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Differential Sensitivity of Lipegfilgrastim and Pegfilgrastim to Neutrophil Elastase Correlates With Differences in Clinical Pharmacokinetic Profile

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Abstract

To assess the basis of the different half-lives of long-acting human granulocyte colony-stimulating factor (G-CSF) drugs, the effect of neutrophil elastase on lipegfilgrastim and pegfilgrastim was investigated. Sensitivity to human neutrophil elastase (HNE) was evaluated by incubating the drugs with HNE followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Drugs were also incubated with isolated human neutrophils followed by Western blot analysis. Lipegfilgrastim was more resistant to degradation with HNE or neutrophils than pegfilgrastim and appeared more intact on SDS-PAGE gels and Western blots. Lipegfilgrastim retained more functional activity than pegfilgrastim after incubation with HNE (67% vs ∼9%, respectively) or neutrophils (80% vs ∼4%, respectively) as assessed in an NFS-60 cell–based [^{3}H]-thymidine incorporation assay. The binding and affinity of untreated lipegfilgrastim and pegfilgrastim for G-CSF receptors were evaluated using an NFS-60 competitive G-CSF receptor-binding assay and surface plasmon resonance. Untreated drugs were also evaluated in the functional NFS-60 thymidine incorporation assay. G-CSF receptor binding, receptor affinity, and functional activity were comparable between untreated drugs. The results showed a greater resistance to neutrophil elastase degradation and concomitant retention of functional activity of lipegfilgrastim compared with pegfilgrastim, which potentially explains the clinical observations of a longer half-life of lipegfilgrastim versus pegfilgrastim.

Keywords

granulocyte colony-stimulating factor, neutrophil elastase, granulocyte colony-stimulating factor receptor, long-acting G-CSF, lipegfilgrastim

Cytotoxic chemotherapy frequently results in neutropenia, which increases the risk of serious or life-threatening infections.[1] Prophylactic treatment with a recombinant granulocyte colony-stimulating factor (G-CSF) promotes neutrophil proliferation, differentiation, and mobilization from the bone marrow into the bloodstream, and can reduce the severity and duration of chemotherapy-induced neutropenia and febrile neutropenia.[2–7]

Pegfilgrastim and lipegfilgrastim are long-acting recombinant G-CSFs administered once per chemotherapy cycle to reduce the duration of neutropenia and the incidence of febrile neutropenia in patients with cancer receiving myelosuppressive or cytotoxic chemotherapy. Conjugation of a polyethylene glycol molecule (ie, PEGylation) to a therapeutic protein has been shown to decrease plasma clearance and extend the terminal elimination half-life ($t_{½}$) of the drug.[8–11] In pegfilgrastim, the PEG moiety is covalently attached to the N-terminus of filgrastim, a recombinant N-methionyl human G-CSF (r-metHuG-CSF) produced in *Escherichia coli*.[12] In lipegfilgrastim, the PEG moiety is attached to the O-glycosylation site (threonine-134) of recombinant N-methionyl human G-CSF through glycoPEGylation, an alternate method of PEGylation that uses site-specific, enzymatic coupling of an

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activated methoxyPEG-sugar conjugate to generate functional glycoPEGylated proteins with increased bioavailability and prolonged duration of action.[13,14] Lipegfilgrastim was approved by the European Medicines Agency in August 2013 for use within the European Union for the reduction of the duration of neutropenia and the incidence of febrile neutropenia in adult patients treated with cytotoxic chemotherapy for malignancy.[15,16] Phase 1 studies in healthy volunteers have demonstrated that lipegfilgrastim has approximately 60% higher bioavailability and approximately 30% greater neutrophil response compared with pegfilgrastim, as measured by absolute neutrophil count (ANC) area over the baseline effect curve,[17] suggesting potential differences in drug metabolism and clearance between the 2 drugs.

Lipegfilgrastim and pegfilgrastim both have a molecular size of ~38–39 kDa and appear to be absorbed via the lymphatic system following subcutaneous administration.[18,19] After administration, neutrophil-mediated clearance involving internalization via G-CSF receptors and degradation within the neutrophil is an important mechanism of plasma clearance for both drugs.[8,20] Extracellular proteolytic degradation by neutrophil elastase is potentially another clearance pathway for G-CSF compounds.[21–23] When exposed to neutrophil elastase, G-CSF is rapidly cleaved and rendered inactive, suggesting that resistance to elastase degradation may result in an extended drug half-life. In addition, purified elastase completely abrogated the proliferation induced by G-CSF in serum-free suspension cultures of $CD34^{+}$ cells.[21]

To evaluate possible mechanisms for the elongated half-life of lipegfilgrastim compared with pegfilgrastim in clinical trials, the current studies were undertaken to compare the sensitivity of the 2 drugs to neutrophil elastase. The sensitivity of the molecules to neutrophil elastase was determined in controlled proteolytic reactions with either purified neutrophil elastase or isolated human neutrophils. Reaction products were analyzed for both the appearance of proteolytic fragments on Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels or Western blots and for proliferative activity in an NFS-60 cell–based [^{3}H]-thymidine incorporation assay.

In addition to assessing the sensitivity of both lipegfilgrastim and pegfilgrastim to neutrophil elastase, the in vitro pharmacodynamic actions of untreated lipegfilgrastim and pegfilgrastim were evaluated in receptor-binding assays and a cell-proliferation assay. G-CSF receptor binding of both drugs was measured in an NFS-60 cell–based iodine-125 ([^{125}I])-G-CSF competitive G-CSF receptor-binding assay[24–26] and through surface plasmon resonance (SPR) technology. The induction of cell proliferation by lipegfilgrastim and pegfilgrastim were determined using an NFS-60 cell–based [^{3}H]-thymidine incorporation assay.

Methods

Effect of Neutrophil Elastase on Lipegfilgrastim and Pegfilgrastim

Effect of Purified Human Neutrophil Elastase (HNE) on G-CSF Drugs. The G-CSF drugs tested were lipegfilgrastim (Lonquex, XM22, Teva Pharmaceutical Industries Ltd., Petach Tikva, Israel), manufactured by Teva/Merckle GmbH; pegfilgrastim (Neulasta, Amgen, Thousand Oaks, California), commercially purchased from Amgen; and filgrastim (XM21, rG-CSF nonPEGylated control), manufactured by Teva/Merckle GmbH. For evaluation of the effect of elastase on the G-CSF drugs, purified elastase from human leukocytes (Sigma-Aldrich, St. Louis, Missouri) was added to a final concentration of 1 U/mL to 1 sample each of lipegfilgrastim, pegfilgrastim, and filgrastim (167 μg/mL for each drug) in phosphate-buffered saline (PBS). A second set of samples without elastase served as controls. These 6 samples were incubated for 15, 30, 60, 120, and 240 minutes at 37°C. At each time point, 2 aliquots were collected from each sample. Samples to be analyzed by SDS-PAGE (30 μL) were mixed with an equal volume of 2× reducing loading dye sample buffer (diluted NuPAGE Loading Dye Sample Buffer [4×] and NuPAGE Sample Reducing Agent [10×]; Invitrogen, Life Technologies, Grand Island, New York) to stop the elastase reaction. Aliquots designated for activity testing (48 μL) were combined with 4.8 μL of 10 mM phenylmethanesulfonyl fluoride (PMSF) to stop the elastase reaction. All samples were frozen at −20°C until analysis.

Effect of Isolated Human Neutrophils. Neutrophils were isolated from freshly drawn, ethylenediaminetetraacetic acid (EDTA)–preserved unspun whole human blood (Biological Specialty Corporation, Colmar, Pennsylvania) by overlaying 20 mL of Polymorphprep solution (Accurate Chemical & Scientific Corp., Westbury, New York) with 20 mL of blood in several tubes. The tubes were centrifuged at 500*g* at room temperature (18°C–20°C) for 50 to 55 minutes in a swing-out rotor (5682; Forma Scientific, Marietta, Ohio), after which 2 clear bands of isolated leukocytes were visible. The lower band, containing polymorphonuclear neutrophils, was extracted by pipette, washed once in PBS, and suspended in prewarmed (37°C) RPMI-1640 media. Viable cells were quantified by Trypan Blue (Sigma-Aldrich, St. Louis, Missouri) staining, the cell concentration was adjusted to 1×10^{7} viable cells per millimeter, and the cells were incubated for 1–2 hours at 37°C with 5% CO_2 and 85% relative humidity until the time of assay.

For evaluation of the effect of neutrophils on the G-CSF drugs, 2×10^{6} viable human neutrophils were

added to 1 sample each of lipegfilgrastim, pegfilgrastim, and filgrastim at 33 μg/mL in RPMI-1640 in a final volume of 360 μL in 0.5-mL polypropylene 96-well assay blocks. A second set of samples without neutrophils served as unexposed drug controls. The 6 samples were incubated for 30, 60, and 120 minutes at 37°C with 5% CO_2 and 85% relative humidity. Neutrophils plus PBS were used as additional controls. At each time point, 1 assay block was removed from the incubator and centrifuged at ~200*g* at room temperature for 4 minutes in a swing-out rotor (RT7 Plus, Sorvall, DuPont, Newtown, Connecticut) to pellet the neutrophils. Reaction supernatants (200 μL of each) were transferred to a new set of assay blocks, and aliquots for analysis were dispensed into fresh tubes. Samples to be analyzed by Western blot (25 μL) were mixed with 25 μL 2 × reducing loading dye sample buffer, and those designated for activity testing (90 μL) were combined with 10 μL of 10 mM PMSF. Samples were frozen at −20°C until analysis.

SDS-PAGE Analysis. Samples from purified human elastase treatment were incubated at 70°C for 10 minutes, and 24 μL of each sample (2 μg of drug) was loaded onto 4%–12% gradient precast gels as per the manufacturer's directions (NuPAGE Novex Bis-Tris Gels; Life Technologies, Grand Island, New York) and run under denaturing conditions at 200 V for 35 minutes in NuPAGE MES SDS Running Buffer (Life Technologies, Grand Island, New York). Molecular weight standards (Mark 12 Unstained and Novex Sharp Pre-stained; Life Technologies, Grand Island, New York) were included on the gels. Gels were stained with Coomassie blue using the Novex Colloidal Blue Staining Kit (Life Technologies, Grand Island, New York). Electronic images of the stained gels were captured with a Kodak EDAS 290 imager and formatted using Kodak 1D Image Analysis Software (Kodak 1D Image Analysis Software v.3.6).

Western Blot Analysis. SDS-PAGE samples incubated with isolated human neutrophils containing 16.7 ng of drug product (DP)/μL were diluted to 8.3 ng/μL in 1× loading dye sample buffer (reducing) and incubated at 70°C for 10 minutes. A 9-μL aliquot (75 ng DP) of each sample was loaded onto 12-well NuPAGE gels and run under denaturing conditions in MES SDS buffer at 200 V for 35 minutes. Nitrocellulose membranes (0.2 μm; Life Technologies, Grand Island, New York) were equilibrated in transfer buffer containing methanol (20% v/v) for 10 minutes. Filter papers were prewet in the buffer for 30 seconds, and proteins were transferred using an XCell II Blot Module (Invitrogen, Life Technologies, Grand Island, New York) at 30 V for 1 hour. After transfer, the membranes were quickly rinsed in 1× PBS and blocked for 20 minutes in SuperBlock (Thermo Scientific, Rockford, Illinois) plus 0.05% Tween 20 (v/v; Bio-Rad Laboratories, Hercules, California). SuperBlock diluted 1:10 plus 0.05% Tween 20 (v/v) was used as the incubation buffer for the subsequent primary and secondary antibody labeling steps, and 1× PBS with 0.05% Tween 20 (v/v) was used as wash buffer. After blocking, the blots were incubated for 1 hour with shaking in 0.1 μg/mL of primary antibody (anti-human G-CSF polyclonal goat IgG; R & D Systems, Minneapolis, Minnesota). They were quickly rinsed and washed 2 times in wash buffer for 5 minutes each. The blots were then incubated with a 1:1000 dilution of HRP-conjugated anti-goat IgG (R & D Systems, Minneapolis, Minnesota) for 1 hour with shaking, then quickly rinsed, and washed 3 times for 5 minutes each in wash buffer. Blots were developed for 90 seconds using the chromogenic CN/DAB substrate kit (Thermo Scientific, Rockford, Illinois) before stopping development with ultrapure distilled water. Once the blots had completely air-dried, electronic images were captured using an Epson scanner, and banding patterns of the samples on each blot were analyzed.

Activity Measurements. The activity of lipegfilgrastim and pegfilgrastim samples following incubation with purified neutrophil elastase or isolated human neutrophils was assessed using the [^{3}H]-thymidine incorporation assay as described below. The final concentrations of G-CSF drug samples (incubated with elastase or neutrophils) and G-CSF drug controls (incubated without elastase or neutrophils) were 0.3–2200 pg/mL. Counts per minute (CPM) values at each concentration point were plotted and analyzed using a 4-parameter logistic curve fitted to generate EC_{50} values. Two dilution curves were run for each sample and control at each incubation point.

To quantify the effect of elastase and neutrophils on the functional activity of the G-CSF drugs, 2 relative activity values for each drug at each time point were calculated as (drug EC_{50} - elastase or neutrophils/drug EC_{50} + elastase or neutrophils) × 100. Each control dose–response curve and its adjacent sample dose–response curve were used for these calculations. The mean ± standard deviation (SD) of the 2 relative activities of each drug at each time point was determined. To assess if elastase or neutrophils differentially affect the functional activities of the G-CSF drugs, the relative activities of lipegfilgrastim and pegfilgrastim at each time point were compared statistically.

Receptor-Binding Assays

[^{125}I]-G-CSF Competitive G-CSF Receptor-Binding Assay. To assess competitive binding, 2.5×10^{6} NFS-60 cells were incubated with 0.25 nM [^{125}I]-G-CSF (Perkin Elmer, Waltham, Massachusetts) in the presence of 0.19 pM to 300 nM unlabeled pegfilgrastim or lipegfilgrastim in 96-well plates for 2 hours at room temperature on a plate shaker. NFS-60 is a murine myeloblastic cell line not known to secrete elastase and is a standard cell line used for assessing the activity of G-CSFs.[27] Samples were transferred to 300-μL microtubes containing 200 μL of a phthalate oil mixture (Sigma Aldrich, St. Louis,

Missouri) and centrifuged through the oil to separate cell-bound [^{125}I]-G-CSF from unbound material. Tubes were flash-frozen in liquid nitrogen, cell pellets were placed into 12×75 mm polystyrene tubes, and cell-bound [^{125}I]-G-CSF counts were measured in a 1480 Wizard Gamma Counter (Perkin Elmer, Waltham, Massachusetts).

Binding data were analyzed by nonlinear regression and curve fitting to a single binding-site model. The half maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism software (v.5.01; GraphPad Software, Inc., La Jolla, California). Binding IC_{50} values for lipegfilgrastim and pegfilgrastim were compared using a 2-tailed t test.

SPR Assay. A BIACORE 3000 (GE Healthcare, Uppsala, Sweden) was used to assess binding of lipegfilgrastim and pegfilgrastim to immobilized recombinant human G-CSF receptor extracellular domain (R&D Systems, Minneapolis, Minnesota) — 4 μg/mL in 10 mM sodium acetate (pH 5.5) — on the surface of CM5 biosensor chips (BIACORE, Uppsala, Sweden). The receptor was immobilized via amine coupling using a flow rate of 10 μL/minute to obtain a final surface density of 180 to 300 resonance units. To measure binding affinities (K_D), lipegfilgrastim and pegfilgrastim (0–5 μM) were diluted in HBS-EP buffer (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania) — 0.01 M HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20 — and flowed twice at 10 μL/minute for 10 minutes at concentrations of 0, 0.04, 0.2, 1.0, and 5.0 μM over reference and active surfaces. Regeneration of the chips was accomplished by using two 15-second pulses each of solution I (HBS-EP containing 0.5 M NaCl, 5 mM NAOH, and 0.05% [v/v] Empigen) and solution II (HBS-EP) at 50 μL/minute. Each drug was tested twice using 2 sets of independent human G-CSF receptor immobilizations on 2 different chips. The order of sample injection was randomized to avoid any bias in data. Binding affinities were determined by averaging the steady-state binding responses recorded for 30 seconds during the equilibrium binding plateau near the end of the association phase at each given concentration.

Cell-Based [^{3}H]-Thymidine Incorporation Assay

Activity of untreated lipegfilgrastim and pegfilgrastim in stimulating NFS-60 cell proliferation was measured using a [^{3}H]-thymidine incorporation assay. For these assays, NFS-60 cells were treated with triplicate serial dilutions of lipegfilgrastim and pegfilgrastim at final concentrations of 0.03–116 pM in each 96-well assay plate. Plates were incubated in a 37°C humidified 5% CO_2 incubator for 20 hours, after which they were pulsed with 0.5 μCi/well [^{3}H]-thymidine for 4 hours. The level of [^{3}H]-thymidine incorporation was measured in CPM using a 1450 MicroBeta TriLux liquid scintillation counter (6-detector, Perkin Elmer, Waltham, Massachusetts). For each assay plate, the triplicate dose–response curves for each drug were analyzed using a 4-parameter logistic curve fit, and a mean EC_{50} value was generated. Four independent assay plates were run for both untreated G-CSF drugs. The mean of the 4 independent EC_{50} values $\pm$ SD was calculated for each drug. The functional activity of untreated lipegfilgrastim and pegfilgrastim was assessed by statistically comparing their mean $\pm$ SD EC_{50}values.

Statistical Analysis

The relative functional activity of lipegfilgrastim and pegfilgrastim after incubation with elastase or neutrophils was compared using 2-tailed t tests in GraphPad Prism (GraphPad Software, La Jolla, California), with the P value cutoff for each analysis at $P < .05$. The receptor binding and functional activity of untreated lipegfilgrastim and pegfilgrastim were also compared using 2-tailed t tests in GraphPad Prism, with the P value cutoff for each analysis at $P < .05$.

Results

Effect of Neutrophil Elastase on Lipegfilgrastim and Pegfilgrastim

To examine the effect of neutrophil elastase on lipegfilgrastim and pegfilgrastim, as well as filgrastim used as a control, the drugs were treated with and without purified neutrophil elastase for 15, 30, 60, 120, and 240 minutes. Cleavage of the drugs and the functional activity of the resulting products were assessed. Data from the 15- and 120-minute incubations are presented.

As seen in the Coomassie-stained SDS-PAGE gels shown in Figure 1A,B, the filgrastim G-CSF control was substantially degraded after 15 and 120 minutes of exposure to elastase. Lipegfilgrastim treated with purified neutrophil elastase for 15 minutes was fractionally degraded as seen by the slightly less intense full-length lipegfilgrastim band and the faint truncated bands in the elastase-treated drug lane compared with the untreated drug lane (compare lanes 4 and 5, Figure 1A). Elastase-treated lipegfilgrastim retained 67% of the activity of untreated lipegfilgrastim in the NFS-60 cell–based [^{3}H]-thymidine incorporation assay (Table 1). In contrast, pegfilgrastim treated under the same conditions was substantially more degraded than lipegfilgrastim and produced multiple lower-molecular-weight species (compare lanes 6 and 7, Figure 1A). The pegfilgrastim degraded under these conditions retained only 9% of the activity of untreated pegfilgrastim in the NFS-60 cell–based [^{3}H]-thymidine incorporation assay (Table 1). After 120 minutes of incubation with neutrophil elastase, lipegfilgrastim was noticeably degraded (compare lanes 4 and 5, Figure 1B). However, at this time point the treated lipegfilgrastim retained approximately 19% of its ability to induce proliferation (Table 1). Pegfilgrastim appeared

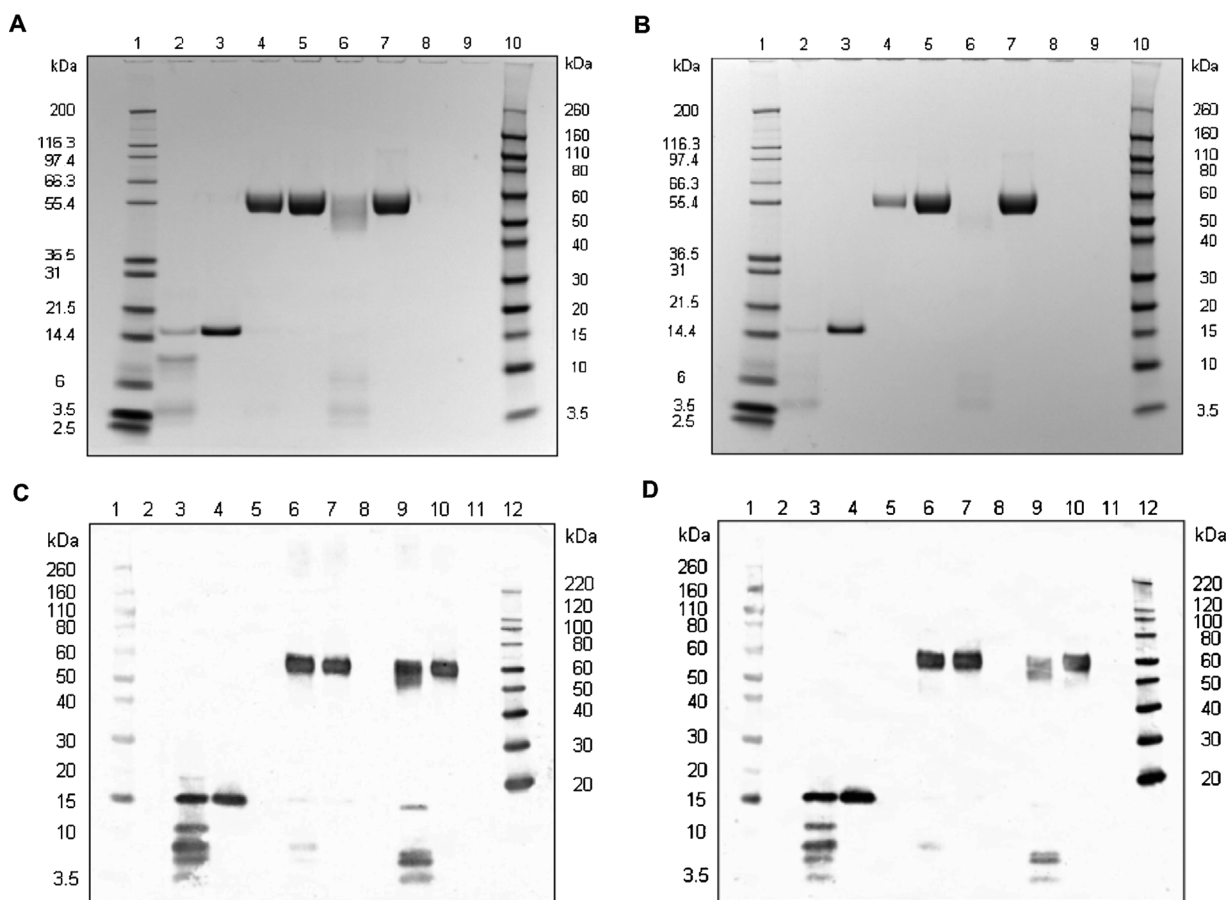

Figure 1. Coomassie-stained SDS-PAGE analysis of lipegfilgrastim, pegfilgrastim, and filgrastim G-CSF control incubated with purified neutrophil elastase for 15 minutes (A) and 120 minutes (B) and Western blot analysis of lipegfilgrastim, pegfilgrastim, and filgrastim G-CSF control incubated with isolated human neutrophils for 60 minutes (C) and 120 minutes (D). Lane 1, Mark 12 Unstained Standard; lane 2, filgrastim + elastase; lane 3, filgrastim; lane 4, lipegfilgrastim + elastase; lane 5, lipegfilgrastim; lane 6, pegfilgrastim + elastase; lane 7, pegfilgrastim; lane 8, elastase; lane 9, PBS (1×); lane 10, Novex Sharp Pre-Stained Standard. Western blot: lane 1, Novex Sharp Pre-Stained Protein Standard; lane 2, blank; lane 3, filgrastim + neutrophils; lane 4, filgrastim; lane 5, blank; lane 6, lipegfilgrastim + neutrophils; lane 7, lipegfilgrastim; lane 8, blank; lane 9, pegfilgrastim + neutrophils; lane 10, pegfilgrastim; lane 11, blank; lane 12, MagicMark XP Western Protein Standard. PBS, phosphate-buffered saline; G-CSF, granulocyte colony-stimulating factor; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

completely degraded after 120 minutes (compare lanes 6 and 7, Figure 1B) and retained no detectable functional activity. After 15 minutes of incubation with elastase, lipegfilgrastim retained significantly more functional activity than pegfilgrastim. The functional activity of lipegfilgrastim was also greater than pegfilgrastim at 120 minutes, but the difference could not be evaluated statistically, as dose response for pegfilgrastim incubated with elastase was flattened to the extent that it would not fit the 4-parameter logistic curve. The level of degradation and functional activity after 30, 60, and 240 minutes of elastase treatment followed the same pattern as the effects of the 15- and 120-minute treatments (data not shown).

To evaluate the drugs in a more closely representative natural physiological environment, the drugs were incubated in the presence of purified human neutrophils for 30, 60, and 120 minutes, and the resulting products were analyzed by Western blots (Figure 1C,D; products of 60- and 120-minute incubations shown) and [^{3}H]-thymidine incorporation in NFS-60 cells (Table 2). After a 60-minute incubation with neutrophils (Figure 1C), the filgrastim control band was visibly less intense compared with the same molecule incubated without neutrophils, and smaller molecular-weight bands were observed on the Western blot. Lipegfilgrastim incubated with isolated human neutrophils for 60 minutes demonstrated a slight change in banding patterns, indicating minimal degradation (compare lanes 6 and 7, Figure 1C) and exhibited no decline in relative activity (100%) compared with lipegfilgrastim incubated without neutrophils (Table 2). Incubation of pegfilgrastim with neutrophils produced multiple bands at lower molecular weights (compare lanes 9 and 10, Figure 1C) and retention of only 25% functional activity (Table 2). After 120 minutes of incubation with neutrophils, faint bands at lower molecular weights were observed for lipegfilgrastim (compare lanes 6 and 7, Figure 1D). The lipegfilgrastim products of the 120-minute incubation retained 81% of

Table 1. Proliferative Activity of Lipegfilgrastim and Pegfilgrastim Following Treatment ± Neutrophil Elastase in the NFS-60 Cell–Based [^{3}H]-Thymidine Incorporation Assay

Time Point	Sample	Mean EC_{50}, pg/mL (SD)	Mean % Relative Activity (SD)
15 minutes	Lipegfilgrastim	40.1 (7.1)	67 (9.4)[a]
	Lipegfilgrastim + elastase	59.4 (2.2)	
	Pegfilgrastim	49.8 (4.5)	9 (0.2)
	Pegfilgrastim + elastase	546 (38.9)	
120 minutes	Lipegfilgrastim	45.6 (11.7)	19 (2.8)
	Lipegfilgrastim + elastase	244 (26.2)	
	Pegfilgrastim	38.5 (4.9)	NC
	Pegfilgrastim + elastase	NC	

EC_{50} calculated as the concentrations at which half maximal proliferative responses were observed in the NFS-60 cell–based [^{3}H]-thymidine incorporation assay.
[a]Relative activity of lipegfilgrastim significantly greater than relative activity of pegfilgrastim after 15-minute incubation with elastase ($P < .05$).
NC, not calculable (data could not be fitted with 4-parameter logistic curve); SD, standard deviation.

the activity observed for the lipegfilgrastim incubated in the absence of neutrophils (Table 2). After 120 minutes of incubation with neutrophils, pegfilgrastim was markedly degraded compared with pegfilgrastim without neutrophils (compare lanes 9 and 10, Figure 1D) and did not retain any functional activity (Table 2). After 60 minutes of incubation with neutrophils, lipegfilgrastim retained significantly more functional activity than pegfilgrastim. The functional activity of lipegfilgrastim was also much higher than pegfilgrastim at 120 minutes, but the difference could not be evaluated statistically.

Table 2. Proliferative Activity of Lipegfilgrastim and Pegfilgrastim Following Incubation ± Isolated Human Neutrophils in the NFS-60 Cell–Based [^{3}H]-Thymidine Incorporation Assay

Time Point	Sample	Mean EC_{50}, pg/mL (SD)	Mean % Relative Activity (SD)
30 minutes	Lipegfilgrastim	76.5 (23.5)	113 (40)
	Lipegfilgrastim + neutrophils	68.3 (3.3)	
	Pegfilgrastim	85.7 (23.1)	53 (17)
	Pegfilgrastim + neutrophils	162 (7.1)	
60 minutes	Lipegfilgrastim	66.1 (11.0)	100 (14)[a]
	Lipegfilgrastim + neutrophils	65.9 (2.2)	
	Pegfilgrastim	81.9 (17.2)	25 (1)
	Pegfilgrastim + neutrophils	326 (51.6)	
120 minutes	Lipegfilgrastim	42.3 (2.2)	81 (13)
	Lipegfilgrastim + neutrophils	52.9 (5.8)	
	Pegfilgrastim	48.6 (13.6)	NC
	Pegfilgrastim + neutrophils	NC	

EC_{50} calculated as the concentrations at which half maximal proliferative responses were observed in the NFS-60 cell–based [^{3}H]-thymidine incorporation assay.
[a]Relative activity of lipegfilgrastim significantly greater than relative activity of pegfilgrastim after 60- and 120-minute incubation with neutrophils ($P < .05$).
NC, not calculable (data could not be fitted with 4-parameter logistic curve); SD, standard deviation.

Receptor Binding and Functional Activity

Binding to the human G-CSF receptor was assessed in competitive [^{125}I]-G-CSF binding assays using NFS-60 cells and SPR. G-CSF receptor-binding data for lipegfilgrastim and pegfilgrastim from 4 independent [^{125}I]-G-CSF competitive binding assays are summarized in Table 3, and representative binding curves are shown in Figure S1. G-CSF receptor binding was equivalent between lipegfilgrastim and pegfilgrastim, as the IC_{50} values for inhibition of [^{125}I]-G-CSF binding to NFS-60 cells was not significantly different (0.70 ± 0.09 nM IC_{50} versus 0.72 ± 0.18 nM IC_{50}, respectively; $P > .05$).

In the SPR binding assay, the K_D for filgrastim, lipegfilgrastim, and pegfilgrastim binding to human G-CSF receptor values were 121.0 ± 14.0, 481.0 ± 84.0, and 516.0 ± 153.0 nM, respectively. The SPR sensorgram profiles for lipegfilgrastim and pegfilgrastim indicated very similar binding affinity of the 2 PEGylated G-CSF species with the receptor (Figure S2).

To evaluate if the similarity in G-CSF receptor binding and affinity for lipegfilgrastim and pegfilgrastim was extended to complete cell response, the effects of the drugs on NFS-60 cell–based [^{3}H]-thymidine incorporation were measured. The functional activity of the 2 drugs was similar (lipegfilgrastim stimulated [^{3}H]-thymidine incorporation mean EC_{50}, 1.8 pM; pegfilgrastim mean EC_{50}, 2.1 pM; Figure S3 and Table 4).

Discussion

Lipegfilgrastim and pegfilgrastim are long-acting G-CSFs developed to reduce the duration of neutropenia and the incidence of febrile neutropenia in patients with cancer receiving myelosuppressive or cytotoxic chemotherapy. In 2 phase 1 studies in healthy volunteers, lipegfilgrastim was associated with cumulative exposures (area under the concentration–time curve, zero to infinity [$AUC_{0-\infty}$]) that were 57% to 64% higher than the exposures observed

Table 3. IC_{50} Values and Relative Binding for Lipegfilgrastim Versus Pegfilgrastim Inhibition of [^{125}I]-G-CSF Binding to NFS-60 Cells

Assay Number	Lipegfilgrastim IC_{50} (nM)	Pegfilgrastim IC_{50} (nM)
1	0.66	0.55
2	0.58	0.58
3	0.76	0.83
4	0.78	0.92
Mean (SD)	0.70 (0.09)[a]	0.72 (0.18)[a]

IC_{50}, half maximal inhibitory concentration; SD, standard deviation.
[a]Mean IC_{50} of lipegfilgrastim and pegfilgrastim not significantly different ($P > .05$).

Table 4. Proliferative Activity of Lipegfilgrastim and Pegfilgrastim in the NFS-60 Cell–Based [^{3}H]-Thymidine Incorporation Assay

Assay Number	Lipegfilgrastim EC_{50} (pM)	Pegfilgrastim EC_{50} (pM)
1	1.56	1.62
2	1.45	1.80
3	2.23	2.55
4	2.04	2.59
Mean (SD)	1.82 (0.37)[a]	2.14 (0.50)[a]

EC_{50}, half maximal effective concentration; SD, standard deviation.
[a]Mean EC_{50} of lipegfilgrastim and pegfilgrastim not significantly different ($P > .05$).

with matched doses of pegfilgrastim.[17] Lipegfilgrastim also had a longer $t_{1/2}$ (32.4 versus 27.2 hours with the 6-mg dose [geometric mean values]) and longer time to maximum plasma concentration (30 versus 21 hours with the 6-mg dose) than pegfilgrastim, suggesting that the increase in exposure may be due to longer body residence (ie, slower clearance) of lipegfilgrastim. Phase 1 pharmacodynamic assays also showed that lipegfilgrastim produced a longer-lasting increase in ANC without a significant increase in ANC peak values.[17]

In a recent phase 3 study in patients with breast cancer receiving doxorubicin/docetaxel chemotherapy, the cumulative exposure (geometric mean $AUC_{0-\infty}$) was higher for lipegfilgrastim compared with pegfilgrastim in the first chemotherapy cycle.[28] In addition, patients treated with lipegfilgrastim had a similar incidence and duration of severe neutropenia compared with patients treated with pegfilgrastim and a safety profile comparable with pegfilgrastim, with no signs of increased toxicity due to lipegfilgrastim treatment.

Clearance pathways for filgrastim, a nonPEGylated G-CSF, include elimination via the kidneys and via neutrophils through binding to cell surface G-CSF receptors, internalization of the growth factor–receptor complexes via endocytosis, and subsequent degradation inside the cells.[8] PEGylation of filgrastim prohibits passage through the kidneys and eliminates this clearance pathway, thereby making the clearance of PEGylated G-CSF dependent on other pathways.[8] Neutrophil receptor binding is a major component of the clearance of PEGylated G-CSF drugs.[20,29] In addition, degradation by neutrophil elastase is another potential clearance pathway for G-CSF.[22] Injected G-CSF drugs may be particularly subject to elastase-mediated clearance, as G-CSF administration has resulted in significant ($P \leq .05$) increases in plasma elastase antigen levels and neutrophil elastase activity in clinical studies.[30,31] To investigate the possibility that the higher drug exposure observed in clinical trials after treatment with lipegfilgrastim compared with pegfilgrastim could be due to differential neutrophil elastase sensitivity, we treated both drugs with purified HNE. Our results clearly show that lipegfilgrastim was less susceptible to elastase degradation and retained greater functional activity after exposure to purified elastase compared with pegfilgrastim. Importantly, in a model designed to more closely mimic the physiological environment in which the G-CSFs act, lipegfilgrastim was much less susceptible than pegfilgrastim to degradation when incubated in the presence of isolated human neutrophils. This differential susceptibility to degradation in the presence of purified HNE and human neutrophils corresponds with the longer pharmacokinetic profile of lipegfilgrastim compared with pegfilgrastim and suggests that it is a possible mechanistic basis for the differences in their pharmacokinetics.

In vitro analysis of the pharmacodynamic actions of lipegfilgrastim and pegfilgrastim using G-CSF receptor binding and induction of cell proliferation was examined for potential differences that could possibly provide a basis for the longer-lasting increase in ANC observed in clinical trials. The binding of lipegfilgrastim and pegfilgrastim to the G-CSF receptor in both an NFS-60 cell–based [^{125}I]-G-CSF competitive binding assay and a human G-CSF receptor SPR binding assay was similar. In addition, the cell-proliferative effect of lipegfilgrastim and pegfilgrastim was equivalent, as measured in the [^{3}H]-thymidine incorporation assay. These results indicate that receptor binding and cell proliferative activity are unlikely to underlie any differences in clinical responses observed for lipegfilgrastim and pegfilgrastim.

PEGylation and methoxyPEGylation are technologies used to extend the half-life of biologic proteins and have generated a number of approved and widely used biotherapeutic agents.[32,33] The present study demonstrated that lipegfilgrastim exhibited substantially increased resistance to degradation and retained greater functional activity than pegfilgrastim after treatment with purified HNE or when incubated with human neutrophils. Given that both lipegfilgrastim and pegfilgrastim are PEGylated proteins, it became evident that PEGylation per se was not responsible for the increased resistance of lipegfilgrastim against elastase. Rather, it seems likely that the different attachment of PEG to each G-CSF may play a role in protection. In this regard, lipegfilgrastim differs from pegfilgrastim in the placement and chemical nature of this covalent modification.

This difference in attachment site offers an explanation for the increased protection of lipegfilgrastim against elastase. Figure 2 depicts initial models of glycoPEGylated G-CSF and N-terminally PEGylated G-CSF derived from an in vacuo molecular dynamics simulation of PEG 20 000 (PEG 20K) and the structural coordinates of human recombinant G-CSF (PDB 1GNC).[34,35] The overall dimensions of the in vacuo PEG model (radius, ∼24 Å) are somewhat smaller than experimentally determined dimensions ($R_h = 32.5$–45 Å)[36] because of missing PEG-bound water[9,37] in the PEG model. Lipegfilgrastim is a covalent conjugate of filgrastim that features a single

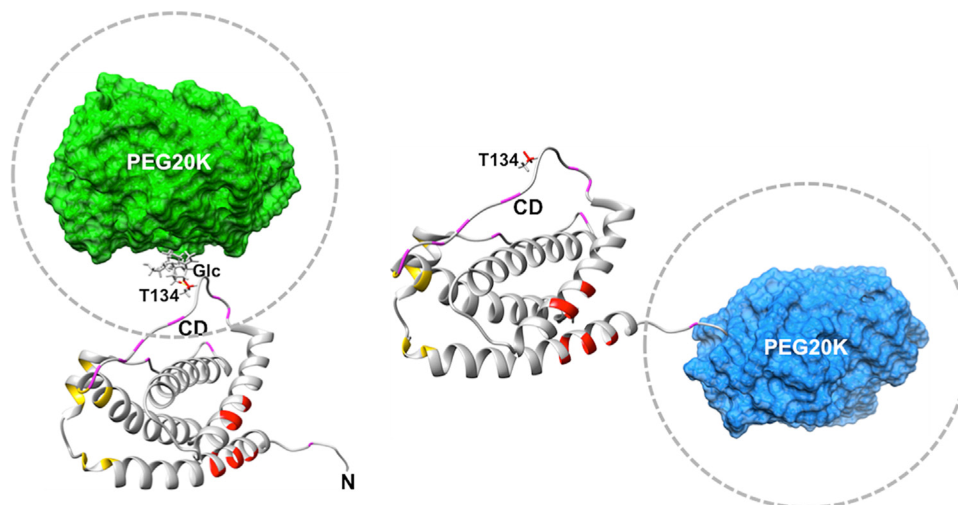

Figure 2. Models of PEGylated recombinant human granulocyte colony-stimulating factor (G-CSF). GlycoPEGylated G-CSF (lipegfilgrastim, N- and C-terminus indicated by N and C, respectively) depicted on the left and N-terminally PEGylated G-CSF (pegfilgrastim, C-terminus indicated by C, N-terminus hidden in blue PEG model) depicted on the right. Hatched lines indicate the size of water-containing PEG 20K. Predicted elastase sites are indicated in magenta and G-CSF receptor interaction sites I and II[40] in yellow and red, respectively. CD, flexible CD-loop; Glc, glucosidic linker; T134, threonine-134. Model building and rendering were carried out with the University of California–San Francisco CHIMERA package.[34]

methoxyPEG molecule attached at the natural glycosylation site at threonine-134 via a carbohydrate linker consisting of glycine, N-acetylneuraminic acid, and N-acetylgalactosamine.[15] In contrast, the PEG moiety of pegfilgrastim is chemically linked directly to the N-terminus of filgrastim.[12,15] The model in Figure 2 provides a rationale for increased protection of lipegfilgrastim from elastase, where the PEG covalently attached at threonine-134 serves as a steric hindrance toward elastase access to the flexible CD-loop of G-CSF, which contains several elastase sites as predicted by the "PeptideCutter" program.[38] It has been previously reported that glycosylated native G-CSF was more resistant to elastase degradation than nonglycosylated G-CSF, supporting the current results for differential elastase sensitivity to lipegfilgrastim and pegfilgrastim.[39] The model also provides an explanation for the very similar binding to the G-CSF receptor, as receptor interaction sites I and II[40] are not occluded by PEG, irrespective of its attachment site at threonine-134 via a glucosidic linker in lipegfilgrastim or the N-terminus in pegfilgrastim.

In summary, the results showing greater resistance of lipegfilgrastim to elastase degradation relative to that of pegfilgrastim is likely due to the position of the PEGylation site in the respective molecules. The results of these studies support differential elastase sensitivity as a likely explanation of the observed clinical pharmacokinetic and pharmacodynamic differences between the 2 drugs.

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Declaration of Conflicting Interests

M.G. has served as a paid consultant for Teva Pharmaceutical Industries Ltd. and contributed to this study through secondary employment with agreement from the Fritz Lipmann Institute, Jena, Germany; all other authors are employees or former employees of Teva Pharmaceutical Industries, Ltd., Merckle GmbH, or Merckle Biotec GmbH.

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