, PBS and PEG-hGH were administered at days 0 and 7 and monitored for the duration of the study. The weights of the rats were measured and the change in body weight can be seen in Figure 2. Animals dosed with compound **11** showed a significant body mass gain in all three dose groups. The group that received **11** at 50 mg/kg/week dose showed comparable weight gain as a group of hypophysectomized rats that received GH at 5 μg/day, s.c.[29] The group that received PEG-hGH gained comparable weight as the group that received 50 mpk of compound **11** during the first week. However, during the 2nd week, the group that received PEG-hGH gained more weight compared to the group that received 50 mpk of compound **11**.

In conclusion, we have developed a long acting human growth hormone analog using CovX technology and demonstrated its efficacy in an in vivo model. The introduction of a cysteine mutant offers a thiol nucleophile which provides a convenient handle to introduce selective modifications. However the above method suffers from the fact that the introduction of cysteine mutant in a protein containing disulphide bonds can potentially cause misfolding. Consequently one has to make several different cysteine mutants to identify a suitable one for development. The reduction of native disulphide bond offers two thiol nucleophiles and a convenient way to introduce a linker. The use of maleimide linker offers several advantages including small size, rapid reaction and re-construction of bridge to mimic the role of natural disulfide bond. We have developed a linker that targets surface exposed disulfide bonds for attaching a linker. These type of protein modifications using the above linker can be accomplished under mild conditions and have potential applications in developing modified proteins for therapeutics. The approach was based on the hypothesis that: (a) one could keep a protein in circulation longer by lowering its affinity towards the receptor thus reducing its internalization rate and; (b) by attaching a protein to an antibody, the protein stays in circulation longer and is protected from proteolysis. By conjugating GH to the CovX antibody, we saw an approximately 45-fold drop in cell activity. However, after a single injection, the compound showed a long duration of action as evidenced by the continued growth of the animals. Following day 5 the weight of the animals were stabilized. When a second dose was given at day 7, the animals continued their weight gain. The compound is currently in pre-clinical stage and additional information will be disclosed in due time.

Table 1
In vitro efficacy of human growth hormone and its analogs were evaluated in MTS proliferation bioassay (Cell titer-glo kit from Promega) using prolactin-dependent Nb2 Tumor Cell Lines (Sigma–Aldrich)

Compound	NB2-11 cell assay, EC_{50}, pM
Wt-GH	25
11	882
12	899

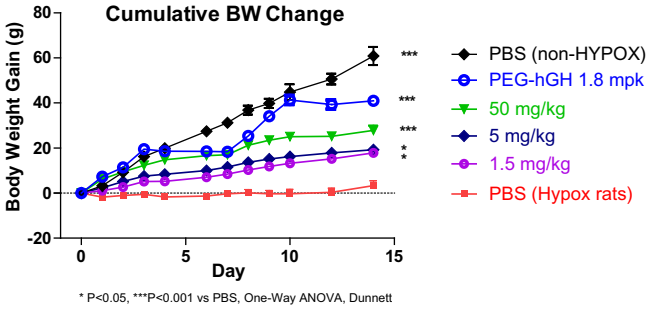


Figure 2. Mean cumulative body weight gain for hypophysectomized rats treated with **11** s.c. using various dosing regimens ($n = 5$ per treatment group).

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