# Albugranin™, a Recombinant Human Granulocyte Colony Stimulating Factor (G-CSF) Genetically Fused to Recombinant Human Albumin Induces Prolonged Myelopoietic Effects in Mice and Monkeys

**Wendy Halpern,[1,6] Todd A. Riccobene,[1,6] Heidi Agostini,[2] Kevin Baker,[3] David Stolow,[2] Mi-Li Gu,[4] Jonathan Hirsch,[1] Angela Mahoney,[5] Jeffrey Carrell,[1] Ernest Boyd,[1] and Krzysztof J. Grzegorzewski[1,7]**

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***Purpose.*** Albugranin™ fusion protein is recombinant granulocyte colony stimulating factor (rG-CSF) genetically fused at its N-terminus to the C-terminus of recombinant serum human albumin and is expected to have a relatively long half-life compared with rG-CSF alone. In this study, the pharmacodynamics and pharmacokinetics of Albugranin were evaluated in BDF1 mice and cynomolgus monkeys.

***Methods.*** Single doses of Albugranin (0.25-5 mg/kg) or Filgrastim (methionyl rG-CSF, 0.25, or 1.25 mg/kg) were administered subcutaneously (SC) to mice and multiple doses of Albugranin (25–100 μg/kg every 4 or 7 days) or Filgrastim (5 μg/kg daily) were administered SC for 14 days to monkeys for hematologic evaluation. For pharmacokinetics studies, mice were injected intravenously (IV) or SC with single doses of Albugranin (0.25–1.25 mg/kg) or Filgrastim (0.25 mg/kg) and monkeys were injected SC with multiple doses of Albugranin (100–1000 μg/kg once weekly for 5 weeks). Plasma levels of Albugranin and Filgrastim were measured by enzyme-linked immunosorbent assay.

***Results.*** In mice, administration of Albugranin effectively increased the number of peripheral granulocytes and mobilized hematopoietic progenitor cells for up to 5 days. The magnitude and duration of this effect were dose-dependent. In contrast, administration of Filgrastim resulted in a small increase in both cell types on day 1 only. Albugranin administered to cynomolgus monkeys caused an increase in peripheral neutrophils, with a less prominent increase in peripheral monocytes. Albugranin-induced neutrophilia peaked 24 h following each dose administration. Administration of Filgrastim daily in monkeys resulted in moderate increases in neutrophils that were maximal on days 8–12 during the course of treatment. Compared with Filgrastim, Albugranin had a longer terminal half-life ($t_{1/2,term}$), and mean residence time (MRT), and slower clearance (CL/F) in mice. The $t_{1/2,term}$, MRT, and CL/F of Albugranin following SC administration to BDF1 mice were 5.6–5.7 h, 16.7–20.7 h, and 6.37–12.2 mL/h/kg, respectively, compared with 2.54 h, 4.9 h, and 164 mL/h/kg, respectively for Filgrastim. In cynomolgus monkeys, the corresponding values of $t_{1/2,term}$, MRT, and CL/F for Albugranin were 7.73–13.3 h, 19.4–27.3 h, and 7.90–27.5 mL/h/kg, respectively, for doses of 100-1000 μg/kg. An exposure-response relationship that could be empirically described with a simple $E_{max}$ model with baseline was found between day 15 absolute neutrophil count and area under the curve following the first dose in cynomolgus monkeys.

***Conclusion.*** The sustained activity of Albugranin in mice and monkeys demonstrated in these studies suggests that this agent could be given less frequently than Filgrastim to achieve similar therapeutic effects in patients.

**KEY WORDS:** G-CSF; fusion protein; granulocyte mobilization; progenitor mobilization.

## INTRODUCTION

Granulocyte colony stimulating factor (G-CSF) is a member of a family of secreted glycoproteins that play a pivotal role in the regulation of the survival, proliferation, differentiation, and functional activation of hematopoietic cells (1,2). G-CSF promotes the proliferation and maturation of neutrophil precursors, and treatment of rodents with G-CSF induces a pronounced neutrophilic leukocytosis (1). Filgrastim is the recombinant methionyl human G-CSF expressed in bacteria that is currently used therapeutically to increase circulating levels of neutrophils. Clinical indications for Filgrastim include cancer patients receiving myelosuppressive chemotherapy, patients with acute myeloid leukemia receiving induction or consolidation chemotherapy, cancer patients receiving bone marrow transplant, patients undergoing peripheral blood progenitor cell collection and therapy, and patients with severe chronic neutropenia (3). The magnitude of the neutrophilic response to administration of Filgrastim is dose-dependent. Daily or twice-daily injections are required for a full beneficial response because of its short circulating half-life of a few hours (3).

The development of a long-acting form of Filgrastim given at weekly intervals would offer the advantage of a more convenient dosing schedule and may enhance compliance and clinical efficacy compared with current G-CSF therapy. A polyethylene glycol-conjugated form of recombinant (r)G-CSF (Neulasta™) has been shown in preclinical and clinical studies to have activity for several days following a single administration without significant toxicity and has recently been approved for marketing (4,5).

[1] Preclinical Development, Human Genome Sciences, Inc., 9410 Key West Avenue, Rockville, Maryland 20850.
[2] Protein Development, Human Genome Sciences, Inc., Rockville, Maryland 20850.
[3] Preclinical Discovery, Human Genome Sciences, Inc., Rockville, Maryland 20850.
[4] Assay Development, Human Genome Sciences, Inc. Rockville, Maryland 20850.
[5] Antibody Development, Human Genome Sciences. Inc. Rockville, Maryland 20850.
[6] Authors equally contributed to this paper.
[7] To whom correspondence should be addressed. (e-mail: kris_grzegorzewski@hgsi.com)

**ABBREVIATIONS:** ANC, absolute neutrophil count; ANOVA, analysis of variance; AUC, area under the curve; AUEC, area under the effect curve; CL, clearance; CL/F, clearance over bioavailability; $E_{max}$, maximum effect; $E_0$, baseline effect; $EC_{50}$, AUC required to produce 50% of the maximum drug-induced effect ($E_{max}$-$E_0$); EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; G-CSF, granulocyte colony stimulating factor; GLP, good laboratory practice; HSA, human serum albumin; IV, intravenous; MRT, mean residence time; PBS, phosphate-buffered saline; Q4D, every 4 days; Q7D, every 7 days; RBC, red blood cell; rG-CSF, recombinant granulocyte colony stimulating factor; rHSA, recombinant human serum albumin; SC, subcutaneous; SEM, standard error of the mean; $t_{1/2,term}$, terminal half-life; $t_{max}$, time of maximum concentration; WBC, white blood cell; Vz, volume of distribution; Vz/F, volume of distribution over bioavailability.

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Albugranin is a genetic fusion protein of human serum albumin (HSA) and rG-CSF and is based on a novel strategy for developing long-acting proteins. HSA was chosen as the stabilizing agent as it is the most prevalent naturally occurring blood protein and has an *in vivo* half-life of several (2–3) weeks (6). HSA is widely distributed *in vivo* and is known to be a natural carrier for molecules in the blood (6). These studies were undertaken to evaluate the pharmacokinetics and pharmacodynamic effects of Albugranin in BDF1 mice and cynomolgus monkeys.

## MATERIALS AND METHODS

### Animals

Specific pathogen-free BDF1 male mice (Charles River Laboratories, Inc.; Wilmington, Mass.) at 6–9 weeks of age were used for efficacy and pharmacokinetics studies. BDF1 mice were chosen for these studies because the pharmacokinetics of G-CSF are known to depend on the strain of mice used (7), and BDF1 mice have been shown to have a robust response to G-CSF (8,9). Mice were allowed to acclimate for 1 week prior to the start of the experiment. All animal experimentation was conducted in accordance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and under the supervision and approval of the Institutional Animal Care and Use Committees.

Male and female experimentally naïve, young adult cynomolgus monkeys (*Macaca fascicularis)* weighing 1.6–3.1 kg were evaluated. Monkeys were individually housed in stainless steel cages containing drop pans with corncob bedding. Water was provided *ad libitum*, and Teklad™ 2055 Certified Monkey diet was provided twice daily. Monkeys also participated in the environmental enrichment program including fruit, puzzles, toys, and audio and visual stimulation during the study.

### Protein and Growth Factor Preparations

Albugranin is rG-CSF genetically fused at its N-terminus to the C-terminus of recombinant human albumin (rHSA) and has a molecular mass of approximately 85 kD. Albugranin was produced using a yeast host system (*Saccharomyces cerevisiae*) engineered to express the Albugranin fusion protein in a similar manner as previously described (6). Protein was harvested from the fermentation medium of the yeast culture and purified using a combination of ion exchange, affinity, and hydrophobic interaction chromatography techniques. The purified protein was greater than 95% pure as determined by N-terminal sequencing and contained less than 7 EU/mg of endotoxin. Albugranin at a concentration of 1.34 mg/mL was used for mouse studies and at 0.41 or 1.0 mg/mL in monkey studies. The working concentrations were obtained by diluting as necessary in phosphate-buffered saline (PBS). Filgrastim (rG-CSF, Neupogen®) (Amgen; Thousand Oaks, Calif.) was purchased commercially as a 300 μg/mL solution and diluted for injection with 5% dextrose. Human serum albumin (HSA) (Baxter; Glendale Calif.) at 25% solution (w/v) was diluted with PBS to a final concentration of 1 mg/mL.

Molar equivalence of the test proteins is based upon an average molecular weight of 85 kD for Albugranin and a reported MW of 19 kD for Neupogen. Table I summarizes the moles (in nmol) for each dose administration used in these studies.

### Hematologic Evaluation of Mouse Blood

Vehicle control mice (n = 6) received a single SC injection of Albugranin formulation buffer (50 mM TRIS-HCl, pH = 7.0) diluted in PBS. Control mice (n = 6) received a single SC injection of HSA at 5 mg/kg, and experimental mice (n = 6) received a single administration of Albugranin at 3 dose levels (0.25 mg/kg, 1.25 mg/kg, 5.0 mg/kg), or a single administration of Filgrastim at 2 dose levels (0.25 mg/kg or 1.25 mg/kg). The number of peripheral granulocytes and hematopoietic progenitor cells in mice was evaluated daily for 5 consecutive days. Blood was collected from the tail vein into capillary tubes pretreated with heparin and transferred into Capiject ethylene diamine tetraacetic acid (EDTA) tubes. For total white blood cell (WBC) count 10 μl of blood was added directly to 10 mLof Isoton II buffer containing four drops of Zapoglobin II for red blood cell (RBC) lysis. WBC count was determined via Coulter Counter (Coulter; Miami, Fla.). The number of granulocytes and hematopoietic progenitor cells was assessed by flow cytometry (FACScan, Becton Dickinson; San Jose, Calif.) using Gr-1 (granulocytes) and c-kit (hematopoietic progenitor cells) antibody markers. For staining, 20–50 μl of whole blood was added to an antibody cocktail containing FITC conjugated Gr.1/8C5 (BDPharmingen; San Diego, Calif.), c-kit/CD117 conjugated to R-PE (BDPharmingen), and Mac-1/CD11b conjugated to PE-Cy5 (Caltag Laboratories; Burlingame, Calif.). Cells were incubated 20 min at room temperature. Erythrocytes were lysed by a brief incubation with 0.5 mL ACK Lysing Buffer (BioWhittaker;Walkersville, Md.). The lysing reaction was stopped with 2 mL of FACS buffer, and the cells were pelleted by centrifugation. The cells were resuspended in FACS buffer (d-PBS with 0.1% sodium azide and 0.1% BSA) and acquired on the FACScan. Data were analyzed using CellQuest software (Becton Dickinson). A region depicting granulocytes was set to include all $Gr.1^+$ cells on a plot of Gr.1 vs. Mac-1. A second region to depict hematopoietic progenitor cells was set to include all $c\text{-}kit^+$ cells on a plot of c-kit vs. Gr.1.

### Hematologic Evaluation of Cynomolgus Monkey Blood

Twenty (10 male and 10 female) cynomolgus monkeys were randomized into five treatment groups of 4 (2 male and 2 female) monkeys each. Group assignments are shown in

**Table I.** Molar Equivalence

| Protein | Dose administered (mg/kg) | Molar equivalent (nmol/kg) |
|---|---|---|
| Albugranin | 0.025 | 0.294 |
| | 0.100 | 1.176 |
| | 0.250 | 2.940 |
| | 1.250 | 14.700 |
| | 5.000 | 58.8 |
| Neupogen | 0.005 | 0.263 |
| | 0.250 | 13.150 |
| | 1.250 | 65.750 |

Table II. Monkeys were injected SC in the mid-scapular region with vehicle, Albugranin, or Filgrastim. Vehicle was administered every 4 days (Q4D); Albugranin was administered at 25 μg/kg (Q4D), as well as at 100 μg/kg (Q4D) or every 7 days (Q7D); and Filgrastim was administered at 5 μg/kg daily during the 14-day treatment phase of the study. Individual doses were calculated based on the most recent body weight (at study initiation, monkeys were 1.6–2.5 kg). Whole blood was collected in EDTA anticoagulant on days 0–5, 8, 9, 12, 13, 16, 20, and 27 except for the Q7D Albugranin group with collection on days 0–5, 7, 8, 12, 13, 16, 20, and 27. If treatment was scheduled for the same day, hematology samples were collected prior to dosing. Samples were stored at room temperature (20–25°C) and evaluated on a Cell-Dyne™ 3500 automated hematology analyzer (Abbott Laboratories; Abbott Park, Il.) within 8 h of collection. The WBC count was determined, as well as leukocyte subsets, erythrocyte and platelet parameters. In addition, a Diff-Spin™ was made for each sample, and a manual differential for leukocyte subsets was performed. The manual differential was used preferentially for subsequent analyses. On day 9, the total WBC count was performed manually, followed by manual differential. On each of days 1, 2, 5, and 13, a single blood sample was clotted and could not be analyzed. Clotted samples were from groups 1, 3, 5, and 3, respectively. Although these individual samples could not be analyzed, at least three samples were analyzed for each group on those days.

### Pharmacokinetic Studies

In the mouse pharmacokinetic study, normal male BDF1 mice were injected intravenously (IV) via the tail vein or SC in the mid-scapular region with 0.25 or 1.25 mg/kg of Albugranin, 0.25 mg/kg Filgrastim or Albugranin vehicle diluted in PBS. Three mice per time point were sacrificed by $C0_2$ overexposure at various times following dosing, and blood was collected via the inferior vena cava. For the IV Albugranin groups, blood was collected at 5 min, 30 min, 2, 6, 24, 48, and 72 h following dosing. For the IV Filgrastim group, blood was collected at 5 min, 30 min, 1, 2, 4, and 8 h following dosing. Blood was collected from mice in the SC Albugranin groups at 30 min, 2, 6, 16, 24, 48, and 72 h after injection, and from mice in the SC Filgrastim group at 15 min, 30 min, 1, 2, 4, 8, and 16 h after injection. Blood was collected from mice in the IV vehicle group at 5 min and 72 h after injection and in the SC vehicle group at 30 min and 72 h after injection. Blood samples were transferred to tubes containing EDTA and centrifuged at 16000g for 10 min to obtain plasma. The plasma was transferred to tubes in a 96 well plate and stored at –80°C until analyzed by enzyme-linked immunosorbent assay (ELISA).

**Table II.** Group Assignments for Pharmacodynamic Study in Cynomolgus Monkeys

| Group | Number of monkeys | Treatment | Route | Schedule (days) | Dose (μg/kg) |
|---|---|---|---|---|---|
| 1 | 2M/2F | Vehicle | SC | 0, 4, 8, 12 | 0 |
| 2 | 2M/2F | Albugranin | SC | 0, 4, 8, 12 | 25 |
| 3 | 2M/2F | Albugranin | SC | 0, 4, 8, 12 | 100 |
| 4 | 2M/2F | Albugranin | SC | 0 and 7 | 100 |
| 5 | 2M/2F | Filgrastim | SC | 0–13 | 5 |

M = male; F = female; SC = subcutaneous.

Samples for pharmacokinetic analysis were taken as part of a Good Laboratory Practice (GLP) toxicology study with Albugranin in cynomolgus monkeys. Monkeys (5 males and 5 females in each group) received SC injections of Albugranin at doses of 100, 500, or 1000 μg/kg once weekly over a 5-week period (on study days 1, 8, 15, 22, and 29). Samples for PK analysis were drawn pre-dose and at 0.5, 2, 8, 24, 48, 96, and 168 h after the first and last doses. Blood was drawn from a femoral vein into tubes containing EDTA and centrifuged at 16000g within 30 min to obtain plasma. Plasma was frozen at –80°C until analyzed by ELISA. Absolute neutrophil counts were measured pre-dose and on days 15, 27, and 42 and were used as a pharmacodynamic marker.

### ELISA for Albugranin and Filgrastim

Albugranin and Filgrastim concentrations in murine or primate plasma samples were analyzed using a sandwich-type ELISA. For murine samples, a monoclonal mouse anti-human G-CSF antibody (R&D Systems; Minneapolis, Minn.) was used for capture of both Albugranin and Filgrastim; a biotinylated polyclonal goat anti-human HSA antibody (United States Biologic; Swampscott, Mass.) was used for detecting Albugranin; and a biotinylated polyclonal goat anti-G-CSF antibody (R&D Systems) was used for detecting Filgrastim. Up to 10% mouse plasma had no effect on the Albugranin standard curves indicating that cross-reactivity of the anti-human HSA antibody for murine albumin was low (PK samples were diluted to contain a maximum of 2% mouse plasma). For primate samples, the same monoclonal mouse anti-human G-CSF antibody was used for capture and the biotinylated polyclonal goat anti-G-CSF antibody was used to detect Albugranin and Filgrastim. There were no measurable levels in pre-dose samples or samples from vehicle-treated mice or monkeys, so the anti-human G-CSF antibody does not appear to cross-react appreciably with endogenous proteins in mouse or monkey plasma.

A signal was generated by incubation of the plates with streptavidin-conjugated peroxidase followed by addition of 3,3′,5,5′ tetramethylbenzidine substrate. The absorbance was measured at 450 nm in a Spectromax ELISA plate reader (Molecular Devices Corp.; Menlo Park, Calif.). The software program Softmax (version 3.1, Molecular Devices Corp.) was used to generate Albugranin and Filgrastim standard curves from the mean absorbance values using a four-parameter fit. The relative amounts of Albugranin or Filgrastim in the samples were interpolated into the appropriate standard curve and corrected by the dilution factor to generate the Albugranin or Filgrastim concentrations in neat plasma. The limit of detection of the assay in mouse plasma was 3 ng/mL for Albugranin and 1.5 ng/mL for Filgrastim, and the limit of quantification was 6 ng/mL. The limit of detection for Albugranin in cynomolgus monkey plasma was 1.0 ng/mL and the limit of quantification was 1.25 ng/mL. The limit of quantification of the assay was determined by spiking sera with known amounts of Albugranin and determining the limit at which 80-120% of the material was detected. Assays were qualified by having different people perform several runs on different days to determine the intra- and inter-day and person-to-person variability. The intra-day coefficient of varia-

tion (CV) was 7.8%, the inter-day CV was 4.6%, and the person-to-person CV was 5.6%. All CV's met acceptance criteria. Any sample with a CV greater than or equal to 20% was retested.

### Pharmacokinetic Analysis

Plasma concentration data were analyzed with the software package WinNonlin (Pharsight Corp.; Mountain View, Calif.) using non-compartmental analysis with the linear up/log down method and uniform weighting of the data. This method utilizes the linear trapezoidal rule when the concentration data are increasing, and the logarithmic trapezoidal rule when the concentration data are decreasing for calculating Area Under the Curve (AUC). Clearance (CL) and CL/F were calculated as Dose/AUC. Terminal half-life ($t_{1/2,term}$) was calculated by regression analysis of the terminal phase of the plasma concentration curve. If there were fewer than 3 data points in the terminal phase of the plasma concentration curve, a terminal phase half-life and any pharmacokinetic parameters derived from the half-life were not calculated. Mean residence time (MRT), or the average time that a molecule resides in the systemic circulation, was calculated as $\int_0^\infty C \cdot t\, dt/AUC$. $C_{max}$ and $T_{max}$ are the maximum observed plasma concentration and the time of the maximum observed plasma concentration, respectively. The volume of distribution from the terminal phase ($V_z$) was calculated as Dose/($\lambda_z$*AUC) where $\lambda_z$ is the rate constant for the terminal phase estimated via linear regression of time vs. log concentration. For the mouse study, because destructive blood sampling was necessary, mean plasma concentration data for each dose group were analyzed. For the monkey study, each animal was analyzed separately, and parameters are reported as mean ± standard error of the mean (SEM). All data above the limit of detection were included in the analysis.

Area under the effect curve (AUEC) for granulocyte counts (mouse study) or absolute neutrophil counts (monkey study) were calculated using the linear trapezoidal rule. For the mouse data, pre-dose granulocyte counts were assumed to be equal to the mean value from 6 untreated mice. Granulocyte counts at all subsequent time points were from individual animals. For the monkey data, absolute neutrophil counts (ANC) on day 15 of the GLP toxicology study were plotted vs. AUC following the first dose and fit to a simple $E_{max}$ model with baseline using WinNonlin. The equation for this model is as follows:

$$E = E_0 + (E_{max}\text{-}E_0) * (AUC/[AUC + EC_{50}])$$

where E is the effect (ANC), $E_{max}$ is the maximum effect, $E_0$ is the baseline effect, and $EC_{50}$ is the AUC required to produce 50% of the drug-induced maximum effect ($E_{max}$-$E_0$). Pre-dose levels (AUC = 0) were included to allow for a better fit of the baseline ANC value.

### Hematology Statistical Methods

For each outcome, a one-way analysis of variance (ANOVA) was performed to compare average response among treatment groups. When the overall ANOVA F-test p value was > 0.05, the conclusion was that there were no significant differences among treatment groups. The equal variance assumption was assessed using Bartlett's test, and the normality assumption was tested using the Shapiro-Wilk test. If the equal variance assumption was violated, then groups were compared using two-sample *t* tests assuming unequal variance. Regardless of the test used, each p value reflected a comparison of average responses between two groups.

## RESULTS

### Hematopoietic Effects of Albugranin in Mice

A single administration of Albugranin to BDF1 mice at 3 dose levels significantly increased the total number of peripheral granulocytes (Fig 1A) and hematopoietic progenitor cells (Fig 1B). This effect was dose dependent. The maximum mobilization of peripheral granulocytes occurred on day 2 for Albugranin at 0.25 mg/kg and 1.25 mg/kg, with a 5.4-fold and 10-fold increase respectively, and on day 4 for Albugranin at 5 mg/kg (24-fold increase) when compared with mice that received vehicle only. Granulocytes returned to normal levels on days 3, 4, and 5 for Albugranin at 0.25, 1.25 and 5.0 mg/kg, respectively (Fig 1A). The maximum mobilization of granulocytes occurred on day 1 for a single administration of Filgrastim (0.25 and 1.25 mg/kg) (3-fold increase at both doses) ($p < 0.0025$ and $p < 0.003$, respectively) compared with control. Granulocytes returned to normal levels by day 2 after a single administration of Filgrastim. AUEC for granulocyte counts are listed in Table III. Following a single SC administration of Albugranin, AUEC increased by 95.3%, 326%, and 679% compared to HSA controls for the 0.25, 1.25, and 5.0 mg/kg doses, respectively. AUEC for a single dose of Filgrastim increased by 21.2% and 45.3% over buffer controls, for the 0.25 and 1.25 mg/kg doses, respectively.

A single administration of Albugranin also resulted in an increase in hematopoietic progenitor cells (c-kit+) at various stages of differentiation, and this effect was dose dependent (Fig 1B). The maximum mobilization of hematopoietic progenitors occurred on day 2 for Albugranin at 0.25 mg/kg (23-fold increase) and 1.25 mg/kg (58.3-fold increase), and on day 4 for Albugranin at 5 mg/kg (244-fold increase) when compared with mice that received control vehicles. Hematopoietic progenitors returned to normal levels on days 3, 4, and 5 for Albugranin groups at 0.25, 1.25, and 5 mg/kg, respectively (Fig 1B). The maximum mobilization of c-kit+ cells occurred on day 1 for Filgrastim at 0.25 and 1.25 mg/kg (4-fold [$p < 0.012$] and 9-fold [$p < 0.001$] increases, respectively). Hematopoietic progenitors returned to normal levels by day 2 after a single administration of Filgrastim (Fig 1B).

### Pharmacokinetics of Albugranin in Mice

Plasma concentrations in male BDF1 mice following a single injection of 0.25 or 1.25 mg/kg Albugranin or 0.25 mg/kg Filgrastim are shown in Figs 2A and 2B, respectively, for the IV and SC routes of administration. The IV curves do not follow multi-exponential decay, but instead appear to have a more rapid rate of decline when the concentration falls below ~2000 ng/mL. This is also observed for the SC curves. Pharmacokinetic parameters derived from noncompartmental analysis of the IV and SC groups are summarized in Table IV.

Following IV administration of 1.25 mg/kg, Albugranin has a clearance (CL) of 3.91 mL/h /kg, a terminal half-life ($t_{1/2,term}$) of 8.32 h, and a terminal volume of distribution ($V_z$)

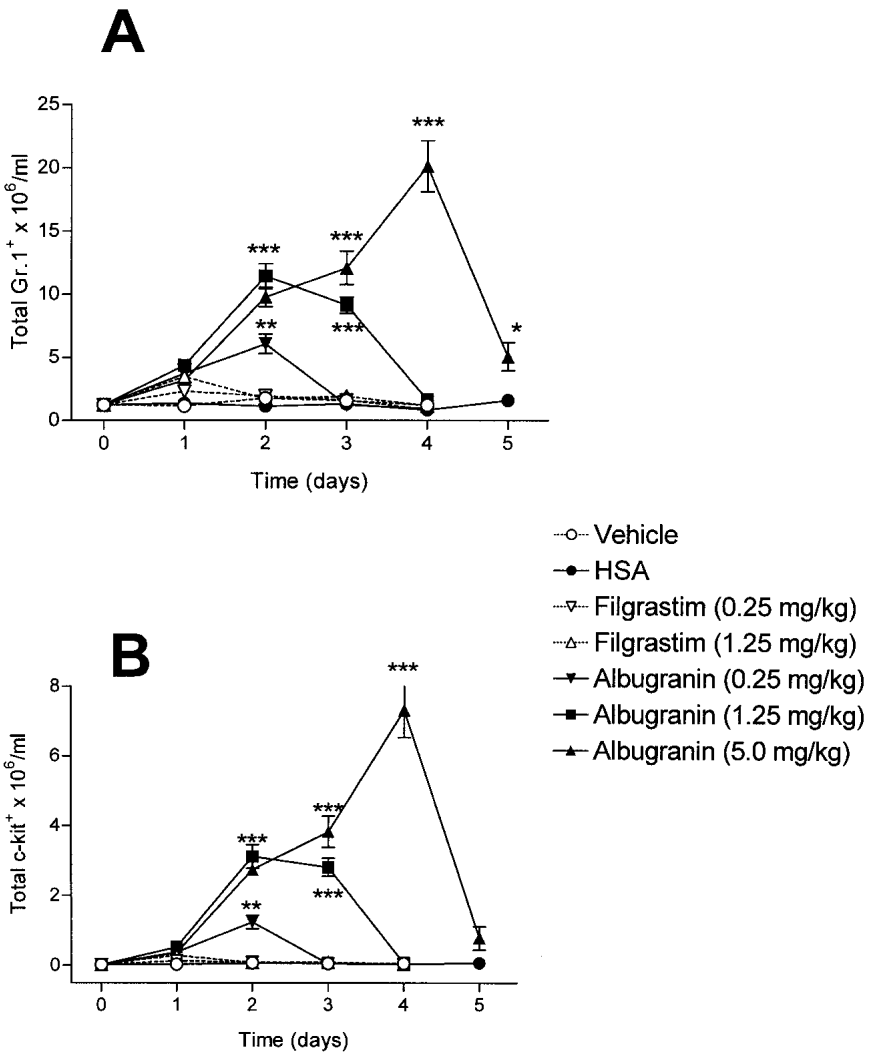


**Fig. 1.** Levels of peripheral blood granulocytes ($Gr.1^+$) (A) and hematopoietic progenitors ($c\text{-}kit^+$) (B) in BDF1 mice after single SC administration of Albugranin (time course). Mice (n = 6) received a single subcutaneous injection of vehicle, human serum albumin (at 5 mg/kg), single administrations of Albugranin at 3 dose levels (0.25 mg/kg, 1.25 mg/kg, or 5.0 mg/kg) or single administrations of Filgrastim at 2 dose levels (0.25 mg/kg or 1.25 mg/kg). The number of peripheral granulocytes and hematopoietic progenitor cells in mice were evaluated daily for 5 consecutive days. The data are presented as the total number of granulocytes (Gr.1+) (A) or hematopoietic progenitors cells (c-kit+) (B) per mL of blood. These values were obtained from the following formula: total cells per mL = cellularity per mL / 100 x % of Gr.1+ (or c-kit+) cells. The total number of $Gr.1^+$ or c-kit+ is expressed as the mean and standard error of the mean calculated for each group. Differences among treatment groups were analyzed using the heteroscedastic *t* test. Administration of single doses of Albugranin increased the total number of $Gr.1^+$ or c-kit+ cells compared with vehicle control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). This effect was dose dependent such that higher doses of Albugranin yielded more dramatic increases in the number of granulocytes and hematopoietic cells and this effect was sustained longer.

of 46.9 mL/kg. Following IV injection of 0.25 mg/kg, the CL, $t_{1/2,term}$, and $V_z$ of Albugranin are 4.74 mL/h/kg, 5.38 h, and 36.8 mL/kg, respectively (Fig 2A and Table IV). The apparent decrease in CL observed at the higher dose is consistent with a saturable clearance mechanism for Albugranin. This is in agreement with the known nonlinear pharmacokinetics of G-CSF, and most likely represents clearance via receptor-mediated endocytosis (10). The CL, $t_{1/2,term}$, and $V_z$ of Filgrastim following IV injection of 0.25 mg/kg are 35.8 mL/h/kg, 2.51 h, and 130 mL/kg, respectively. The CL of Filgrastim is approximately 8 times faster than that of Albugranin, and the terminal half-life is less than half that of Albugranin. The half-life of Filgrastim measured here is comparable to other reported values (11).

Following SC injection of 0.25 or 1.25 mg/kg, Albugranin has a clearance over bioavailability (CL/F), $t_{1/2,term}$, and volume of distribution over bioavailability ($V_z$/F) of 6.37-12.2 mL/h/kg, 5.58-5.68 h, and 52.2-98.2 mL/kg, respectively (Fig 2B and Table IV). The SC bioavailability of Albugranin was 38.9% at 0.25 mg/kg and 61.4% at 1.25 mg/kg. Filgrastim has

**Table III.** Area under the Effect Curve (±SEM) for Granulocyte Counts in BDF1 Mice Receiving a Single SC Dose of Albugranin or Filgrastim

| Treatment | Dose (mg/kg) | AUEC[a] (h × $10^6$/mL) | % Increase over control[b] |
|---|---|---|---|
| HSA | 0 | 149 ± 6.36 | N/A |
| Buffer | 0 | 137 ± 8.81 | N/A |
| Albugranin | 0.25 | 291 ± 28.2 | 95.3 |
| Albugranin | 1.25 | 634 ± 34.8 | 326 |
| Albugranin | 5.0 | 1160 ± 97.4 | 679 |
| Filgrastim | 0.25 | 166 ± 7.99 | 21.2 |
| Filgrastim | 1.25 | 199 ± 16.1 | 45.3 |

[a] For calculating AUEC, pre-dose granulocyte counts were assumed to be equal to the mean value from 6 untreated mice. Granulocyte counts at all subsequent time points were from individual animals.
[b] For calculating % increase over control, HSA controls were used for Albugranin-treated mice and buffer controls were used for Filgrastim-treated mice.
Abbreviations: SEM = standard error of the mean, AUEC = area under the effect curve, N/A = not applicable, human serum albumin (HSA).

a CL/F, $t_{1/2,term}$, and $V_z$/F of 163.8 mL/h/kg, 2.54 h, and 601 mL/kg, respectively following SC injection of 0.25 mg/kg, and a SC bioavailability of 21.8%. This value of bioavailability for Filgrastim is lower than reports in the literature (12, reported bioavailabilty in mice~100%). However, because of the nonlinear nature of G-CSF pharmacokinetics, true bioavailability is difficult to assess. As with the IV route of administration, Albugranin seems to have a much slower clearance and longer terminal half-life and mean residence time than Filgrastim following SC administration, resulting in a much greater exposure (AUC) following a single dose.

### Pharmacodynamic Effects of Albugranin on Hematopoiesis in Monkeys

A comparison between the single-dose effects of Albugranin (100 μg/kg) vs. daily Filgrastim (5 μg/kg) is illustrated for ANC in Fig 3. In the 6 days following a single SC administration of Albugranin at 100 μg/kg, there were significant increases in the absolute neutrophil count (ANC) that were maximal approximately 24 h following dosing and gradually decreased to baseline over the following 5 days. The ANC response after a second SC administration of Albugranin (100 μg/kg) on day 7 was similar in magnitude and duration to the first (data not shown).

Multidose effects of Albugranin vs. Filgrastim on ANC are illustrated in Fig 4. When Albugranin was administered SC every 4 days at either 25 or 100 μg/kg, the peak ANC 24 h after administration of Albugranin increased slightly over the first 3 injections, then decreased for the fourth injection. Filgrastim administered at 5 μg/kg daily for 14 days resulted in a consistent but modest increase in ANC during the treatment period. The maximum Filgrastim effect was observed on days 8–12.

AUEC for ANC are listed in Table V. When Albugranin was given every 4 days for two weeks at 25 and 100 μg/kg, the AUEC increased by 118 and 262%, respectively, compared to vehicle controls. When Albugranin was given every 7 days, AUEC increased by 160% compared to controls. In comparison, administration of Filgrastim daily for 14 days at 5 μg/kg resulted in a 93% increase in AUEC over controls.

The effect of Albugranin on total WBC paralleled but was smaller in magnitude than the increase in neutrophil levels. In monkeys treated with Albugranin at 100 μg/kg, the WBC at 24 h post administration was increased 4-fold from 13.09 ± 1.87 K/μl in the time-matched vehicle control to 50.7 ± 8.54 K/μl (mean ± SEM) in the Albugranin treated group (maximal response). Monocyte numbers, which are reported to increase in the periphery in response to G-CSF (13), were modestly increased only in the high dose Albugranin group (100 μg/kg) administered every 4 days. Absolute numbers of monocytes peaked at 2.93 ± 0.52 K/μl compared with 1.36 ± 0.55 K/μl in the time matched vehicle control. The absolute numbers of lymphocytes were not affected by treatment with Albugranin. Since there was little to no effect of Albugranin on other hematopoietic cells, the increase in WBC at 24 h after Albugranin (100 μg/kg) is essentially due to the increase in ANC.

### Pharmacokinetics of Albugranin in Monkeys

Plasma concentration data following subcutaneous injection of Albugranin at 100, 500, and 1000 μg/kg are shown in Fig 5, and the corresponding pharmacokinetic parameters are listed in Table VI. Following a single SC administration, CL/F of Albugranin was 27.5 ± 4.31, 10.9 ± 1.15, and 7.90 ± 1.12 mL/h/kg for the 100, 500, and 1000 μg/kg doses, respectively. The observed decrease in CL/F with increasing dose of Albugranin is similar to what was observed in BDF1 mice, and is consistent with a saturable clearance mechanism or a dose-dependent change in bioavailability. Albugranin has a $t_{1/2,term}$ of 7.73 ± 0.15 to 13.3 ± 1.61 h However, because of the apparent change in shape of the plasma concentration curve with increasing dose, the mean residence time (MRT, the average amount of time that a molecule spends in the circulation) offers a more robust parameter for time comparisons. Albugranin had an MRT of 19.4 ± 2.38, 19.4 ± 0.85, and 27.3 ± 0.87 for the 100, 500, and 1000 μg/kg dose groups, respectively.

The ANC on day 15 (24 h after the third dose in this study) are plotted in Fig 6 against the AUC following the first dose. These data were fit to a simple $E_{max}$ model with baseline, and model parameters are listed in Table VII. The $E_0$ is 5.2 x$10^3$/μL, which is within the normal range for ANC. The $E_{max}$ is 89.5 x$10^3$/μL, and the $EC_{50}$ is 6981 h-ng/mL. The 100 μg/kg dose group, with a mean day 15 ANC of 34.0 ± 1.5 x$10^3$/μL and an AUC of 4137 ± 435 h-ng/mL, has a response that is approximately 32% of $E_{max}$. The 500 and 1000 μg/kg groups (AUC = 51447 ± 6630 and 141462 ± 12588 h-ng/mL, respectively) have near maximal responses (ANC= 73.5 ± 3.67 and 87.9 ± 6.16 x$10^3$/μL, respectively).

## DISCUSSION

In these studies, we have shown that the *in vivo* effects of Filgrastim can be markedly improved in mice and monkeys by fusion with albumin and that the fusion product, Albugranin, has a decreased clearance and longer circulating half-life than the parent drug. These changes in pharmacokinetics are associated with a sustained neutrophilic leukocytosis in both species.

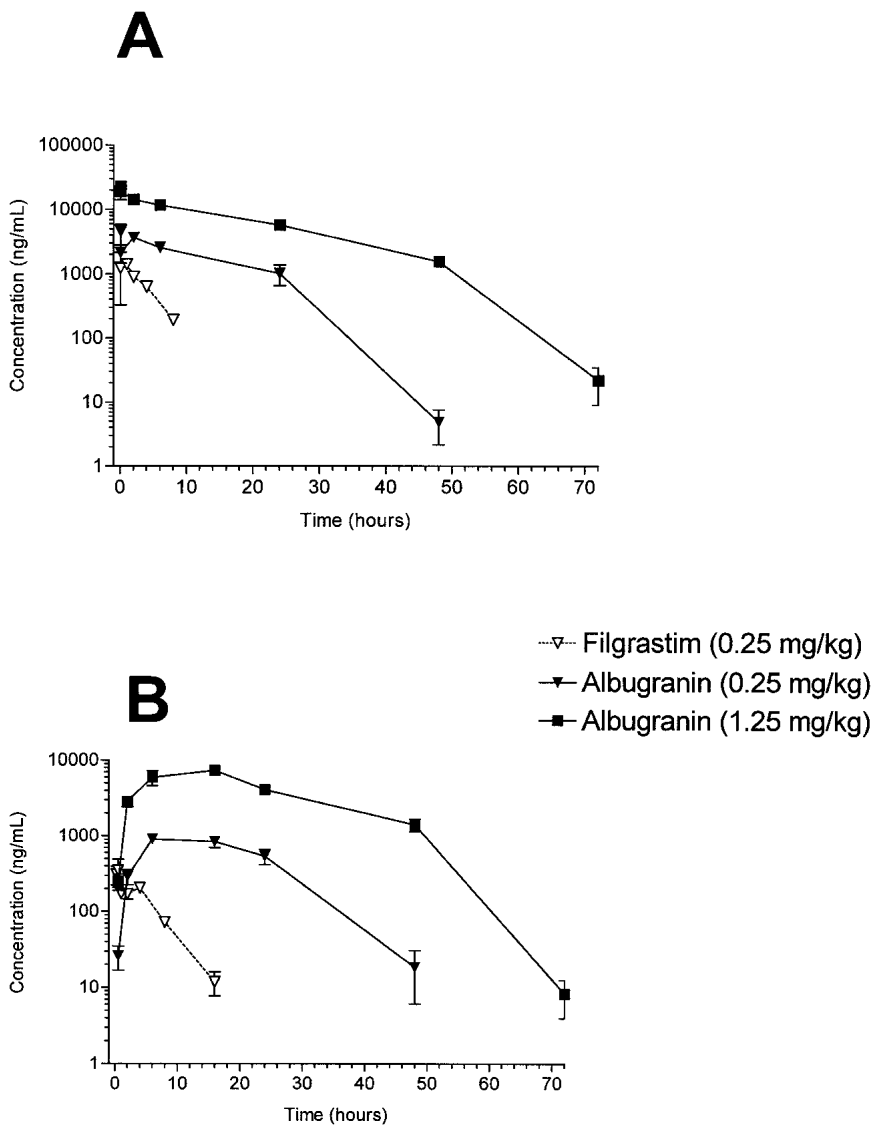


**Fig. 2.** Plasma concentrations in BDF1 mice following intravenous (IV) or subcutaneous (SC) injection of Albugranin or Filgrastim. Male BDF1 mice (n = 3) were injected IV (Fig 2A) or SC (Fig. 2B) with single doses of either 1.25 or 0.25 mg/kg Albugranin or 0.25 mg/kg Filgrastim. Plasma concentrations were followed out to 72 h. Each point represents the mean value and error bars represent the standard error of the mean.

In BDF1 mice, the clearance of Albugranin is approximately eight times slower than that of Filgrastim and the terminal half-life is more than twice that of Filgrastim for both IV and SC administration. A decrease in clearance was observed at the higher dose of Albugranin and, although not statistically significant, is consistent with a saturable clearance mechanism, in agreement with the known nonlinear pharmacokinetics of G-CSF (10). However, because the decrease in CL at the higher dose was greater for SC administration than IV administration, it might also reflect differences in bioavailability (e.g., absorption form the SC site). Albugranin produced a prolonged hematopoietic activity in mice with an increase in the number of peripheral granulocytes and hematopoietic progenitors for up to 5 days after a single dose of Albugranin vs. 1 day after a single dose of Filgrastim. The magnitude of the neutrophilic response to Albugranin was dose dependent.

Albugranin was well tolerated by cynomolgus monkeys when administered every 4 (25 or 100 μg/kg, SC) or 7 days (100 μg/kg, SC) over a 14-day period. Multiple days of dosing with Albugranin caused an increase in the absolute number of granulocytes and in WBC. This increase was maintained for a longer period after cessation of treatment in the Albugranin-treated monkeys (up to 5 days) vs. the Filgrastim-treated monkeys (less than 3 days). In addition, Albugranin had a long MRT ranging from 17.9 to 27.2 h in cynomolgus monkeys when administered at doses of 100–1000 μg/kg.

The response in ANC after each dose administration in monkeys was variable, leading to an apparent decrease in effect on ANC following the fourth dose administration at 100 μg/kg. There was a relatively large standard error for the 100 μg/kg/dose group at this timepoint, in part due to the fact that one sample, from a previously strong responder, was clotted and could not be evaluated. However, the response

**Table IV.** Pharmacokinetic Parameters from Noncompartmental Analysis for Albugranin and Filgrastim following Single IV and SC Injection in BDF1 Mice

| Parameter | Albugranin | Albugranin | Filgrastim |
|---|---|---|---|
| Route | | IV | |
| Dose (mg/kg) | 1.25 | 0.25 | 0.25 |
| AUC (h-ng/ml) | 319857 | 52704 | 6990 |
| CL (mL/h/kg) | 3.91 | 4.74 | 35.8 |
| $V_z$ (mL/kg) | 46.9 | 36.8 | 130 |
| $t_{1/2,term}$ (h) | 8.32 | 5.38 | 2.51 |
| MRT (h) | 11.2 | 16.7 | 3.3 |
| $C_{max}$ (ng/mL) | 23390 | 5810 | 6098 |
| Route | | SC | |
| Dose (mg/kg) | 1.25 | 0.25 | 0.25 |
| AUC (h * ng/mL) | 196250 | 20513 | 1526 |
| CL/F (mL/h/kg) | 6.37 | 12.2 | 163.8 |
| $V_z$/F (mL/kg) | 52.2 | 98.2 | 601 |
| $t_{1/2,term}$ (h) | 5.68 | 5.58 | 2.54 |
| MRT (h) | 20.7 | 16.2 | 4.87 |
| $C_{max}$ (ng/mL) | 7339 | 901 | 308.6 |
| $T_{max}$ (h) | 16 | 6 | 0.25 |
| Bioavailability (%) | 61.4 | 38.9 | 21.8 |

Abbreviations: IV = intravenous; SC = subcutaneous; AUC = area under the curve; CL = clearance; $V_z$ = volume of distribution using the terminal phase; $t_{1/2,term}$ = terminal half-life; $C_{max}$ = maximum concentration; $T_{max}$ = time of maximum concentration; MRT = mean residence time; CL/F = clearance over bioavailability; $V_z$/F = volume of distribution over bioavailability.

was similar to that observed following the first and second dose administrations. In addition, the ANC on day 16, 4 days after the fourth dose administration, remained slightly elevated as compared to the ANC 4 days after the previous dose administrations.

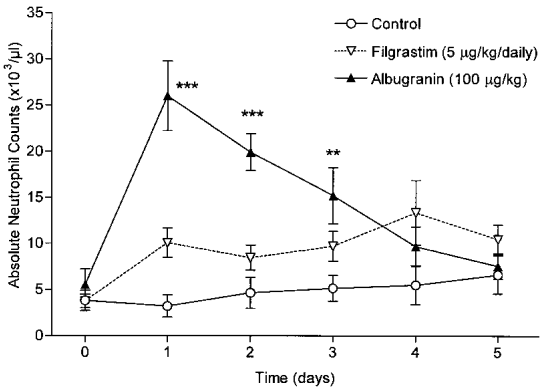


**Fig. 3.** Effect of Albugranin on monkey absolute neutrophil count (ANC) after a single administration. This figure depicts the first 5 days following a single subcutaneous (SC) administration of Albugranin (100 µg/kg; closed triangles); monkeys were additionally administered Albugranin on day 7 with a similar response (data not shown). Vehicle (open circles) was administered SC on days 0, 4, 8 and 12, with days 0–5 shown here. Filgrastim (open triangles and dashed line) was administered subcutaneously at 5 µg/kg/day on days 0–13, with days 0–5 shown here. ANC is reported in thousands of cells per microliter (K/µl). Each point represents the mean of 3 or 4 monkeys, with error bars representing the standard error of the mean. The ANC was significantly increased compared with vehicle on days 1 through 3 following a single administration of Albugranin at 100 µg/kg (**$p < 0.01$, ***$p < 0.001$).

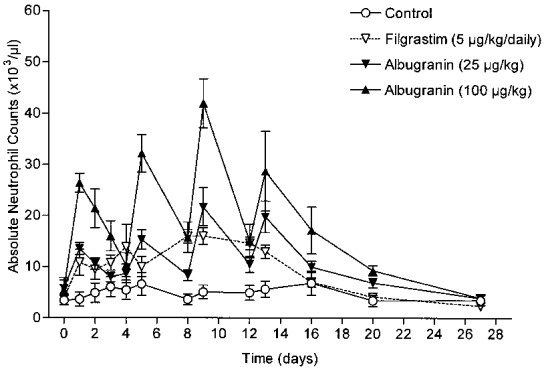


**Fig. 4.** Effect of Albugranin on monkey absolute neutrophil count (ANC) after multiple administrations. Albugranin (25 or 100 µg/kg; closed triangles) or vehicle (open circles) was administered subcutaneous (SC) every 4 days (Q4D), corresponding to days 0, 4, 8, and 12. Filgrastim (open triangles and dashed line) was administered SC at 5 µg/kg/day on each of days 0–13. ANC is reported in thousands of cells per microliter (K/µl). Each point represents the mean of 3 or 4 monkeys, with error bars representing the standard error of the mean. The ANC was significantly increased in Albugranin-treated monkeys compared with vehicle control on days 9 and 13 when administered at 25 µg/kg, and on days 1, 2, 3, 5, 9, 12, 13, and 20 when administered at 100 µg/kg ($p < 0.01$). Filgrastim-treated monkeys had a significant elevation in ANC as compared with vehicle controls on days 8 and 12 ($p < 0.01$).

**Table V.** Area under the Effect Curve (±SEM) for Granulocyte Counts in Cynomolgus Monkeys Receiving SC Doses of Albugranin or Filgrastim

| Treatment | Dose (µg/kg/dose) | AUEC (h × $10^3$/µL) | % Increase over vehicle control |
|---|---|---|---|
| Vehicle | 0 | 129 ± 36.2 | N/A |
| Albugranin Q4D | 25 | 281 ± 28.7 | 118 |
| Albugranin Q4D | 100 | 467 ± 44.2 | 262 |
| Albugranin Q7D | 100 | 335 ± 35.6 | 160 |
| Filgrastim QD | 5 | 249 ± 18.5 | 93.0 |

Abbreviations: SEM = standard error of the mean, AUEC = area under the effect curve, N/A = not applicable.

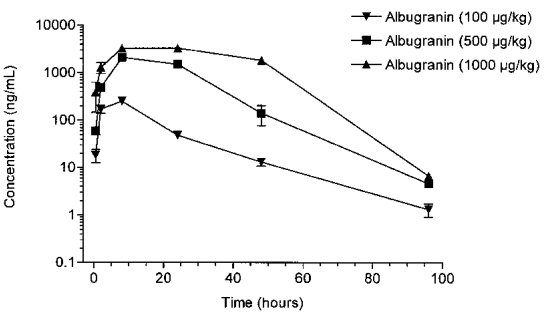


**Figure 5.** Plasma concentrations in cynomolgus monkeys following a single subcutaneous (SC) injection of Albugranin. Cynomolgus monkeys (5 males and 5 females in each group) were injected SC with either 100, 500, or 1000 µg/kg Albugranin. Each point represents the mean, and error bars represent the standard error of the mean. At time points where some samples had detectable levels of Albugranin but others were below the limit of detection of the enzyme-linked immunosorbent assay, a value of zero was assigned to each of the samples below the detection limit before calculating mean values for the purpose of graphing only.

**Table VI.** Pharmacokinetic Parameters (±SEM) for Albugranin following a Single SC Dose to Cynomolgus Monkeys

| Parameter | Albugranin | Albugranin | Albugranin |
|---|---|---|---|
| Dose (μg/kg) | 100 | 500 | 1000 |
| n | 10 | 10 | 10 |
| AUC (h-ng/mL) | 4217 ± 436 | 51507 ± 6629 | 141537 ± 12586 |
| CL/F (mL/h/kg) | 27.5 ± 4.31 | 10.9 ± 1.15 | 7.90 ± 1.12 |
| Vz/F (mL/kg) | 591 ± 156 | 146 ± 18.1 | 90.1 ± 15.3 |
| $t_{1/2,term}$ (h) | 13.3 ± 1.61 | 9.07 ± 0.24 | 7.73 ± 0.15 |
| MRT (h) | 19.4 ± 2.38 | 19.4 ± 0.85 | 27.3 ± 0.87 |
| $C_{max}$ (ng/mL) | 249 ± 38.8 | 2158 ± 235 | 3661 ± 1073 |
| $T_{max}$ (h) | 8 | 11.2 | 16 |

Abbreviations: SEM = standard error of the mean; SC = subcutaneous; AUC = area under the curve; CL/F = clearance over bioavailability; $V_z/F$ = volume of distribution from the terminal phase over bioavailability; $t_{1/2,term}$ = terminal half-life; $C_{max}$ = maximum concentration; $T_{max}$ = time of maximum concentration; MRT = mean residence time.

**Table VII.** Model Parameters from fit of Day 15 ANC vs. First Dose AUC in Cynomolgus Monkeys

| Parameter | Estimate | Standard error of estimate |
|---|---|---|
| $E_{max}$ ($\times 10^3/\mu L$) | 89.5 | 3.79 |
| $EC_{50}$ (h-ng/mL) | 6981 | 1215 |
| $E_0$ ($\times 10^3/\mu L$) | 5.2 | 0.58 |

Abbreviations: $E_{max}$ = maximum effect (ANC), $EC_{50}$ = AUC required to produce 50% of the drug-induced maximum effect ($E_{max}$-$E_0$), $E_0$ = baseline effect.

In a separate study, the pharmacokinetics of Albugranin at 100, 500, and 1000 μg/kg were determined after a single dose, and the peak neutrophil response was determined on day 15, 24 h after the third weekly SC administration. Day 15 ANC vs. AUC data from cynomolgus monkeys were fit to a simple $E_{max}$ model with baseline. While the model fit the data reasonably well, the relationship is only empirical, not mechanistic, since the pharmacokinetics of G-CSF are know to be nonlinear and dependent on neutrophil count (10,15). A mechanistic model involving multiple pathways has been successfully used to describe the PK/PD of G-CSF (16), but it requires a much more extensive data set for parameter specification than was available in this study. The $E_{max}$ model does, however, allow an approximation of the neutrophil response at given levels of exposure. In cynomolgus monkeys 500 and 1000 μg/kg doses result in near maximal responses while 100 μg/kg induces a response that is approximately 32% maximal.

Although Albugranin has an extended plasma half-life relative to G-CSF, it does not remain in the circulation as long as HSA ($t_{1/2}$ ~19 days in humans, 6). Thus the pharmacokinetics of Albugranin appear to depend on the characteristics of both of its constituent molecules. The primary clearance mechanisms for G-CSF are renal clearance and receptor-mediated endocytosis by neutrophils (8), whereas HSA distributes between vascular and extravascular compartments and is degraded in a number of tissues including liver, muscle, and skin (14). The relatively large molecular weight (85 kDa) of Albugranin imparted by the fusion with HSA should result in reduced renal clearance, while the presence of the G-CSF portion of the molecule allows Albugranin to be cleared via the receptor-mediated route. As the dose of G-CSF is increased, the receptor-mediated component of clearance presumably becomes saturated, resulting in decreased total body clearance, and this decrease in clearance with increasing dose was observed in both mice and monkeys. Because of reduced renal clearance, Albugranin may be more dependent on receptor-mediated clearance than G-CSF.

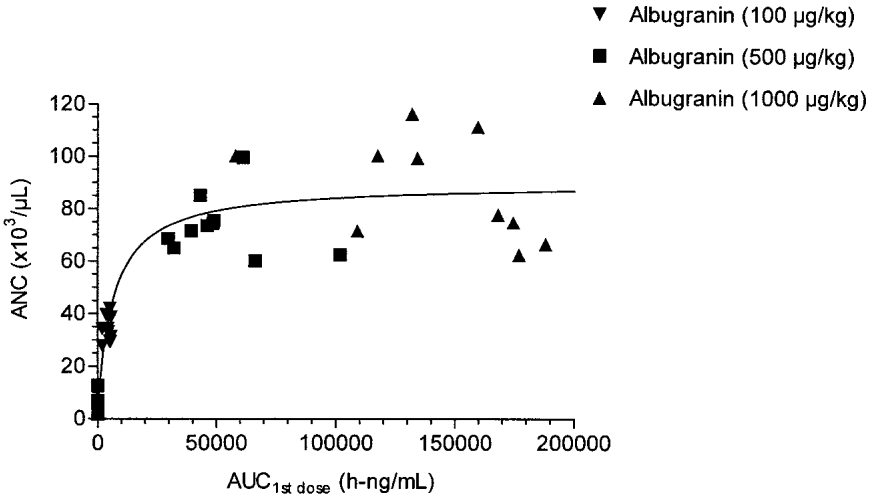


**Fig. 6.** Maximum effect model fit to absolute neutrophil count vs. area under the curve (AUC) data in cynomolgus monkeys. Cynomolgus monkeys (5 males and 5 females in each group) were injected subcutaneously (SC) with either 100, 500, or 1000 μg/kg Albugranin on study days 0, 7, 14, and 21. The absolute neutrophil count on day 15 is plotted verses the AUC following the first dose of Albugranin. Each point represents an individual monkey. Pre-dose levels (AUC = 0) for all animals were included to allow for a better fit of the baseline absolute neutrophil count value.

A long-acting form of Filgrastim given at weekly intervals would greatly add to the convenience of its administration and possibly enhance therapeutic benefit. A polyethylene glycol-conjugated G-CSF (Neulasta) has been shown in preclinical and clinical studies to have activity for several days following a single administration without significant toxicity, and has been recently approved for marketing (4,5). Since Albugranin is a protein comprising rHSA fused to rG-CSF, it may be simpler to manufacture and result in a more uniform chemical entity. The preclinical studies presented here have demonstrated that Albugranin has good potential for being developed into a long-acting form of rG-CSF.

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