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Chemical Synthesis of Homogeneous Human Glycosyl-interferon-β That Exhibits Potent Antitumor Activity in Vivo

Izumi Sakamoto,[†] Katsunari Tezuka,[†,‡] Kazuhiro Fukae,[†] Kazuyuki Ishii,[†] Keisuke Taduru,[†] Masatoshi Maeda,[†] Masaki Ouchi,[†] Kenta Yoshida,[†] Yuri Nambu,[§] Jun Igarashi,[§] Naohiro Hayashi,[§] Takashi Tsuji,[‡,∥] and Yasuhiro Kajihara*[,⊥]

[†]Glycotechnology Laboratory, Otsuka Chemical Co., Ltd., 134, Chudoji minami-machi, Shimogyo-ku, Kyoto 600-8815, Japan

[‡]Research Project for Glycoprotein Engineering funded by Otsuka Chemical Co., Ltd., Research Institute for Science and Technology, Tokyo University of Science, Noda, Chiba, 278-8510, Japan

[§]Glycotechnology Laboratory, Otsuka Chemical Co., Ltd., 463, Kagasuno, Kawauchi, Tokushima, 771-0193, Japan

[∥]Department of Biological Science and Technology, Tokyo University of Science, Noda, Chiba, 278-8510, Japan

[⊥]Department of Chemistry, Graduate School of Science, Osaka University, 1-1, Machikaneyama, Toyonaka, 560-0043, Japan

Supporting Information

ABSTRACT: Chemical synthesis of homogeneous human glycoproteins exhibiting bioactivity in vivo has been a challenging task. In an effort to overcome this long-standing problem, we selected interferon-β and examined its synthesis. The 166 residue polypeptide chain of interferon-β was prepared by covalent condensation of two synthetic peptide segments and a glycosylated synthetic peptide bearing a complex-type glycan of biological origin. The peptides were covalently condensed by native chemical ligation. Selective desulfurization followed by deprotection of the two Cys(Acm) residues gave the target full-length polypeptide chain of interferon-β bearing either a complex-type sialyl biantennary oligosaccharide or its asialo form. Subsequent folding with concomitant formation of the native disulfide bond afforded correctly folded homogeneous glycosyl-interferon-β. The chemically synthesized sialyl interferon-β exhibited potent antitumor activity in vivo.

Cell expression systems have enabled the preparation of bioactive protein drugs such as erythropoietin, interferon, and G-CSF.[1] Many of these protein drugs are glycosylated proteins, also known as glycoproteins, and the glycan moieties have been shown to be essential for activity in vivo. This is because the glycosylation pattern alters the physical and chemical properties of proteins.[1−4] Glycoproteins have thus far been solely prepared by means of cell expression systems, but this often results in considerable heterogeneity of the oligosaccharide structure.[5,6] Furthermore, this biological production technology requires an effective means for the removal of undesired contaminants, such as viruses, bacteria, or unknown genomic materials to be able to produce safe protein drugs. To date, it has been difficult to chemically synthesize homogeneous human glycoproteins that exhibit bioactivity *in vivo*. However, a recently developed approach for the semisynthetic incorporation of complex-type glycan moieties of biological origin potentially enables us to use chemistry to prepare homogeneous bioactive glycoproteins and their analogues.[7,8] Here we demonstrate that this chemical approach affords bioactive glycosyl-interferon-β that exhibits potent antitumor activity *in vivo*.

Human Interferon-β (IFN-β) is a cytokine which possesses immunomodulating, antiviral, and cytostatic activities.[9] IFN-β is classified into the subtypes IFN-β-1a and IFN-β-1b, which plays an active role in host cell defense:[10] IFN-β-1a is a glycoprotein (e.g., **10**), while IFN-β-1b does not bear any oligosaccharide. It is noteworthy that IFN-β-1a, which does bear an oligosaccharide, exhibits significantly more potent immunomodulating, antiviral, and antitumor activities compared to IFN-β-1b.[11] Furthermore, the carbohydrate components in the oligosaccharide structure strongly influence the pharmacokinetics: it has been reported that asialo-IFN-β, which has an acidic sialic acid removed (e.g., **2**), is rapidly metabolized in the liver.[12]

IFN-β comprises a polypeptide chain of 166 amino acid residues and has a single complex-type *N*-linked oligosaccharide (e.g., **1** or **2**) attached to Asn80 (Figure.1a).[13] In order to synthesize homogeneous sialyl-IFN-β and asialo-IFN-β, we used a novel method of dealing with complex-type sialo- (**1**) as well as asialo- (**2**) oligosaccharides of biological origin.[14] Using these methods combined with native chemical ligation,[15] a synthetic strategy for the preparation of glycosylated IFN-β was designed. Native chemical ligation involves the chemoselective condensation of unprotected peptide segments at Xaa-Cys sites. There are three Cys residues in the IFN-β sequence (Figure 1a), but the positions of these Cys residues are not suitable for facile synthesis of the full length IFN-β polypeptide chain from synthetic peptide segments. Instead, we used native chemical ligation at Xaa-Cys sites, followed by desulfurization to give the Xaa-Ala sequences found in the target polypeptide chain.[16] This strategy enabled us to design a three-segment (1Met-67Phe **3**, 68Ala-88Leu **4**, and 89Ala-166Asn **5**) coupling strategy by means of a conversion of cysteine residues at the 68 and 89 positions into alanine residues after native chemical ligation (Figure 1c). Selective desulfurization in the presence of

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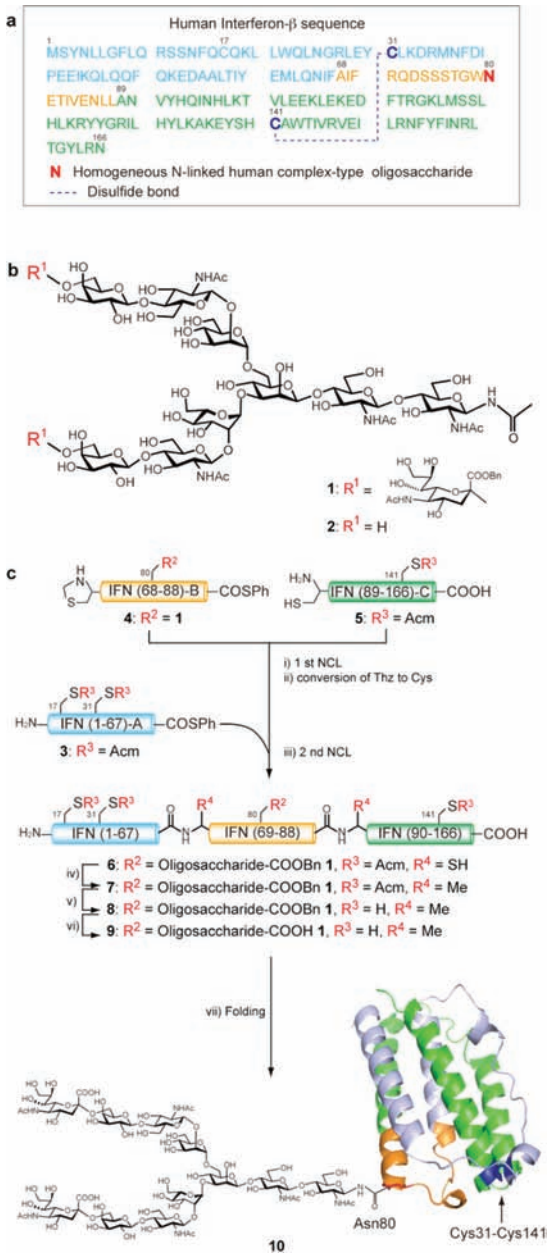

Figure 1. Full chemical synthesis of human IFN-β. (a) Amino acid sequence of human IFN-β indicating the disulfide bond. (b) Structure of human complex-type asparagine linked oligosaccharide. The R^1 position is the acidic sialic acid or hydrogen. (c) The synthesis of glycosyl-IFN-β by chemical ligation and selective desulfurization. (i) Sodium phosphate buffer solution containing 8 M Guanidine-HCl, 20 mM tris(carboxyethyl)phosphine (TCEP), and 3.0% thiophenol; (ii) sodium phosphate buffer solution containing 0.2 M methoxyamine-HCl (pH 4.0); (iii) the conditions are the same in the 1st NCL (i); (iv) sodium phosphate buffer solution containing 8 M Guanidine-HCl, TCEP, 2-methyl-2-propanethiol, and 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride; (v) AgOAc/90% AcOH; (vi) 50 mM NaOH; (vii) folding process.

protected Cys(Acm) enabled the preservation of the three native Cys residues.[17] A recently reported metal-free desulfurization protocol[18,19] was used for converting cysteine into alanine residues after native chemical ligation. We also designed two types of IFN-β bearing either an acidic sialyloligosaccharide **1** or a neutral asialooligosaccharide **2**.[14]

The synthetic results are shown in Figure 1. Each segment **3**−**5** was prepared by 9-fluorenylmethyloxycarbonyl-solid-phase peptide synthesis (Fmoc-SPPS) using HMPB-PEGA resin, as previously described:[20] the segments were released from the HMPB linker in a protected form, converted to peptide-thioesters, and then deprotected.[21] The sialylglycopeptide-segment-B (68Thz-88Leu) **4** and segment-C (89Ala −166Asn) **5** were condensed by native chemical ligation, followed by the conversion of Thz to Cys at the *N*-terminus of the ligation product using methoxyamine hydrochloride. Subsequently, the second native chemical ligation between this segment (68Ala-166Asn) and segment-A (1Met-67Phe) **3** afforded the desired full length IFN-β polypeptide chain **6**. Mild metal-free desulfurization[18,19] of this product afforded polypeptide chain **7**. Finally, polypeptide **7** was treated with silver acetate to remove the Acm protecting groups at the 17Cys, 31Cys, and 141Cys positions.[22] In terms of sialyl-IFN-β **8** thus obtained, the benzyl ester of the oligosaccharide-carboxylic acid was removed with 50 mM sodium hydroxide.[21] The total synthetic yields of IFN-β bearing sialyloligosaccharide **9** and asialooligosaccharide **15** (Supporting Information SI-Figure 8) obtained after purification by HPLC were ca. 5% and 10%, respectively, based on segment-C (89Ala-166Asn) **5** as a starting peptide segment. Dialysis under redox conditions[23] afforded correctly folded sialyl-IFN-β **10**.

Analytical data for the synthetic sialyl-IFN-β **10** bearing the sialyloligosaccaride is shown in Figure 2. HPLC analysis gave a single major peak (Figure 2a). The ESI mass spectrum (Figure 2b) gave a mass of 22229.5 Da. (calculated: 22230.8 Da, average isotope composition). In addition, the CD spectrum showed highly helical secondary structure (Figure 2c), and SDS−PAGE (Figure 2d) under both reductive (lane 2) and

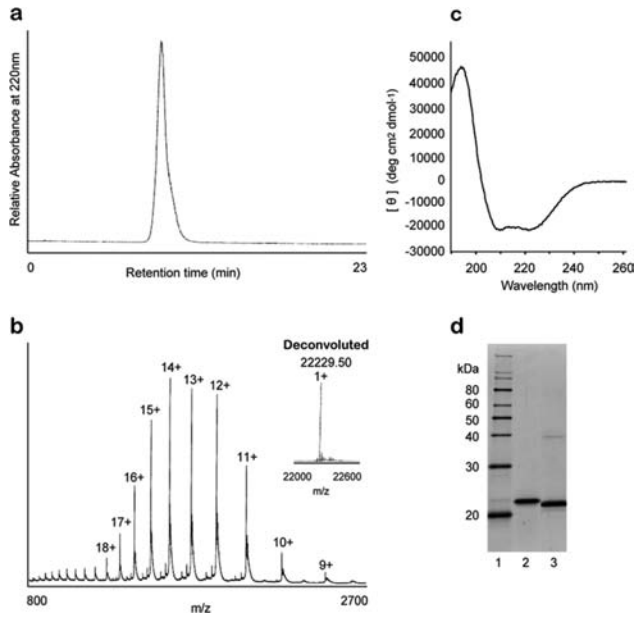

Figure 2. Analytical characterization of synthetic human IFN-β **10** containing a complex-type disialyloligosaccharide. (a) Reversed-phase HPLC profile of **10** after folding and purification. (b) Electrospray ionization mass spectrum (calcd m/z 22230.8: average isotope composition; found m/z 22229.5). (c) Circular dichroism (CD) spectrum in 10 mM AcOH, pH 3.4. (d) SDS-PAGE gel (15%). Lane 1, the molecular mass standard; lane 2, **10** under reducing conditions with dithiothreitol (DTT); lane 3, **10** under nonreducing conditions.

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nonreductive (lane 3) conditions reveals a single band of approximately 22 kDa. These data suggest that the sialyl-IFN-β **10** has the correct protein tertiary structure and is monomeric. To confirm the disulfide bond formation, proteolysis and subsequent mass/mass analysis were performed (SI-Figures 20–22).[24] The resultant peptide and glycopeptide fragments were consistent with the expected structure, including the disulfide-bonded segments. In terms of asialo-IFN-β **15** bearing a neutral asialooligosaccharide **2**, analytical data indicated that the synthesis and folding process afforded correctly folded, homogeneous asialo-IFN-β **15** (SI-Figures 8, 19, and 22).

The biological activity of the two synthetic IFN-β forms with different oligosaccharides was evaluated (Figure 3, SI-Figure 28 and SI-Table 1). In terms of antitumor activity *in vitro*, the relative proliferation of Daudi cells, a human Burkitt's lymphoma cell line, was monitored in the presence of IFN-β (SI-Figure 28). The synthetic sialyl-IFN-β **10** and asialo-IFN-β **15** displayed similar antitumor activity toward lymphoma cells. The results suggested that the *in vitro* activity[25] was not much influenced by the difference in oligosaccharide structure. For the assessment of the antitumor activity *in vivo*,[26] a mouse xenograft model established using Daudi cells in SCID mice (Figure 3a) was employed. The tumor-bearing mice were subcutaneously (*s.c.*) injected with equivalent unit amounts (2×10^7 Units/kg/day) of the synthetic IFN-β (red, sialyl-IFN-β; blue, asialo-IFN-β) or the commercially available fibroblast-derived human IFN-β[27] (green) that has an oligosaccharide at the same 80-Asn position. It is known that fibroblast-derived human IFN-β[27] exhibits ca. 80% homogeneity of its dibranched disialyloligosaccharide at the 80-Asn position, and recombinant IFN-β derived from CHO cell culture exhibits 95% glycan homogeneity.[28] The amount of the injected synthetic or commercially available IFN-β was adjusted to 2×10^7 Units/kg/day based on the estimated *in vitro* antitumor activity. The treatment with the daily administration of either the synthetic sialyl-IFN-β **10** (red) or asialo-IFN-β **15** (blue) diminished the tumor growth. In particular, synthetic sialyl-IFN-β **10** (red) completely suppressed tumor growth and exhibited potent antitumor activity *in vivo*. Sialyloligosaccharide may confer a long half-life on INF-β *in vivo*. To the best of our knowledge, this is the first report to demonstrate that synthetic glycoproteins bearing homogeneous oligosaccharides exhibit significant bioactivity *in vivo*.

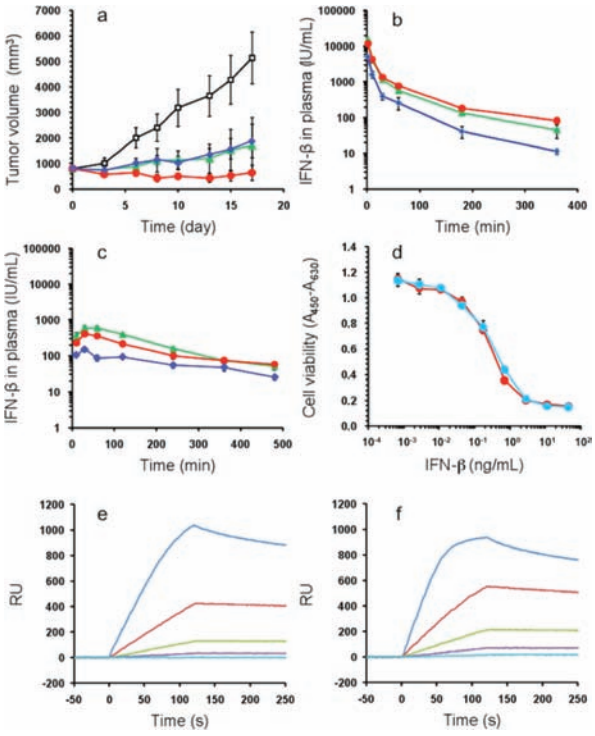

Figure 3. Biological characterization of chemically synthesized human IFN-β. (a–d) Synthetic sialyl-IFN-β **10** (red lines) and asialo-IFN-β **15** (blue lines) were compared with the commercial IFN-β (green lines) or vehicle control (black line in a). (a) The antitumor activity of the synthesized IFN-β was evaluated *in vivo* in a tumor-bearing xenograft mouse model. The measurement of the IFN-β levels in mouse plasma after a single administration via the intravenous (b) or subcutaneous (c) route. (d) The *in vitro* antitumor activity of heat-treated (red) or untreated (cyan) synthesized IFN-β. The reactivity of heat-treated (e) or untreated (f) synthesized IFN-sialyl-IFN-β **10** to IFNα/β receptor 2 was measured using surface plasmon resonance (SPR) at 0.31 nM (cyan line), 0.93 nM (violet line), 2.8 nM (green line), 8.3 nM (red line), and 25 nM (blue line), respectively. The K_d values estimated for heat-treated (e) and untreated (f) were 5.3×10^{-10} and 4.9×10^{-10} M, respectively.

In addition to this assay, the influence of the oligosaccharide structure on IFN-β pharmacokinetics was evaluated. Mice were injected with synthetic or commercially available IFN-β via an intravenous (*i.v.*) route or a subcutaneous (*s.c.*) route at the dosage of 2×10^6 Unit/kg. The concentration of IFN-β in plasma was periodically estimated, and the results are summarized in Figure 3b and 3c. The pharmacokinetic parameters were also summarized (SI-Table 1). Synthetic sialyl-IFN-β **10** (red) exhibited a similar pattern to that of the commercially available IFN-β (Figure 3b, 3c and SI-Table 1). Regardless of whether the administration was via the *i.v.* or *s.c.* route, the concentration of asialo-IFN-β **15** (without sialic acid: blue) was lower than those of the commercial and synthetic sialyl-IFN-β **10**. It is known that a galactose-binding receptor is expressed on hepatocytes, and this receptor may trap asialo-IFN-β **15**[29] in order to metabolize it. The results of this investigation using a homogeneous acidic sialyl-glycoprotein support this suggestion concerning the role of the galactose-binding receptor.

We also carried out a heat treatment of synthetic sialyl-IFN-β **10**, and this experiment provided a critically important result. Because glycoprotein biologics are prepared by mammalian cell expression systems, they normally require further processing to eliminate contamination with viruses, bacteria, or other unknown materials. In order to remove such undesirable contaminants, various systems have been developed,[30] because the most effective heat treatment for disrupting enveloped viruses[31] has proven to not be applicable to glycoprotein drugs because of the resulting denaturation or decomposition of the biologics. Because even the solutions of synthetic glycoprotein are easily fermented when the solutions are left due to bacteria, heat treatment for synthetic glycoproteins is also a desirable process. Our idea was to apply heat before the folding process in order to overcome this problem. We performed a liquid heat treatment (the "pasteurization method") on the synthesized sialyl-IFN-β polypeptide **7** at 60 °C for 10 h.[32] This process is usually employed for the purification of blood products.

It is known that the cysteine thiol may be involved in elimination or other side reactions during the course of heat treatment. Therefore, we examined the heat treatment of the Cys-Acm-protected glycosylated polypeptide chain **7**. During the heat treatment process, the protected IFN-β-polypeptide

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chain **7** was monitored by both HPLC and ESI-mass spectrometry (SI-Figures 23−27). Based on the results of these extensive studies, the Cys-Acm-protected polypeptide chain of glycosylated IFN-β **7** did not exhibit any decomposition, and there was no difference in purity before and after the heat treatment. After this heat treatment, the folding process was examined to evaluate the *in vitro* antitumor activity (Figure 3d) as well as the binding affinity for the IFN-α/β-receptor 2 using a Surface Plasmon Resonance device (SPR: Figure 3e and f). The folding experiment was performed under the same conditions for sialyl-IFN-β **10**, and the folding pattern and yield were unchanged compared with the original synthetic IFN-β (SI-Figure 27).

In conclusion, we have shown that chemical synthesis is able to afford a homogeneous bioactive sialyl-IFN-β **10** that exhibits potent antitumor activity *in vivo*. Thus far, the preparation of glycoproteins has only been possible using mammalian cell expression systems, and therefore the regulation of the oligosaccharide structure, as well as the tertiary protein structure, has been a challenge. However, our chemical approach not only helped in overcoming this problem but also resulted in a more homogeneous glycoprotein than that obtained by biotechnology. Chemical synthesis using other oligosaccharides will be developed, and the substitution of native amino acids with "unnatural" amino acids can be performed during the course of the chemical synthesis of glycoproteins.[33−37] These chemical approaches to the preparation of glycoproteins provide a means not only to obtain insight into the relationship between the oligosaccharide structure and glycoprotein bioactivity but also to systematically alter the physical and chemical properties of these glycoproteins.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

All experimental details of the chemical synthesis of two glyosylated forms of interferon β, its analytical data, biological evaluations, and complete information for ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

kajihara@chem.sci.osaka-u.ac.jp

Notes

The authors declare no competing financial interest.

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