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Intact bioactivities and improved pharmacokinetic of the SL335-IFN-β-1a fusion protein that created by genetic fusion of SL335, a human anti-serum albumin fab, and human interferon-β

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ABSTRACT

Recombinant human interferon beta (rIFN-β) has long been used as a first-line treatment for multiple sclerosis (MS), and any attempt to develop a long-acting rIFN-β is desirable since only one pegylated version of long-acting rIFN-β-1a (Plegridy) is currently available in clinics. Previously, we reported that SL335, a human Fab molecule specific to serum albumin, exhibits an extended serum half-life *via* utilizing the FcRn recycling mechanism. With the ultimate goal of developing a long-acting rIFN-®, we generated a fusion construct by linking human IFN-β cDNA to the C-terminus of the SL335 H chain at the DNA level followed by expression of the fusion protein, referred to as SL335-IFN-β-1a, in Chinese hamster ovary-S (CHO-S) cells. In its N-linked glycosylated form, the resulting fusion protein was easily purified from the culture supernatant *via* a three-step chromatography process. *In vitro* functional assays revealed that the fusion protein retained its intrinsic binding capabilities to human serum albumin (HSA) and interferon α/β receptor (IFNAR) that were almost identical to those of parental SL335 and rIFN-β-1a (Rebif). In addition, the fusion protein possessed an antiviral potency and antiproliferation activity comparable to those of Rebif. In pharmacokinetic (PK) analyses using Lewis rats and cynomolgus monkeys, SL335-IFN-β-1a exhibited at least a two-fold longer serum half-life and a significantly reduced renal clearance rate compared to those of Rebif. Finally, a four-week repeated dose toxicity study revealed no abnormal toxicological signs. In conclusion, our results clearly demonstrated that SL335-IFN-β-1a is worthy of further development as an alternative long-acting IFN-β therapeutic.

1. Introduction

Interferon beta (IFN-β) is a glycoprotein composed of 166 amino acids with N-linked glycans at a single glycosylation site (Asn-80). IFN-β is produced by a variety of cell types, including fibroblasts, in the body in response to virus infection or to other biological stimulants [1], and its antiviral potency is well-known to have a clinical effect on both RNA and DNA viruses [2]. In addition, IFN-β plays a role as an immune modulating molecule, exhibiting anti-inflammatory activities [3]. binding of IFN-β to the interferon-α/β receptor (IFNAR), indeed, induces complex transcriptional responses in immune cells, resulting in reduced antigen presentation and T cell proliferation, altered cytokine and matrix metalloproteinase (MMP) expression, and restored immunosuppressive function [4–6]. *Via* these multifactorial modulating effects on the immune system, IFN-β has been demonstrated to be effective, particularly for inhibiting the recurrence of multiple sclerosis (MS), a type of autoimmune disease that affects nerve cells in the brain and spinal cord, and has been used as a first-line therapeutic agent for MS for more than 20 years [7].

There are two types of recombinant IFN-β (rIFN-β), rIFN-β-1a and rIFN-β-1b, depending on the expression system. rIFN-β-1a is expressed in Chinese hamster ovary (CHO) cells, resulting in the production of a glycosylated form similar to that of natural IFN-β [8], whereas rIFN-β-1b is expressed as a nonglycosylated protein in *Escherichia coli* (*E. coli*) [9,10]. rIFN-β-1a and rIFN-β-1b are slightly different due to the presence or absence of N-linked glycans; rIFN-β-1a has better antiviral and

Abbreviation: IFN-β, interferon beta; HSA, human serum albumin; IFNAR, interferon α/β receptor; CHO, Chinese hamster ovary; FcRn, neonatal Fc receptor; Fab, fragment of antigen binding; MS, multiple sclerosis; mAb, monoclonal antibody; pAb, polyclonal antibody

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anti-proliferative activities, a reduced protein aggregation, and a lower immunogenicity *in vivo* than rIFN-β-1b. Currently, two rIFN-β-1a and two rIFN-β-1b drugs with different dosing regimens are available on the market. The two rIFN-β-1a drugs are Avonex (Biogen Idec), which requires intramuscular (I.M.) injection once a week, and Rebif (Merck KGaA), which requires subcutaneous (S.C.) injection three times a week [11,12]. The two rIFN-β-1b drugs are Betaferon (Bayer Healthcare), which requires S.C. injection every other day, and Extavia (Novartis), which requires daily S.C. injection.

Considering that patients with MS require steady treatment for an extended period of time, developing a rIFN-β-1a therapeutic with an improved protein stability and a prolonged efficacy would be worthwhile. PEGylation is the process by which polyethylene glycol (PEG) is chemically conjugated to a small protein to enlarge the molecular weight (MW) of the protein and has been widely used to increase the serum half-life *via* reducing protein turnover by the kidney [13]. This platform technology has been successfully applied to IFN-β to meet the urgent need to maximize drug effectiveness. The resulting PEGlylated IFN-β-1a (Plegridy, Biogen Idec) with increased blood stability and sustained high blood drug concentrations enables the dosing scheme of S.C. administration once every two weeks [14]. The disadvantage of PEGylated IFN-β-1a related to the manufacturing processes, however, is the low production yield because of the intrinsic tendency of IFN-β to form aggregates and the complicated manufacturing procedures that eventually contribute to the high medication cost [15]. Therefore, it can be assumed that development of an alternative version of long-acting IFN-® is appropriate if it showed an improvement over PEGylated IFN-β-1a in terms of serum longevity and/or production yields.

Serum albumin, the most abundant protein in the blood of mammals, is known to be essential for maintaining osmotic pressure and acts as both a carrier and a transporter for steroid hormones, hemin and fatty acids [16]. Interestingly, human serum albumin (HSA) is also often used as a vehicle either directly or indirectly to increase the half-life of proteins because the *in vivo* serum half-life of HSA, along with that of IgG, is particularly long through the FcRn recycling mechanism [17,18]. We had previously reported that SL335, an anti-human serum albumin Fab, exhibits the extended *in vivo* pharmacokinetic (PK) value in rats *via* cross-species binding to rat serum albumin (RSA) [19]. In this study, we utilized SL335 to create the SL335-IFN-β-1a fusion protein and tested whether the fusion protein has a potential as a novel long-acting therapeutic for MS.

2. Materials and methods

2.1. Molecular cloning of SL335-IFN-β-1a

Molecular cloning experiments were carried out according to the standard procedures [20]. SL335 Fd (V_H + C_{H1}) and L (V_L + $C_{L\kappa}$) genes were synthesized from Cosmogenetech (Seoul, South Korea), the human INF-β gene was synthesized by Bioneer, (Daejeon, South Korea), and the primers for polymerase chain reaction (PCR) were synthesized from Macrogen (Seoul, South Korea). PCR was performed under the following conditions unless otherwise noted: 25 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min. The SL335 Fd gene fragment was obtained by PCR amplification from the synthesized gene using PCR primers #1 (5'-gctggaattcgccaccatg-gagtggtcc-3') and #2 (5'-gagctggtgcagaaccggaggacttgggctccac-3'). The human INF-β gene fragment containing a short peptide linker was PCR amplified from the synthesized human INF-β gene using PCR primers #3 (5'-ggttctgcaccagctcctggatcttcatacaacctgctgggctt-3') and #4 (5'-tcgagcggccgcttagttgcgcagatagccggtcaggcggttaat-3'). The gene fragment corresponding to SL335 Fd + short linker + IFN-β was obtained by assembly PCR using primers #1 and #4. The resulting ~1250 bp PCR product was treated with *EcoR* I/*Not* I (Takara, Shiga, Japan) and ligated with pdCMV-dhfrc mammalian expression vector (kindly provided by Prof. Hong at Kangwon National University, South Korea) using T4 DNA ligase (Takara). Standard gene cloning procedure was carried out using electrocompetent DH-5α *E. coli* strain. The SL335 L chain gene (~750 bp) was then treated with *Hind* III/*Xba* I (Takara) and ligated to the pdCMV-dhfrc vector containing the SL335 Fd-IFN-β gene fragment.

2.2. Generation of a CHO-S stable cell line

CHO-S cells (cGMP banked) (Gibco, Grand Island, NY, USA) were cultured in CD OptiCHO Medium (Gibco) in a humidified 5% CO_2 shaking incubator at 37 °C and 140 rpm. CHO-S transfections were performed using Neon transfection system (Life Technology, Carlsbad, CA, USA) according to the device's protocol. Briefly, 10^6/ml CHO-S cells were transfected with 30 μg of the recombinant pdCMV-dhfrC vector at the condition of 1400 V pulse, 10 ms, three pulses, and cultured using T75 flasks for 2 days in a humidified 5% CO_2 incubator at 37 °C. The cells were then seeded into T75 flasks with fresh medium containing 550 μg/ml of G418 (Thermofisher Scientific, Waltham, MA, USA) and 20 nM MTX (Invitrogen, Carlsbad, CA, USA) at 5 × 10^5 cells/ml, followed by incubation in a humidified CO_2 incubator at 37 °C. After methotrexate (MTX) selection, stable cell lines were established according to the protocol provided by the CHO-S protocol (ThermoFisher Scientific) with slight modification. In short, the cells were plated in the semisolid medium (StemCell Technologies, Vancouver, Canada) containing CD OptiCHO liquid medium, 4 mM glutaMAX (Invitrogen, Carlsbad, CA, USA), Cell Boost 5 (GE healthcare, Chicago, IL, USA) and 20 nM MTX. After 10–14 days, each monoclonal cell colony was transferred to the liquid medium in a new well of the 96-well plate, and cells were sequentially transferred to 24- and 6-well plates based upon the cell growth. Lastly, productivities of the clones in suspension culture were compared by ELISA that measures the quantity of SL335-IFN-β-1a fusion protein.

2.3. Enzyme-linked immunosorbent assay (ELISA)

For quantitative ELISA, MaxiSorp ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 ng/well of goat anti-human IgG F(ab)′$_2$ (Jackson Immunoresearch Laboratories, West Grove, PA, USA), and the serial dilutions of CHO-S cell culture supernatant were allowed to bind to the antibody for 1 h at 37 °C. Anti-IFN-β mAb, clone A1, (EMD Millipore, Burlington, MA) was used as a primary antibody to detect IFN-β. Goat anti-mouse IgG-horseradish peroxidase (HRP) conjugated (Jackson Immunoresearch Laboratories) was used as a secondary antibody, and the binding signals were visualized with 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate (BDscience, Franklin Lakes, NJ, USA). Absorbance at 450 nm was then measured using an ELISA reader (BMG Labtech, Ortenberg, Germany). Purified SL335-IFN-β-1a with known concentration was used to draw the standard curve to quantitate SL335-IFN-β-1a in the culture supernatant.

ELISA for the rat PK analysis was performed as follow. MaxiSorp ELISA plates (Nunc) were coated with 50 ng/well of rabbit anti-human IFN-β antibody (Peprotech, Rocky Hill, NJ, USA). The serial dilutions of rat sera were allowed to bind to the antibody for 2 h while shaking at 400 rpm at room temperature (RT). A mouse anti-human IFN beta-conjugated with biotin (eBioscience, San Diego, CA, USA) was used as a detection antibody for rIFN-β-1a (Rebif) (Merck Serono, Darmstadt, Germany) and a donkey anti-human IgG (H + L)-conjugated with biotin (Jackson Immunoresearch Labs) was used as a detection antibody for SL335-IFN-β-1a. Streptavidin-conjugated with horseradish peroxidase (HRP) (Thermofisher) and TMB were used to visualize the ELISA signals. For the monkey PK analysis, ELISA was carried out as above except for using a goat anti-human kappa (monkey absorbed)-conjugated with biotin (IBL, Hamburg, Germany) to detect SL335-IFN-β-1a. Again, Rebif and purified SL335-IFN-β-1a with known concentration was used to draw the standard curve to quantitate Rebif or SL335-IFN-β-1a in the serum samples.

2.4. Purification of SL335-IFN β-1a fusion protein

Soluble SL335-IFN β-1a protein was produced by growing the stable CHO-S cell clone (termed 4–37) in 5 L of PowerCHO media (Lonza, Basel, Switzerland) using a WAVE bioreactor (GE Healthcare) [21] (Rodriguez and others 2010). The culture supernatant was harvested by centrifugation (3500 rpm, 20 min) at 4 °C and filtered through a 0.2 μm filter, and soluble SL335-IFN β-1 was purified by a 3-step purification; capture for affinity chromatography, intermediate purification for cation exchange chromatography and polishing for anion exchange chromatography. In brief, affinity purification was performed using CaptureSelect IgG-CH1 Affinity Matrix resins (Life technologies). After washing the matrix with 10 column volumes (CVs) of phosphate-buffered saline (PBS) with or without 1 M sodium chloride, soluble SL335-IFN β-1 was eluted by using elution buffer (20 mM sodium citrate, pH 5.0) at flow rate = 6 ml/min. Cation exchange purification was carried out using CM sepharose Fast Flow (GE Healthcare) that equilibrated with 20 mM sodium citrate pH 5.5. Binding buffer (20 mM sodium citrate pH 5.0) and elution buffer (20 mM sodium citrate pH 5.0, 1 M NaCl) were used at flow rate = 3 ml/min during purification. Finally, anion exchange purification was performed using Capto Adhere ImpRes (GE Healthcare). For binding, washing and elution, buffer compositions were as follow at wash flow rate = 3 ml/min and elution flow rate = 2 ml/min: binding buffer (20 mM sodium citrate pH 5.5), wash buffer (20 mM sodium citrate pH 5.5, 1 M NaCl) and elution buffer (50 mM sodium citrate pH 2.6).

2.5. SDS-PAGE

Protein samples were mixed with 4x sample buffer [250 mM Tris-HCL, pH 6.8, 8% sodium dodecyl sulfate (SDS), 40% glycerol, 0.02% bromophenol blue and 5% 2-mercaptoethanol (2-ME)] and treated at 100 °C for 5 min under the reducing condition. For the native condition, SDS and 2-ME were omitted from the sample buffer, and heat treatment was also skipped. SDS-PAGE analyses were then performed by loading the protein samples onto the 12% acrylamide gel (Biorad) at 1 μg/well and electrophoresed at 200 V for 40 min, and the protein bands were visualized by using Coomassie Blue staining (Elpisbio, Daejeon, South Korea). SDS-PAGE analysis of the deglycosylated SL335-IFN-β-1a was performed using the protein sample pre-treated with Peptide:N-glycosidase F (PNGase F) (New England Biolabs, Essex, MA, USA) according to the supplier's protocol for reducing condition.

2.6. High-performance liquid chromatography (HPLC)

Size exclusion high-performance liquid chromatography (SE-HPLC) was carried out by Kangwon National University Central Laboratory. In short, Agilent 1260 series (Agilent Technologies, Santa Clara, CA, USA) and the TSKgel SuperSW mAb HR column (Tosoh Bioscience, Tokyo, Japan) with a mobile phase of 100 mM Na_2HPO_4, 200 mM NaCl, 0.05% (w/v) NaN_3 (pH 6.7) at a flow rate of 0.5 mL/min were used to analyze the fusion protein. The sample was diluted to 1 mg/mL using 100 mM Na_2HPO_4, 100 mM Na_2SO_4, 0.05% (w/v) NaN_3 (pH 6.7), and 20 μg of sample was injected using an auto sample injector at 4 °C. Elution was monitored using UV absorption at 280 nm. For reversed-phase high-performance liquid chromatography (RP-HPLC), the Shimadzu Prominence system HPLC and a POROSHELL 300SB-C3 (3.1 i.d × 75 mm) were used along with mobile phase A (0.1% TFA in Milli-Q water) and mobile phase B (0.1% TFA in acetonitrile), and the experiments were performed Dongil SHIMADZU corp., Technical Research Center (Seoul, South Korea). The flow rate was set at 1 ml/min and oven temperature was at set 40 °C. Elution was monitored to observe the protein peaks as above.

2.7. Capillary electrophoresis (CE)

CE was performed using ProteomeLab PA 800 and Launch 32 Karat 10.2 software according to the Application Guide of PA 800 plus Pharmaceutical Analysis System (Beckman Coulter, Brea, CA, USA). For isoelectric point (pI) analysis, capillary isoelectric focusing (cIEF) was performed on 50 μm ID × 67 cm (total length) [30 cm (length to detector)] neutral (polyacrylamide)-coated capillaries. The sample was prepared [3 μl of Urea-cIEF solution, 12 μl of pharmalyte 3–10, 20 μl of cathodic stabilizer, 2 μl of anodic stabilizer, 2 μl of cIEF peptide marker kit (A58481) (pI 4.1, 5.5, 7.0, 9.5, 10.0) and 10 μl of 4 mg/ml SL335-IFN β-1a in Tris buffer pH 8.0], loaded by low-pressure injection (25 psi, 99 s), focused 15 min at 25 kV, and then mobilized by low pressure while maintaining a high voltage (30 kV, 30 min). Before and between each analysis the capillary was rinsed with deionized water (50 psi, 2 min). For separation program, the capillary was thermostated at 25 °C, and the instrument was set up with the anode at the inlet end of the capillary (anolyte: 200 mM H_3PO_4) and the cathode at the outlet (catholyte: 300 mM NaOH). For the glycosylation analysis, capillary electrophoresis sodium dodecyl sulfate (CE-SDS) was performed under the reducing condition. To prepare samples, 95 μl of SL335-IFN-β-1a with or without PNGase F treatment was mixed with SDS-MW sample buffer (SCIEX, Concord, ON, Canada), 2 μl of Internal Standard (10 kDa) (SCIEX) and 5 μl 2-ME, and heated at 100 °C for three minutes. Bare Fused-silica Cap 50 μm ID × 57 cm (30 cm) (SCIEX) was used for all separations. Samples were introduced into the polymer-filled capillary by electrophoretic injection (5.0 KV, 20 s), and separation was performed at 15.0 KV for 30 min. Prior to each run, the capillary was flushed with 0.1 N HCl and 0.1 N NaOH to remove any contaminants residual SDS and protein. Electropherogram scan was carried out at 190–400 nm and channel data was obtained at 220 nm.

2.8. Bilayer interferometry analysis

Real-time binding assays between HSA and SL335 moiety, and between IFN-β and IFNAR2 were carried out by bilayer interferometry using an Octet RED system (ForteBio, Menlo Park, CA, USA). To determine the binding affinity to HSA, the predetermined concentration of HSA was coupled to kinetics grade AR2G biosensors (Amine reactive second generation), and unbound molecules were removed by incubating in the kinetics buffer (1 M ethanolamine, pH 8.5). The probes were then allowed to bind to SL335-IFN-β-1a at the predetermined concentrations under pH 6.0 or pH 7.4 conditions, followed by dissociation in PBS containing 0.1%BSA, pH 6 or pH 7.4. To determine the binding affinity to IFNAR2, the predetermined concentrations of SL335-IFN-β-1a and Rebif were coupled to the AHC biosensors (anti-human Fc capture) (PALL Corporation, Port Washington, NY, USA) (Capito and others 2013), and unbound molecules were removed as above. The probes were then allowed to bind to IFNAR2 at the predetermined concentrations under pH 7.4 conditions, followed by dissociation in PBS containing 0.1% BSA, pH 7.4. The binding and dissociation kinetics were calculated using the Octet QK software package, which fit the observed binding curves to a 1:1 binding model to calculate the association rate constants. The association and dissociation rate constants were calculated using at least three different concentrations of the antigens.

2.9. Antiviral-based cytopathic effect (CPE) assay

This experiment was carried out in PBL Assay Science (Piscataway, NJ, USA) according to their internal protocols using A549 cells (ATCC CCL-185), the encephalomyocarditis virus (EMCV) (ATCC VR-1479) and WHO International Standard IFN-β (HUMAN, rDNA, Glycosylated) (NIBSC code: 00/572) (IS IFN-β). Briefly, serial two-fold dilutions of the samples (Rebif and SL335-IFN-β-1a) including IS IFN-β were added onto A549 cells (ATCC CCL-185) in 96-well plates. The plate are then

incubated 18–24 hours at 37 °C, 5% CO_2. Appropriate titer of EMCV was added to the plates except for the cell control wells. Approximately 40 to 56 h later, the media was removed from the wells, and the live cells were fixed and stained with crystal violet solution. After washing the wells with cold tap water, the wells were examined visually to determine which dilution protects 50% of the cells from cytopathic effect, and this dilution was calibrated to a standard IS IFN-β solution to obtain Units/mg of the samples.

2.10. Anti-proliferative activity assay

Daudi cells [Korean Cell Line Bank (KCLB), South Korea] were grown in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). Cells were seeded at 4×10^5/ml (50 µl/well) in 96-well plates (SPL Life Sciences, Pocheon, South Korea). IS IFN-β, rIFN-β-1a (Rebif) and SL335-IFN-β-1a were diluted in medium and added in 50 µl aliquots to the cultures. Cells were incubated for 5 days (120 h) in a humidified 5% CO_2 incubator, followed by adding 10 ml of cell counting kit-8 (CCK-8) solution (Dojindo, Tokyo, Japan) and 2 h incubation (Sung and others 2003). The absorbance at 450 nm was measured using a 96-well plate reader (Molecular Devices, San Jose, CA, USA) to determine the cell viability.

2.11. Pharmacokinetic analysis

The pharmacokinetic (PK) studies were performed using Wistar Lewis rats (6 weeks old) and cynomolgus monkeys (2–3 years of age) at Korea Institute of Toxicology (KIT) (earned AAALAC International accreditation in 1998) (Daejeon, South Korea), and the experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of KIT. Briefly, the rats (four females/group) received 0.06 mg/kg Rebif as a positive control or 0.18 mg/kg SL335-IFN-β-1a by intravenously (I.V.) administration. Blood samples were obtained from the jugular vein at the specified time points after injection as follow: 0, 0.083, 0.25, 0.5, 1.25, 3, 5 and 24 h for Rebif; 0, 0.083, 0.25, 0.5, 1.25, 3, 5, 24, 48 and 72 h for SL335-IFN-β-1a. For subcutaneously (S.C.) administration, the rats (four females/group) received 0.11 mg/kg Rebif or 0.33 mg/kg SL335-IFN-β-1a. Blood samples were obtained from the jugular vein at the specified time points after injection as follow: 0, 0.33, 1, 1.5, 3, 5, 7, 12 and 24 h for Rebif; 0, 0.33, 1, 1.5, 3, 5, 7, 12, 24, 48, 72 and 144 h for SL335-IFN-β-1a. Plasma samples were analyzed for Rebif or SL335-IFN-β-1a levels by ELISA. Three times higher dosage of SL335-IFN-β-1a was injected to the rats compared to Rebif to administer the same mole of the proteins.

For the non-human primate (NHP) PK studies, the cynomolgus monkeys received 0.011 mg/kg Rebif (one male) or 0.034 mg/kg SL335-IFN β-1a (two males) by I.V. administration. Blood samples were obtained from the jugular vein at the specified time points after injection as follow: 0, 0.083, 0.25, 0.5, 1, 3, 6, 12, 24, 48 and 72 h for Rebif; 0, 0.083, 0.25, 0.5, 1, 3, 6, 12, 24, 48, 96, 144 and 192 h for SL335-IFN-β-1a. In S.C. administration, the cynomolgus monkeys received 0.034 mg/kg Rebif (one male) or 0.102 mg/kg SL335-IFN β-1a (two males), and blood samples were obtained after administration as follow: 0, 0.5, 1, 3, 6, 12, 24, 48, 72 and 96 h for Rebif; 0, 0.5, 1, 3, 6, 12, 24, 48, 96, 144, 168, 192, 240 and 288 h for SL335-IFN-β-1a. Serum concentrations of Rebif and SL335-IFN-β-1a were determined by ELISA, and analyzed by one-compartment model for I.V. PK parameters and non-compartment model for S.C. PK parameters using Phoenix WinNonlin (Version 6.4) (Certara Inc., Princeton, NJ. USA). Again, three times higher dosage of SL335-IFN-β-1a was injected to the monkeys compared to Rebif to administer the same mole of the proteins. For the preliminary pharmacodynamics (PD) analysis, the concentration of neopterin was determined with the Neopterin ELISA kit (IBL, Hamburg, Germany) according to the manufacture's protocol using the plasma samples of the monkeys received S.C. administration of Rebif or SL335-IFN β-1a.

2.12. Preliminary toxicology study

A four-week repeated dose toxicity study using cynomolgus monkeys (2–3 years of age) was performed at KIT, which was again approved by IACUC of KIT. One male and one female monkeys were given 0.09 mg/kg of SL335-IFN-β-1a once a week for 4 weeks. The animals were checked for food consumption, physical and ophthalmic exams, body weight, hematology and clinical chemistry and urinalysis every week. After 4 week study, the animals were sacrificed, organ/tissues (16 organs from male and 13 organs from female) were collected and the full range of tissues (42 tissues - 38 common tissues and 4 gender-specific tissues) was examined microscopically.

3. Results

3.1. Construction and CHO cell expression of SL335-IFN-β-1a fusion protein

The SL335-IFN-β fusion construct was generated by genetic linkage of human IFN-β to the C-terminus of Fd (V_H + C_{H1}) in an $SL335_{\Delta ds}$ form *via* a short flexible peptide linker, as previously described in the creation of a long-acting version of hGH [22]. The resulting DNA constructs were subcloned into the mammalian expression vector and transfected into CHO-S cells to produce the SL335-IFN-β-1a fusion protein. The stable CHO-S cell lines were established *via* MTX selection and monoclonal cell expansion, and finally, the stable CHO-S clone, referred to as 4–37, was chosen based on the cell stability and the production yield (data not shown). The maximum yield of SL335-IFN-β-1a from 4 to 37 in the culture supernatant was approximately 32 mg/L under our laboratory conditions without any procedure optimizations (data not sown). The fusion protein in the culture supernatant was purified *via* a three-step purification process that included affinity purification using the IgG-CH1 affinity resin followed by two additional IEX chromatography steps. The recovery yields of the fusion protein after each step of chromatography were 99% after affinity chromatography, 71% after cation exchange chromatography and 67% after anion exchange chromatography, implying that the final fusion protein yield from the culture supernatant was 67% after the three-step purification (data not shown). After purification, the final elute was analyzed with SDS–PAGE under reducing or native conditions (Fig. 1A). Under the reducing condition (lane 1), two protein bands corresponding to the SL335 Fd-IFN-β-1a fusion protein (46 kDa) and the SL335 L chain polypeptide (23 kDa) were visible as expected. Under the native condition (lane 2), a single protein band corresponding to the heterodimeric form of the SL335-IFN-β-1a fusion protein was visible, suggesting the appropriate heterodimeric folding of the fusion protein. The arrows lead to the illustrations depicting a molecular structure of the corresponding protein bands. In the illustrations, the 233th amino acid of C_{H1} and the 214th amino acid of $C_{L|}$ of SL335 were denoted as Ser because SL335 without the interchain disulfide bond (IDB), referred to as $SL335_{\Delta}ds$, in which the Cys233 of C_{H1} and the Cys214 of $C_{L|}$ are replaced with Ser, was used in this study. To measure the purity of the fusion protein, SE-HPLC (Fig. 1B, upper panel) and RP-HPLC (Fig. 1B, lower panel) analyses were performed under native or reducing conditions, respectively. The SE-HPLC result showed a single protein peak corresponding to the intact SL335-IFN-®-1a, and the two distinct protein bands corresponding to the SL335 L chain and the SL335 Fd-IFN-β-1a fusion were shown by RP-HPLC analysis. These HPLC results confirmed that the SL335-IFN-β-1a fusion protein at > 98.5% purity was obtained after the three-step purification process.

3.2. Molecular characterization of SL335-IFN-β-1a

The SL335 Fd-IFN-β fusion is composed of 396 amino acids and its theoretical MW is 44 kDa. Whereas, the SL335 L chain is consisted of 215 amino acids and its theoretical MW is 23 kDa. Thus, the theoretical

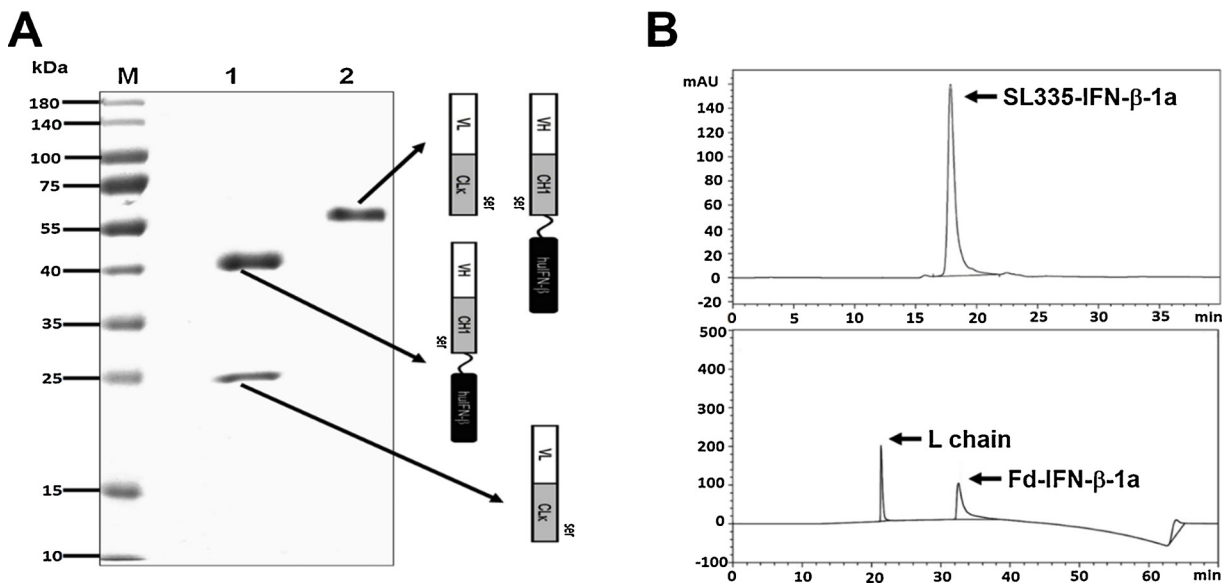

Fig. 1. SDS-PAGE and HPLC analyses of SL335-IFN-β-1a fusion protein. The purified SL335-IFN-β-1a from CHO-S cell culture supernatant was characterized by **(A)** SDS-PAGE on a 12% acrylamide gel under the reducing (lane 1) and native (lane 2) conditions, and **(B)** HPLC (upper panel: SE-HPLC and lower panel: RP-HPLC). **(A)** Protein bands in the SDS-PAGE gel were visualized with Coomassie Blue staining, and the arrows lead to the illustrations depicting a molecular structure of the corresponding protein band. **(B)** In the HPLC analyses, the protein sample was analyzed under the native condition (without presence of DTT) for the SE-HPLC, and under the reduced condition (with presence of DTT) for the RP-HPLC. The arrows denotes the nature of molecules corresponding to each protein peaks on the HPLC analyses (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

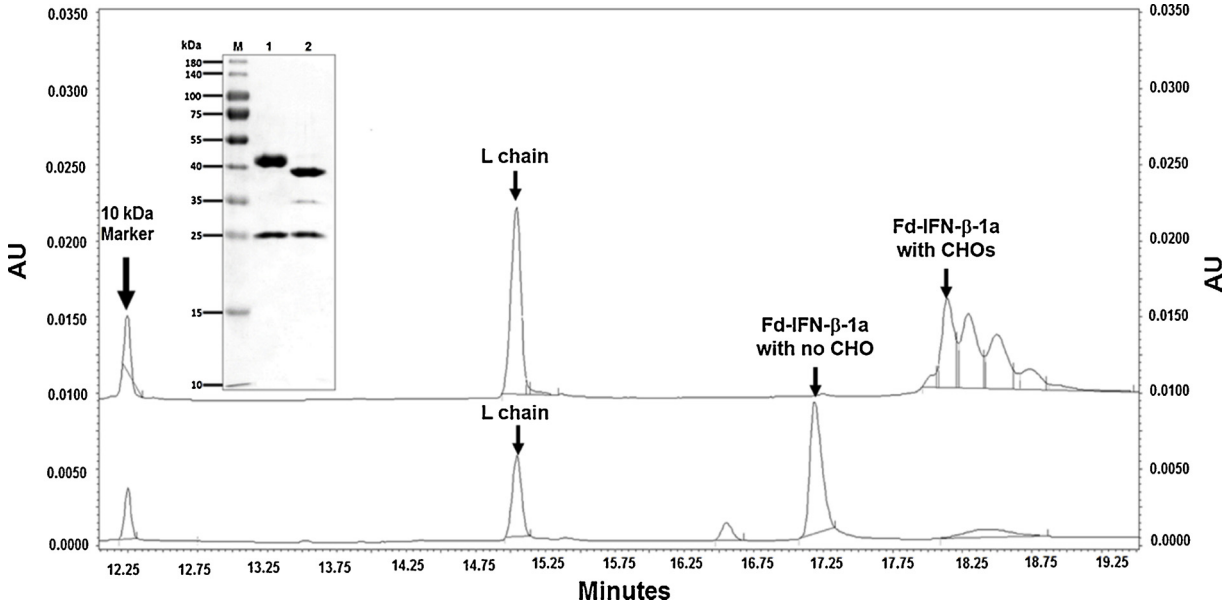

Fig. 2. N-linked glycosylation of SL335-IFN-β-1a analyzed by SDS-PAGE and CE-SDS analyses.SL335-IFN-β-1a with or without PNGase F treatment were mixed with SDS sample buffer containing 2-ME, and subjected to **(A)** SDS-PAGE on a 12% acrylamide gel (lane 1: SL335-IFN-β-1a without PNGase F treatment; lane 2: SL335-IFN-β-1a with PNGase F treatment), or **(B)** CE-SDS (upper electropherogram: SL335-IFN-β-1a without PNGase F treatment; lower electropherogram: SL335-IFN-β-1a with PNGase F treatment). **(A)** Protein bands in the SDS-PAGE gel were visualized with Coomassie Blue staining. **(B)** The electropherogram scan was carried out at 190–400 nm for CE-SDS. The arrows on the electropherogram indicate the nature of polypeptides corresponding to each peak, and CHO stands for carbohydrates (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

MW of SL335- IFN-β-1a is calculated to be 67 kDa. However, the MW of SL335-IFN-β-1a was determined to be 70 kDa by MALDI-TOF and Q-TOF analyses (data not shown), which is slightly larger than the theoretical MW. Natural IFN-® has one N-glycosylation site at the 80th amino acid (Arg). To verify the presence of N-linked glycans on SL335-IFN-β-1a, the fusion protein was treated with PNGase F and subjected to SDS-PAGE and CE-SDS analyses under the reducing condition (Fig. 2). The results revealed that the MW of the protein band corresponding to the SL335 Fd-IFN-β fusion but not the SL335 L chain was decreased upon PNGase F treatment, suggesting the presence of the N-linked glycans on the SL335 Fd-IFN-β fusion. In addition, the multiple protein peaks produced by SL335 Fd-IFN-β fusion without PNGase F treatment (Fig. 2, upper electropherogram) indicated the heterogeneity of N-linked glycans. Although the corresponding data are not shown, the pI value of SL335-IFN-β-1a was measured as 8.59–9.56 by cIEF analysis (the theoretical pI is 9.06).

Table 1
Comparative measurement of the binding affinity between SL335-IFN-β-1a fusion protein and HSA, or the IFN-α/β R2.

Binder	Ligand	pH	K_d (M)	K_{on} (1/Ms)	K_{dis} (1/s)
HSA	SL335	pH 6.0	2.94E-10	6.69E+05	1.97E-04
		pH 7.4	5.33E-10	5.65E+05	3.01E-04
	SL335-IFN-β-1a	pH 6.0	4.09E-10	9.89E+05	4.04E-04
		pH 7.4	3.18E-10	1.02E+05	3.24E-05
IFNAR2	rIFN-β-1a (Rebif)	pH 7.4	1.05E-09	1.14E+05	1.20E-04
	SL335-IFN-β-1a	pH 7.4	1.15E-09	1.25E+05	1.45E-04

*The binding kinetics and the dissociation kinetics were calculated using the Octet QK software package.

3.3. In vitro functional assays

To determine whether SL335-IFN-β-1a retained the antigen binding affinities to both HSA and IFN-α/β R2, a bilayer interferometry assay was performed (Table 1). The dissociation constants (K_d) of SL335-IFN-β-1a to HSA were 4.09×10^{-10} M at pH 6 and 3.18×10^{-10} M at pH 7.4, and these values were comparable to those of SL335 to HSA (2.94×10^{-10} M at pH 6 and 5.33×10^{-10} M at pH 7.4). In addition, the K_d of SL335-IFN-β-1a to IFN-α/β R2 was measured to be 1.15×10^{-9} M, which correlated well with that of the positive control rIFN-β-1a (Rebif) ($K_d = 1.05 \times 10^{-9}$ M). These results implied that the genetic linkage of SL335 to IFN-β neither harmed the binding affinity of the SL335 moiety to HSA nor that of the IFN-β-1a moiety to IFN-α/β R2 of the fusion protein.

The *in vitro* IFN-β bioactivity of SL335-IFN-β-1a was further analyzed by antiviral potency and anti-proliferative assays (Fig. 3A and B). The antiviral potencies of SL335-IFN-β-1a and Rebif were measured using A549 cells infected with EMCV (Fig. 3A). A standard curve was created using IS IFN-® with known units of activity/mg, and the dilutions of SL335-IFN-β-1a and Rebif were calibrated to the standard curve to obtain their units/mg. The EC_{50} of SL335-IFN-β-1a was 7.47 ng/ml (Log dilution), which corresponded to 7.15×10^7 units/mg, and the EC_{50} of Rebif was 4.07 ng/ml (Log dilution), which corresponded to 1.16×10^8 units/mg. Because the MW of SL335-IFN-β-1a is three times greater than that of Rebif, the antiviral potency of SL335-IFN-β-1a appeared to be approximately two-fold stronger than that of Rebif at the equivalent molar ratio. In the anti-proliferative assay using Daudi Burkitt's lymphoma cells, both Rebif and SL335-IFN-β-1a showed concentration-dependent anti-proliferative activity with an apparent EC_{50} of 3.94 pM. (Fig. 3B). Again, the IS IFN-® with known units of activity/mg was included as an internal bioactivity control. Almost identical degrees of anti-proliferative activity were produced by all three rIFN-® preparations, suggesting that they possess a similar biological potency.

3.4. In vivo pharmacokinetic (PK) assays

The PK profiles of SL335-IFN-β-1a were studied using rat and monkey models that were administered a single I.V. or S.C. injection. Rebif (rIFN-®-1a) was included in the study as a reference molecule. Because the MW of SL335-IFN-β-1a (~67 kDa) is 3.7 times larger than that of Rebif (18 kDa), the dosage of SL335-IFN-β-1a was increased to three times that of Rebif to make the IFN-® molar ratio similar. The plasma concentrations of Rebif and SL335-IFN-β-1a were measured by quantitative ELISA.

The mean serum concentration-time profiles of the samples in rats are shown in Fig. 4A (I.V. injection) and B (S.C. injection), and the PK parameters are summarized in Table 2. Upon I.V. administration (0.06 mg/kg Rebif or 0.18 mg/kg SL335-IFN-β-1a, Fig. 4A), a prolonged serum half-life ($t_{1/2}$) value of SL335-IFN-β-1a compared to that of Rebif was clearly observed (10.8 h *vs.* 3.9 h). The systemic clearance rates of SL335-IFN-β-1a and Rebif were found to be 1.9 and 15.1 mL/hr/kg, respectively, and the volume of distribution at the steady state (Vss) of SL335-IFN-β-1a was 29.8 mL/kg, while that of Rebif was 84.2 mL/kg. The S.C. route injection (0.11 mg/kg Rebif or 0.33 mg/kg SL335-IFN-β-1a) also clearly showed a prolonged $t_{1/2}$ of SL335-IFN-β-1a compared to that of Rebif (Fig. 4B and Table 2) (19.7 h *vs.* 9.3 h). The systemic clearance rates of SL335-IFN-β-1a and Rebif were 50.8 and 151 mL/hr/kg, respectively, and the volume of distribution at the steady state (Vss) of SL335-IFN-β-1a was 1446.2 mL/kg and that of Rebif was 2043.3 mL/kg. These results confirmed the prolonged serum half-life of SL335-IFN-β-1a in rats.

To determine whether SL335-IFN-β-1a further exhibits an extended serum half-life in NHP, preliminary PK analyses were carried out using cynomolgus monkeys (one male for Rebif and two males for SL335-IFN β-1a), and the results are shown in Fig. 5A & B and Table 3. Upon I.V. administration (0.011 mg/kg Rebif or 0.034 mg/kg SL335-IFN β-1a), the $t_{1/2}$ of SL335-IFN-β-1a was 48.9 h, and that of Rebif was 6.0 h. The systemic clearance rates of SL335-IFN-β-1a and Rebif were 1.04 and 50.24 mL/hr/kg, respectively, and the volume of distribution at the steady state (Vss) of SL335-IFN-β-1a was 58.4 mL/kg, while that of

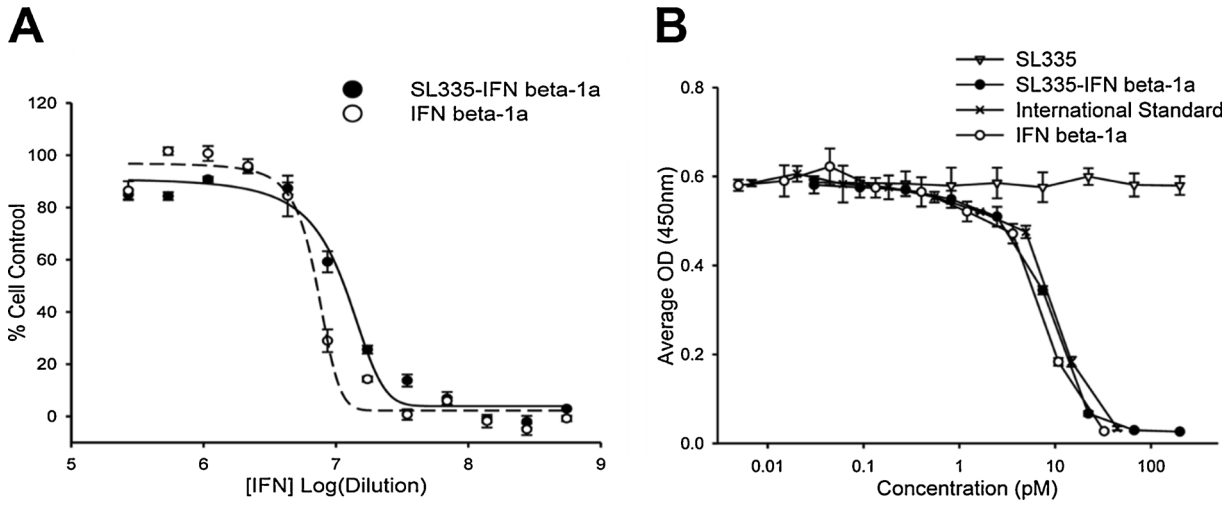

Fig. 3. The *in vitro* bioactivities of SL335-IFN-β-1a determined by the antiviral and anti-proliferative assays. The *in vitro* bioactivities of rIFN-β-1a (Rebif) and SL335-IFN-β-1a were assessed in **(A)** the antiviral assays using A549 cells challenged with EMCV and **(B)** the anti-proliferative activity Daudi cells as described in the Materials and Methods. WHO International Standard IFN-β (IS IFN-β) was also included in the experiments to draw a standard curve, and used to deduce the specific activity unit of Rebif and SL335-IFN-β-1a.

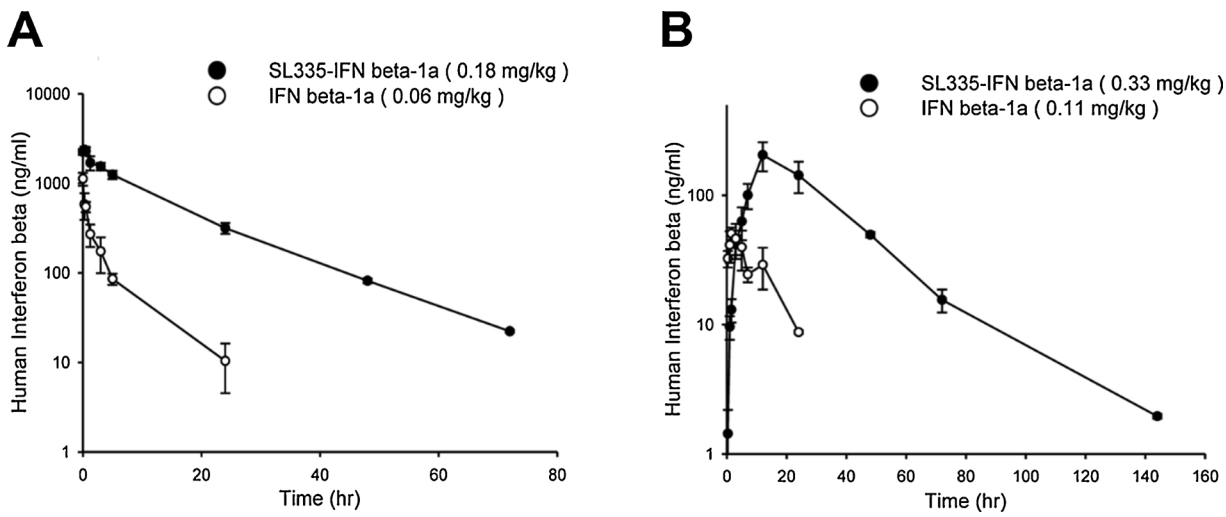

Fig. 4. Pharmacokinetics for SL335-IFN-β-1a in rats. Pharmacokinetic properties after a single dose I.V. **(A)** or S.C. **(B)** administration of rIFN-β-1a (Rebif) (open circle) or SL335-IFN-β-1a (closed circle) were studied using Wistar Lewis rats (four females/group). The plasma concentrations of the samples at each time points were measured by ELISA, and data were plotted as mean ± standard deviation.

Table 2
Comparison of pharmacokinetic parameters for rIFN-β-1a (Rebif) and SL335-IFN-β-1a in rats following different routes of administration.

Species	Dosing Route	Treatment	$T_{1/2}$ (h)	C_{max} (ng/mL)	AUC_{inf} (h·ng/mL)	CL (mL/h/kg)	V_{ss} (mL/kg)
Rat	I.V.	rIFN-β-1a (Rebif)	3.9	712.6	3962.6	15.1	84.2
		SL335-IFN-β-1a	10.8	2014.9	31389.3	1.9	29.8
	S.C.	rIFN-β-1a (Rebif)	9.3	50.6	728.5	151	2043.3
		SL335-IFN-β-1a	19.7	68.3	2164.3	50.8	1446.2

Rebif was 270.9 mL/kg (Fig. 5A, Table 3). Upon S.C. injection (0.034 mg/kg Rebif or 0.102 mg/kg SL335-IFN β-1a), the $t_{1/2}$ of SL335-IFN-β-1a was 22.4 h, and that of Rebif was 12.3 h. The systemic clearance rates of SL335-IFN-β-1a and Rebif were 2.18 and 37.29 mL/hr/kg, respectively, and the volume of distribution at the steady state (Vss) of SL335-IFN-β-1a was 71.8 mL/kg, while that of Rebif was 660.6 mL/kg (Fig. 5B, Table 3). These results also demonstrated a longer SL335-IFN-β-1a $t_{1/2}$ compared to that of Rebif in cynomolgus monkeys.

Neopterin, a catabolic product of guanosine triphosphate (GTP), serves as a serum biomarker of IFN-β *in vivo* [23]. To determine the pharmacodynamic properties of SL335-IFN-β-1a, the neopterin concentrations in the plasma samples obtained from the PK study above following S.C. injection were analyzed by ELISA. Fig. 6 shows the mean plasma concentration-time curve of neopterin after a single injection of Rebif or SL335-IFN-β-1a. Although there was a variation in the neopterin concentration among individual animals, the neopterin concentration was increased after the administration of both Rebif and SL335-IFN-β-1a, indicating the *in vivo* IFN-® biological activity of SL335-IFN-β-1a. The neopterin concentration reached its maximum at 48 h postinjection and declined at different rates in the animals; the neopterin concentration decreased at a slower rate in monkeys treated with SL335-IFN-β-1a than in the monkey administered Rebif. Along

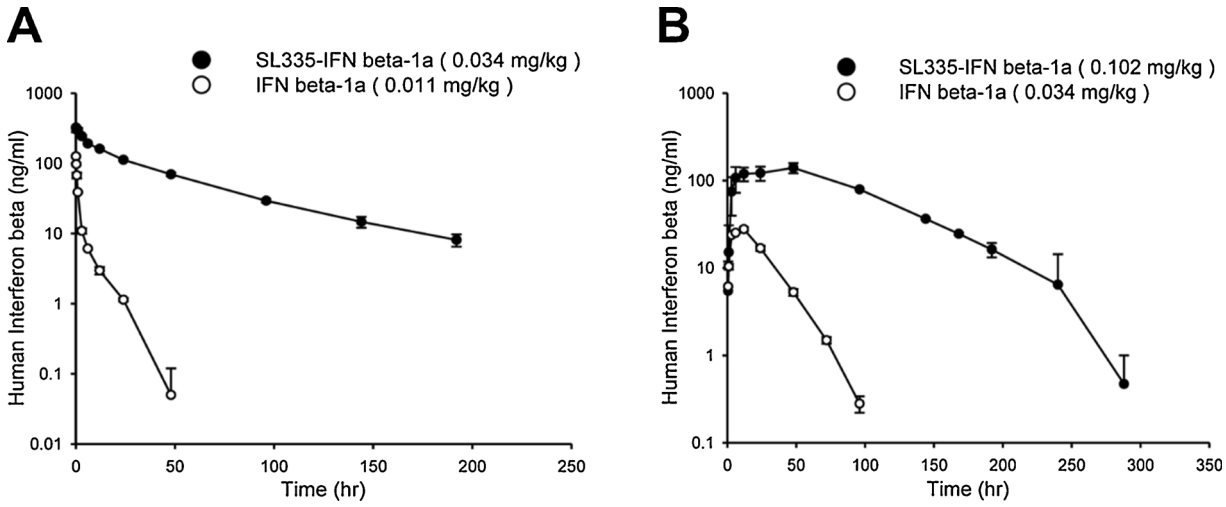

Fig. 5. Pharmacokinetics for SL335-IFN-β-1a in cynomolgus monkeys. Pharmacokinetic properties after a single dose I.V. **(A)** or S.C. **(B)** administration of rIFN-β-1a (Rebif) (open circle) or SL335-IFN-β-1a (closed circle) were studied using cynomolgus monkeys (one male for Rebif and two males for SL335-IFN-β-1a). The plasma concentrations of the samples at each time points were measured by ELISA, and data were plotted as mean ± standard deviation.

Table 3
Comparison of pharmacokinetic parameters for rIFN-β-1a (Rebif) and SL335-IFN-β-1a in cynomolgus monkeys following different routes of administration.

Species	Dosing Route	Treatment	$T_{1/2}$ (h)	C_{max} (ng/mL)	AUC_{inf} (h·ng/mL)	CL (mL/h/kg)	V_{ss} (mL/kg)
Monkey	I.V.	rIFN-β-1a (Rebif)	6.0	143.0	218.9	50.24	270.9
		SL335-IFN-β-1a	48.9	1002.5	32917.1	1.04	58.4
	S.C.	rIFN-β-1a (Rebif)	12.3	27.8	911.9	37.29	660.6
		SL335-IFN-β-1a	22.4	418.5	46858.1	2.18	71.8

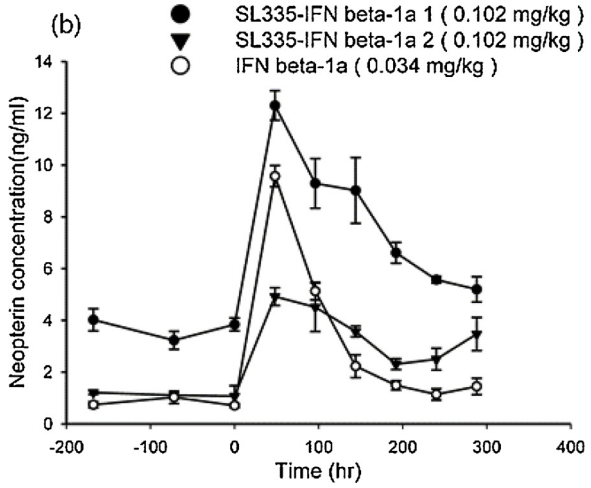

Fig. 6. Pharmacodynamics assay in cynomolgus monkeys. The plasma samples were obtained from the cynomolgus monkeys used in the PK study following S.C. administration, and the plasma neopterin concentrations were measured by ELISA to analyze the pharmacodynamics property of rIFN-β-1a (Rebif) (one male; open circle) and SL335-IFN-β-1a (two males; closed circle and triangle). The curves represent the value of data from each of three monkeys, and data were expressed as mean ± standard deviation.

with the PK results, these data further supported the long-acting efficacy of SL335-IFN-β-1a.

3.5. Pilot repeated toxicology study in NHP

A high dose of SL335-IFN-β-1a (90 mcg/kg) was injected *via* the S.C. route each week into cynomolgus monkeys (one male and one female) for four weeks, and diverse clinical parameters, such as general clinical signs, body weight, food consumption, ophthalmology, hematology, coagulation, clinical chemistry and urinalysis, were observed regularly. No specific changes related to SL335-IFN-β-1a administration were observed in the clinical analyses (data not shown). After the study, the weights of 16 organs from males and 13 organs from females were measured, and no specific abnormalities were found (data not shown). Additionally, microscopic examinations were performed on 42 male tissues (38 common tissues and 4 gender-specific tissues), and no abnormalities were found (data not shown).

3.6. A pilot repeated toxicology study in NHP

High dose of SL335-IFN-β-1a (90 mcg/kg) was weekly injected with the S.C. route to cynomolgus monkeys (one male and one female) for four weeks, and diverse clinical parameters such as general clinical signs, body weight, food consumption, ophthalmology, hematology, coagulation, clinical chemistry and urinalysis were observed regularly. No specific changes related to SL335-IFN-β-1a administration were observed by the clinical analyses (data not shown). After the study, weight of 16 organs from male and 13 organs from female was measured, and no specific abnormality was found (data not shown). Additionally, microscopic examinations were made for 42 tissues for male for female (38 common tissues and 4 gender-specific tissues), and couldn't find any abnormalities, either (data not shown).

4. Discussion

We had previously reported the isolation of SL335, a human anti-serum albumin Fab, and its long serum half-life in rats [19] by utilizing the FcRn recycling mechanism like other albumin binders [24,25]. We further utilized SL335 to construct a functional and long-acting SL335-human growth hormone (SL335-hGH) fusion protein and produced using the *E. coli* periplasmic expression system [22]. In the SL335-hGH study, we demonstrated that the removal of the interchain disulfide bond (IDB) from SL335 ($SL335_{\Delta}ds$) led to the improved soluble expression of a functional SL335-hGH fusion protein. Herein, an identical approach was utilized in an attempt to generate the SL335-IFN-β fusion protein using $SL335_{\Delta}ds$, of which the Cys233 of C_{H1} and the Cys214 of $C_{L|}$ are replaced with Ser as shown in Fig. 1. Nonglycosylated rIFN-β (rIFN-β-1b) has been produced in *E. coli* and successfully commercialized under the product names Betafefon and Extavia. In this study, however, we used mammalian CHO cells to produce the glycosylated form of SL335-IFN-β fusion protein, designated as SL335-IFN-β-1a, because the presence of glycosylation on rIFN-β is favorable in reducing the level of aggregation and sensitivity to thermal denaturation [26]. Furthermore, the *in vitro* bioactivity of rIFN-β-1b was also found to be 10–14 times lower than that of glycosylated rIFN-β (rIFN-β-1a) that has long been available on the market under the names Avonex and Rebif [27,28].

Nevertheless, manufacturing rIFN-β-1a in CHO cells was still problematic due to low host cell viability and low productivity; thus, improving production yield by using an optimal culture process would be mandatory for drug development [21,29]. It is uncertain whether the production yield of SL335-IFN-β-1a by 4–37 CHO-S cell line under our laboratory conditions is superior or inferior to those of other CHO cell lines producing rIFN-β because of the lack of published information. One study using a complicated macroporous microcarrier CHO cell culture reported the production of 26.8×10^6 units/mL of rIFN-® [30]. Because our yield of SL335-IFN-β-1a corresponds to 2.3×10^6 units (the bioactivity of SL335-IFN-β-1a is 7.15×10^7 units/mg), our yield is only 1/10th of the microcarrier culture. Nonetheless, the yield of our CHO-S cell line is far from disappointing because media and process optimizations usually increase the production yield substantially. The advantage of SL335-IFN-β-1a seems quite obvious in terms of proteins purification. The purification yield of SL335-IFN-β-1a was 67% in our experimental setup, which is considerably higher than that of PEGylated IFN-β-1a (> 27%) (Baker and others 2006), not to mention of a considerably simpler production process than that of PEGylated IFN-β-1a.

Natural IFN-β has a single N-glycosylation site (Asn-80), and the presence of heterogeneous N-glycans has been reported [31]. In accordance with the previous report, the CE-SDS analysis of SL335-IFN-β-1a prior to but not after the PNGase F treatment also produced multiple peaks corresponding to SL335 Fd-IFN-β-1a fusions of diverse sizes (Fig. 2), confirming the presence of heterogeneous N-glycans on our fusion protein. Although glycosylation somewhat reduces the aggregation, the aggregated form of IFN-β tends to form an antidrug antibody (ADA) upon repetitive *in vivo* administration, resulting in a faster

decline in drug efficacy [32–34]. Therefore, formulation conditions are important, especially for therapeutic rIFN-β preparations. Although data are not presented, we carried out a preliminary formulation of SL335-IFN-β-1a without adding HSA as an excipient, and the melting temperature (Tm) of SL335-IFN-β-1a was determined to be 57.8 °C, which is approximately 2.5 °C higher than that of Rebif (55.3 °C), a commercial rIFN-β-1a therapeutic. Although data are not shown, SL335-IFN-β-1a was subjected to stability testing, revealing that the fusion protein remained approximately 90% intact when incubated at 4 °C for 90 days and that the *in vitro* activity was maintained by up to 70% under our formulation conditions. These data suggest that our SL335-IFN-β-1a has the potential to become a drug candidate.

As shown in Table 1 and Fig. 3, the bi-functionality of SL335-IFN-β-1a was well-preserved because it retained the binding affinities to both HSA and IFN-α/β R2 as well as the *in vitro* IFN-® bioactivity. In the antiviral potency assay, the specific activity of SL335-IFN-β-1a was found to be 7.15×10^7 U/mg, while that of Rebif was 1.16×10^8 U/mg. The Rebif drug packaging describes its specific activity as 2.7×10^8 U/mg, implying that our specific antiviral units were slightly underestimated. This probably originates from the difference in the experimental setups since the antiviral potency of Rebif reported on its drug packaging was determined by the CPE assay using WISH cells and vesicular stomatitis virus (VSV) (Rebif Drug Description) [35]. Or the difference is merely within an experimental error range. At any rate, our results clearly demonstrates that SL335-IFN-β-1a fully retains the antiviral and anti-proliferative activities of Rebif. It would have been informative to conduct a comparative study with a pegylated version of IFN-®-1a (Plegridy). Unfortunately, however, we were not able to purchase the drug legitimately for research purposes in Korea. The data packaging of Plegridy denotes its specific antiviral activity as being 100 million international units (MIU) per mg (10^8 U/mg), which is approximately three-fold lower than that of Rebif. Because SL335-IFN-β-1a exhibits a degree of antiviral potency similar to that of Rebif, it can be inferred that SL335-IFN-β-1a may possess an approximately three-fold higher potency than Plegridy.

rIFN-® therapeutics, except for Avonex, are administered *via* the S.C. route in clinical settings. Our rat PK study utilizing the S.C. injection revealed that SL335-IFN-β-1a has an approximately 2-fold longer serum half-life than Rebif in rats (the $t_{1/2}$ = 19.7 h *vs.* 9.3 h). This result seems unimpressive because our unpublished PK analytical data with the SL335-hGH fusion showed an approximately 24-fold increase in the $t_{1/2}$ of SL335-hGH compared to that of hGH (52.9 h *vs.* 2.25 h) in rats. Additionally, it has been reported that the $t_{1/2}$ of PEG IFN-β-1a is approximately seven times longer than that of rIFN-β-1a upon S.C. injection in rats (10.56 h *vs.* 1.47 h) [36]. It is interesting to note that our $t_{1/2}$ value of Rebif appears unexpectedly long considering its small MW (18 kDa). As shown in Table 2, our $t_{1/2}$ PK parameters following I.V. administration were 3.9 h for Rebif and 10.8 h for SL335-IFN-β-1a, whereas values of 1.47 h for Avonex and 10.13 h for PEG IFN-β-1a were observed in rats [36], further suggesting that the $t_{1/2}$ of Rebif was overestimated in our study. We assume that this discrepancy might be derived from the small number of animals used or the difference in the analytical method for determining the serum concentration of IFN-β-1a. Indeed, the antiviral potency assay was used in the study with Avonex, whereas we used ELISA, which is considerably less sensitive than the antiviral potency assay in determining the serum concentration of IFN-β-1a.

Pepinsky et al. further reported the PK measurements following S.C. administration in rhesus monkeys, and their $t_{1/2}$ parameters were 10 h for Avonex and 22 h for PEG IFN-β-1a [36]. Our PK results using cynomolgus monkeys were in accordance with their data (12 h for Rebif and 20 h), suggesting that SL335-IFN-β-1a has a serum half-life similar to that of PEG IFN-β-1a, at least in monkeys. Considering the difference in the long-acting mechanism *in vivo* between PEG IFN-β-1a and SL335-IFN-β-1a, it is uncertain whether they also exhibit similar PK parameters in humans. In this regard, it is noteworthy that the *in vivo* longevity of SL335 would be proportional to the half-life of serum albumin because it indirectly uses the FcRn-mediated recycling mechanism. Therefore, it can be assumed that SL335-IFN-β-1a may show longer PK parameters in humans because the serum half-life of HSA is almost three times longer than that of monkeys (19 days *vs.* 7 days). The feasibility of our approach in extending the serum half-life of IFN-β was supported by the PK study using an IFN-®-albumin fusion protein (IFN-®-HSA, Albuferon) in rhesus monkeys, revealing that the terminal half-life of Albuferon was 36–40 h, while that of rIFN-® was 8 h [37]. The approximately two-fold longer terminal half-life of Albuferon compared to that of SL335-IFN-β-1a might be due to the difference in dosages (300μg/kg IFN-®-HSA *vs.* 34 μg/kg SL335-IFN-β-1a).

By comparing the $t_{1/2}$ PK parameters following I.V. administration, the $t_{1/2}$ of Rebif was 6 h, and that of SL335-IFN-β-1a was 48.9 h in our study, whereas that of Avonex was 3.2 h, and that of PEG IFN-β-1a was 9.5 h [36], suggesting that SL335-IFN-β-1a has almost a five-fold longer serum-half life than PEG IFN-β-1a. However, it is hard to believe that the $t_{1/2}$ of SL335-IFN-β-1a exhibited upon I.V. administration is longer than that exhibited upon S.C. administration. We would like to point out that a very limited number of animals were used in our preliminary PK studies to observe the long-acting trend of SL335-IFN-β-1a rather than to obtain statistically accurate PK parameters. Notwithstanding, our PK studies clearly demonstrate the serum longevity of SL335-IFN-β-1a compared to that of Rebif *in vivo*. This finding is further supported by the preliminary pharmacodynamics study based on measuring the neopterin concentration, a typical serum biomarker of IFN-β [23], revealing that the neopterin concentration decreased more slowly in monkeys treated with SL335-IFN-β-1a than in the monkey treated with Rebif. Finally, our pilot 4-week repeated toxicology study in cynomolgus monkeys demonstrated the *in vivo* safety of SL335-IFN-b-1a.

In summary, we have attempted to use SL335, a human antiserum albumin Fab molecule, to create a modified rIFN-β fusion protein, named SL335-IFN-β-1a, and demonstrated the potential of our fusion protein as a novel long-acting IFN-β therapeutic in terms of its protein productivity, physical properties, *in vitro* bioactivity, improved *in vivo* serum half-life and *in vivo* safety.

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