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RESEARCH PAPER

Differences in Proportion of N-acetyllactosamine and O-acetylated Sialic Acid Have No Significant Effect on the Pharmacokinetics and Biological Activity of Darbepoetin Alfa

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Abstract Darbepoetin alfa is used to treat anemia in patients with chronic renal failure and is a therapeutic glycoprotein with five N-glycosylation sites and one O-glycosylation site. Since the glycosylation of therapeutic proteins is known to affect pharmacokinetics and biological activity, it was wondered how the certain glycosylation structure of darbepoetin alfa affects pharmacokinetics and biological activity. To investigate the effects of glycosylation structures, several darbepoetin alfa samples were generated through the processes slightly modified from HK-DPO production process. Analysis of N-linked glycosylation profiles showed that darbepoetin alfa samples have different proportions of N-acetyllactosamine and O-acetylated sialic acids. Since these structures were known to affect the stability and efficacy of glycoproteins, we evaluated the pharmacokinetics and biological activities of darbepoetin alfa samples. As a result of pharmacokinetics, the time course of the concentration in plasma was similar to that of NESP®, commercially available darbepoetin alfa. Also, the relative AUC_{last} ratio (sample/reference) was ranged from 84% to 114%, which indicated they did not have meaningful differences. For *in vitro* biological activity, a cell proliferation test was conducted in the EPO-dependent F36E cell-line. An *in vivo* biological activity test was performed with EP-based assay using B6D2F1 female mice. As a result, the biological activities of each samples were similar to those of reference drug, NESP®. In conclusion, different proportions of N-acetyllactosamine and O-acetylation of sialic acid in darbepoetin alfa did not show any meaningful effects on pharmacokinetics and biological activities.

Keywords: darbepoetin alfa, N-acetyllactosamine, O-acetylated sialic acid, pharmacokinetics, biological activity

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1. Introduction

Human erythropoietin (hEPO) is a glycoprotein with 165 amino acids, which controls red blood cell (RBC) production. After it is produced in kidney, hEPO moves to bone marrow and activates survival, proliferation, and differentiation of RBC precursor cells. Patients with chronic renal failure are often anemic, due to poor production of endogenous EPO following kidney damage. Some cancer patients also suffer from anemia following chemotherapy or radiotherapy [1-5]. To treat these anemias, recombinant human EPO (rhEPO) is administered to patients 2-3 times

a week. Darbepoetin alfa (DPO) was developed to be administered once a week, thereby increasing the dosing interval to enhance convenience. This was made possible by introducing two additional N-glycosylation sites into rhEPO by substitution of five amino acid residues (A30N, H32T, P87V, W88N, and P90T). Since it can contain up to four sialic acid residues per N-glycan and two sialic acids per O-glycan, DPO including five N-linked and one O-linked glycosylation sites can contain up to 22 sialic acid residues [6]. It is known that sialic acid residues have several functions including protection from proteases and modulation of receptor binding affinity [7]. The higher sialic acid content allows DPO to sustain in the serum approximately three times longer than hEPO, which contains up to 14 sialic acid residues [5,8-11].

In addition to the sialic acid contents, glycosylation structures are known to have effects on glycoprotein's function or stability. Commercially available rhEPO products have various glycan structures and each of products show different glycosylation profiles even though they have the same amino acid sequences. This is because glycosylation is finally determined by post-translational modification, which can vary depending on cell-lines and culture condition. Previous studies have shown that, in particular, N-acetyllactosamine (LacNAc) and O-acetylated sialic acid have different ratios for different rhEPO products [4]. O-acetylated sialic acid can be present in tetra-antennary oligosaccharide up to eight because one sialic acid residue has two O-acetylation sites. O-acetylated sialic acid is known to affect the efficacy and safety of bio-therapeutics, so it is important to control contents of O-acetylated sialic acids when producing therapeutic glycoproteins [12,13]. The antennary oligosaccharides with zero to two LacNAcs are also present in most rhEPO produced in Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. Di-, tri-, and tetra-antennary oligosaccharides have a1-6-linked mannose residues to which can be attached one or two LacNAc [12,14-19]. LacNAc extension can increase the half-life of EPO by increasing the overall size of the protein molecule [11]. Therefore, the proportion of LacNAc can be one of the quality attributes which should be monitored during commercial production of pharmaceuticals.

Erythropoiesis stimulating agents (ESAs) such as EPO and DPO are so effective that they have been widely used around the world. Patients with chronic renal failure may have to take ESA for their lifetime unless they have a kidney transplant. These patients can reduce their treatment costs by using biosimilar products with similar efficacy and safety. Since biosilmilars are required to have the similar efficacy as the original, their structure and biological properties should be developed highly similar to those of original. To obtain a biosimlar's approval from the Ministry of Food and Drug Safety, the comparable similarity between original and biosimilar must be intensively verified. As the technology has advanced, it is possible to develop the primary to higher order structures and the physicochemical properties almost identically, but there are still differences in glycosylation pattern by post-translational modification. It is known that the types of glycan structures of proteins produced by species such as humans, insects, and plants are different. When producing biopharmaceuticals, human-derived cells or mammalian-derived cells are used because the glycan structures of proteins from the cells are the same or most similar to those in human. However, even though the proteins are produced in the same cell-lines, the post-translational modification can be affected by the clone and culture condition, and the glycosylation profiles in the final drug substance are various by prufication processes. Previous study has compared commercially availble EPO products to confirm how different the glycan profiles they have are. However, they did not make a head-to-head comparison of how the efficacy of EPO products depends on differences in specific glycan structures. Also, unlike more studies on the glycosylation of EPO so far, less studies on the glycosylation of DPO may have a more complex glycosylation profile by two additional N-glycans.

We wondered how differently glycosylated DPOs would be produced when the production process was slightly modified. To investigate the effect of slightly different glycosylation profiles, we first produced several DPO variants by making some changes to the production process of HK-DPO. HK-DPO refers to DPO produced by our laboratory for this research and is in order to distinguish it from the commercially available DPO. After preparing HK-DPOs, the glycan structures such as LacNAc and O-acetylated sialic acid, which are known to affect the biological activities of EPO were analyzed. After confirming the glycosylation profiles, we compared the pharmacokinetics and biological activities of HK-DPOs with various glycosylation profiles. In this study, NESP®, the original drug and commercially available DPO, was compared together as a reference drug to determine whether the differences in the glycan structures were significant for efficacy.

2. Materials and Methods

2.1. Cell-line development

The expression vector was constructed with synthesized *darbepoetin-alfa* and *dihydrofolate reductase* (*dhfr*) genes. It was transfected into *dhfr*-deficient CHO-DG44 cells by electroporation and the transfected genes sequentially amplified by methotrexate. Recombinant CHO cells expressing DPO (DPO-CHO cells) were maintained in Erlenmeyer

flasks at 37°C in a 5% CO_2, humidified shaking incubator. The cell density and viability were measured using a Vi-cell counter (Beckmann Coulter, USA).

2.2. Production of darbepoetin alfa

DPOs were expressed by culturing DPO-CHO cells in 1 L scale flasks (Corning®, CLS431147) or 2 L scale bioreactors (NBS, CG115), and the culture supernatant was harvested by centrifugation. Purification was conducted through ion exchange column chromatography in AKTA Avant (Cytiva, USA).

Chemically-defined media (Lonza) containing 4 mmol/L L-glutamine (HyClone™, SH30034) was used for cultivation of DPO-CHO cells. Two of the DPO samples were produced by adding 10 mM lithium chloride or 200 mM sodium butyrate to culture medium, respectively, at initial day. One sample was obtained by supplying 75 mM sodium chloride on the 3rd day after seeding, and the other sample was produced in culture media substituting L-glutamine to 4 mM α-ketoglutarate.

2.3. Isoform distribution analysis

Isoelectric focusing (IEF) was conducted using a horizontal gel (pH 2-4, SERVA). Samples were desalted via centrifugation, and the gel was pre-focused for 30 min at 4°C. Prepared samples were electrophoresed using 2,000 V, 4 mA, and 8 W at 4°C for 180 min. Afterward, the gel was stained with Coomassie Brilliant Blue and then destained.

2.4. N-Glycan analysis

Hydrophilic interaction liquid chromatography-high performance liquid chromatography/fluorescence detection-mass spectrometry (HILIC-HPLC/FLD-MS) was conducted using a UPLC with a fluorescence detector (Shimadzu) and an on-line Q-TOF MS 5600+ (Sciex). The columns were a 2.1 × 100 mm ACQUITY UPLC BEH glycan column (Waters) for amine-based HILIC/FLD-MS and a 2.1 × 150 mm GlycanPac AXH-1 column (Thermo Scientific) for amide-based HILIC/FLD-MS. Several peaks were separated depending on the number of antennary structure, sialic acid residues, and carbohydrates and then identified by MS. After that, the area of each peak was calculated to determine the proportion of each structure.

2.5. Pharmacokinetics

Comparative pharmacokinetics (PK) were evaluated in 8-week-old male Sprague-Dawley rats (n = 4 per sample) with subcutaneous (SC) administration of 3 μg/kg at the back of the neck. The rats were randomly divided into each group for HK-DPO samples and reference DPO. Blood samples were collected from the tail vein at pre-dose and 3, 6, 9, 12, 15, 18, 24, 32, 48, and 72 h after administration. The concentration of DPO in plasma was measured by ELISA using human EPO antibody (R&D Systems, primary antibody MAB287, secondary antibody AB-286-N). The PK parameters were calculated using a non-compartmental analysis with WinNolin (Pharsight, Mountain View).

The correlation of relative AUC_{last} was assessed by analysis of variance (ANOVA) from bivariant fit test using JMP software (SAS Institute, USA), and a p value of $\leq$ 0.05 was considered statistically significant.

2.6. *In vitro* biological activity study

Human leukemia F-36E cells (Riken BRC Cell Bank, Japan) were cultivated in RPMI 1640 (5% FBS, 1% Penicillin/Streptomycin) including rhEPO (EP BRP, 5 U/mL). After cells were washed twice, they were incubated with increasing concentrations (0.5, 1.5, and 4.5 ng/mL) of DPO. Then, they were grown for 72 h at 37°C in 5% CO_2 incubators. MTS assay was conducted using CellTiter 96® AQueous Non-radioactive cell proliferation assay kit.

The correlation of *in vitro* relative potency was assessed by analysis of variance (ANOVA) from bivariant fit test using JMP software (SAS Institute, USA), and a p value of $\leq$ 0.05 was considered statistically significant.

2.7. *In vivo* biological activity study

HK-DPO samples were injected subcutaneously into 8-week-old B6D2F1 female mice (n = 8 per sample). The mice were randomly divided into each group for HK-DPO samples and reference DPO. After 4 days, blood was drawn from the orbital vein using heparinized capillary tubes. After stained, reticulocytes were measured using ADVIA2120i (Siemens). The potency was calculated using a parallel line analysis comparing the assay response to log concentration with CombiStats (EDQM). The suitability for 95% CI was 64 to 156%. *In vivo* biological activity was assessed according to European Pharmacopoeia (EP) monograph for *Erythropoietin-concentrated solution*.

The correlation of *in vivo* relative potency was assessed by analysis of variance (ANOVA) from bivariant fit test using JMP software (SAS Institute, USA), and a p value of $\leq$ 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Darbepoetin alfa with various glycosylation profiles

3.1.1. Preparation of various Darbepoetin alfa samples

In this study, DPO was produced utilizing the cell line and the process of DPO biosimilar production. To investigate the effects of different glycosylation patterns on pharmacokinetics and biological activity, darbepoetin alfa samples

were prepared through modified production processes. Some HK-DPOs were obtained by supplementing additives such as lithium chloride, sodium butyrate, α-ketoglutarate, and sodium chloride (HK-DPO 1, 2, 4, and 5) in flask culture. Lithium chloride has anti-apoptosis functions, and sodium butyrate arrests cell cycle and increases productivity. Also, α-ketoglutarate is one of the intermediates in the TCA cycle, and sodium chloride inhibits sialate O-acetyltransferase. These additives were choosen because they were expected to affect the glycosylation of recombinant proteins [20-22]. HK-DPO 6 was produced by culturing DPO-CHO cells without additional supplements in a 2 L scale bioreactor under well-controlled pH and DO. Each HK-DPO expressed in different culture process was purified by the same method. Finally, we obtained that HK-DPOs from three different fractions in last step of purification process (HK-DPO 3, 7, and 8). In this purification, the culture conditioned media was prepared in a 200 L scale bioreactor. In order to obtain various glycosylated DPOs, eight HK-DPOs were produced in slightly different processes. In addition, a commercially available darbepoetin-alfa product, NESP® by Kyowa Kirin Co., Ltd., was purchased and prepared as a reference drug [6].

After preparing DPO samples, IEF analyses were performed to confirm the pattern of purified HK-DPOs. As shown in Fig. 1, all DPOs were seperated into six isoforms on the horizontal IEF gel, and pI value of each isoform was the same between DPO samples. The more terminal sialic acid the isoform has, the higher it has a negative charge, so it appears in the more acidic region. This is why there were various ratios of six isoforms in eight HK-DPOs. These results were expected because HK-DPOs were designed to have different glycosylation profiles.

3.1.2. Analysis of Glycosylation profile

To determine the N-glycan profile including LacNAc and O-acetylated sialic acid, DPO samples were analyzed by HILIC-HPLC/FLD-MS. Each N-glycan isoform was seperated into a peak, and the peaks were identified by MS. The peak area was also calculated to find the relative proportion of each structure. The results were summerized in Table 1.

It was confirmed that all DPO samples had mono-, di-, tri-, and tetra-sialo N-glycans, sorted according to the number of sialic acids from 1 to 4. It is known that the *in vivo* biological activity of rhEPO increases as the number of carbohydrates and sialic acids on the protein increases [23,24]. Because of this, we first checked the proportions of tri- and tetra-sialo N-glycans. The ratios of tetra-sialo N-glycan with the highest number of carbohydrates and sialic acids were above 75% and the proportion of tri-sialo N-glycan were between 10-20% in all samples. The sum of tetra- and tri-sialo N-glycan proportion were approximately 94% or more. The results indicated that HK-DPO samples were highly glycosylated forms even though their production processes were all different. This can be thought of as

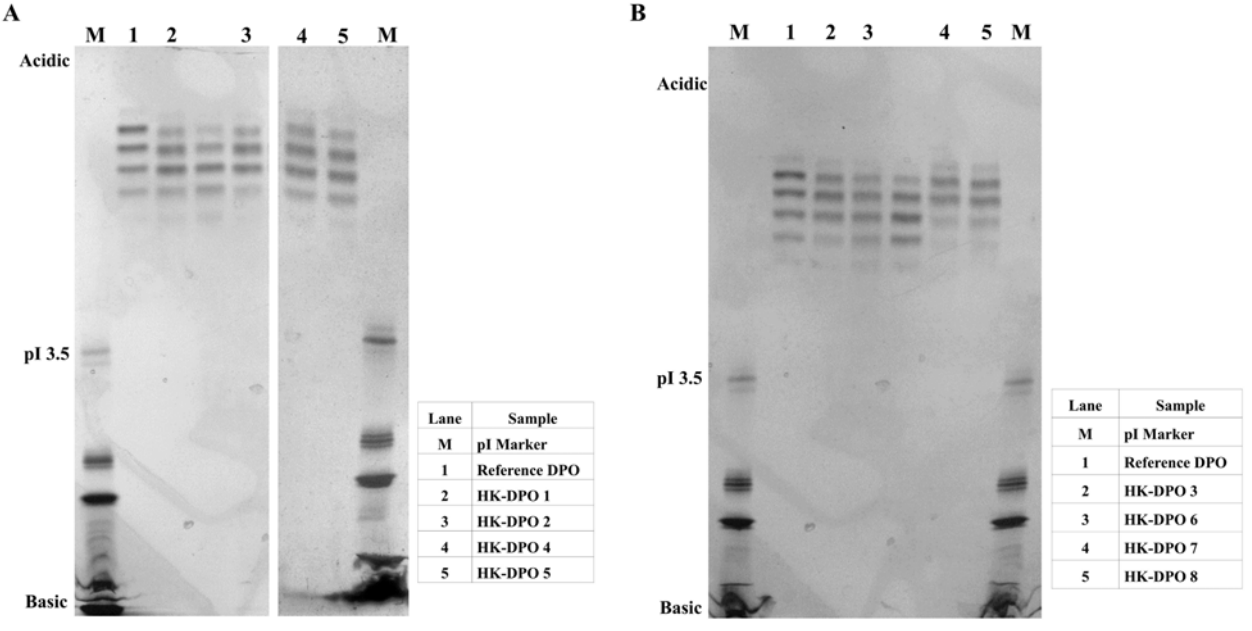

Fig. 1. Confirmation of electropherograms of HK-DPOs. Isoelectric focusing (IEF) was performed to confirm the expression patterns of purified HK-DPOs. As DPO is highly glycosylated protein and the pI value is acidic, IEF analysis was conducted using horizontal gels (pH 2-4) to clearly separate each isoform band. All HK-DPOs showed six isoforms, like the reference DPO, but the proportions of isoforms varied from each other. This result showed that HK-DPOs were expressed and purified as expected. (A) HK-DPO 1-2, 4-5. (B) HK-DPO 3, 6-8.

Table 1. N-glycan profiles of darbepoetin alfa analyzed by hydrophilic interaction liquid chromatography-high performance liquid chromatography/fluorescence detection-mass spectrometry

N-Glycan (Area%)			Reference DPO	HK-DPO1	HK-DPO2	HK-DPO3	HK-DPO4	HK-DPO5	HK-DPO6	HK-DPO7	HK-DPO8
Mono sialo	FA2G2S1	1	0.0	0.1	0.1	0.1	0.3	0.9	0.1	0.2	0.0
	FA3G3S1	2	0.1	0.1	0.1	0.2	0.8	0.7	0.1	0.2	0.0
	Total Mono-sialo N-Glycan		0.1	0.2	0.2	0.3	1.2	1.6	0.2	0.4	0.1
Di sialo	FA2G2S2	3	0.0	0.2	0.4	0.6	0.6	0.9	0.1	0.6	0.1
	FA3G3S2Ac1	4	0.0	0.5	0.4	0.4	1.0	0.2	-	0.4	0.1
	FA3G3S2	5	0.4	0.8	0.6	0.7	0.8	0.6	0.3	0.8	0.3
	FA4G4S2Ac1	6	1.2	0.5	0.6	0.5	2.7	2.1	1.1	0.5	0.8
	FA4G4S2	7	0.1	1.1	-	-	-	-	-	-	-
	Total Di-sialo N-Glycan		1.7	3.0	2.1	2.1	5.1	3.9	1.5	2.3	1.3
Tri sialo	FA3G3S3Ac2	8	0.6	0.9	1.7	2.0	-	2.1	1.2	2.0	0.5
	FA3G3S3Ac1	9	0.3	2.6	1.7	3.6	4.4	3.0	1.3	3.6	-
	FA3G3S3	10	0.5	4.0	1.3	4.5	4.2	5.6	3.5	4.5	2.6
	FA4G4S3Ac1 FA4G4S3Ac2	11	2.6	3.8	4.0	5.3	4.3	6.8	3.7	2.4	3.6
	FA4G4S3	12	9.6	5.8	4.3	0.7	1.6	1.8	8.5	0.4	4.4
	Total Tri-sialo N-Glycan		13.6	17.1	13.0	16.1	14.5	19.3	18.2	12.9	11.1
Tetra sialo	FA4G4S4Ac6 FA4G4S4Ac7	13	5.1	2.5	3.3	1.7	2.7	2.9	-	2.1	5.1
	FA4G4S4Ac4 FA4G4S4Ac5	14	2.3	7.4	7.5	6.2	6.7	5.0	4.6	6.8	5.0
	FA4G4S4Ac2 FA4G4S4Ac3	15	8.4	11.7	13.3	12.7	13.9	12.3	10.2	12.9	11.0
	FA4G4S4Ac1	16	23.8	16.9	19.0	21.2	19.5	19.8	20.2	21.2	19.6
	FA4G4S4	17	39.2	16.8	17.0	20.4	17.3	19.8	27.7	20.4	22.1
	FA4G4Lac1S4Ac1	18	1.8	6.3	7.6	5.2	5.7	4.8	5.5	6.1	8.0
	FA4G4Lac1S4Ac2	19	3.1	7.4	7.3	6.5	5.8	6.2	7.8	7.3	8.3
	FA4G4Lac1S4	20	1.0	2.9	1.0	1.1	2.3	1.5	2.0	1.7	3.0
	FA4G4Lac2S4Ac1	21	-	3.5	2.2	1.1	2.7	1.7	1.8	1.1	4.0
	FA4G4Lac2S4	22	-	1.7	3.3	2.4	0.8	0.9	-	2.6	1.5
	FA4G4Lac3S4Ac1	23	-	2.5	1.5	1.4	1.3	0.2	-	0.5	-
	FA4G4Lac3S4	24	-	-	1.7	1.5	0.5	0.2	0.4	1.8	-
	Total Tetra-sialo N-Glycan		84.6	79.7	84.7	81.4	79.3	75.2	80.1	84.4	87.6
Total (Tri+Tetra)-sialo N-Glycan			98.2	96.8	97.7	97.5	93.8	94.5	98.3	97.3	98.7
Total LacNAc (Lac1+Lac2+Lac3)			5.9	24.4	24.6	19.2	19.2	15.5	17.5	21.1	24.8
Total Acetylated Sialic Acid (Ac1-Ac7)			49.2	66.5	70.1	67.8	70.7	67.0	57.3	66.9	66.0

F: presence of fucose, A: number of antennae, G: number of galactose, Lac: number of N-acetyllactosamine (LacNAc), S: number of sialic acid, Ac: acetylation.

showing that the DPO biosimilar production process is robust for producing well-qualified DPO.

Next, we confirmed the proportions of LacNAc and O-acetylated sialic acid, the glycan structures known to have different ratios for several rhEPO. The reference DPO had only 1 LacNAc extension (Lac1) as 5.9% ratio in tetra-sialo N-glycans. HK-DPO samples had LacNAc upto 3 extensions (Lac1-Lac3) in tetra-sialo N-glycans, and the proportion of LacNAc extensions was different from sample to sample, ranging from 15.5-24.8%. In particular, HK-DPO 1, 2, 7, and 8 had the highest LacNAc proportions, above 20%.

Since one sialic acid can contain up to two O-acetylations, a tetra-sialo N-glycan potentially has from 0 to 8 O-acetylations in sialic acids. The results of N-glycan analysis exhibited that mono-sialo N-glycan had no O-acetylated sialic acid residues and di-sialo N-glycan had zero or one O-acetylated sialic acid in HK-DPOs including the reference. Besides, tri-sialo N-glycan contained up to two and tetra-sialo N-glycan up to seven O-acetylations in sialic acid per N-glycan. The total proportion of N-glycans with O-acetylated sialic acids was 49.2-70.7% depending on the sample; the reference DPO had the lowest percentage of O-acetylated sialic acid residues at 49.2%.

3.2. Comparison of biological activities

3.2.1. Analysis of pharmacokinetics profiles

Pharmacokinetics (PK) of HK-DPOs were evaluated in female SD rats with single subcutaneous (SC) administration, and the results were summarized in Table 2. To accurately assess the PK profile, the reference DPO was tested together in each set of experiments. Unlike EPO, there is no international standard, so NESP®, commercially available DPO, was used as a reference drug. The PK parameters measured to determine the rate and extent of HK-DPO systemic absorption were the maximum plasma concentration (C_{max}), the area under the concentration-time curve (AUC_{last}), the terminal half-life (HL_Lambda_z), and the time to

Table 2. Pharmacokinetics parameters of HK-DPO samples and reference DPO following administration of 3 μg/kg to male Sprague-Dawley rats (n = 4, data presented as mean ± SD)

Sample	HL_Lambda_z (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	Relative AUC_{last}
Reference DPO	13.7 ± 3.5	16.5 ± 1.7	8.0 ± 0.4	319.6 ± 31.7	-
HK-DPO 1	14.0 ± 1.9	15.8 ± 1.5	8.7 ± 0.5	364.1 ± 7.3	1.14
HK-DPO 2	18.2 ± 8.1	18.0 ± 4.2	8.1 ± 0.8	318.6 ± 19.1	1.00
Reference DPO	13.7 ± 3.5	16.5 ± 1.7	8.0 ± 0.4	319.6 ± 31.7	-
HK-DPO 3	11.2 ± 0.3	22.5 ± 3.0	7.3 ± 0.7	287.6 ± 48.5	0.90
HK-DPO 4	12.8 ± 5.2	18.0 ± 4.2	7.0 ± 0.8	278.9 ± 34.3	0.87
Reference DPO	15.9 ± 3.3	18.0 ± 4.2	8.5 ± 1.0	331.2 ± 41.1	-
HK-DPO 5	18.6 ± 3.3	13.5 ± 2.1	7.2 ± 1.0	287.5 ± 26.4	0.87
HK-DPO 6	17.6 ± 3.2	21.0 ± 3.5	7.0 ± 0.7	297.9 ± 32.3	0.90
Reference DPO	16.8 ± 2.1	18.0 ± 4.2	5.3 ± 0.2	190.5 ± 28.3	-
HK-DPO 7	19.6 ± 8.9	18.0 ± 4.2	4.6 ± 0.5	159.7 ± 28.3	0.84
HK-DPO 8	17.9 ± 4.2	15.0 ± 0.0	4.5 ± 0.3	181.5 ± 19.1	0.95

HL_Lambda_z: Terminal half-life, T_{max}: Time of C_{max}, C_{max}: maximum observed concentration, AUC_{last}: area under the plasma concentration-time curve from time 0 to 72 h, Relative AUC: $AUC_{last\ HK\text{-}DPO}$ / $AUC_{last\ Reference\ DPO}$ SD: standard deviation.

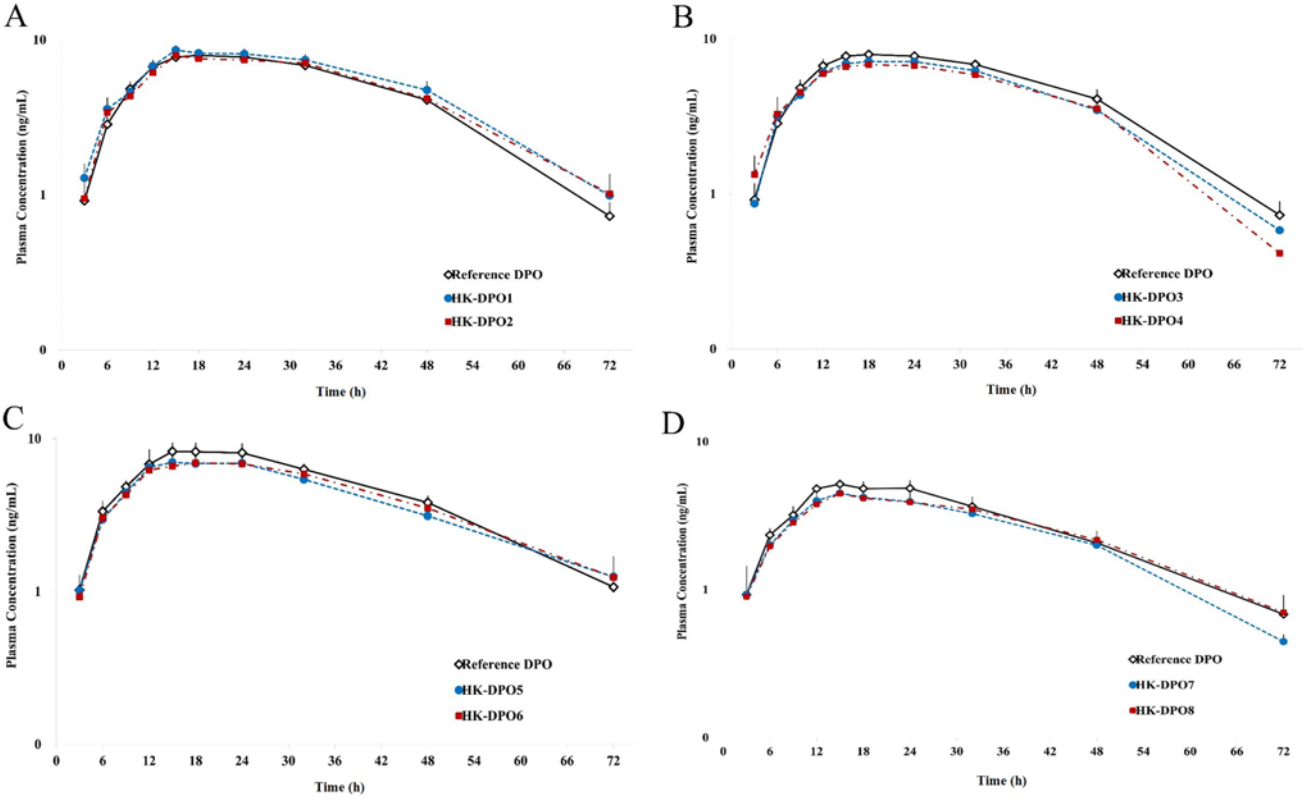

Fig. 2. Mean plasma darbepoetin alfa concentration-time profiles after single subcutaneous injections of HK-DPO samples and reference DPO in 8-week-old male Sprague-Dawley rats (n = 4 per sample). (A) HK-DPO 1-2. (B) HK-DPO 3-4. (C) HK-DPO 5-6. (D) HK-DPO 7-8.

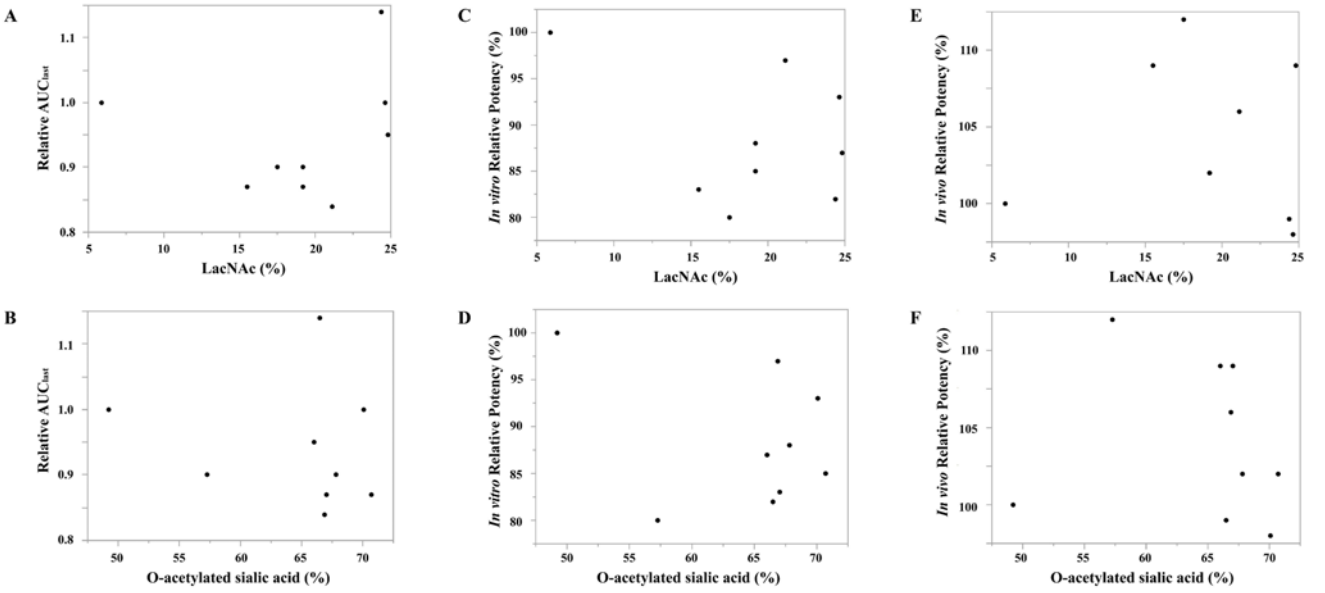

Fig. 3. Bivariate fit of the relative AUC_{last} and potencies by the proportion of N-acetyllactosamine (LacNAc) and O-acetylated sialic acid. To determine the correlation between two continuous variables, analysis of variance was performed with JMP software using the test results as shown in Table 1. (A, B) Bivariate fit of the relative AUC_{last}. (A) Linear Fit, Relative AUC_{last} = 0.8900396 + (0.0026692 x % LacNAc), *p* value 0.6622 (> 0.05). (B) Linear Fit, Relative AUC_{last} = 1.0857105 - (0.002238 x % O-acetylated sialic acid), *p* value 0.6703 (> 0.05). (C, D) Bivariate fit of *in vitro* relative potency. (C) Linear Fit, *in vitro* relative potency = 96.400796 - (0.4216444 x % LacNAc), *p* value 0.3370 (> 0.05). (D) Linear Fit, *in vitro* relative potency = 108.70785 - (0.3153408 x % O-acetylated sialic acid), *p* value 0.4080 (> 0.05). (E, F) Bivariate fit of *in vivo* relative potency. (E) Linear Fit, *in vivo* relative potency = 104.47639 - (0.0190911 x % LacNAc), *p* value 0.9540 (> 0.05). (F) Linear Fit, *in vivo* relative potency = 109.8727 - (0.0891733 x % O-acetylated sialic acid), *p* value 0.7530 (> 0.05).

maximum plasma concentration (T_{max}).

As shown in Fig. 2, The time course of the plasma erythropoietin concentration was similar among all test samples. To compare together how similar or different the PK characteristics of all test groups were, the relative AUC_{last} (AUC_{last} of sample/AUC_{last} of reference) was calculated, and the values ranged from 0.84 to 1.14 depending on the HK-DPO sample when the relative AUC_{last} of reference DPO was 1.00 (Table 2). It was confirmed whether the relative AUC_{last} showed correlation with the ratio of LacNAc and O-acetylated sialic acids using JMP software. As a result of bivariate fit analysis, there was no statistically significant correlation between the relative AUC_{last} and the proportion of specific glycan structures (Fig. 3). When fitting the regression line to the data, the *p* values of the analysis of variance (ANOVA) were > 0.05. These results indicated that changes in the proportion of specific glycan structures did not meaningfully affect to the rates and extent of DPO's systemic absorption.

3.2.2. Analysis of biological activities

Biological activity studies were performed both *in vitro* and *in vivo* to compare the efficacy of HK-DPOs head-to-head. First, a cell proliferation assay was conducted in the EPO-dependent F36E cell-line to confirm *in vitro* biological activity. Second, an *in vivo* biological activity test was performed with EP-based assay using B6D2F1 female mice. In all biological activity tests, NESP®, as a reference DPO, was used to adjust for test deviations measuring the activities of HK-DPOs (Table 3). Calculating the relative potency with the reference DPO, the *in vitro* relative potencies of HK-DPOs were 80-97% and the *in vivo* relative potencies were 98-112%. There was no proportional or inverse correlation between the relative potency *in vitro* and *in vivo*. According to the part of erythropoietin concentrated solution in EP, they suggested a criterion that the *in vivo* biological activity is equivalent if the estimated potency is 80% or more and 125% or less of the stated potency. Thus, it can be concluded that the *in vivo* biological activity is similar among all HK-DPO samples including the reference DPO.

Additionally, a bivariate fit analysis was performed to statistically understand the relationship between biological activity and the specific glycosylation patterns. When fitting Y by X using JMP software, the ratios of LacNAc and O-acetylated sialic acid were set as X and the *in vitro* and *in vivo* relative potency were set as Y. As a result, the distribution of Y for each X showed there were no certain patterns and all *p* values of ANOVA were found to be over 0.05 in linear fit (Fig. 3). This result indicated that

Table 3. *In vitro* biological activity using F-36E cells and *in vivo* biological activity with B6D2F1 female mice (n = 8)

Sample	*In vitro*		*In vivo*	
	Relative Potency	95% CI	Relative Potency	95% CI
Reference DPO	100	-	100	-
HK-DPO 1	82	75-89	99	83-120
HK-DPO 2	93	74-116	98	81-118
HK-DPO 3	88	75-102	102	85-123
HK-DPO 4	85	69-103	102	84-124
HK-DPO 5	83	71-96	109	93-128
HK-DPO 6	80	71-90	112	91-139
HK-DPO 7	97	86-109	106	91-125
HK-DPO 8	87	73-103	109	93-127

In vitro Relative potency: ED_{50} of HK-DPO / ED_{50} of reference DPO, ED_{50}: concentration produced 50% effect, CI: Confidential Interval, *In vivo* Relative potency: Each activity is reported as the relative potency compared to the reference DPO.

differences in the ratios of LacNAc and O-acetylated sialic acid did not have meaningful effects on their efficacy *in vitro* and *in vivo*.

4. Conclusion

In this study, we investigated whether specific glycosylation patterns of DPO influence pharmacokinetic characteristics and biological activities. DPO has a complex glycosylation profile with micro-heterogeneity in glycans. Previous studies have reported that rhEPO products on the market have differences in their glycosylation profile, but they did not compare how pharmacokinetics and efficacy vary with changing the ratio of specific glycan structures. Meanwhile, the function of specific glycan structures has been studied by genetically modified or chemically processed proteins. These studies were able to clarify the function of the specific structure as it allowed comparative testing by completely altering the structure of the protein as intended. They cannot determine the effect resulting from minor differences in glycosylation, which may vary depending on the production process of the therapeutic glycoprotein. In particular, biosimilars are developed to be almost identically to the original drug, so the overall structure is highly similar, except for subtle differences in specific glycan structures. Therefore, when developing therapeutic glycoproteins, it is important to determine if these subtle differences in specific glycan structures affect the efficacy.

Here, we tried to diversify the glycan structure of DPO through some modification of production process and to see if the differences from the various production process affect the PK profile and efficacy. As a result, we obtained eight HK-DPOs in the modified process and one reference DPO on the market, and they showed LacNAc extensions in the 15-25% range and O-acetylated sialic acids in the 55-70% range. Comparable PK confirmed that the ratio of O-acetylated sialic acid proportion in the 55-70% range did not influence the relative AUC_{last} of DPO although previous reports have been reported that O-acetylation of sialic acids decreases susceptibility to sialidase and sustaines EPO circulation [11,25].

It is known that the binding affinity of EPO to the cognate receptor decreases as the size of the carbohydrate increases [4]. It has also been observed that as the number of glycans increases, the *in vitro* efficacy as well as binding affinity decreases [1,26]. Thus, it was expected that the *in vitro* biological activity would decrease as the proportions of LacNAc extension and O-acetylated sialic acid increased. However, LacNAc extensions in the 15-25% range and O-acetylated sialic acids in the 55-70% range did not correlate with *in vitro* biological activity. Unlike *in vitro*, the *in vivo* biological activity of EPO is known to proportionally correlate to the number of carbohydrates and sialic acids [27]. However, in this study we cannot also find any relationship between *in vivo* biological activity and the proportion of LacNAc extension or O-acetylated sialic acid residue within the ranges described above.

Therapeutic glycoproteins normally contain micro-heterogeneity in their glycan profiles since they are produced by living organisms. When evaluating glycosylation structures, it is important to determine its effect at a given proportion rather than the presence or absence of the structure. This study is meaningful because it demonstrated that in the reported proportions, the glycan structures-LacNAc extension and O-acetylated sialic acid-had little effect on the Pharmacokinetics and biological activity of DPO.

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The study protocol of animal research was reviewed and

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