***In Vivo* Pharmacokinetics and Pharmacodynamics of Positional Isomers of Mono-PEGylated Recombinant Human Granulocyte Colony Stimulating Factor in Rats**

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In this study, the pharmacokinetic and pharmacodynamic properties of Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated recombinant human granulocyte colony stimulating factor (rhG-CSF) positional isomers were evaluated in rats. The *in vitro* biological activities of Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF were determined by examining NFS-60 cell proliferation. Plasma concentrations of rhG-CSF and white blood cell (WBC) counts and absolute neutrophil count (ANC) were measured and pharmacokinetic and pharmacodynamic properties were determined after a single subcutaneous administration of the Lys³⁵, Met^{N-terminal}, or Lys¹⁷ isomers at 0.1 mg/kg in rats. The *in vitro* biological activities of Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF individual positional isomers were 20.1%, 37.4%, and 15.3%, respectively, that of rhG-CSF. However, all three mono-PEGylated rhG-CSF isomers had a greater blood half-life ($T_{1/2}$) and *in vivo* efficacy as determined by WBC counts and ANC than rhG-CSF, but no significant difference was observed between the three isomers. In conclusion, Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF individual positional isomers exhibit an enhanced the *in vivo* pharmacokinetics and pharmacodynamics. Furthermore, three isomers have comparable *in vivo* pharmacokinetic and pharmacodynamic properties, but their *in vitro* biological activities are PEGylation site dependent.

Key words random PEGylation; covalent conjugation; individual positional isomer; *in vitro* biological activity; blood half-life

PEGylation describes the chemical modification of therapeutic proteins by covalent conjugation with polyethylene glycol (PEG), a non-toxic, non-immunogenic polymer that is approved by U.S. Food and Drug Administration (FDA) for parental administration, that is used as a strategy to overcome the disadvantages associated with therapeutic proteins with a short half-life due to rapid clearance from the body.^{1,2)} PEGylation is a well established technology and a common practice during the development of therapeutic proteins, because it prolongs serum half-life and improves the pharmacokinetics of therapeutic proteins by decreasing renal clearance and enhancing proteolytic resistance due to increased steric hindrance.^{3–6)}

Several therapeutic proteins, such as Adagen[®], Oncaspar[®], PEG-Intron[®], PEGASYS[®], Somavert[®], Neulasta[®], Macugen[®], Mircera[®], Cimzia[®], and Krystexxa[®], are conjugated by alkylation, acylation, or thiol modification.^{7,8)} Methoxy polyethylene glycols (mPEG) activated with aldehyde (ALD), succinimidyl carbonate (SC), *N*-hydroxy succinimide (NHS), or malaimide (MAL) are available for the specific conjugation of amino acid side chains and N-terminal amines on proteins.^{9–12)} mPEG activated with NHS (mPEG-NHS) is frequently used to modify amino groups of target proteins and produce stable amide linkages between PEG and primary amines, such as, N-terminal α -amine and/or lysine ϵ -amine residues. In most cases, when mPEG-NHS and mPEG-SC are used, PEGylation occurs randomly at multiple lysine, histidine residues, and N-terminals in therapeutic proteins depending on reaction pH.^{10,13)} The PEGylated therapeutic proteins using mPEG-NHS are consequently heterogeneous and in the number of individual positional isomers and/or multiple isomers that could be

obtained after random PEGylation.^{13,14)} The structural characterization of individual PEG positional isomers is a significant chemistry and manufacturing control (CMC) challenges in the manufacturing process for PEGylated therapeutic proteins.¹⁵⁾

We previously described the preparation of a long-acting, mono-PEGylated recombinant human granulocyte colony stimulating factor (rhG-CSF) using trimeric-structured mPEG-NHS¹⁶⁾ and the structural characterization of individual positional isomers isolated from mono-PEGylated rhG-CSF using a preparative two-step chromatography method developed in our laboratory. In this previous study, we demonstrated that mono-PEGylated rhG-CSF is a mixture, of three positional isomers in which PEG is linked to Lys³⁵, Met^{N-terminal}, or Lys¹⁷.¹⁷⁾ However, no report has previously described on the pharmacokinetic and pharmacodynamic properties of individual positional isomers of PEGylated proteins.

In this study, we examined the pharmacokinetic and pharmacodynamic properties of Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF individual positional isomers in Sprague-Dawley rats.

MATERIALS AND METHODS

Materials rhG-CSF was obtained from the Biopharmaceuticals Production Department of Dong-A Pharm. Co., Ltd. Trimeric-structured methoxy polyethylene glycol activated with *N*-hydroxy succinimide (mPEG-NHS) was purchased from the NOF Corporation (Tokyo, Japan). All reagent grade chemicals and buffer components were purchased from Sigma-Aldrich (St. Louis, U.S.A.).

Preparation of Mono-PEGylated rhG-CSF and the Isolation of Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Positional Isomers We previously described the

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preparation of mono-PEGylated rhG-CSF using a 23 kDa trimeric-structured mPEG-NHS¹⁶⁾ and the isolation of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers formed using a preparative two-step chromatography method developed in our laboratory.¹⁷⁾ The isolated Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF isomers were concentrated to approximately 1 mg/mL and diafiltrated with 10 mM sodium acetate (pH 4.0) containing 5% (w/v) sorbitol, and 0.004% (w/v) Tween 20, and stored at 4°C until required.

***In Vitro* Biological Activities of Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Positional Isomers** The *in vitro* biological activities of the three positional isomers were determined using a cell proliferation assay using the G-CSF dependent cell line, NFS-60.^{18,19)} NFS-60 cells (ATCC CRL-1838) were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1 ng/mL of interleukin-3 (IL-3) in a humid CO₂ incubator at 37°C. Cells were then washed twice with phosphate-buffered saline (PBS) and re-suspended at 3.5 × 10⁵ cells/mL in assay media (RPMI 1640 medium containing 5% FBS). A 100 μL aliquot of the prepared NFS-60 cell suspension was then plated into each well of a 96-well micro plate. rhG-CSF and the Lys³⁵, Met^{N-terminal}, and Lys¹⁷ isomers were prepared at a concentration of 8 ng/mL and then a series of 10 twofold dilutions serially with assay media and 50 μL aliquots were added to wells. After 48 h of incubation, cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay kits (Promega, Madison, WI, U.S.A.). Sample-treated NFS-60 cells in the 96-well plate were then further incubated for 2 h after adding 30 μL of MTS solution per well and wells were read at 490 nm using an ELISA reader (TECAN, Sunrise™, Korea). The potencies of the three positional isomers were calculated using the parallel line assay (a statistical method), and results were expressed as relative activities (%) *versus* rhG-CSF. Data are presented as the means ± S.E.M.s of triplicate experiments.

Animals Male Sprague-Dawley (SD) rats (8–10 weeks old, mean weight 200 g) were purchased from Orient Bio (Korea). Four groups of six rats (randomly selected) were housed individually in cages and maintained under controlled conditions (21–25°C/RH 40–70%/12-h light and dark cycle) before and during the experiment. Food and water were provided *ad libitum*. All experiments were performed in accordance with the “Standard Operation Procedure for Animal Care and Experiments” (SOP-ANC) of Dong-A Pharmaceutical, and with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health.

***In Vivo* Pharmacokinetic and Pharmacodynamic Analysis of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Positional Isomers** The *in vivo* pharmacokinetics and pharmacodynamics of the three positional isomers were studied in male SD rats. After a week of acclimation, three groups of 6 SD rats received a single subcutaneous (s.c.) injection of the Lys³⁵, Met^{N-terminal}, or Lys¹⁷ isomers at a dose of 0.1 mg/kg (1 mL/kg) and one group was s.c. administered rhG-CSF at 0.4 mg/kg (1 mL/kg). Two hundred microliter of blood were withdrawn from tail veins and placed in Microtainer tubes (BD, U.S.A.) containing dipotassium ethylenediaminetetraacetate (K₂EDTA) at 0, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168 h post-injection rats treated with the three

positional isomers and at 0, 0.2, 0.4, 0.6, 1, 1.5, 3, 6, 8, 10, 24 h post-injection for rats treated with rhG-CSF and then immediately centrifuged at 12000 rpm for 10 min. Separated plasma was stored at –80°C prior to enzyme-linked immunosorbent assay (ELISA) analysis. Concentrations of rhG-CSF in plasma samples were measured using G-CSF ELISA kits (Bender Medsystems, Austria). Individual pharmacokinetics (PK) parameters ($C_{\max}$, $T_{\max}$, AUC_{last} , $T_{1/2}$, MRT_{last}) were calculated using WinNonlin software (Pharsight Inc., U.S.A.) by non-compartmental analysis. Maximum plasma concentrations ($C_{\max}$) and times to reach maximum concentrations ($T_{\max}$) were determined directly from the data. The area under plasma concentration time curve (AUC) from time zero to the last day of experiment ($AUC_{0-168\text{h}}$) was calculated using a standard method.²⁰⁾ The first-order terminal rate constant (k_{el}) was measured using linear regression of the terminal log-linear decay phase. The terminal half-life ($T_{1/2}$) was calculated as $\ln(2)$ divided by k_{el} . Data are presented as means ± S.E.M.s.

The pharmacodynamic effects of the three isomers and of rhG-CSF were assessed by measuring white blood cell (WBC) counts and absolute neutrophil counts (ANC) after a single s.c. administration with PEGylated individual positional isomers at 0.1 mg/kg and after daily s.c. administrations of rhG-CSF at 0.1 mg/kg/day for 5 d. Two hundred microliters of blood were collected from tail veins and placed in Microtainer tubes (BD, U.S.A.) containing K₂EDTA at 0, 1, 2, 3, 4, 5, 6 d after administration for the *in vivo* pharmacodynamic analysis. Peripheral blood WBC counts and ANC were determined using ADVIA-120 (Bayer, German). Maximum cell counts and areas under cell count *vs.* time curves (AUC) for WBC counts and ANC were calculated for each treatment group. AUC s were calculated using the linear trapezoidal rule from 0 to 6 d post-injection. Data are presented as means ± S.E.M.s.

Statistical Analysis Statistical analysis was carried out using SigmaStat Software (SPSS Inc., Chicago, IL, U.S.A.). The statistical significances of differences between the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers were examined by one-way analysis of variance (ANOVA) followed by Dunnett’s method for multiple pairwise comparisons. Differences between groups were considered significant when p values were <0.05.

RESULTS

Preparation of Mono-PEGylated rhG-CSF and the Isolation of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Positional Isomers As illustrated in Fig. 1, the α -amino group of the N-terminal (Met) and the ϵ -amino groups of lysine residues (Lys¹⁷, Lys²⁴, Lys³⁵, and Lys⁴¹) in rhG-CSF are potential sites for covalent modification by mPEG-NHS.²¹⁾ We previously described the preparation of mono-PEGylated rhG-CSF using trimeric-structured mPEG-NHS¹⁶⁾ and the structural characterization of individual positional isomers isolated from mono-PEGylated rhG-CSF using a preparative two-step chromatography method developed. The Lys³⁵, Met^{N-terminal}, Lys¹⁷-mono-PEGylated rhG-CSF positional isomers used in the present *in vitro* and *in vivo* pharmacokinetic and pharmacodynamic study had purities >98% after size-exclusion chromatography.¹⁷⁾

***In Vitro* Biological Activities of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Positional Isomers**

The *in vitro* biological activity of rhG-CSF is commonly determined by cell proliferation assay using the NFS-60 cell line. The *in vitro* biological activities of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers were compared with rhG-CSF (9.5×10^7 IU/mg) (Table 1). The *in vitro* biological activities of these isomers were retained at the 20.1% (1.91×10^7 IU/mg), 37.4% (3.55×10^7 IU/mg), and 15.3% (1.45×10^7 IU/mg) levels, respectively, for the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers *versus* rhG-CSF. Furthermore, the *in vitro* biological activities of the three isomers were found to differ signifi-

cantly.

***In Vivo* Pharmacokinetics of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Positional Isomers** The pharmacokinetics of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers and of rhG-CSF were evaluated in SD rats after a single administration at 0.1 or 0.4 mg/kg s.c. for the three isomers and rhG-CSF, respectively. Plasma rhG-CSF concentrations in SD rats following these administrations are shown in Fig. 2, and the pharmacokinetics parameters ($C_{\max}$, $T_{\max}$, AUC_{last} , $T_{1/2}$, MRT_{last}) derived by non-compartmental analysis are summarized in Table 2.

All three mono-PEGylated rhG-CSF positional isomers had substantially greater blood half-lives ($T_{1/2}$) than rhG-CSF, that is, 18.9–21.2 h *versus* 2.9 h. As illustrated in Fig. 2, the three positional isomers had similar PK profiles. The concentration of rhG-CSF in plasma rapidly increased immediately after administering 0.1 mg/kg s.c., peaked at 24 h ($T_{\max}$), and then decreased slowly to baseline at 168 h. No significant difference was observed between the $C_{\max}$ and AUC_{last} values of the three positional isomers. As shown in Table 2, for the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers $C_{\max}$ values were 134.7, 124.0, 119.0 ng/mL, respectively, and AUC_{last} values were 4535, 4450, and 4495 ng·h/mL, respectively.

***In Vivo* Pharmacodynamics of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Positional Isomers** Peripheral blood WBC counts and ANC were determined after a single administration of 0.1 mg/kg (s.c.) of Lys³⁵, Met^{N-terminal}, or Lys¹⁷-mono-PEGylated rhG-CSF positional isomers or the daily administration of 0.1 mg/kg (s.c.) of rhG-CSF for 5 d

Fig. 1. Ribbon Representation of Three Dimensional (3D) Structure of Recombinant Human Granulocyte-Stimulating Factor (rhG-CSF) and Positions PEGylated

Table 1. *In Vitro* Biological Activities of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Individual Positional Isomers

Sample	Biological activity (10 ⁷ IU/mg)	Relative activity (%)
rhG-CSF	9.5	100
Lys ³⁵ -mono-PEGylated rhG-CSF	1.91 ± 0.15	20.1 ± 1.6* [†]
Met ^{N-terminal} -mono-PEGylated rhG-CSF	3.55 ± 0.12	37.4 ± 1.2* [†]
Lys ¹⁷ -mono-PEGylated rhG-CSF	1.45 ± 0.19	15.3 ± 2.0* [†]

Relative activity (%) was defined as residual *in vitro* biological activity expressed as a percentage of rhG-CSF activity. Results are presented as means ± S.E.M.s of triplicate experiments. * $p < 0.05$ vs. rhG-CSF treated group. [†] $p < 0.05$ vs. each other mono-PEGylated rhG-CSF treated group.

Fig. 2. Plasma rhG-CSF Concentration-Time Profiles after a Single Subcutaneous Injection of the Lys³⁵-Mono-PEGylated rhG-CSF (■), Met^{N-terminal}-Mono-PEGylated rhG-CSF (▲), Lys¹⁷-Mono-PEGylated rhG-CSF (●), and rhG-CSF (◇) in SD Rats

The Lys³⁵, Met^{N-terminal}, and Lys¹⁷ isomers were injected at a dose of 0.1 mg/kg and rhG-CSF at 0.4 mg/kg. Results are expressed as means ± S.E.M.s.

Table 2. Pharmacokinetic Parameters of Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Individual Positional Isomers in SD Rats

Parameters	$C_{\max}$ (ng/mL)	$T_{\max}$ (h)	AUC_{last} (ng·h/mL)	$T_{1/2}$ (h)	MRT_{last} (h)
rhG-CSF ^{a)}	1383 ± 243	1.6 ± 0.7	5643 ± 306	2.9 ± 0.1	4.0 ± 0.1
Lys ³⁵ -mono-PEGylated rhG-CSF ^{b)}	134.7 ± 4.3	24.0 ± 0.0*	4535 ± 109	19.1 ± 0.3*	28.9 ± 0.4*
Met ^{N-terminal} -mono-PEGylated rhG-CSF ^{b)}	124.0 ± 9.1	24.0 ± 0.0*	4450 ± 227	18.9 ± 0.2*	29.3 ± 0.2*
Lys ¹⁷ -mono-PEGylated rhG-CSF ^{b)}	119.0 ± 8.3	24.0 ± 0.0*	4495 ± 220	21.2 ± 0.7*	29.4 ± 0.2*

Results are presented as means ± S.E.M.s. a) The administered dose was 0.4 mg/kg. b) The administered dose was 0.1 mg/kg. * $p < 0.05$ vs. rhG-CSF treated group.

to SD rats. Both WBC counts and ANC in peripheral blood increased after treatment with the three positional isomers, remained at an elevated level for 1 to 2 d, and then gradually decreased to baseline at 4–5 d. However, 5 daily administrations of rhG-CSF at 0.1 mg/kg/d s.c. failed to elicit WBC counts and ANC from baseline (Fig. 3). For the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers peak WBC counts (WBC_{max}) were 38.8×10^9 , 39.5×10^9 , and 40.1×10^9 cells/L, respectively, AUC_{WBC} values were 149.7×10^9 , 152.4×10^9 and 156.8×10^9 cells·d/L, peak ANC (ANC_{max}) were 19.8×10^9 , 21.2×10^9 and 20.5×10^9 cells/L, and AUC_{ANC} values were 53.2×10^9 , 58.5×10^9 , and 59.6×10^9 cells·d/L, respectively. These data show that the three mono-PEGylated rhG-CSF po-

sitional isomers have similar pharmacodynamic effects.

DISCUSSION

rhG-CSF is rapidly cleared from circulation by renal clearance and receptor-mediated endocytosis by neutrophils, which result in short-term pharmacological effects.^{22–24} Conjugation with PEG alters the *in vivo* efficacies of drugs by altering their pharmacokinetic (PK) and pharmacodynamic (PD) properties.⁶ Several research groups have shown that the *in vivo* half-life and activity of rhG-CSF or its mutant can be increased by PEG conjugation using several different conjugation chemistry strategies.^{9,16,25–31} The results of conjugation between target therapeutic proteins and PEG, such as, the extent of modification, molecular weight, the structure of the PEG moiety (linear or branched), and the locations of PEG attachment, importantly affect *in vitro* biological activity and pharmacological properties.^{6,32,33} It has been well established that inverse relationships exist between *in vitro* biological activity and the average molecular weights of PEG and the number of PEG units, whereas positive relationships exist between *in vivo* pharmacokinetics and pharmacodynamics and these average molecular weights.^{34,35} Furthermore, in previous studies, no advantages have been attributed to different conjugation chemistries in the *in vivo* pharmacokinetics and pharmacodynamics⁹ whereas selective advantage was attributed to different types of PEG moieties.³⁶

We previously reported on the preparation of mono-PEGylated rhG-CSF using 23 kDa trimer-structured methoxy polyethylene glycol with *N*-hydroxy succinimide (mPEG-NHS).¹⁶

Meanwhile, there has been a growing interest in CMC and in the regulatory aspects of PEG-modified protein drugs. In particular, the structural characterization of individual positional isomers poses significant CMC challenges concerning the manufacture of PEGylated therapeutic proteins.¹⁵ Therefore, in the present study, individual positional isomers were successfully isolated and characterized with respect to site of PEGylation by enzymatic digestion with endoproteinase Lys-C and N-terminal sequencing, which showed that the three positional isomers were PEGylated at Lys³⁵, Met^{N-terminal} and Lys¹⁷ of rhG-CSF.¹⁷ However, no previous report has described the pharmacokinetic and pharmacodynamic properties of positional isomers of PEGylated therapeutic proteins.

In the present study, we evaluated the *in vitro* biological activities and *in vivo* pharmacokinetic/pharmacodynamic properties of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers in SD rats. Animals were

Fig. 3. Total White Blood Cell (WBC) (A) and Absolute Neutrophil Counts (ANC) (B) after a Subcutaneous Injection of Lys³⁵-Mono-PEGylated rhG-CSF (■), Met^{N-terminal}-Mono-PEGylated rhG-CSF (▲), Lys¹⁷-Mono-PEGylated rhG-CSF (●), or rhG-CSF (◇) into SD Rats

Lys³⁵, Met^{N-terminal}, or Lys¹⁷ isomers were injected once at 0.1 mg/kg s.c. and rhG-CSF was injected daily for 5 d at 0.1 mg/kg per d. Results are expressed as means ± S.E.M.s.

Table 3. Pharmacodynamics Parameters (ANC Levels and WBC Counts) of Lys³⁵, Met^{N-terminal}, and Lys¹⁷-Mono-PEGylated rhG-CSF Positional Isomers in SD Rats after a Single Administration at 0.1 mg/kg s.c.

	Absolute neutrophil count		White blood cell count	
	ANC_{max} (10^9 cells/L)	AUC_{ANC} (10^9 cells·d/L)	WBC_{max} (10^9 cells/L)	AUC_{WBC} (10^9 cells·d/L)
rhG-CSF	8.3 ± 2.4	39.1 ± 6.5	24.2 ± 4.6	124.9 ± 7.3
Lys ³⁵ -mono-PEGylated rhG-CSF	$19.8 \pm 0.8^*$	$53.2 \pm 7.2^*$	$38.8 \pm 2.6^*$	$149.7 \pm 2.6^*$
Met ^{N-terminal} -mono-PEGylated rhG-CSF	$21.2 \pm 2.0^*$	$58.5 \pm 4.2^*$	$39.5 \pm 3.5^*$	$152.4 \pm 14.1^*$
Lys ¹⁷ -mono-PEGylated rhG-CSF	$20.5 \pm 2.0^*$	$59.6 \pm 2.0^*$	$40.1 \pm 3.2^*$	$156.8 \pm 3.3^*$

ANC_{max} =maximum observed absolute neutrophil count; AUC_{ANC} =area under the absolute neutrophil counts vs. time curve; WBC_{max} =maximum observed white blood cell count; AUC_{WBC} =area under the white blood cell count vs. time curve. Results are presented as means ± S.E.M.s. * $p < 0.05$ vs. rhG-CSF treated group.

administered 0.1 mg/kg s.c. of the three individual positional isomers in the *in vivo* pharmacokinetic and pharmacodynamic because mono-PEGylated rhG-CSF exhibited dose-dependent responses in dosage range 10–1000 μ g/kg in a preliminary study (data not shown).

The *in vitro* biological activities of the Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers in the cell proliferation assay were 20.1% (1.91×10^7 IU/mg), 37.4% (3.55×10^7 IU/mg), and 15.3% (1.45×10^7 IU/mg), respectively, versus rhG-CSF. In general, the *in vitro* biological activities of PEGylated proteins are reduced due to steric hindrance caused by PEG at receptors, and the extents of these reductions depend on the extent of PEGylation, and the type (linear or branched) and molecular weight of the PEG used.^{33–36} Of the three positional isomers examined, the Met^{N-terminal}-mono-PEGylated rhG-CSF showed the highest *in vitro* biological activity, which may have been caused by steric hindrance of receptor binding or of active sites on rhG-CSF. It has been reported that Glu¹⁹ and Glu⁴⁷ of G-CSF interact with specific residues in G-CSF receptor.^{37,38} In addition, the *in vitro* biological activities of mono-PEGylated rhG-CSF have been reported to be highly dependent on PEG molecular weight^{34,35} and the number of PEG units attached to rhG-CSF,³³ especially in the G-CSF receptor binding region. However, losses of *in vitro* biological activity shown by the PEGylated rhG-CSF was compensated for by reduced renal clearance due to increased hydrodynamic volume and reduced protease degradation.^{8,32} It has been well established that the *in vitro* biological activities of PEGylated proteins decrease with increasing PEG molecular weight (M_w), but that *in vivo* activity is enhanced by PEGylation.

The Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers showed enhanced pharmacokinetic and pharmacodynamic performance in SD rats (Figs. 2, 3), and as was expected, the three isomers had markedly increased blood half-lives ($T_{1/2}$) and WBC counts and ANC than rhG-CSF treated rats despite their lower *in vitro* biological activities. Furthermore, no significant difference was observed between the pharmacokinetic and pharmacodynamic properties of the three isomers. However, their *in vitro* biological activities were found to depend significantly on PEGylation site (Table 3).

This study demonstrates that the *in vivo* pharmacokinetics and pharmacodynamics of mono-PEGylated rhG-CSF positional isomers are independent of PEGylation sites for a given PEG moiety, but that their *in vitro* biological activities are PEGylation site-dependent.

CONCLUSION

We report for the first time the pharmacokinetics and pharmacodynamics of individual positional isomers of mono-PEGylated rhG-CSF. This study demonstrates that Lys³⁵, Met^{N-terminal}, and Lys¹⁷-mono-PEGylated rhG-CSF positional isomers have different *in vitro* biological activities but that the *in vivo* pharmacokinetic and pharmacodynamic properties of the PEGylated rhG-CSF positional isomers are comparable.

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