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Pharmacodynamics and Pharmacokinetics of Single Doses of Subcutaneous Pegylated Human G-CSF Mutant (Ro 25-8315) in Healthy Volunteers: Comparison With Single and Multiple Daily Doses of Filgrastim

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Ro 25-8315 is produced by conjugation of rhG-CSF mutant with polyethylene glycol (PEG). The purpose of this study was to examine the pharmacodynamics and pharmacokinetics of Ro 25-8315 in comparison with Filgrastim (rhG-CSF). Subjects received single subcutaneous doses of Ro 25-8315 ranging from 10 to 150 μg/kg using a double-blind, randomized, placebo-controlled design. Filgrastim was administered as a single dose (5 or 10 μg/kg) and, following a 14-day washout period, daily for 7 days. Ro 25-8315 increased absolute neutrophil count (ANC) by 6- to 8-fold and $CD34^+$ cell count more than 30-fold at the highest doses tested. Single doses (60–150 μg/kg) of Ro 25-8315 and multiple doses of Filgrastim had similar effects on ANC and $CD34^+$, although Ro 25-8315 had a greater effect on CFU-GM. The pharmacokinetics of Ro 25-8315 were dose-dependent, with peak concentrations and area under the serum concentration–time curve (AUC) increasing 100-fold over the range of doses studied. Time to reach peak concentration (T_{max}) and half-life of Ro 25-8315 averaged 20–30 hr at all doses, approximately three times longer than with Filgrastim. Adverse events were not serious and occurred with similar frequency with both products. Pegylation of rhG-CSF mutant results in more desirable pharmacokinetic properties and a longer duration of action with effective increases in ANC and measures of peripheral blood progenitor cell mobilization for at least 1 week. Am. J. Hematol. 66:245–251, 2001.

Key words: pegylation; G-CSF; mutant; pharmacokinetics; pharmacodynamics

INTRODUCTION

Recombinant human granulocyte-colony stimulating factor (rhG-CSF, Filgrastim) stimulates the proliferation and differentiation of neutrophil precursors leading to an increase in the number of circulating neutrophils [1]. It has become a useful therapeutic agent in the treatment of myelosuppression associated with chemotherapy. Subcutaneous administration of Filgrastim at doses of 5–10 μg/kg/day increases the absolute neutrophil count several fold and decreases the duration of neutropenia. This may reduce the risk of infection as well as the morbidity and mortality associated with treatment of patients with cancer [2–4]. Other indications include the treatment of neutropenia in AIDS or following bone marrow transplantation in patients with severe chronic neutropenia as well as the mobilization of peripheral blood progenitor cells, with or without chemotherapy, for autologous or allogenic transplantation [1,5]. A number of derivatives of human G-CSF have been created using in vitro mutagenesis. KW-2228 is a mutant in which five amino acids have been replaced at the N-terminal region of intact human G-CSF [1]. The compound appears to have 2–4 times higher specific activity than the native protein and exhibits improved stability at 37°C in human plasma [6].

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The administration of proteins as drugs presents a challenge since they are subject to proteolysis, rapid removal from the systemic circulation, and a relatively short duration of action. Filgrastim has a half-life of approximately 4–8 hr in humans [7–9] and requires daily administration to produce a sustained increase in circulating granulocytes. The conjugation of proteins to the water-soluble polymer polyethylene glycol (PEG) offers the potential to enhance systemic exposure to the protein. Pegylation reduces the sensitivity of the protein to the action of proteases, decreases systemic clearance, and invariably increases the half-life of the protein [10]. The increase in plasma concentration and half-life may result in an increase in the magnitude and duration of pharmacologic effect. However, these benefits may be partially offset by a decrease in bioactivity because PEG-linked protein may interact less efficiently with its specific receptor molecule. A number of proteins have been pegylated, including asparaginase, tumor necrosis factor (TNF), human megacaryocyte growth and development factor (MGDF), interferon, brain-derived neurotrophic factor (BDNF), and interleukin-6 (IL-6) [11–16].

Ro 25-8315 is the pegylated form of the recombinant human G-CSF mutant KW-2228. The protein is pegylated at the amino terminus and at the four lysine residues [17]. The final product is a mixture of one, two, three, or four PEG molecules attached to each molecule of KW-2228 with the di-pegylated protein predominating (60%). Studies in animals have suggested that the average elimination half-life of pegylated G-CSF is 2–4 times longer than G-CSF [18]. The purpose of this study was to examine the pharmacodynamic effects, pharmacokinetics, and tolerability of single subcutaneous doses of Ro 25-8315 in healthy human subjects. For comparison, similar parameters were assessed following single and multiple daily doses of Filgrastim.

MATERIALS AND METHODS

Study Design

The study involved 52 healthy male volunteers ranging from 20 to 45 years of age (mean 25.7 years). Subjects averaged 70.7 kg and were within 20% of their ideal body weight. The study was divided into two parts in order to accommodate the different assessment schedules required for compounds (Ro 25-8315 and Filgrastim) with highly different pharmacokinetic and pharmacodynamic properties. Part 1 was a double-blind study in which 40 subjects were randomized to receive either placebo or a single subcutaneous dose of 10, 30, 60, 100, or 150 μg/kg of pegylated rhG-CSF mutant (Ro 25-8315). Subjects were assigned in blocks of 8 with 2 subjects in each block receiving placebo and the other 6 receiving the same dose of active drug. As a safety precaution, the study was initiated at the lowest dose (10 μg/kg) with subsequent doses studied in an ascending manner at weekly intervals.

In Part 2, 12 volunteers received either 5 or 10 μg/kg (6 in each group) of rhG-CSF (Filgrastim). A single subcutaneous dose of Filgrastim was administered on Day 1 followed by a 2-week washout period. Beginning on Day 15, subjects received daily injections of Filgrastim for 7 days. A multiple-dosing scheme was included in Part 2 since this is the standard treatment regimen and provides a more valid comparison with the expected clinical effects of a pegylated protein such as Ro 25-8315.

Pharmacodynamic Assessments

The pharmacodynamic effects of Ro 25-8315 and Filgrastim were assessed by measuring absolute neutrophil count (ANC) and several indicators of peripheral blood progenitor cell (PRBC) mobilization ($CD34^+$ and $CD34^+38^-$ cells in peripheral blood and colony counts of CFU-GM [colony forming unit-granulocyte-macrophage] and BFU-E [burst forming unit-erythroid]). In Part 1 of the study, blood samples for pharmacodynamic assessment were collected into glass Vacutainer tubes containing EDTA at 0, 6, 24, 48, 72, 96, 120, 144, 168, and 192 hr after administration of Ro 25-8315 or placebo. In Part II, ANC was assessed at 0, 1, 3, 6, 12, 24, 36, 48, and 72 hr following single-dose administration of Filgrastim. After the 2-week washout period, ANC as well as measures of PRBC mobilization were assessed 6 hr after administration of each daily dose and at 1, 3, 6, 12, 24, 48, and 72 hr following the final dose of Filgrastim on Day 21.

$CD34^+$ and $CD34^+38^-$ cell counts were determined by a double-labeling technique using FITC-labeled CD34 (clone HPCA2) and anti phycoerythrin CD38 (Leu 17) with a control of FITC or phycoerythrin-labeled IgG1. Samples were analyzed using a FACScan™ with a single argon laser and reviewed by a single observer. For determination of CFU-GM and BFU-E colony counts, mononuclear cells were separated from the samples by gradient centrifugation. Cells (5×10^4) were plated on a 24-well culture plate with 500 μl of semisolid culture medium (Methocult GF H4534 or Methocult H4433) per well. The plates were incubated at 37°C in the presence of 5% CO_2 for 15 days. Colonies were numbered at J15 by inverted microscopy according to the criteria of Eaves and expressed as number of colonies per 10^5 plated cells.

Maximum cell count, time to reach the maximum cell count (T_{max}), and the area under the cell count vs. time curve (AUC) were calculated for each treatment. AUC was calculated for ANC and $CD34^+$ using the linear trapezoidal rule from 0 to 168 hr after Ro 25-8315 administration. This allowed for direct comparison to the AUC_{0-168} with daily administration of Filgrastim for 1 week (Days 15–21). AUC_{ANC} was calculated over a 72-

TABLE I. Effect of Subcutaneous Ro 25-8315 (Peg-rhG-CSF Mutein), Filgrastim, or Placebo on Absolute Neutrophil Count (ANC) and CD34$^+$ Cells

	Absolute neutrophil count (/mm^3)			CD34$^+$ count (/mm^3)[a]		
Treatment	ANC_{max}	AUC_{ANC} ($\times 10^3$)	$T_{max\ ANC}$	$CD34^+_{max}$	AUC_{CD34^+}	$T_{max\ CD34^+}$
Placebo	4,970 (1416)	648 (199)	–	2.5 (1.2)	516 (244)	–
Ro 25-8315						
10 μg/kg	20,404 (5951)	2,481 (524)	30 (10)	10.8 (4.9)	743 (357)	136 (45)
30 μg/kg	30,246 (9235)	3,936 (917)	62 (22)	30.7 (18.7)	2,157 (1123)	96 (37)
60 μg/kg	37,240 (2035)	4,878 (472)	58 (16)	83.9 (19.3)	7,172 (1355)	96 (0)
100 μg/kg	33,054 (8849)	4,304 (1004)	82 (39)	87.7 (39.5)	7,023 (1996)	104 (20)
150 μg/kg	41,922 (8785)	5,636 (754)	104 (29)	89.0 (33.0)	8,356 (2288)	104 (33)
Filgrastim						
5 μg/kg	23,042 (8430)	977 (322)	12 (0)	–[b]	–	–
10 μg/kg	25,163 (5877)	1,231 (233)	18 (7)	–	–	–
5 μg/kg daily for 1 week	46,447 (12197)	5,443 (1099)	–	70.1 (20.8)	7,125 (2331)	–
10 μg/kg daily for 1 week	44,097 (8849)	6,145 (1226)	–	90.1 (34.2)	9,764 (3519)	–

[a]Data are expressed as mean (standard deviation).
[b]CD34$^+$ counts were not measured after single doses of Filgrastim.

hr period following administration of the single dose of Filgrastim on Day 1.

Pharmacokinetic Assessments

Blood samples for measurement of Ro 25-8315 were obtained at 0, 3, 6, 12, 24, 36, 48, 72, 96, 120, 144, 168, and 192 hr after administration in Part 1 of the study. In Part 2, blood was collected at 1, 3, 6, 12, 24, 36, 48, and 72 hr after administration of Filgrastim on Day 1. Blood was allowed to clot for 30 min, and the serum was harvested following centrifugation. Serum concentrations of Ro 25-8315 and Filgrastim were measured by separate sandwich ELISAs. The sensitivity of the method was 100 pg/ml for both compounds. The coefficient of variation for the assay averaged 11% for Ro 25-8315 and 5% for Filgrastim.

The pharmacokinetic parameters for Ro 25-8315 and Filgrastim following single-dose administration were determined using noncompartmental methods. Maximum plasma concentration (C_{max}) and time to reach maximum plasma concentration (T_{max}) were obtained directly from the data. The elimination rate constant and terminal half-life were calculated from the terminal portion of the log concentration–time profile using linear regression. The area under the plasma concentration–time curve from zero time to the time of the last quantifiable concentration was calculated using the logarithmic trapezoidal rule. This was added to the extrapolated area (last measurable concentration divided by the elimination rate constant) to obtain the total AUC.

Safety Assessments

Subjects were monitored throughout the study for clinical adverse events. These were characterized as mild, moderate, or severe and were classified as to likely relationship to treatment. Laboratory testing of blood (hematology, chemistry) and urine (urinalysis, drug screening) was conducted on Day 1, Day 4, and at follow-up (Days 9–10) in Part 1 of the study. In Part 2, laboratory testing occurred on Days 1, 4, 15, 18, 21, and at follow-up (Days 25–26). Vital signs were monitored daily in Part 1 and on Days 1–6 and 15–25 in Part 2.

RESULTS

Administration of single doses of Ro 25-8315 resulted in substantial increases in ANC (Fig. 1, Table I). ANC_{max} and AUC_{ANC} increased 6- to 8-fold over placebo at the highest doses tested. Both parameters increased with dose, although further increases were modest at doses above 60 μg/kg (Table I). The time to reach maximum ANC ($T_{max\ ANC}$) was clearly dose-dependent, increasing from 30 hr at 10 μg/kg to 104 hr at 150 μg/kg.

Single doses of Filgrastim produced more rapid changes in ANC with the maximum value occurring at 12 and 18 hr with 5 and 10 μg/kg doses, respectively (Fig. 1). Figure 1 also illustrates the more rapid decline in ANC with Filgrastim compared to Ro 25-8315. AUC_{ANC} with a single 10 μg/kg dose of Filgrastim was approximately half that obtained with the same dose of Ro 25-8315. With daily administration of 5 or 10 μg/kg Filgrastim for 1 week, AUC_{ANC} was comparable to that observed with single doses of 60–150 μg/kg of Ro 25-8315 (Table I).

The effect of Ro 25-8315 and Filgrastim on CD34$^+$ is presented in Table I and Figure 2. Maximum cell counts for CD34$^+$ occurred at 4–5 days after administration of Ro 25-8315 (Fig. 2). Compared to baseline, increases ranged from 4-fold at 10 μg/kg to more than 30-fold at doses of 60–150 μg/kg. The AUC_{CD34^+} was increased by

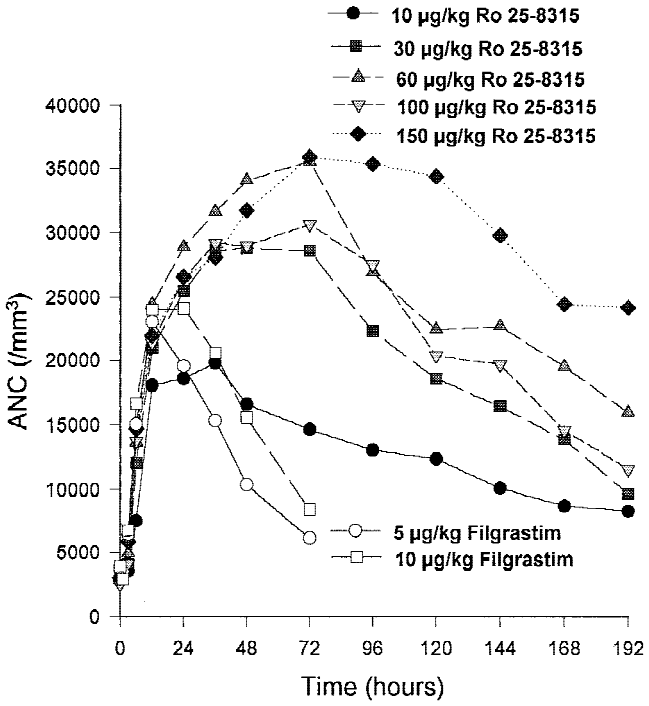

Fig. 1. Absolute neutrophil count (ANC) after single subcutaneous doses of Ro 25-8315 (10–150 µg/kg) and Filgrastim (5 and 10 µg/kg).

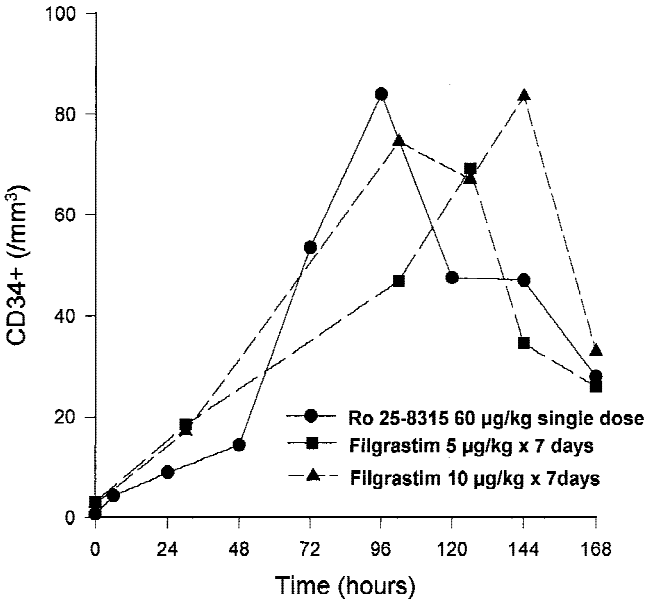

Fig. 2. Effect of a single 60 µg/kg dose of Ro 25-8315 and multiple daily doses (5 µg/kg) of Filgrastim on CD34$^+$ cell counts.

about 15-fold at the highest doses tested (Table I). Daily administration of Filgrastim for 1 week resulted in increases in the CD34$^+$ cell count that were comparable to those seen with 60 µg/kg doses of Ro 25-8315 (Fig. 2). CD34$^+$38$^-$ mobilization was observed at a dose of 60

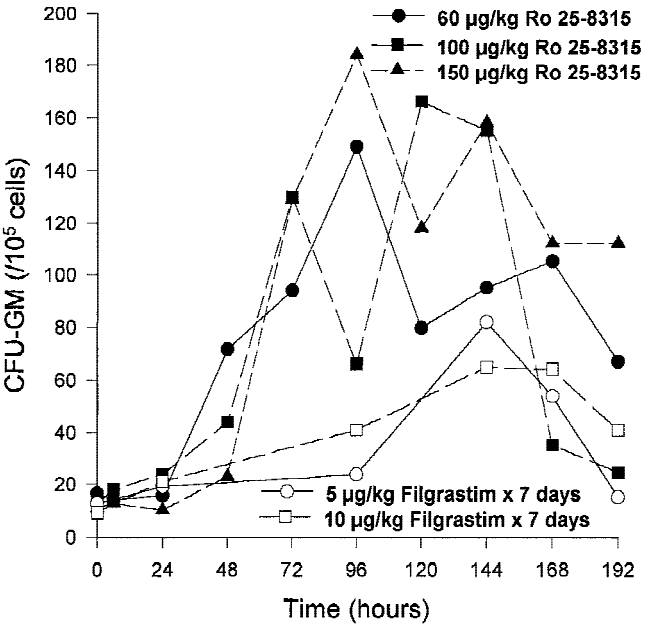

Fig. 3. CFU-GM colony counts with single doses of Ro 25-8315 (60–150 µg/kg) compared with multiple daily doses of Filgrastim (5–10 µg/kg).

µg/kg but could not be characterized in all subjects. Mobilization occurred more consistently at higher doses. Single doses of Ro 25-8315 (60–150 µg/kg) had a greater effect on the colony forming cells CFU-GM (Fig. 3) and BFU-E (data not shown) than multiple doses of Filgrastim.

The pharmacokinetic data are presented in Table II. For Ro 25-8315, a greater than proportional increase in both C_{max} and AUC was observed with increasing dose (Fig. 4, Table II). Over the 15-fold range of doses studied, C_{max} and AUC increased approximately 100-fold. From 30 to 150 µg/kg, increases in these parameters were 10- to 15-fold. T_{max} was shorter and the half-life longer with the 10 µg/kg dose compared to all others. At doses from 30 to 150 µg/kg, T_{max} and half-life did not exhibit significant dose-dependence and were both relatively consistent in the range of 20–30 hr. With Filgrastim, T_{max} was achieved much earlier (5–6 hr) and the half-life was shorter (6–12 hr) relative to Ro 25-8315 (Fig. 5, Table II). The increase in AUC was also more than proportional to the 2-fold increase in dose.

Of the 52 subjects enrolled in the study, 45 had at least one adverse event, including 93% of subjects receiving Ro 25-8315 and 100% of subjects receiving Filgrastim. None of the adverse events were serious or unexpected, and no subject had to withdraw from the study due to an adverse event. The most common events were headache, back pain, and bone pain, which occurred at a similar frequency with both compounds and could generally be controlled with acetaminophen. At a dose of 150 µg/kg of Ro 25-8315, 3 subjects had severe back pain requiring

TABLE II. Pharmacokinetics of Subcutaneous Ro 25-8315 (PEG-rhG-CSF Mutein) and Filgrastim

Treatment	Parameter			
	C_{max} (ng/ml)	T_{max} (hr)	AUC (ng × hr/ml)	Half-life (hr)
Ro 25-8315				
10 μg/kg	2.5 (1.6)[a]	13 (5.9)	184 (77)	59.3 (36.5)
30 μg/kg	28.1 (31.6)	24 (11)	1,054 (932)	35.1 (40.2)
60 μg/kg	66.7 (37.2)	20 (14)	2,577 (1567)	30.4 (13)
100 μg/kg	173 (55)	24 (7.6)	5,525 (1637)	19.9 (7.2)
150 μg/kg	260 (124)	30 (6.6)	14,010 (7255)	25.3 (6)
Filgrastim				
5 μg/kg	15.1 (8.2)	5.5 (1.2)	133 (39)	9.8 (4.7)
10 μg/kg	36.7 (11.4)	6.0 (0)	369 (125)	7.4 (2.9)

[a]Data are expressed as mean (standard deviation).

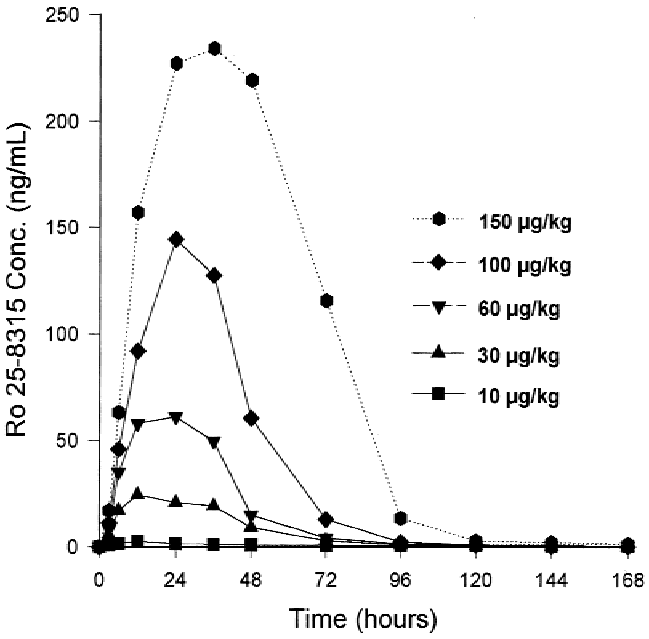

Fig. 4. Plasma concentrations of Ro 25-8315 following single subcutaneous doses of 10–150 μg/kg.

bed rest for 1 day. Laboratory abnormalities were also relatively common with both drugs. Increases in alkaline phosphatase occurred in approximately 80% of subjects receiving Filgrastim or Ro 25-8315 at doses from 60–150 μg/kg. SGPT was also elevated in a number of subjects (6 of 40 in Part 1; 1 of 12 in Part 2). These changes occurred typically about 1 week after dosing, were transient, and judged to be of no clinical significance in all cases. Mild decreases in platelet count was observed with both drugs (platelets remained above 100,000/mm^3 in all cases) and returned to normal within 2–3 weeks after treatment.

DISCUSSION

The results of this investigation indicate that Ro 25-8315 is effective in stimulating the mobilization of peripheral blood progenitor cells and increasing the absolute neutrophil count. Pegylated proteins do not always retain full biologic activity. However, this does not appear to be a concern with Ro 25-8315. Both the intensity of effect (as assessed by the ANC_{max}) and the extent of activity (as measured by AUC_{ANC}) with doses of 60–150 μg/kg were greater than that observed with single doses of Filgrastim used clinically (5–10 μg/kg). In addition, the increase in ANC with a single subcutaneous dose of Ro 25-8315 in the range of 60–150 μg/kg is comparable to the effect produced by daily doses of Filgrastim for 1 week (Table I).

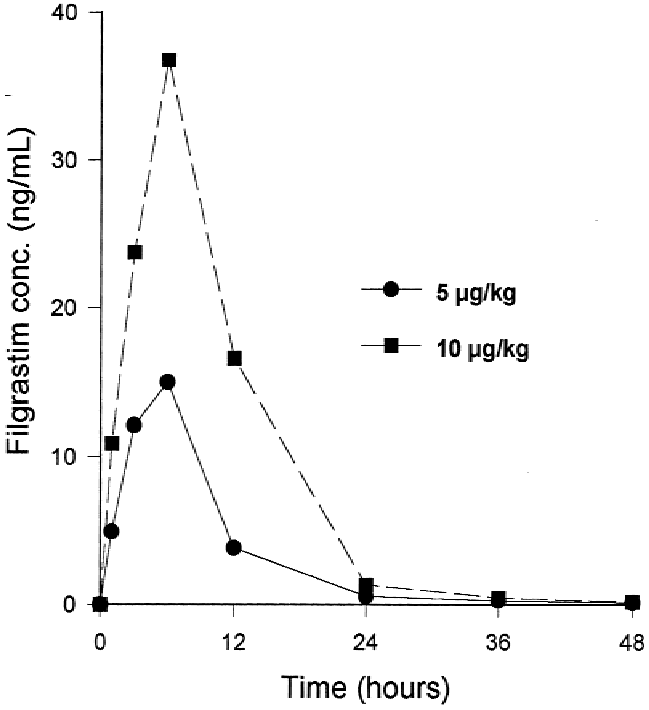

Fig. 5. Plasma concentrations of Filgrastim following single subcutaneous doses of 5–10 μg/kg.

As indicated in Table I, the time to reach a maximal increase in ANC is several-fold longer with Ro 25-8315 than with Filgrastim, while inspection of Figure 1 also suggests that the decline in ANC after reaching the peak effect is much slower with Ro 25-8315. This is consistent with data in male Sprague-Dawley rats where the neutrophil count after a 100 μg/kg dose of rhG-CSF returned to normal within 48 hr compared to 168 hr with pegylated G-CSF [18]. With doses of 30 μg/kg and above, mean ANC remained above 10,000 (more than three times baseline in the placebo group) for the full 192-hr (8-day) study period. Increasing doses of Ro 25-8315 beyond 60 μg/kg did not result in a further increase in maximum ANC, which remained in the range of 30,000–40,000/mm^3. However, the time to reach the maximum effect and the duration of effect were dose-dependent (Fig. 1). From a clinical standpoint, the difference in

ANC response between 60 and 150 μg/kg may not be significant.

Many of the same conclusions can be drawn with respect to the effects of Ro 25-8315 and Filgrastim on $CD34^+$ counts. Clearly, a single dose of Ro 25-8315 produces a dramatic increase in the maximum $CD34^+$ count of more than 30-fold with doses of 60 μg/kg and above. $CD34^+$ levels above 20, often considered to trigger leukopheresis, were consistently measured after 72 hr (Fig. 2). Doses of this magnitude produce a comparable effect on $CD34^+$ to the daily administration of Filgrastim for 1 week as assessed by AUC_{CD34^+}. In the case of the colony-forming profiles, the effect of Ro 25-8315 at doses above 60 μg/kg was superior to that of multiple doses of Filgrastim 10 μg/kg/day on CFU-GM (Fig. 3) and BFU-E. In addition, circulating $CD34^+38^-$ cells were detected more consistently with 100–150 μg/kg doses of Ro 25-8315.

The prolonged duration of action of single doses of Ro 25-8315 compared to Filgrastim is directly related to the pharmacokinetic differences between the products. As expected following pegylation of rhG-CSF mutant, the elimination half-life was much longer (approximately 24 hr), representing about a 3-fold increase over the average half-life of about 8 hr for Filgrastim. Similar differences have been reported in rats, where pegylation of G-CSF increased the half-life from 1.79 to 7.05 hr [18]. There were no statistically significant differences in half-life related to dose with Ro 25-8315. The largest value was actually observed with the smallest dose (Table II), possibly due to the fact that peak concentrations were approximately 100-fold lower compared to the 150 μg/kg dose and rapidly declined to values approaching the sensitivity of the analytical method. In addition, the time to reach a maximum concentration was increased about 4-fold with Ro 25-8315 (about 24 hr vs. 6 hr). The increase in half-life and time to reach peak concentrations both contribute to a duration of effect that appears to be at least 1 week with Ro 25-8315.

The pharmacokinetics of Ro 25-8315 are dose-dependent (Fig. 4, Table II). Although both half-life and time to reach peak concentration (T_{max}) are similar across the range of doses tested, peak concentration and AUC increase more than proportionately with dose. Dose-dependence was also evident with Filgrastim, where the AUC with a single 10 μg/kg dose averaged about three times that of the 5 μg/kg dose. The mechanism for the nonlinear disposition of Ro 25-8315 cannot be determined from this study but may relate in part to increased availability. This explanation is consistent with an increase in C_{max} and AUC with no change in elimination half-life.

The safety of single-dose Ro 25-8315 appeared similar to that of Filgrastim in the short time frame of this study. All subjects were able to complete the study, and no serious adverse events were observed. Headache and bone pain occurred frequently with both compounds as expected. The severity of bone and back pain appeared to be dose-dependent with Ro 25-8315 but was controlled by acetaminophen at all dose levels other than 150 μg/kg, where 3 subjects required bed rest for 1 day. Almost all laboratory abnormalities were transient, returning to baseline by the end of the trial period. In subjects experiencing thrombocytopenia, platelet counts remained above 100,000 in all cases and returned to normal within 2–3 weeks.

The results of this study support the conclusion that Ro 25-8315 is effective in increasing the absolute neutrophil count and mobilization of peripheral blood progenitor cells. The pegylated product exhibits improved pharmacokinetic properties compared to Filgrastim, with a longer time to reach maximum plasma concentration and a longer elimination half-life. These characteristics translate to a much longer duration of action with effective increases in ANC for at least 1 week following single doses of 60–150 μg/kg. This offers the potential clinical advantage of dosing Ro 25-8315 once per week compared to the requirement for daily administration of Filgrastim. Adverse events with these products are similar in frequency and severity.

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