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(54) **Title:**
NEW STABLE FORMULATIONS OF RECOMBINANT HUMAN ALBUMIN-HUMAN GRANULOCYTE COLONY STIMULATING FACTOR FUSION PROTEIN

(57) **Abstract:**
Disclosed are compositions and methods for treating, preventing and ameliorating conditions and diseases characterized by a lowered white blood cell count. The methods and compositions described herein include a fusion polypeptide formed from human serum albumin protein ("HSA") and human granulocyte- colony stimulating factor ("G-CSF").

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[Continued on next page]

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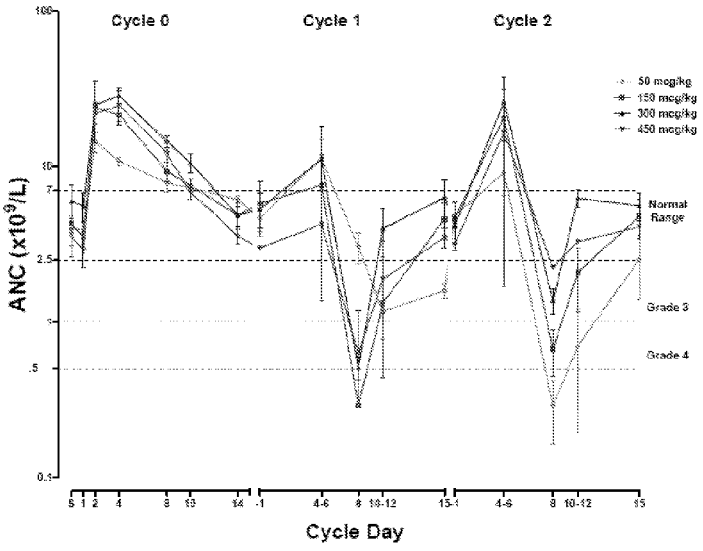
FIGURE 7A



(57) Abstract: Disclosed are compositions and methods for treating, preventing and ameliorating conditions and diseases characterized by a lowered white blood cell count. The methods and compositions described herein include a fusion polypeptide formed from human serum albumin protein ("HSA") and human granulocyte- colony stimulating factor ("G-CSF").

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Recombinant Human Albumin-Human Granulocyte Colony Stimulating Factor for the Prevention of Neutropenia

[0001] The present application claims the benefit of U.S. Provisional Application No. 61/145,440 filed on January 16, 2009 and U.S. Provisional Application No. 61/145,436 filed on January 16, 2009. The contents of U.S. Provsional Application No. 61/145,440 and U.S. Provisional Application No. 61/145,436 are hereby incorporated by reference in their entirety.

BACKGROUND

[0002] Leukopenia is a reduction in the circulating White Blood Cells (WBC) and is often defined as a WBC count < 4000/mL. The main cells involved in leukopenia are neutrophils. However a reduced number of lymphocytes, monocytes, eosinophils, or basophils may also contribute to the decreased total cell count (Merck Manual, 17th edition).

[0003] Neutropenia is characterized by a reduction in the blood neutrophil count, often leading to increased susceptibility to bacterial and fungal infections. Neutropenia is classified by the neutrophil count and the relative risk of infection: mild (1000 to 1500/mL), moderate (grade 3, 500 to 1000/mL), or severe (grade 4, < 500/mL). Acute and severe neutropenia is a life-threatening condition as it predisposes the patient to rapidly fatal infections (Merck Manual, 17th edition).

[0004] Neutropenia can be caused by impaired production of neutrophils in the bone marrow, or by accelerated destruction of neutrophils. Acute neutropenia may occur over a few days when neutrophil use is rapid and production is severely impaired. Chronic neutropenia may last for many months and is often caused by reduced production or sequestration of neutrophils in the spleen. Neutropenia may be classified by whether it arises secondary to factors extrinsic to marrow myeloid cells or whether an intrinsic defect appears to be present in the myeloid progenitors (Merck Manual, 17th edition).

[0005] Neutropenia and its infectious complications are among the most common and serious adverse effects of cytotoxic chemotherapy and other cancer therapies such as radiation therapy, biotherapy, targeted therapy and bone marrow transplantation. Cytotoxic chemotherapy, which works by destroying fast-growing cells, induces neutropenia because of

the high proliferative rate of neutrophil precursors and the rapid turnover of blood neutrophils (Merck Manual, 17th edition). The most common symptoms of neutropenia in patients with undergoing chemotherapy include fever, mouth sores, and ear infections. Patients with profound neutropenia often suffer from pyogenic infections such as septicemia, cutaneous cellulitis, liver abscesses, furunculosis, pneumonia, stomatitis, gingivitis, perirectal inflammation, colitis, sinusitis, and otitis media. Chemotherapy may have to be delayed until the body can produce more neutrophils and a lower dosage may have to be given, resulting in the treatment being less effective.

SUMMARY

[0006] Described herein are methods and compositions useful for the treatment, amelioration and prevention of conditions characterized by a lower than normal white blood cell count. Such conditions include but are not limited to leukopenia and neutropenia.

[0007] In a first embodiment, described is a method of treating or preventing neutropenia in a human subject comprising administering to a human subject exhibiting neutropenia or at risk of developing neutropenia, recombinant human albumin-human granulocyte colony stimulating factor in an amount effective to treat the subject. In an exemplary embodiment, the human subject can be suffering from a non-myeloid malignancy and receiving at least one myelosuppressive anti-cancer drug associated with a clinically significant incidence of febrile neutropenia.

[0008] In a second embodiment, described is a method of treating or preventing leukopenia in a human subject comprising administering to a human subject exhibiting leukopenia or at risk of developing leukopenia, recombinant human albumin-human granulocyte colony stimulating factor in an amount effective to treat the subject.

[0009] In a third embodiment, described is a method of decreasing the incidence of infection, as manifested by febrile neutropenia, in a human subject with non-myeloid malignancies and receiving at least one myelosuppressive anti-cancer drug associated with a clinically significant incidence of febrile neutropenia, comprising administering to the subject recombinant human albumin-human granulocyte colony stimulating factor in an amount effective to treat the subject.

[0010] In some methods, compounds described herein are useful for decreasing the incidence of infection, such as infection manifested by febrile neutropenia. In some embodiments, the compositions and methods include a fusion polypeptide formed from human serum albumin protein ("HSA") and human granulocyte-colony stimulating factor ("G-CSF"). The fusion polypeptide is 759 amino acids in length; amino acids 1-585 of the fusion correspond to amino acids from the mature form of HSA, and amino acids 586-759 of the fusion correspond to amino acids of the mature form of human G-CSF. The amino acid sequences of the fusion protein is presented in **FIG. 1.** The fusion polypeptide, termed Neugranin™ ("NEUG") is administered to patients exhibiting or at risk of exhibiting leukopenia or neutropenia. For example, in some embodiments, methods include treating leukopenia or neutropenia in a human subject by administering recombinant human albumin-human granulocyte colony stimulating factor in an amount effective to treat the subject.

[0011] In some embodiments, the neutropenia is primary neutropenia, acute neutropenia, severe chronic neutropenia (SCN), severe congenital neutropenia (Kostmann's syndrome), severe infantile genetic agranulocytosis, benign neutropenia, cyclic neutropenia, chronic idiopathic neutropenia, secondary neutropenia, syndrome associated neutropenia, or immune-mediated neutropenia.

[0012] In other embodiments, the neutropenia is caused or associated with radiation, alcoholism, drugs, allergic disorders, aplastic anemia, autoimmune disease, T-γ lymphoproliferative disease (T-γ LPD), myelodysplasia, myelofibrosis, dysgammaglobulinemia, paroxysmal nocturnal hemoglobinuria, cancer, vitamin B_{12} deficiency, folate deficiency, viral infection, bacterial infection, spleen disorder, hemodialysis, transplantation, leukemia, myeloma, lymphoma, metastatic solid tumors which infiltrate and replace the bone marrow, toxins, bone marrow failure, Schwachman-Diamond syndrome, cartilage-hair hypoplasia, dyskeratosis congenita, glycogen storage disease type IB, splenomegaly of any cause, and intrinsic defects in myeloid cells or their precursors. In some embodiments, the neutropenia is caused or associated with cytotoxic chemotherapy.

[0013] In some embodiments, the human subject is suffering from a non-myeloid malignancy, for example, breast cancer, and is receiving cytotoxic chemotherapy. For example, in some embodiments the patient is receiving at least one myelosuppressive anti-cancer drug associated with a clinically significant incidence of febrile neutropenia. In some

embodiments, the myelosuppressive anticancer drugs are doxorubicin and docetaxel. In further embodiments, about 50 mg/m^2 doxorubicin and about 75 mg/m^2 docetaxel are administered sequentially by intravenous infusion on the same day for at least one treatment cycle. In still other embodiments, about 60 mg/m^2 doxorubicin and about 75 mg/m^2 docetaxel are administered sequentially by intravenous infusion on the same day for at least one treatment cycle.

[0014] In other embodiments, methods include decreasing the incidence of infection, as manifested by febrile neutropenia, in human subjects. In some embodiments, the human subject is suffering from non-myeloid malignancies and is receiving at least one myelosuppressive anti-cancer drug associated with a clinically significant incidence of febrile neutropenia. In some embodiments, recombinant human albumin-human granulocyte colony stimulating factor is administered to the subject in an amount effective to treat the neutropenia in the subject.

[0015] In some embodiments, the duration or severity of neutropenia is reduced or neutropenia is eliminated in a subject. For example, in some embodiments, grade 4 or grade 3 neutropenia in the subject is eliminated. In other embodiments, the duration of grade 4 or grade 3 neutropenia is reduced. For example, in some embodiments the duration of grade 4 neutropenia in the subject is less than 5 days; in some embodiments, the duration of grade 4 neutropenia in the subject is less than 4 days, less then 3 days or less than 2 days. In other embodiments, the duration of grade 3 neutropenia in the subject is eliminated, and/or the duration of grade 3 neutropenia in the subject is decreased as compared to subjects who do not receive treatment with human albumin-human granulocyte colony stimulating factor.

[0016] In some embodiments, administering recombinant human albumin-human granulocyte colony stimulating factor induces a rise in white blood cells ("WBC") or decreases a loss of WBC in a subject. For example, in some embodiments, the number of neutrophils is increased in the subject; the decrease in the number of neutrophils is inhibited in the subject, the nadir absolute neutrophil count ("ANC") is increased in the subject, the recovery ANC is increased in the subject, and/or the time to ANC recovery is reduced in the subject.

[0017] In some embodiments, the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is from about 40μg/kg to

about 500 μg/kg; in other embodiments, the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is about 50μg/kg to about 450 μg/kg. In still other embodiments, the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is about 50 μg/kg, about 100 μg/kg, about 150μg/kg, about 200 μg/kg or about 250 μg/kg. In further embodiments, the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is about 300μg/kg, about 350 μg/kg or about 400 μg/kg. In alternative embodiments, the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is about 450μg/kg. In yet other embodiments, the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is from about 20 to about 100 mg. In further embodiments, the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is from about 30 mg to about 60 mg. In further embodiments, the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is about 30mg, about 40mg, about 50 mg, or about 60mg.

[0018] In some embodiments, recombinant human albumin-human granulocyte colony stimulating factor is administered after chemotherapy (*e.g.*, administration of a myelosuppressive anti-cancer drug). For example, in some embodiments, recombinant human albumin-human granulocyte colony stimulating factor is administered during chemotherapy, or is administered within 2 hours, within 4 hours, within 6 hours, within 12 hours, within 18 hours, within 24 hours or within 48 hours of chemotherapy administration.

[0019] In another embodiment of the invention, recombinant human albumin-human granulocyte colony stimulating factor can be administered after myelosuppressive anti-cancer drug administration. For example, the recombinant human albumin-human granulocyte colony stimulating factor can be administered at a time selected from the group consisting of: (a) at least 2 hours after administration of the myelosuppressive anti-cancer drug; (b) at least 4 hours of administration of the myelosuppressive anti-cancer drug; (c) at least 6 hours after administration of the myelosuppressive anti-cancer drug; (d) at least 12 hours after administration of the myelosuppressive anti-cancer drug; (e) at least 18 hours after administration of the myelosuppressive anti-cancer drug; (f) at least 24 hours after

administration of the myelosuppressive anti-cancer drug; (g) at least 48 hours after administration of the myelosuppressive anti-cancer drug; or (h) during, or substantially concurrently with, the administration of the myelosuppressive anti-cancer drug.

[0020] In some embodiments, administering recombinant human albumin-human granulocyte colony stimulating factor during or after chemotherapy treatment induces a rise in WBC and/or induces a rise in ANC. For example, in some embodiments, ANC and WBC return to normal by day 10 after chemotherapy. In other embodiments ANC and WBC return to normal by day 11 after chemotherapy, by day 12 after chemotherapy, by day 13 after chemotherapy, by day 14 after chemotherapy or by day 15 after chemotherapy. In some embodiments, on day 14 after chemotherapy administration the rise in ANC in patients treated with recombinant human albumin-human granulocyte colony stimulating factor is lower than the rise in ANC in patients treated with an equivalent dose of pegfilgrastim.

[0021] In some embodiments, administering recombinant human albumin-human granulocyte colony stimulating factor induces a rise in lymphocytes, monocytes, eosinophils, or basophils. For example, in some embodiments, the number lymphocytes, monocytes, eosinophils, or basophils is increased in the subject. In other embodiments, the decrease in the number of lymphocytes, monocytes, eosinophils, or basophils is inhibited in the subject.

[0022] In some embodiments, particularly for methods of treating or preventing neutropenia, a result achieved can be selected from the group consisting of: (a) grade 4 neutropenia in the subject is eliminated; (b) grade 4 neutropenia in the subject is reduced; (c) the duration of severe neutropenia is reduced in the subject; (d) the duration of grade 3 neutropenia in the subject is eliminated; (e) the duration of grade 3 neutropenia in the subject is decreased; or (f) any combination thereof.

[0023] In some embodiments, particularly for methods of treating or preventing neutropenia, administering recombinant human albumin-human granulocyte colony stimulating factor induces a rise in white blood cells (WBC). In yet other embodiments, particularly for methods of treating or preventing neutropenia, the result achieved is selected from the group consisting of (a) the number of neutrophils is increased in the subject; (b) a decrease in the number of neutrophils is inhibited in the subject; (c) the nadir absolute neutrophil count (ANC) is increased in the subject; (d) the recovery ANC is increased in the

subject; (e) the time to ANC recovery is reduced in the subject; or (f) any combination thereof.

[0024] In some embodiments the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is selected from the group consisting of: (a) from about 50μg/kg to about 450 μg/kg; (b) about 50μg/kg; (c) about 150μg/kg; (d) about 300μg/kg; (e) about 450μg/kg; (f) from about 30 mg to about 60 mg; (g) about 30mg; (h) about 40mg; (i) about 50mg; (j) about 60mg; or (k) any combination thereof.

[0025] In some embodiments, the neutropenia to be treated or prevented is selected from the group consisting of primary neutropenia, acute neutropenia, severe chronic neutropenia (SCN), severe congenital neutropenia (Kostmann's syndrome), severe infantile genetic agranulocytosis, benign neutropenia , cyclic neutropenia, chronic idiopathic neutropenia, secondary neutropenia, syndrome associated neutropenia, and immune-mediated neutropenia. In addition, the eutropenia can be caused or associated with, for example, radiation, alcoholism, drugs, allergic disorders, aplastic anemia, autoimmune disease, T-γ lymphoproliferative disease (T-γ LPD), myelodysplasia, myelofibrosis, dysgammaglobulinemia, paroxysmal nocturnal hemoglobinuria, cancer, vitamin B_{12} deficiency , folate deficiency, viral infection, bacterial infection, spleen disorder, hemodialysis, or transplantation, leukemia, myeloma, lymphoma, metastatic solid tumors which infiltrate and replace the bone marrow, toxins, bone marrow failure, Schwachman-Diamond syndrome, cartilage-hair hypoplasia, dyskeratosis congenita, glycogen storage disease type IB, splenomegaly of any cause, intrinsic defects in myeloid cells or their precursors.

[0026] In embodiments of the invention where a the human subject is suffering from a non-myeloid malignancy, the non-myeloid malignancy can comprise breast cancer.

[0027] In embodiments of the invention where a myelosuppressive anticancer drugs is administered, the myelosuppressive anticancer drugs can comprise doxorubicin and docetaxel. For example, about 50 mg/m^2 doxorubicin and about 75 mg/m^2 docetaxel can be administered sequentially by intravenous infusion on the same day for at least one treatment cycle. Alternatively, about 60 mg/m^2 doxorubicin and about 75 mg/m^2 docetaxel can be administered sequentially by intravenous infusion on the same day for at least one treatment cycle.

[0028] In some embodiments of the invention following treatment ANC and WBC return to normal at a time period selected from the group consisting of: (a) by day 10 after chemotherapy; (b) by day 11 after chemotherapy; (c) by day 12 after chemotherapy; (d) by day 13 after chemotherapy: (e) by day 14 after chemotherapy; or (f) by day 15 after chemotherapy. In yet another embodiment, on day 14 after chemotherapy administration the rise in ANC in patients treated with recombinant human albumin-human granulocyte colony stimulating factor is lower than the rise in ANC in patients treated with an equivalent dose of pegfilgrastim.

[0029] In some embodiments of the invention administering recombinant human albumin-human granulocyte colony stimulating factor induces a rise in lymphocytes, monocytes, eosinophils, basophils, or any combination thereof. In other embodiments, the number of lymphocytes, monocytes, eosinophils, basophils or any combination thereof is increased in the subject. In yet further embodiments of the invention, a decrease in the number of lymphocytes, monocytes, eosinophils, or basophils is inhibited in the subject.

[0030] Both the foregoing general description and the following brief description of the drawings and the detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. Other objects, advantages, and novel features will be readily apparent to those skilled in the art from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] **FIG. 1A-1C: FIG 1A** shows the nucleic acid sequence and the amino acid sequence of the recombinant human albumin-granulocyte colony stimulating factor ("rHA-G-CSF") fusion protein termed "Neugranin™" ("NEUG"); **FIG 1B** shows the amino acid sequence of human G-CSF; **FIG 1C** shows the amino acid sequence of human serum albumin.

[0032] **FIG. 2** is a graph showing the absolute neutrophil count ("ANC") for subjects in Phase I. Subjects received 300 μg/kg NEUG (n=19), 450 μg/kg NEUG (n=20) or 6 mg pegfilgrastim (Neulasta®) (n=9) in cycle 1 following study chemotherapy.

[0033] **FIG. 3** is a graph showing the pharmacokinetics of NEUG in the Phase I study in human subjects. The serum concentration of NEUG administered subcutaneously at the indicated doses (450 μg/kg, 300 μg/kg or 150 μg/kg) was measured in subjects with breast

cancer in the absence of chemotherapy. Squares: 450 μg/kg Cycle 0; triangles: 300 μg/kg Cycle 0; circles: 150 μg/kg Cycle 0.

[0034] **FIG. 4** is a graph showing the pharmacokinetics/pharmacodynamics ("PK/PD") of NEUG in cycle 1 of chemotherapy (Phase I study). Patients received 450 μg/kg of NEUG one day after doxorubicin/docetaxel administration in cycle 1. ANC is shown by the open diamonds; NEUG concentration is shown by closed squares. Cut-offs for neutropenia grades 3 and 4 are shown by the dashed lines. The Lower Limit of Quantitation ("LLOQ") for NEUG is shown as a dotted line at 6 ng/ml.

[0035] **FIG. 5** is a graph showing the ANC profile for patients who received either 30 mg of NEUG or 6 mg of pegfilgrastim (Neulasta®) one day after starting cycle 1 of chemotherapy (Phase II study). Grade 3 and 4 neutropenia cut-off values are shown by dashed lines.

[0036] **FIG. 6** illustrates the chemotherapy cycles for the Phase I studies.

[0037] **FIG. 7A and 7B** are graphs showing the ANC and white blood cell ("WBC") count for subjects in the Phase I study.

[0038] **FIG. 8** shows a graph of NSF-60 cell proliferation with either NEUG (Albugranin) or Neupogen®.

[0039] **FIG. 9** show a graph of NSF-60 cell proliferation with either NEUG or Neulasta.

[0040] **FIG. 10** shows a graph of levels of peripheral blood neutrophils (Gr.1+) in BDF-1 mice after single SC administration of NEUG (Albugranin) and Neupogen (time course). The total number of Gr.1+ cells is expressed as the group mean +/- SEM.

[0041] **FIG. 11** shows a graph of levels of peripheral hematopoietic progenitors (c-kit+) after single SC administration of NEUG (Albugranin) and Neupogen (time course). The total number of c-kit+ cells is expressed as the group mean +/- SEM.

[0042] **FIG. 12 A and 12 B** are graphs showing the levels of peripheral blood granulocytes (Gr.1+) after single subcutaneous ("SC") administration of NEUG (Albugranin) or Neulasta® in BDF-1 mice. 12A shows a time course of response following single dose of Neulasta or NEUG and 12B show relative potency of NEUG or Neulasta.

[0043] **FIG. 13** is a table showing the composition of the NEUG drug product used in Phase I.

[0044] **FIG. 14** is a table showing the composition of the NEUG drug product used in Phase II.

[0045] **FIG. 15** is a graph showing levels of peripheral hematopoietic progenitor cells (c-kit+) after single subcutaneous administration of NEUG (Albugranin) or Neulasta® (time course). The total number of c-kit+ is expressed as the mean and standard error of the mean calculated for each group. Differences among treatment groups were analyzed by using heteroscedastic t-test.

[0046] **FIG. 16** is a graph showing levels of peripheral blood neutrophils (Gr.1+) after single subcutaneous administration of NEUG (Albugranin) or Neulasta 1 day after 5-FU (150 µg/kg) IP injection. The total number of GR.1+ cells enumerated daily is expressed as the mean and standard error of the mean calculated for each group. Difference among treatment groups were assessed with the 2-sample t-test with unequal variance. Treatment with either agent at all dose levels resulted in statistically significant increases in neutrophil count compared with the vehicle control.

[0047] **FIG. 17** is a graph showing the effect of NEUG (Albugranin) on the relative percent of peripheral blood neutrophils. The relative percent of neutrophils on each study day is presented as the group mean +/- SEM. Data from days 8 and 9 for NEUG 100 µg/kg Q7 are presented as days 9 and 10 respectively to facilitate comparison with other groups. Controls were saline vehicle administered SC every 4 days x 4 or Neupogen® administered SC daily x 14. The treatment period is considered days 1-14, and the recovery period is days 15-28.

[0048] **FIG. 18** is a graph showing a comparison of repeated dose-administration of NEUG (Albugranin) SC, NEUG IV, or Neulasta® SC on neutrophil mobilization in monkeys. The number of neutrophils (K/µl) on each study day through day 22 is presented as the group mean +/- SEM. The arrows indicate dose administration. NEUG was administered SC (n=6) or IV (n=6) at 1.0 mg/kg/dose, and Neulasta was administered SC (n=6) at 0.22 mg/kg/dose (equimolar dose to 1.0 mg/kg NEUG). The NEUG vehicle was administered SC as a control (n=2).

[0049] **FIG. 19A** and **19B** is a table showing a summary of *in vivo* pharmacokinetic studies.

[0050] **FIG. 20** is a table showing a summary of *in vivo* non-clinical studies that provide safety data.

[0051] **FIG. 21** a flow chart showing an exemplary overview of fermentation and purification of NEUG.

[0052] **FIG. 22** is a graph showing the median absolute neutrophil count (ANC) for subjects in Phase I, part A (cycle 0, or pre-chemotherapy) from treatment to 14 days. At day 4, the lines, from highest to lowest are: 300 μg/kg, 450 μg/kg, 150 μg/kg and 50 μg/kg.

[0053] **FIG. 23A** and **23 B** show the area the curve (AUC) for each subject treated in Phase I, Part B, based on the ANC values obtained for days 0 to 15. Figure **23A** is a graph; the data from Figure 23A is summarized in the table, **23B**.

[0054] **FIG. 24** is a graph showing the area under the curve (AUC) for subjects treated in Phase II, based on the ANC values obtained for days 0 to 15 in cycle 1 (fixed dose cohorts). For all subjects in Phase II, AUC_{ANC} (days 0-15) was calculated. Patients treated with Neugranin received a range of doses from 0.3 to 1 mg/kg (calculated as dose divided by baseline weight). The weight adjusted dose range was divided into quartiles and plotted vs. AUC_{ANC} (left panel). For all subjects treated with pegfilgrastim (N=112), 30 mg (N=10), 40 mg (N=105), or 50 mg (N=105) of Neugranin AUC_{ANC} was also calculated and compared. Data shown in means $\pm$ SEM.

DETAILED DESCRIPTION

[0055] Disclosed herein are compositions and methods for treating, preventing and ameliorating conditions and diseases characterized by a lowered white blood cell count. The methods and compositions described herein include a fusion polypeptide formed from human serum albumin protein ("HSA") and human granulocyte-colony stimulating factor ("G-CSF"). In a preferred embodiment, the fusion polypeptide is about 759 amino acids in length; amino acids 1-585 of the fusion correspond to amino acids from the mature form of HSA, and amino acids 586-759 of the fusion correspond to amino acids of the mature form of human G-CSF. The amino acid sequence of the fusion protein is presented in **FIG. 1.**

[0056] The invention also encompasses fusion proteins comprising variants or fragments of G-CSF, and fusion proteins comprising albumin or fragments or variants of albumin. The

invention also encompasses polynucleotides encoding the therapeutic albumin fusion proteins of the invention, therapeutic albumin fusion proteins, compositions, pharmaceutical compositions, formulations and kits. Host cells transformed with the polynucleotides encoding therapeutic albumin fusion proteins are also encompassed by the invention, as are methods of making the albumin fusion proteins of the invention using these polynucleotides, and/or host cells.

[0057] In one embodiment, an albumin fusion protein according to the present invention has extended shelf fife.

[0058] In a second embodiment, an albumin fusion protein according to the present invention is more stable than the corresponding unfused G-CSF molecule.

[0059] The present invention further includes transgenic organisms modified to contain the nucleic acid molecules of the invention, preferably modified to express an albumin fusion protein of the invention.

[0060] The present invention relates generally to polynucleotides encoding albumin fusion proteins; albumin fusion proteins; and methods of treating, preventing, or ameliorating diseases or disorders using albumin fusion proteins or polynucleotides encoding albumin fusion proteins. As used herein, "albumin fusion protein" refers to a protein formed by the fusion of at least one molecule of albumin (or a fragment or variant thereof) to at least one molecule of G-CSF (or fragment or variant thereof). An albumin fusion protein of the invention comprises at least a fragment or variant of a G-CSF and at least a fragment or variant of human serum albumin, which are associated with one another by genetic fusion (i.e., the albumin fusion protein is generated by translation of a nucleic acid in which a polynucleotide encoding all or a portion of G-CSF is joined in-frame with a polynucleotide encoding all or a portion of albumin). The G-CSF and albumin protein, once part of the albumin fusion protein, may each be referred to as a "portion", "region" or "moiety" of the albumin fusion protein (e.g., a "G-CSF protein portion" or an "albumin protein portion"). In a highly preferred embodiment, an albumin fusion protein of the invention comprises at least one molecule of G-CSF or fragment or variant of thereof (including, but not limited to a mature form of the G-CSF protein) and at least one molecule of albumin or fragment or variant thereof (including but not limited to a mature form of albumin).

[0061] In a further preferred embodiment, an albumin fusion protein of the invention is

processed by a host cell and secreted into the surrounding culture medium. Processing of the nascent albumin fusion protein that occurs in the secretory pathways of the host used for expression may include, but is not limited to signal peptide cleavage; formation of disulfide bonds; proper folding; addition and processing of carbohydrates (such as for example, N- and O-linked glycosylation); specific proteolytic cleavages; and assembly into multimeric proteins. An albumin fusion protein of the invention is preferably in the processed form. In a most preferred embodiment, the "processed form of an albumin fusion protein" refers to an albumin fusion protein product which has undergone N-terminal signal peptide cleavage, herein also referred to as a "mature albumin fusion protein".

[0062] In one embodiment, the invention provides a polynucleotide encoding an albumin fusion protein comprising, or alternatively consisting of, G-CSF and a serum albumin protein. In a further embodiment, the invention provides an albumin fusion protein comprising, or alternatively consisting of, G-CSF protein and a serum albumin protein. In other embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, a biologically active and/or therapeutically active fragment of G-CSF protein and a serum albumin protein. In other embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, a biologically active and/or therapeutically active variant of G-CSF protein and a serum albumin protein. In preferred embodiments, the serum albumin protein component of the albumin fusion protein is the mature portion of serum albumin. The invention further encompasses polynucleotides encoding these albumin fusion proteins.

[0063] In further embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, G-CSF protein, and a biologically active and/or therapeutically active fragment of serum albumin. In further embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, G-CSF protein and a biologically active and/or therapeutically active variant of serum albumin. In preferred embodiments, the G-CSF protein portion of the albumin fusion protein is the mature portion of the G-CSF protein. In a further preferred embodiment, the G-CSF protein portion of the albumin fusion protein is the extracellular soluble domain of the G-CSF protein. In an alternative embodiment, the G-CSF protein portion of the albumin fusion protein is the active form of the G-CSF protein. The invention further encompasses polynucleotides encoding

these albumin fusion proteins.

[0064] In further embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, a biologically active and/or therapeutically active fragment or variant of G-CSF protein and a biologically active and/or therapeutically active fragment or variant of serum albumin. In preferred embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, the mature portion of G-CSF protein and the mature portion of serum albumin. The invention further encompasses polynucleotides encoding these albumin fusion proteins.

I. Definitions

[0065] As used herein, "polynucleotide" refers to a nucleic acid molecule having a nucleotide sequence encoding a fusion protein comprising, or alternatively consisting of, at least one molecule of albumin (or a fragment or variant thereof) joined in frame to at least one molecule of Granulocyte-colony stimulating factor (G-CSF) (or fragment or variant thereof).

[0066] As used herein, "albumin fusion construct" refers to a nucleic acid molecule comprising, or alternatively consisting of, a polynucleotide encoding at least one molecule of albumin (or a fragment or variant thereof) joined in frame to at least one polynucleotide encoding at least one molecule of G-CSF (or fragment or variant thereof); and, further comprising, for example, one or more of the following elements: (1) a functional self-replicating vector (including but not limited to, a shuttle vector, an expression vector, an integration vector, and/or a replication system), (2) a region for initiation of transcription (e.g., a promoter region, such as for example, a regulatable or inducible promoter, a constitutive promoter), (3) a region for termination of transcription, (4) a leader sequence, and (5) a selectable marker. The polynucleotide encoding the G-CSF and albumin protein, once part of the albumin fusion construct, may each be referred to as a "portion," "region" or "moiety" of the albumin fusion construct.

[0067] By a G-CSF polypeptide displaying a "therapeutic activity" or a G-CSF protein that is "therapeutically active" is meant a G-CSF polypeptide that possesses one or more known biological and/or therapeutic activities associated with G-CSF protein. As a non-limiting example, a "G-CSF therapeutic protein" is a G-CSF protein that is useful to treat, prevent or ameliorate a disease, condition or disorder. As a non-limiting example, a "G-CSF therapeutic

protein" may be one that binds specifically to a particular cell type (normal (e.g., lymphocytes) or abnormal e.g., (cancer cells)) and therefore may be used to target a compound (drug, or cytotoxic agent) to that cell type specifically.

II. Granulocyte-colony stimulating factor

[0068] Granulocyte-colony stimulating factor (G-CSF) is a hematopoietic growth factor that stimulates the production of neutrophils. Administration of G-CSF results in rapid induction of a neutrophilic leukocytosis when there are viable precursor cells to stimulate. Another important *in vivo* activity of G-CSF is mobilization of hematopoietic progenitor cells into the peripheral blood (Duhrsen *et al.*, 1988; Molineux *et al.*, 1999; Roberts *et al.*, 1994). This effect includes not only the neutrophil lineage but extends to other single lineage and multi-lineage progenitors and pluripotent hematopoietic stem cells (Molineux *et al.*, 1999). G-CSF also increases the cellular events that are part of the defense mechanism against infections by priming neutrophils, thereby increasing both their phagocytic and anti-bacterial activities against opsonized *Staphylococcus aureus*. G-CSF also induces chemotaxis of neutrophils and monocytes and adhesion of neutrophils (Yuo *et al.*, 1989; Wang *et al.*, 1988).

[0069] Recombinant G-CSF products are currently approved for a number of clinical indications to stimulate the proliferation and differentiation of neutrophils. In clinical trials, filgrastim (recombinant methionyl human G-CSF; Neupogen®, Amgen, Thousand Oaks, CA) increased the number of peripheral neutrophils and thereby reduced the duration of neutropenia after myelosuppressive chemotherapy. Recombinant G-CSF (filgrastim) is given by daily subcutaneous (SC) injection.

[0070] Another recombinant form of G-CSF is pegfilgrastim, a polyethylene glycol-conjugated rG-CSF (Neulasta®), which has proven safe and effective as a once-per-cycle alternative to daily rG-CSF therapy to decrease the incidence of febrile neutropenia in patients receiving myelosuppressive anti-cancer drugs (Holmes, O'Shaughnessy *et al.*, 2002; Green *et al.*, 2003; Neulasta® SmPC 2007).

[0071] Primary prophylaxis with G-CSF is recommended for the prevention of febrile neutropenia in patients who are at high risk based on age, medical history, disease characteristics, and myelotoxicity of the chemotherapy regimen. The American Society of Clinical Oncology and the European Organization for Research and Treatment of Cancer recommend the use of G-CSF when the risk of febrile neutropenia is approximately 20%.

The U.S. National Comprehensive Cancer Center Network recommends an optional indication of G-CSF prophylaxis when the risk of febrile neutropenia is 10% to 20% and a definite indication of G-CSF prophylaxis when the risk of febrile neutropenia is at least 20%. (Smith *et al.*, 2006, Vogel *et al.*, 2005, Timmer-Bonte *et al.*, 2006, NCCN Guidelines).

[0072] Prophylaxis with colony-stimulating factors is recommended to alleviate the toxicity of certain chemotherapy regimens. However, the added cost of these treatments is a significant consideration both in the U.S. and especially in parts of the E.U. and may lead to under-use of prophylactic G-CSF treatment and may also limit patient eligibility for dose-intensive chemotherapy regimens (Timmer-Bonte *et al.*, 2006; Adams *et al.*, 2006, NCCN Guidelines).

[0073] The G-CSF protein may be encoded by a wild type polynucleotide sequence (e.g., either full length or mature), or in some instances the sequence may be a variant of the wild type polynucleotide sequence (e.g., a polynucleotide which encodes the wild type G-CSF protein, wherein the DNA sequence of the polynucleotide has been optimized, for example, for expression in a particular species; or a polynucleotide encoding a variant of the wild type G-CSF protein (i.e., a site directed mutant; an allelic variant)).

III. Human serum albumin

[0074] Human serum albumin (HSA or HA) is the most prevalent naturally occurring blood protein in the human circulatory system, measured at approximately 40 grams of albumin/liter and persisting in the circulation for over 20 days. Albumin is a carrier protein with minimal activity at physiological concentrations. Even though HSA lacks enzymatic or immunological function it is widely distributed *in vivo,* and is know to be a carrier for various substances in the blood (*e.g.*, hormones, fatty acids, unconjugated bilirubin, etc (Yeh *et al.*, 1992). Both HSA and recombinant HA (rHA) have the same long circulating half-life in humans.

[0075] Research has shown that therapeutic proteins genetically fused to human albumin are able to take on the circulating half-life characteristics of albumin (Syed *et al.*, 1997). For example, in rabbits, the half-life of CD4 fused to albumin is 140 fold greater than non-fused CD4 (Yeh *et al.*, 1992).

[0076] Human serum albumin, a protein of 585 amino acids in its mature form (as shown in FIG. 1 of U.S. Patent No. 7,592,010, is responsible for a significant proportion of the osmotic

pressure of serum and also functions as a carrier of endogenous and exogenous ligands. At present, HA for clinical use is produced by extraction from human blood. The production of recombinant HA (rHA) in microorganisms has been disclosed in EP 330 451 and EP 361 991.

IV. Polypeptide and Polynucleotide Fragments and Variants

A. Fragments

[0077] The present invention is further directed to fragments of G-CSF protein, albumin proteins, and/or albumin fusion proteins of the invention. The present invention is also directed to polynucleotides encoding fragments of the G-CSF protein, albumin proteins, and/or albumin fusion proteins of the invention. Even if deletion of one or more amino acids from the N-terminus of a protein results in modification or loss of one or more biological functions of the G-CSF protein, albumin protein, and/or albumin fusion protein of the invention, other therapeutic activities and/or functional activities (e.g., biological activities, ability to multimerize, ability to bind a ligand) may still be retained. For example, the ability of polypeptides with N-terminal deletions to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptides generally will be retained when less than the majority of the residues of the complete polypeptide are removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete polypeptide retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that a mutein with a large number of deleted N-terminal amino acid residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six amino acid residues may often evoke an immune response.

[0078] Accordingly, fragments of G-CSF protein corresponding to a G-CSF protein portion of an albumin fusion protein of the invention include the full length protein as well as polypeptides having one or more residues deleted from the amino terminus of the amino acid sequence of the reference polypeptide (i.e., a G-CSF protein, or a G-CSF protein portion of an albumin fusion protein encoded by a polynucleotide or albumin fusion construct). In particular, N-terminal deletions may be described by the general formula m to q, where q is a whole integer representing the total number of amino acid residues in a reference polypeptide (e.g., a G-CSF protein, or a G-CSF protein portion of an albumin fusion protein of the invention), and m is defined as any integer ranging from 2 to q minus 6. Polynucleotides

encoding these polypeptides are also encompassed by the invention.

[0079] In addition, fragments of serum albumin polypeptides corresponding to an albumin protein portion of an albumin fusion protein of the invention, include the full length protein as well as polypeptides having one or more residues deleted from the amino terminus of the amino acid sequence of the reference polypeptide (i.e., serum albumin, or a serum albumin portion of an albumin fusion protein). In preferred embodiments, N-terminal deletions may be described by the general formula m to 585, where 585 is a whole integer representing the total number of amino acid residues in mature human serum albumin, and m is defined as any integer ranging from 2 to 579. Polynucleotides encoding these polypeptides are also encompassed by the invention. In additional embodiments, N-terminal deletions may be described by the general formula m to 609, where 609 is a whole integer representing the total number of amino acid residues in full length human serum albumin, and m is defined as any integer ranging from 2 to 603. Polynucleotides encoding these polypeptides are also encompassed by the invention.

[0080] Moreover, fragments of albumin fusion proteins of the invention, include the full length albumin fusion protein as well as polypeptides having one or more residues deleted from the amino terminus of the albumin fusion protein. In particular, N-terminal deletions may be described by the general formula m to q, where q is a whole integer representing the total number of amino acid residues in the albumin fusion protein, and m is defined as any integer ranging from 2 to q minus 6. Polynucleotides encoding these polypeptides are also encompassed by the invention.

[0081] Also as mentioned above, even if deletion of one or more amino acids from the N-terminus or C-terminus of a reference polypeptide (e.g., a G-CSF protein; serum albumin protein; or albumin fusion protein of the invention) results in modification or loss of one or more biological functions of the protein, other functional activities (e.g., biological activities, ability to multimerize, ability to bind a ligand) and/or therapeutic activities may still be retained. For example, the ability of polypeptides with C-terminal deletions to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptide generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the C-terminus. Whether a particular polypeptide lacking the N-terminal and/or C-terminal residues of a reference polypeptide retains therapeutic activity

can readily be determined by routine methods described herein and/or otherwise known in the art.

[0082] The present invention further provides polypeptides having one or more residues deleted from the carboxy terminus of the amino acid sequence of a G-CSF protein corresponding to a G-CSF protein portion of an albumin fusion protein of the invention. In particular, C-terminal deletions may be described by the general formula 1 to n, where n is any whole integer ranging from 6 to q minus 1, and where q is a whole integer representing the total number of amino acid residues in a reference polypeptide (e.g., a G-CSF protein, or a G-CSF protein portion of an albumin fusion protein encoded by a polynucleotide or albumin fusion construct). Polynucleotides encoding these polypeptides are also encompassed by the invention.

[0083] In addition, the present invention provides polypeptides having one or more residues deleted from the carboxy terminus of the amino acid sequence of an albumin protein corresponding to an albumin protein portion of an albumin fusion protein of the invention. In particular, C-terminal deletions may be described by the general formula 1 to n, where n is any whole integer ranging from 6 to 584, where 584 is the whole integer representing the total number of amino acid residues in mature human serum albumin minus 1. Polynucleotides encoding these polypeptides are also encompassed by the invention. In particular, C-terminal deletions may be described by the general formula 1 to n, where n is any whole integer ranging from 6 to 608, where 608 is the whole integer representing the total number of amino acid residues in serum albumin minus 1. Polynucleotides encoding these polypeptides are also encompassed by the invention.

[0084] Moreover, the present invention provides polypeptides having one or more residues deleted from the carboxy terminus of an albumin fusion protein of the invention. In particular, C-terminal deletions may be described by the general formula 1 to n, where n is any whole integer ranging from 6 to q minus 1, and where q is a whole integer representing the total number of amino acid residues in an albumin fusion protein of the invention. Polynucleotides encoding these polypeptides are also encompassed by the invention.

[0085] In addition, any of the above described N- or C-terminal deletions can be combined to produce a N- and C-terminal deleted reference polypeptide. The invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl

termini, which may be described generally as having residues m to n of a reference polypeptide (e.g., a G-CSF protein, or a G-CSF protein portion of an albumin fusion protein of the invention, or serum albumin, or an albumin protein portion of an albumin fusion protein of the invention, or an albumin fusion protein, or an albumin fusion protein encoded by a polynucleotide or albumin fusion construct of the invention) where n and m are integers as described above. Polynucleotides encoding these polypeptides are also encompassed by the invention.

[0086] The present application is also directed to proteins containing polypeptides at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical to a reference G-CSF polypeptide or a reference albumin polypeptide set forth herein, or fragments thereof. In preferred embodiments, the application is directed to proteins comprising polypeptides at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical to reference polypeptides having the amino acid sequence of N- and C-terminal deletions as described above. Polynucleotides encoding these polypeptides are also encompassed by the invention.

[0087] Preferred polypeptide fragments of the invention are fragments comprising, or alternatively, consisting of, an amino acid sequence that displays a therapeutic activity and/or functional activity (e.g. biological activity) of the polypeptide sequence of the G-CSF protein or serum albumin protein of which the amino acid sequence is a fragment.

[0088] Other preferred polypeptide fragments are biologically active fragments. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of the polypeptide of the present invention. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity.

B. Variants

[0089] "Variant" refers to a polynucleotide or nucleic acid differing from a reference nucleic acid or polypeptide, but retaining essential properties thereof. Generally, variants are overall closely similar, and, in many regions, identical to the reference nucleic acid or polypeptide.

[0090] As used herein, "variant", refers to a G-CSF protein portion of an albumin fusion

protein of the invention, albumin portion of an albumin fusion protein of the invention, or albumin fusion protein of the invention differing in sequence from a G-CSF protein, albumin protein, and/or albumin fusion protein, respectively, but retaining at least one functional and/or therapeutic property thereof as described elsewhere herein or otherwise known in the art. Generally, variants are overall very similar, and, in many regions, identical to the amino acid sequence of the G-CSF protein corresponding to a G-CSF protein portion of an albumin fusion protein, albumin protein corresponding to an albumin protein portion of an albumin fusion protein, and/or albumin fusion protein. Nucleic acids encoding these variants are also encompassed by the invention.

[0091] The present invention is also directed to proteins which comprise, or alternatively consist of, an amino acid sequence which is at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100%, identical to, for example, the amino acid sequence of a G-CSF protein corresponding to a G-CSF protein portion of an albumin fusion protein of the invention , albumin proteins corresponding to an albumin protein portion of an albumin fusion protein of the invention, and/or albumin fusion proteins. Fragments of these polypeptides are also provided. Further polypeptides encompassed by the invention are polypeptides encoded by polynucleotides which hybridize to the complement of a nucleic acid molecule encoding an albumin fusion protein of the invention under stringent hybridization conditions (e.g., hybridization to filter bound DNA in 6x. Sodium chloride/Sodium citrate (SSC) at about 45 degrees Celsius, followed by one or more washes in 0.2 x SSC, 0.1% SDS at about 50-65 degrees Celsius), under highly stringent conditions (e.g., hybridization to filter bound DNA in 6 x sodium chloride/Sodium citrate (SSC) at about 45 degrees Celsius, followed by one or more washes in 0.1 x SSC, 0.2% SDS at about 68 degrees Celsius), or under other stringent hybridization conditions which are known to those of skill in the art (see, for example, Ausubel, F. M. et al., eds., 1989 Current protocol in Molecular Biology, Green publishing associates, Inc., and John Wiley & Sons Inc., New York, at pages 6.3.1-6.3.6 and 2.10.3). Polynucleotides encoding these polypeptides are also encompassed by the invention.

[0092] By a polypeptide having an amino acid sequence at least, for example, 95%- "identical" to a query amino acid sequence, it is intended that the amino acid sequence of the

subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, or substituted with another amino acid. These alterations of the reference sequence may occur at the amino- or carboxy-terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

[0093] As a practical matter, whether any particular polypeptide is at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% identical to, for instance, the amino acid sequence of an albumin fusion protein of the invention or a fragment thereof (such as a G-CSF protein portion of the albumin fusion protein or an albumin portion of the albumin fusion protein), can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. 6:237-245 (1990)). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of the global sequence alignment is expressed as percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

[0094] If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-

terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

[0095] For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to made for the purposes of the present invention.

[0096] The variant will usually have at least about 75% (in other embodiments at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99%) sequence identity with a length of normal HA or G-CSF protein which is the same length as the variant. Homology or identity at the nucleotide or amino acid sequence level is determined by BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp,

blastn, blastx, tblastn and tblastx (Karlin et al., Proc. Natl. Acad. Sci. USA 87: 2264-2268 (1990) and Altschul, J. Mol. Evol. 36: 290-300 (1993), fully incorporated by reference) which are tailored for sequence similarity searching.

[0097] The approach used by the BLAST program is to first consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul et al., (Nature Genetics 6: 119-129 (1994)) which is fully incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (i.e., the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff et al., Proc. Natl. Acad. Sci. USA 89: 10915-10919 (1992), fully incorporated by reference). For blastn, the scoring matrix is set by the ratios of M (i.e., the reward score for a pair of matching residues) to N (i.e., the penalty score for mismatching residues), wherein the default values for M and N are 5 and -4, respectively. Four blastn parameters may be adjusted as follows: Q-10 (gap creation penalty); R=10 (gap extension penalty); wink=1 (generates word hits at every wink.sup.th position along the query); and gapw=16 (sets the window width within which gapped alignments are generated). The equivalent Blastp parameter settings were Q=9; R=2; wink=1; and gapw=32. A Bestfit comparison between sequences, available in the GCG package version 10.0, uses DNA parameters GAP=50 (gap creation penalty) and LEN=3 (gap extension penalty) and the equivalent settings in protein comparisons are GAP=8 and LEN=2.

[0098] The polynucleotide variants of the invention may contain alterations in the coding regions, non-coding regions, or both. Especially preferred are polynucleotide variants containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. Nucleotide variants produced by silent substitutions due to the degeneracy of the genetic code are preferred. Moreover, polypeptide variants in which less than 50, less than 40, less than 30, less than 20, less than 10, or 5-50, 5-25, 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination are also preferred. Polynucleotide variants can be produced for a variety of

reasons, e.g., to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host, such as, yeast or E. coli).

[0099] In a preferred embodiment, a polynucleotide of the invention which encodes the albumin portion of an albumin fusion protein is optimized for expression in yeast or mammalian cells. In a further preferred embodiment, a polynucleotide of the invention which encodes the G-CSF protein portion of an albumin fusion protein is optimized for expression in yeast or mammalian cells. In a still further preferred embodiment, a polynucleotide encoding an albumin fusion protein of the invention is optimized for expression in yeast or mammalian cells.

[0100] In an alternative embodiment, a codon optimized polynucleotide which encodes a G-CSF protein portion of an albumin fusion protein does not hybridize to the wild type polynucleotide encoding the G-CSF protein under stringent hybridization conditions as described herein. In a further embodiment, a codon optimized polynucleotide which encodes an albumin portion of an albumin fusion protein does not hybridize to the wild type polynucleotide encoding the albumin protein under stringent hybridization conditions as described herein. In another embodiment, a codon optimized polynucleotide which encodes an albumin fusion protein does not hybridize to the wild type polynucleotide encoding the G-CSF protein portion or the albumin protein portion under stringent hybridization conditions as described herein.

[0101] In an additional embodiment, a polynucleotide which encodes a G-CSF protein portion of an albumin fusion protein does not comprise, or alternatively consist of, the naturally occurring sequence of that G-CSF protein. In a further embodiment, a polynucleotide which encodes an albumin protein portion of an albumin fusion protein does not comprise, or alternatively consist of, the naturally occurring sequence of albumin protein. In an alternative embodiment, a polynucleotide which encodes an albumin fusion protein does not comprise, or alternatively consist of, the naturally occurring sequence of a G-CSF protein portion or the albumin protein portion.

[0102] Using known methods of protein engineering and recombinant DNA technology, variants may be generated to improve or alter the characteristics of the polypeptides of the present invention. For instance, one or more amino acids can be deleted from the N-terminus or C-terminus of the polypeptide of the present invention without substantial loss of

biological function.

[0103] In preferred embodiments, the variants of the invention have conservative substitutions. By "conservative substitutions" is intended swaps within groups such as replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl residues Ser and Tar; replacement of the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln, replacement of the basic residues Lys, Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp, and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly.

[0104] Guidance concerning how to make phenotypically silent amino acid substitutions is provided, for example, in Bowie et al., "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," Science 247:1306-1310 (1990), wherein the authors indicate that there are two main strategies for studying the tolerance of an amino acid sequence to change.

[0105] The first strategy exploits the tolerance of amino acid substitutions by natural selection during the process of evolution. By comparing amino acid sequences in different species, conserved amino acids can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions where substitutions have been tolerated by natural selection indicates that these positions are not critical for protein function. Thus, positions tolerating amino acid substitution could be modified while still maintaining biological activity of the protein.

[0106] The second strategy uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene to identify regions critical for protein function. For example, site directed mutagenesis or alanine-scanning mutagenesis (introduction of single alanine mutations at every residue in the molecule) can be used. See Cunningham and Wells, Science 244:1081-1085 (1989). The resulting mutant molecules can then be tested for biological activity.

[0107] As the authors state, these two strategies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at certain amino acid positions in the protein. For example, most buried (within the tertiary structure of the protein) amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Moreover,

tolerated conservative amino acid substitutions involve replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl residues Ser and Thr; replacement of the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln, replacement of the basic residues Lys. Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp, and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly. Besides conservative amino acid substitution, variants of the present invention include (i) polypeptides containing substitutions of one or more of the non-conserved amino acid residues, where the substituted amino acid residues may or may not be one encoded by the genetic code, or (ii) polypeptides containing substitutions of one or more of the amino acid residues having a substituent group, or (iii) polypeptides which have been fused with or chemically conjugated to another compound, such as a compound to increase the stability and/or solubility of the polypeptide (for example, polyethylene glycol), (iv) polypeptide containing additional amino acids, such as, for example, an IgG Fc fusion region peptide. Such variant polypeptides are deemed to be within the scope of those skilled in the art from the teachings herein.

[0108] For example, polypeptide variants containing amino acid substitutions of charged amino acids with other charged or neutral amino acids may produce proteins with improved characteristics, such as less aggregation. Aggregation of pharmaceutical formulations both reduces activity and increases clearance due to the aggregate's immunogenic activity. See Pinckard et al., Clin. Exp. Immunol. 2:331-340 (1967); Robbins et al., Diabetes 36: 838-845 (1987); Cleland et al., Crit. Rev. Therapeutic Drug Carrier Systems 10:307-377 (1993).

[0109] In specific embodiments, the polypeptides of the invention comprise, or alternatively, consist of, fragments or variants of the amino acid sequence of an albumin fusion protein, the amino acid sequence of a G-CSF protein and/or human serum albumin, wherein the fragments or variants have 1-5, 5-10, 5-25, 5-50, 10-50 or 50-150, amino acid residue additions, substitutions, and/or deletions when compared to the reference amino acid sequence. In preferred embodiments, the amino acid substitutions are conservative. Nucleic acids encoding these polypeptides are also encompassed by the invention.

[0110] The polypeptide of the present invention can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain amino acids other than the 20 gene-encoded amino acids. The polypeptides may be

modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, PROTEINS--STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993); POST-TRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al., Meth. Enzymol. 182:626-646 (1990); Rattan et al., Ann. N.Y. Acad. Sci. 663:4862 (1992)).

C. Functional Activity

[0111] "A polypeptide having functional activity" refers to a polypeptide capable of displaying one or more known functional activities associated with the full-length, pro-protein, and/or mature form of a G-CSF protein. Such functional activities include, but are not limited to, biological activity, antigenicity [ability to bind (or compete with a polypeptide for binding) to an anti-polypeptide antibody], immunogenicity (ability to generate antibody

which binds to a specific polypeptide of the invention), ability to form multimers with polypeptides of the invention, and ability to bind to a receptor or ligand for a polypeptide.

[0112] "A polypeptide having biological activity" refers to a polypeptide exhibiting activity similar to, but not necessarily identical to, an activity of a G-CSF protein of the present invention, including mature forms, as measured in a particular biological assay, with or without dose dependency.

[0113] In preferred embodiments, an albumin fusion protein of the invention has at least one biological and/or therapeutic activity associated with the G-CSF protein portion (or fragment or variant thereof) when it is not fused to albumin.

[0114] The albumin fusion proteins of the invention can be assayed for functional activity (e.g., biological activity) using or routinely modifying assays known in the art, as well as assays described herein. Additionally, one of skill in the art may routinely assay fragments of a G-CSF protein corresponding to a G-CSF protein portion of an albumin fusion protein. Further, one of skill in the art may routinely assay fragments of an albumin protein corresponding to an albumin protein portion of an albumin fusion protein, for activity using assays known in the art and/or as described in the Examples section below.

[0115] For example, in one embodiment where one is assaying for the ability of an albumin fusion protein to bind or compete with a G-CSF protein for binding to an anti- G-CSF polypeptide antibody and/or anti-albumin antibody, various immunoassays known in the art can be used, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

[0116] In a preferred embodiment, where a binding partner (e.g., a receptor or a ligand) of a G-CSF protein is identified, binding to that binding partner by an albumin fusion protein which comprises that G-CSF protein as the G-CSF protein portion of the fusion can be assayed, e.g., by means well-known in the art, such as, for example, reducing and non-reducing gel chromatography, protein affinity chromatography, and affinity blotting. See generally, Phizicky et al., Microbiol. Rev. 59:94-123 (1995). In another embodiment, the ability of physiological correlates of an albumin fusion protein to bind to a receptor(s) of the G-CSF polypeptide corresponding to the G-CSF protein portion of the fusion can be routinely assayed using techniques known in the art.

[0117] In an alternative embodiment, where the ability of an albumin fusion protein to multimerize is being evaluated, association with other components of the multimer can be assayed, e.g., by means well-known in the art, such as, for example, reducing and non-reducing gel chromatography, protein affinity chromatography, and affinity blotting. See generally, Phizicky et al., supra.

[0118] Immunoassays which can be used to analyze protein binding, cross-reactivity or identity include, but are not limited to, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see, e.g., Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety).

[0119] Antibodies that bind a G-CSF protein corresponding to the G-CSF protein portion of an albumin fusion protein may also be described or specified in terms of their binding affinity for a given protein or antigen, preferably the antigen which they specifically bind. Preferred binding affinities include those with a dissociation constant or Kd less than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M. More preferred binding affinities include those with a dissociation constant or Kd less than 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M or 10^{-8} M. Even more preferred binding affinities include those with a dissociation constant or Kd less than 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-}

^{11} M, 5 x 10^{-12} M, 10^{-12} M, 5 x 10^{-13} M, 10^{-13} M, 5 x 10^{-14} M, 10^{-14} M, 5 x 10^{-15} M, or 10^{-15} M. In addition, assays described herein and otherwise known in the art may routinely be applied to measure the ability of albumin fusion proteins and fragments, variants and derivatives thereof to elicit biological activity and/or G-CSF activity (either in vitro or in vivo) related to either the G-CSF protein portion and/or albumin portion of the albumin fusion protein. Other methods will be known to the skilled artisan and are within the scope of the invention.

V. Fusion proteins of G-CSF and HSA

[0120] Recombinant human albumin-human granulocyte colony stimulating factor (rHA-G-CSF) is a G-CSF analogue. Examples of rHA-G-CSFs are described in U.S. Patent No. 5,665,863 and in U.S. Patent No. 7,041,478, both of which are hereby incorporated by reference.

[0121] Another example of rHA-G-CSF is Neugranin™ ("NEUG") developed by Teva Biopharmaceuticals USA LTD. NEUG is a fusion polypeptide with a molecular mass of approximately 85 kDa. NEUG is a 759 amino acid single chain polypeptide, with residues 1-585 corresponding to the mature form of HSA, and residues 586-759 corresponding to the mature form of human G-CSF. The amino acid sequence of the NEUG fusion protein is shown in **FIG. 1**.

VI. Producing the fusion protein

[0122] Exemplary methods of synthetic processes of manufacture of rHA-G-CSF are described in U.S. Patent Application Serial No. 11/929,828 hereby incorporated by reference in its entirety. In some embodiments, NEUG is produced using a yeast host system (*e.g.*, *Saccharomyces cerevisiae*) genetically engineered to express the NEUG fusion protein. NEUG is harvested from the fermentation medium of the yeast culture and purified using methods well known in the art (*e.g.*, by a series of chromatography and filtration steps, such as affinity chromatography and ion exchange chromatography).

[0123] In one non-limiting example, a NEUG fusion construct was developed as follows. The full-length albumin cDNA was isolated from a human cDNA library in the laboratory of Dr. F.E. Baralle at the University of Oxford, UK. This clone was sent to Delta Biotechnology Limited, Nottingham, UK, as the plasmid pAT153ALB. In addition, the 6-

amino acid HSA pro-peptide (RGVFRR) was modified to facilitate more efficient processing in yeast (RSLDKR).

[0124] The NEUG production plasmid, a modified pSAC35-based expression vector, is based on the 2-μ plasmid found in wild type *Saccharomyces cerevisiae*. The pSAC35-based expression vector (*see e.g.*, patents EP 286 424 B, U.S. Patent No. 5,637,504) contains the LEU2 gene from *Saccharomyces cerevisiae* as a selectable marker that complements the leucine-deficiency of the *S. cerevisiae* production host. This production plasmid also contains a strong yeast promoter, PRB1, and sequences from plasmid pUC9 that permit cloning and propagation in *E. coli*. In addition, the plasmid eliminates the pUC9-derived sequences required for propagation in *E. coli* once transformed into yeast. This is accomplished by flanking FLP recognition targets (FRT) and the expression of the yeast FLP recombinase from the plasmid once in yeast. Thus, no bacterial DNA is present in the organism used for production of NEUG. This is confirmed by rescue and sequence of the 2μm plasmid from the yeast after the master cell bank is generated.

[0125] As described above, the NEUG production plasmid, termed CID1643 (pSAC35:HSA.GCSF(T31-P204)), was derived from the pSAC35-based expression vector. The region corresponding to T31-P204 of human G-CSF was amplified by PCR, while adding the appropriate 5' and 3' restriction sites to permit a seamless fusion to the 3'-end of the HSA open reading frame.

[0126] NEUG seed vials were used to prepare a cGMP master cell bank at Human Genome Sciences, Inc., in Rockville, MD. The testing and characterization of the NEUG master cell bank was undertaken at Charles River Laboratories (Malvern, PA, USA) and Lark Technologies (Houston, TX, USA) in compliance with the ICH guideline Q5D (Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biologicals Products).

[0127] A cGMP working cell bank derived from this master cell bank was subsequently generated and tested at Charles River Laboratories (Malvern, PA, USA).

[0128] All media components used in the manufacture of the NEUG cell line banks were synthetic, biosynthetic or plant derived. No components of animal or human origin were used during cell line or cell bank preparation.

[0129] The cell banks is stored at < -135°C in a cryopreservation media in pre-sterilized 1.8 mL Nunc polypropylene tubes with internally threaded caps.

[0130] A non-limiting, exemplary method of isolating, purifying and preparing the rHSA-G-CSF fusion protein for pharmaceutical use is shown in **FIG. 21**. The formulated drug substance is aseptically filtered using a 0.2 μm filter into autoclaved Teflon bottles. The liquid filled drug substance is stored frozen at about -80°C (nominal value, acceptable range of storage temperature is about -65°C).

[0131] To improve the robustness of the formulation for shipping and storage at clinical sites as well as to provide a stable product with an expected long shelf life, NEUG may also be lyophilized by methods well known in the art.

VII. Exemplary causes of leukopenia and neutropenia

[0132] As described above, leukopenia is a reduction in the circulating white blood cells (WBC) count and neutropenia is characterized by a reduction in the blood neutrophil count, often leading to increased susceptibility to bacterial and fungal infections. The following is a non-comprehensive list of factors that can place a human subject at risk of developing leukopenia or neutropenia: drugs (*e.g.* phenytoin, chloramphenicol, sulfa drugs, and chemotherapy); vitamin B12 or folate deficiency; excessive alcohol consumption; cancer or other diseases which involve the bone marrow (*e.g.* aplastic anemia, dysgammaglobulinemia, paroxysmal nocturnal hemoglobinemia, myelodysplasia, myelodysplastic syndromes, myelofibrosis, leukemia, myeloma, lymphoma, or metastatic solid tumors which infiltrate and replace the bone marrow); viral infections (*e.g.* influenza, HIV, early-stage infectious mononucleosis, childhood viral diseases); bacterial infections (*e.g.* tuberculosis); radiation; toxins (*e.g.*, benzene and insecticides); bone marrow failure (*e.g.* Schwachman-Diamond syndrome, cartilage-hair hypoplasia, dyskeratosis congenita, glycogen storage disease type IB); spleen disorder, splenomegaly of any cause; intrinsic defects in myeloid cells or their precursors; allergic disorders; autoimmune disease; T-γ lymphoproliferative disease (T-γ LPD); hemodialysis or transplantation; toxins.

[0133] Numerous drugs, such as many chemotherapy regimens (*e.g.*, cytotoxic chemotherapy regimens), are associated with a high risk of febrile neutropenia (*e.g.*, > than 20% risk). In some chemotherapy regimens, the incidence of febrile neutropenia in the absence of G-CSF treatment is about 40% (*e.g.*, a chemotherapy regimen of intravenous

doxorubicin and docetaxel). Non-limiting examples of various cancers and treatment regimens associated with febrile neutropenia risk are provided below in Table 1. In some embodiments, the HSA-G-CSF fusion protein of **FIG. 1** is administered to a patient to prevent, treat or ameliorate neutropenia associated with the administration of such drug therapies.

Table 1: Exemplary cancers and treatment regimens associated with febrile neutropenia

Cancer	Treatment
Bladder Cancer	MVAC (methotrexate, vinblastine, doxorubicin, cisplatin) (neoadjuvant, adjuvant, metastatic)
Breast Cancer	Docetaxel + trastuzumab (metastatic or relapsed) Dose dense AC- T (doxorubicin, cyclophosphamide, paclitaxel) (adjuvant) AT (doxorubicin, paclitaxel) (metastatic or relapsed) AT (doxorubicin, docetaxel) (metastatic or relapsed) TAC (docetaxel, doxorubicin, cyclophosphamide) (adjuvant)
Esophageal and Gastric Cancer	Docetaxel/cisplatin/fluorouracil
Non-Hodgkin's Lymphoma	ICE (ifosfamide, carboplatin, etoposide) (Diffuse Large B-Cell Lymphoma, Peripheral T cell Lymphomas, 2nd line, salvage) RICE (rituximab, ifosfamide, carboplatin, etoposide) CHOP-14 (cyclophosphamide, doxorubicin, vincristine, prednisone) MINE (mesna, ifosfamide, novantrone and etoposide) (Diffuse Large B-Cell Lymphoma, Peripheral T cell Lymphomas, 2nd line, refractory) DHAP (dexamethasone, cisplatin, cytarabine) (Peripheral T cell Lymphomas, Diffuse Large B-Cell Lymphoma, 2nd line) ESHAP (etoposide, methylprednisolone, cisplatin, cytarabine) (Diffuse Large B-Cell Lymphoma, Peripheral T cell Lymphoma, 2nd line, recurrent) BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone) HyperCVAD + Rituximab (cyclophosphamide, vincristine, doxorubicin, dexamethasone + rituximab) (Burkitt's Lymphoma)
Melanoma	Dacarbazine-based combination (dacarbazine, cisplatin, vinblastine) (advanced, metastatic, or recurrent) Dacarbazine-based combination with IL-2, interferon alfa (dacarbazine, cisplatin, vinblastine, IL-2, interferon alfa) (advanced, metastatic, or recurrent)
Myelodysplastic syndrome	Decitabine
Ovarian Cancer	Topotecan Paclitaxel

Table 1: Exemplary cancers and treatment regimens associated with febrile neutropenia	
Cancer	**Treatment**
	Docetaxel
Pancreatic Cancer	Gemcitabine/docetaxel
Sarcoma	MAID (mesna, doxorubicin, ifosfamide, dacarbazine) Doxorubicin
Small Cell Lung Cancer	Topotecan
Testicular Cancer	VeIP (vinblastine, ifosfamide, cisplatin) VIP (etoposide,ifosfamide, cisplatin) BEP (bleomycin, etoposide, cisplatin) TIP (paclitaxel, ifosfamide, cisplatin)

[0134] Cytotoxic treatment regimens for small cell lung carcinoma *e.g.*, cisplatin plus etoposide, as well as CAE, are also associated with febrile neutropenia.

[0135] Various netropenias are know, and in some embodiments, the HSA-G-CSF fusion protein of **FIG. 1** is used to prevent, treat or ameliorate one or more neutropenias, including, but not limited to chemotherapy induced neutropenia, primary neutropenia, acute neutropenia, severe chronic neutropenia (SCN), severe congenital neutropenia (Kostmann's syndrome), severe infantile genetic agranulocytosis, benign neutropenia, cyclic neutropenia, chronic idiopathic neutropenia, secondary neutropenia, syndrome associated neutropenia, or immune-mediated neutropenia.

VIII. Experimental Examples

[0136] The following examples are given to illustrate the present invention. It should be understood, however, that the invention is not to be limited to the specific conditions or details described in these examples. All publically available documents referenced herein, including but not limited to US patents, are hereby incorporated by reference.

[0137] In the following non-limiting examples, Neugranin™ ("NEUG") was tested on cells and in mice and monkeys, and was also used to prevent, treat or ameliorate neutropenia and/or leukopenia in human subjects caused by drug therapy (*e.g.*, chemotherapy) for the treatment of breast cancer.

[0138] NEUG is a G-CSF analog with a reduced plasma clearance rate attributed to fusion of the active G-CSF moiety to human serum albumin. The resulting fully recombinant

protein retains the pharmacologic activity of G-CSF in vivo, *i.e.* it stimulates neutrophil and hematopoietic stem cell mobilization from the bone marrow to the peripheral blood stream. The activity of this protein was assessed in mice and in monkeys (see Experimental Examples, below). The onset of the observed increases in neutrophil and hematopoietic progenitor counts was rapid and persisted for several days after a single administration. The effects were dose-dependent, with higher doses including a greater magnitude and duration of the response to NEUG compared with lower does. Clearance of NEUG was slower than Neupogen® (filgrastim). The kinetics of blood neutrophilia following single and repeat administration of NEUG was nearly identical to that observed in animals treated with Neulasta® (pegfilgrastim). Because NEUG is 4.5-fold larger than Neulasta® (pegfilgrastim) (by mass), a larger dose (by weight) but an equimolar dose of NEUG can be used to achieve similar effect *in vivo*. In monkeys, equimolar doses of NEUG had equivalent effect to pegfilgrastim, and in mice a 1.5-fold higher does of NEUG was shown to achieve equivalent AUC_{ANC}. In these studies, 1 mg of Neulasta® (pegfilgrastim) was equivalent in effect to 4.5-7.7 mg of NEUG. For context, the No Adverse Effect Level ("NOAEL") of NEUG demonstrated in monkey was greater than 1 mg/kg/week. A 1 mg/kg dose in monkeys resulted in an exposure (Cmax and AUC) ~12-fold higher than was observed in human patients receiving 0.45 mg/kg NEUG. Thus, the NOAEL demonstrated in monkey is higher than the dose range used for clinical evaluation of NEUG (see below).

[0139] In aggregate, the studies show that equimolar doses of NEUG provide similar pharmacological effect to Neulasta® (pegfilgrastim) and have a similar effects on granulocyte populations in human.

A. *In vitro* and *in vivo* studies of NEUG

[0140] Results of *in vitro* and *in vivo* studies of NEUG are summarized as follows and are detailed in the Experimental Examples, below.

[0141] In vitro pharmacology studies have shown the following:

1. NEUG induces proliferation of NFS-60 cells in a dose-dependent fashion.
2. NEUG is ~3-fold less potent than Neupogen® (filgrastim) *in vitro*.
3. NEUG is equipotent with Neulasta® (pegfilgrastim) when evaluated on molar basis (1 mg of Neulasta® (pegfilgrastim) is equivalent in effect to 4.5 of NEUG).

[0142] In vivo pharmacology studies have demonstrated the following properties of NEUG:

1. NEUG was well-tolerated in mice and cynomolgus monkeys.
2. In mice, a single administration of NEUG induces a dose-dependent, rapid and prolonged increase of neutrophils and hematopoietic progenitor cells in the peripheral blood. When compared with current marketed G-CSF products, the rise in neutrophil and progenitor cell count was longer in duration than that achieved by an equimolar dose of Neupogen® (filgrastim) and similar in duration and magnitude to an equimolar dose of Neulasta® (pegfilgrastim).
3. In mice, equivalent and AUC_{ANC} were achieved with a 7.7-fold higher milligram dose of NEUG than Neulasta® (pegfilgrastim).
4. In 5-FU-induced neutropenic mice, single injections of equimolar doses of NEUG and Neulasta® (pegfilgrastim) effectively accelerated neutrophil recovery with similar kinetics and magnitude of effect.
5. Single and repeat (once weekly) doses of NEUG and Neulasta® (pegfilgrastim) induce similar increases in peripheral neutrophil count in monkeys. At equimolar doses, both the magnitude and the duration of neutrophil elevation in monkeys were similar in animals treated with Neulasta® (pegfilgrastim) and animals treated with NEUG.
6. In both mice and monkeys, NEUG has a slower clearance, a longer terminal half-life, and a greater mean residence time than Neupogen® (filgrastim) when administered IV or SC.
7. In cynomolgus monkeys, the terminal half-life of NEUG (12.6 hours) is approximately 33% longer than that of Neulasta® (pegfilgrastim) (9.49 hours) following SC injection and the clearance over bioavailability ("CL/F") of NEUG is about half that of Neulasta® (pegfilgrastim).
8. The clearance of both NEUG and Neulasta® (pegfilgrastim) are slower in mice that have undergone 5-FU-induced cytopenia than in normal mice suggesting that receptor-mediated clearance of both proteins contributes to their clearance.

9. Renal excretion (assessed in rats) contributes significantly to the clearance of Neupogen® (filgrastim), has a small effect on Neulasta® (pegfilgrastim), and is without substantial contribution to the clearance of NEUG.
10. NEUG, a human protein composed of human serum albumin and human colony stimulation factor, is immunogenic in mice and cynomolgus monkeys. Neulasta® (pegfilgrastim) induced a similar incidence and titer of antibodies in monkeys. Antibodies from some monkeys treated with NEUG and Neulasta® (pegfilgrastim) were neutralizing in vitro and for both NEUG and Neulasta® (pegfilgrastim), the neutrophil response diminished with repeat exposure, through antibody positive animals had basal levels of neutrophils similar to antibody negative animals during recovery.

[0143] Taken together, the *in vitro* and *in vivo* pharmacological properties of NEUG suggest that it acts in a manner similar to Neupogen® (filgrastim) and Neulasta® (pegfilgrastim) in that it similarly promotes mobilization of neutrophils and hematopoietic cells into the bloodstream. Non-limiting *in vitro* and *in vivo* Experimental Examples are provided below.

Example 1: NFS-60 cell proliferation

[0144] NSF-60 is a cell line routinely used in bioassay for the measurement of G-CSF activity. This cell line increases proliferation rate in response to G-CSF. The relative potency of recombinant C-CSF (Neupogen®, filgrastim) and NEUG were compared.

[0145] To measure the effectiveness of NEUG and Neupogen® (filgrastim) in stimulation of NFS-60 cell proliferation, ^{3}H-thymidine incorporation was measured following 24-hours of exposure to a range of concentrations of these analogs. EC_{50} values were obtained and expressed in units of mass (ng/ml).

[0146] Briefly, $1x10^{5}$ NFS-60 cells/well were seeded in 96-well plate in a final volume of 200 µl of complete medium containing the indicated amount of NEUG (also termed Albugranin) or Neupogen® (filgrastim). All samples were run in triplicate. The cells were incubated at 30° C for 24 hours and pulsed during the last 4 hours with 0.5 µCi ^{3}H-thymidine/well. Incorporation of thymidine was used as a measure of proliferation. (**FIG. 8**).

[0147] NEUG and Neupogen® (filgrastim) stimulated proliferation in a dose-dependent fashion. In this assay, Neupogen® (filgrastim) was 15-fold more potent that NEUG when compared on a mass basis. NEUG is ~4.5 fold larger (in mass) than Neupogen® (filgrastim), thus expressed on a molar basis, in this assay, NEUG was ~3 –fold less potent than Neupogen® (filgrastim) *in vitro*.

[0148] Neulasta® (pegfilgrastim) is vialed based on the weight of recombinant G-CSF, the mass of the polyethylene glycol modification is not included in dosage calculations. NEUG is 4.5x larger than recombinant G-CSF, because of the mass contribution from HSA, and thus to compare the ability of NEUG and Neulasta® (pegfilgrastim) to induce NFS-60 cell proliferation, a titration of equimolar doses of NEUG and Neulasta® (pegfilgrastim) were compared and EC_{50}s were expressed as a molar concentration. Briefly, $1x10^5$ NFS-60 cells/well were seeded in 96-well plate in a final volume of 200 µl of complete medium containing the indicated amount of NEUG (Albugranin) or Neulasta® (pegfilgrastim). All samples were run in triplicate. The cells were incubated at 37° C for 20 hours and pulsed during the last 4 hours with 0.5 µCi ^{3}H-thymidine/well. Incorporation of thymidine was used as a measure of proliferation. Results are shown in **FIG. 9**.

Example 2: Comparison of NEUG and Neupogen® (filgrastim) in BDF-1 mice

[0149] The objective of this study was to assess the effect of single subcutaneous doses of NEUG on peripheral blood neutrophil and hematopoietic stem cells in BDF-1 mice. BDF-1 mice were injected subcutaneously ("SC") with a single administration of NEUG at 3 dose levels (0.25 mg/kg, 1.25 mg/kg or 5.0 mg/kg) or Neupogen® (filgrastim) at 2 dose levels (0.25 mg/kg and 1.25 mg/kg). Peripheral granulocytes (Gr.1+) and hematopoietic progenitor cells (c-kit+) were quantified by flow cytometry daily from day 1 until day 5 and were compared to the levels obtained in vehicle treated animals.

[0150] Both NEUG and Neupogen® (filgrastim) caused an elevation in peripheral neutrophil counts when compared to vehicle treated animals, but the kinetics and the magnitude of the responses were different (**FIG. 10**). In the Neupogen® (pegfilgrastim) groups, a maximum 3-fold increase in neutrophil count occurred on day 1 and neutrophils returned to normal levels by day 2. In contrast, while a single administration of NEUG elevated neutrophil counts to a similar extent as comparable doses of Neupogen® (filgrastim), neutrophil counts continued to rise in the NEUG groups peaking with a kinetic

and magnitude that was dose dependent. Doses of 0.25, 1.25 and 5.0 mg/kg NEUG resulted in peak neutrophil counts 5.4, 10 and 24 fold over those obtained in vehicle treated animals. The lower two doses caused a peak elevation in neutrophil count on day 2, while the highest dose tested resulted in a peak on day 4. Neutrophils returned to normal levels on days 3, 4 and 5 for NEUG at 0.25, 1.25 and 5.0 mg/kg respectively (**FIG. 10**). As shown in **FIG. 10**, the NEUG induced increases in peripheral blood neutrophils were of greater magnitude and longer duration than those induced by Neupogen® (filgrastim).

[0151] The results of NEUG and Neupogen® (filgrastim) treatment on peripheral hematopoietic (c-kit+) stem cell counts in this study were very similar to those obtained for peripheral neutrophils **(FIG. 11)**. The effect of NEUG was dose-dependent, similar to comparable doses of Neupogen® (filgrastim) on day 1, but continued to rise on days 2-4 with all treatment groups returning to vehicle-defined baseline by day 5. As shown in **FIG. 11**, the NEUG-induced increases in c-kit+ cells were of greater magnitude and longer duration than those induced by Neupogen® (filgrastim).

Example 3: Comparison of NEUG and Neulasta® (pegfilgrastim) in BDF-1 mice

[0152] The objective of this study was to compare the effect of single subcutaneous (SC) injections of NEUG and Neulasta® (pegfilgrastim) on peripheral blood neutrophils and hematopoietic progenitor cells in peripheral blood of BDF-1 mice. This was evaluated by injecting BDF-1 mice (n=5) with a single dose of NEUG at 5 or 10 mg/kg. The effect of NEUG was compared with the effect of equimolar doses of pegfilgrastim (Neulasta®) (1.12 mg/kg and 2.24 mg/kg) given as a single administration. These two doses of Neulasta® (pegfilgrastim) and NEUG are approximately equimolar.

[0153] Results are shown in Figure 12. A single administration of NEUG (5 and 10 mg/kg) or Neulasta® (1.12 mg/kg and 2.24 mg/kg) effectively increased the number of peripheral granulocytes and hematopoietic progenitor cells in BDF-1 mice. In this experiment the maximum mobilization of peripheral granulocytes occurred on day 3 for NEUG at 5 mg/kg and on day 4 for NEUG at 10 mg/kg. Granulocytes returned to normal levels on day 6 post-NEUG treatment. In mice administered with a single dose of Neulasta® (pegfilgrastim), the maximum mobilization of granulocytes occurred on day 4. ANC in mice receiving Neulasta® (pegfilgrastim) at 2.25 mg/kg was still significantly (p=0.036) higher than the

baseline on day 6 post drug administration, while the absolute neutrophil count (ANC) in mice receiving 1.12 mg/kg returned to normal on day 6 (**FIG. 12A**).

[0154] To evaluate relative potency in mice, areas under the PD curves (AUC_{ANC}) versus molar equivalent dose (nmol/kg) were calculated (**FIG 12B**). The lines for the dose response are parallel, suggesting that AUC_{ANC} provides an appropriate means of comparison in this model. AUC_{ANC} was clearly dose-dependent for both G-CSF analogs. In this experiment, the relative potency of Neulasta to NEUG on weight bases was 7.7. That is, 1 mg or Neulasta® (pegfilgrastim) (as dosed in clinic on the bases of rhG-CSF weight) is equivalent in effect to 7.7 mg of NEUG. This is ~1.5 fold higher than the 4.5 fold molecular weight difference between NEUG and Neulasta® (pegfilgrastim).

[0155] A single administration of NEUG or Neulasta® (pegfilgrastim) significantly ($p<0.05$) increased the total number of hematopoietic progenitors in peripheral blood (**FIG. 15**). The maximum mobilization of hematopoietic progenitor cells occurred on day 4 in both NEUG and Neulasta® (pegfilgrastim)-treated groups. NEUG at 10 mg/kg and Neulasta at 1.12 and 2.24 mg/kg induced a similar increase in c-kit+ cells ($p < 0.0001$). A 5 mg/kg dose of NEUG resulted in statistically significant ($p<0.0001$) increased in c-kit+ cells compared to HSA control, however, this increase was about 50% less than that observed in both Neulasta® (pegfilgrastim) groups and appeared sub-maximal since a 2-fold higher dose resulted in an increase in maximal c-kit+ cells count (**FIG. 15**).

Example 4: Comparison of NEUG and Neulasta® (pegfilgrastim) in 5-FU induced neutropenic BDF-1 mice

[0156] G-CSF products are used clinically to accelerate the recovery of neutrophils after myelosuppressive chemotherapy. The objective of this study was to compare the effect of single subcutaneous (SC) injection of NEUG and Neulasta® (pegfilgrastim) on neutrophil recovery in a model where neutropenia was induced by a sub-lethal dose of 5-FU (150 mg/kg). BDF1 mice were given a single administration of NEUG at 5 or 10 mg/kg. The effect of NEUG was compared with the effect of a single administration of Neulasta (1.12 mg/kg – equimolar dose to NEUG at 5 mg/kg). Both agents were given 1 day after a single dose of 5-FU. The number of peripheral blood neutrophils was determined daily from day 6 until day 10. In this period of time, mice receiving 5-FU were characterized by a neutrophil nadir followed by a slow recovery phase. The experiment was designed to determine the effects of NEUG on the time and magnitude of neutrophil recovery.

[0157] Mice injected with vehicle control or HSA 1 day post 5-FU administration reached a neutropenic nadir by day 6 (**FIG. 16**). Neutrophil levels began to recover by day 10. In contrast, recovery from neutropenia was accelerated when mice were treated with either NEUG or Neulasta on day 1 post 5-FU administration. Treatment with either agent at all dose levels resulted in statistically significant increases in neutrophil count compared with the vehicle control. On day 9 the effect of NEUG given at 5 mg/kg was lower ($p = 0.0048$) compared with the effect achieved by an equimolar dose of Neulasta (1.12 mg/kg). However, by day 10 both agents caused similar increases in the total peripheral neutrophils.

[0158] To summarize the mouse study data, a single administration of NEUG to normal mice effectively induced a dose-dependent, rapid and prolonged increase of neutrophils (Gr.1+ cells) and hematopoietic progenitor cells (c-kit+) in the peripheral blood. The response to NEUG was similar to that induced by Neulasta® (pegfilgrastim), however in this study the maximal response to NEUG was slightly delayed relative to Neulasta® (pegfilgrastim). A single administration of Neupogen® (filgrastim) elicited only a transient increase in neutrophil and hematopoietic progenitor cell count in peripheral blood. Using a clinically relevant mouse model of cytopenia in which a sub-lethal dose of 5-FU injected IP induced myelosuppression and peripheral neutropenia, a single administration of NEUG or Neulasta® (pegfilgrastim) effectively enhanced neutrophil recovery.

Example 5: NEUG test in cynomolgus monkeys

[0159] Cynomolgus monkeys were chosen to determine the effects of repeated administration of NEUG. Two monkey studies were performed with serial evaluation of hematology after repeated administrations of NEUG: a 2-week pharmacology study comparing subcutaneous doses of NEUG and Neupogen® (filgrastim), and a longer (5 month) immunogenicity study comparing the effect of subcutaneous and intravenous NEUG with subcutaneous Neulasta® (filgrastim). Both studies show that NEUG causes a prolonged elevation in peripheral blood neutrophils in monkeys with a potency and pharmacodynamic profile similar to Neulasta® (pegfilgrastim).

Example 6: 2-week pharmacology study of NEUG in monkeys

[0160] To evaluate the pharmacodynamics of NEUG in monkeys, a 2-week repeat dose study was performed with hematology parameters as an efficacy endpoint. Twenty

experimentally naïve male and female cynomolgus monkeys were randomized into 5 treatment groups of 2 male and 2 female monkeys each. Monkeys were injected subcuteaneously ("SC") in the mid-scapular region with vehicle, NEUG, or Neupogen® (filgrastim). During the 14 day treatment phase of the study, vehicle was administered every 4 days (Q4D), NEUG was administered at 25 μg/kg every 4 days (Q4D), or 100 μg/kg every 4 or 7 days (Q4D or Q7D, respectively), and Neupogen® (filgrastim) was administered at 5 μg/kg daily.

[0161] NEUG was well tolerated by cynomolgus monkeys at 25 μg/kg or 100 μg/kg administered as frequently as every 4 days, and resulted in no adverse effects. The hematologic changes primarily consisted of NEUG-induced increases in peripheral blood neutrophils, with a less prominent increase in peripheral blood monocytes. The increase in neutrophils peaked 24 hours following SC administration of 100 μg/kg (**FIG. 17**). Administration of NEUG at 25 μg/kg every 4 days, or of 5 μg/kg Neupogen® (filgrastim) daily, resulted in moderate increases in neutrophils that reached significance when compared with vehicle during the second week of administration. All hematology changes attributed to NEUG completely reversed during the 2-week treatment-free recovery period.

Other observations

[0162] Monocyte numbers are reported to increase in the periphery in response to G-CSF, but to a lesser degree than is observed with neutrophils. In this study, only NEUG at 100 μg/kg administered every 4 days induced increases in absolute numbers of monocytes. The absolute number of peripheral blood lymphocytes were not affected by treatment with either NEUG or Neupogen® (filgrastim).

Example 7: Comparison of IV and SC NEUG with SC Neulasta® (pegfilgrastim) in monkeys

[0163] A non-GLP repeat dose administration study of NEUG in cynomolgus monkeys was conducted with the primary objective of assessing immunogenicity (Covance Study No. 6962-129). Hematology parameters were evaluated as a study endpoint and this study also provides useful pharmacology information in comparing equimolar doses of NEUG and Neulasta® (pegfilgrastim). Both NEUG and Neulasta were administered once weekly for 3 weeks. **FIG. 18** illustrates the ANC following each of the first 3 dose administrations of NEUG or Neulasta® (pegfilgrastim).

[0164] In this study, Neugranin administered SC and IV and Neulasta® (pegfilgrastim), administered SC at an equimolar dose, resulted in significant (<0.0001 compared to vehicle) elevation of peripheral blood neutrophils. Both the magnitude and the kinetics of neutrophil response were nearly identical among these three groups.

Example 8: Pharmacokinetics

[0165] The pharmacokinetics of NEUG were evaluated in normal BDF-1 mice following a single IV or SC injection, in 5-FU-treated, neutropenic BDF-1 mice following SC injection, in nephrectomized rats following IV injection, and in cynomolgus monkeys following single and multiple IV and SC injections. In addition, comparisons were made to the PK of rhG-CSF (Neupogen®) and pegylated rhG-CSF (Neulasta®). These studies are summarized below.

[0166] In all studies, plasma NEUG concentration was measured by "sandwich" ELISA with a G-CSF capture and HSA detection. This assay format allows intact NEUG to be quantified without interference or cross reactivity with endogenous G-CSF and albumin. The pharmacokinetics studies are summarized in tabular form in **FIG. 19.**

[0167] In these studies, NEUG has a slower clearance and longer terminal half-life and mean residence time (MRT) than Neupogen® (filgrastim) when administered IV or SC to BDF-1 mice. The clearance of NEUG is approximately 8 times slower than the clearance of Neupogen® (filgrastim) and the MRT (11.2-20.7 hours) is approximately 4 times longer. The clearance of both NEUG and Neulasta® (pegfilgrastim) are slower in mice that have undergone 5-FU induced cytopenia than in normal mice. This is most likely due to the smaller number of neutrophils (following 5-FU treatment), which play a role in the clearance of G-CSF. In cynomolgus monkeys, the MRT for NEUG is 17.9-27.2 hours. In addition, Cmax following the last of 5 weekly SC doses appears to decrease compared with the Cmax following the first dose. The SC bioavailability of NEUG in cynomolgus monkeys is approximately 22%. In cynomolgus monkeys, the terminal half-life of NEUG (12.6 hours) is approximately 33% longer than that of Neulasta® (pegfilgrastim) (9.49 hours) following SC injection. Renal clearance does not appear to play a significant role in the elimination of NEUG (determined in rats).

Example 9: Non-clinical toxicology summary

[0168] NEUG was well tolerated in mice and monkeys. There were no adverse finding in monkeys administered NEUG subcutaneously at 100, 500, or 1000 µg/kg/dose once weekly for 4 weeks. Pharmacodynamic responses to NEUG treatment were observed after multiple SC or IV dose administrations in cynomolgus monkeys, and were consistent with previously reported effects of G-CSF. NEUG consistently induced a marked and dose-dependent leukocytosis and neutrophilia, with less pronounced increases in monocytes, eosinophils and basophils, and inconsistent increases in lymphocytes. A no observable effect level (NOEL) for NEUG in monkeys was not identified in the GLP or non-GLP studies, and is therefore considered to be less than 25 µg/kg/dose for subcutaneous administration. No adverse effects were observed in SC NEUG-treated monkeys, therefore the no observable adverse effect level (NOAEL) for subcutaneous administration of NEUG in monkeys is greater than 1000 µg/kg/dose. Additional findings consistent with the pharmacology of NEUG included: increased splenic weight, microscopic evidence of myeloid hyperplasia and leukocytosis.

[0169] Non-clinical safety studies are summarized in the tables of **FIG. 20**.

Example 10: Immunogenicity

[0170] NEUG could (in theory) induce an immune response in patients that was neutralizing to G-CSF. Antibodies to HSA are also possible, though their clinical significance is uncertain given the extremely high concentration of HSA in the blood (40 mg/mL). A series of highly sensitive assays able to detect antibodies to all components of NEUG was used to assess immunogenicity in man.

[0171] To determine the safety and toxicology for NEUG, immunogenicity was assessed in several studies. These studies demonstrate that human G-CSF (Neulasta®, pegfilgrastim), human albumin, and NEUG are all immunogenic in monkeys. In an immunogenicity study that included a Neulasta® (pegfilgrastim) treatment arm, the majority of animals treated with weekly IV or SC doses of NEUG developed antibodies to Neulasta® (pegfilgrastim). Antibodies to NEUG (or Neulasta® pegfilgrastim) were first detected on or after day 22 (following the 3rd weekly dose). In many cases, these antibodies had a neutralizing effect in an *in vitro* assay, though the presence of neutralizing antibodies did not cause neutropenia and did not prevent the pharmacological effects of NEUG or Neulasta® (pegfilgrastim) in monkeys. Furthermore, following a non-dosing periods of 2 weeks and 2 months, ANCs in

all groups were within a normal range and there was no significant difference in ANC profiles regardless of antibody status.

[0172] Human albumin, like most human proteins, is immunogenic in animals. Experience with other albumin fusion proteins has demonstrated that immunogenicity in monkeys is not predictive of the incidence (or consequence) of immunogenicity in man. For example, Albuferon® (a fusion protein composed of human serum albumin and interferon-alpha) was highly immunogenic in monkeys (10/12 monkeys positive for antibody following a single injection) and the immune response was both neutralizing and substantially impacted exposure. In contrast, in a recent 458 patient Phase 2 study of Albuferon®, the rate of emergent antibodies was very low and was significantly lower in the Albuferon® treatment groups (3%) compared with a pegylated interferon treatment group (18%) through the first 12 weeks of treatment. Furthermore, antibodies were without apparent consequence.

[0173] Antibodies to NEUG have not been observed in human patients receiving up to 3 doses of NEUG (see e.g., section Phase I.5.c and Phase II,.5.e, below). With regard to NEUG risk assessment, available human and animal data suggest that neutralization of G-CSF would not preclude response to pharmacologic doses of G-CSF, nor would it preclude normal response to challenge by an infectious agent. In mouse models in which G-CSF is eliminated it has been shown that neutrophilia can still develop in response to challenge by an infectious agent, suggesting redundancies in the granulopoietic system. In addition, there are reports of humans with auto-antibodies against G-CSF in cases of Felty's syndrome and systemic lupus erythematosus. These patients develop neutropenia; however, treatment with G-CSF or GM-CSF remains effective in the majority of patients.

[0174] In summary, the *in vitro* and *in vivo* data and the pharmacokinetic characteristics of NEUG support NEUG use as a single dose prophylactic against febrile neutropenia in patients undergoing myelosuppressive anti-tumor therapy. Its ability to induce high levels of hematopoietic progenitors in peripheral blood following a single dose may also be beneficial in patients or donors for both autologous and allogenic hematopoietic stem cell transplantation.

B. Human studies of NEUG

[0175] The following examples are provided in two main sections entitled "Phase I" and "Phase II." Each phase includes two parts, Part A and Part B. The Phase I and Phase II examples are summarized in Table 2 below.

TABLE 2: Summary of human clinical NEUG studies

Trial	(Part)/Tumor Type	Objective	Chemo.	No. of Subjects	Treatment Arms
Phase I	(A)/breast	Initial dose-finding in absence of chemotherapy	none	13	50, 150, 300, 450 µg/kg NEUG
	(B)/breast	Initial dose-finding in presence of chemotherapy	Doxorubicin Docetaxel 2 Cycles	51	300 or 450 µg/kg NEUG vs. 6 mg pegfilgrastim
Phase II	(A)/breast	Dose-finding for fixed doses of Neugranin	Doxorubicin Docetaxel 4 Cycles	78	30, 40, 50 mg NEUG vs. 6 mg pegfilgrastim
	(B)/breast	Demonstration of non-inferiority of NEUG vs pegfilgrastim	Doxorubicin Docetaxel 4 Cycles	256	40 and 50 mg NEUG vs. 6 mg pegfilgrastim

[0176] Each Phase is divided into five sections: 1) objectives, 2) patient characteristics, 3) study agent, 4) study characteristics, and 5) results of Parts A and B.

Example 11: PHASE I

1. Objective

[0177] The Phase IA/B, IIA/B study was performed to evaluate the safety, tolerability, immunogenicity, pharmacokinetics and pharmacodynamics of subcutaneously administered Neugranin™ ("NEUG") (recombinant human albumin-human granulocyte colony stimulating factor) in subjects receiving myelosuppressive chemotherapy (doxorubicin/docetaxel).

[0178] For Phase I, the primary study objectives were to assess the safety profile of NEUG given subcutaneously over a range of potential therapeutic doses compared to pegfilgrastim by measuring the frequency, severity, and duration of treatment-emergent adverse events and correlating them with the time and dose of NEUG administration.

[0179] Secondary study objectives were to assess the pharmacokinetics and immunogenicity of NEUG, and to compare the effect of NEUG administration on the incidence, severity and duration of neutropenia to pegfilgrastim in patients receiving doxorubicin/docetaxel.

[0180] Phase I was performed as two parts, Part A and Part B as noted in Table 2 above.

2. Patient characteristics

[0181] For Phase I, patients were screened based on the following characteristics or parameters:

[0182] Inclusion:

1. Patients with histologically-confirmed breast cancer scheduled to receive doxorubicin and docetaxel.
2. 18 years of age or older.
3. Adequate hematologic function.
4. ANC > 1500/mm^3
5. Platelets > 100,000/mm^3
6. Adequate hepatic and renal function:
7. Serum creatinine < 2.0 x upper limit normal
8. Total bilirubin within normal limits (WNL) for local laboratory
9. Serum transaminases (SGOT/SGPT) < 1.5 x upper limit normal
10. Alkaline phosphatase < 2.5 x upper limit normal
11. ECOG performance status 0 or 1.
12. Eligible to receive doxorubicin based on a left ventricular ejection fraction (LVEF) within normal limits.
13. Have the ability to understand the requirements of the study, provide written informed consent (including consent for use and disclosure of research-related health information) and comply with the study protocol procedures.

[0183] Exclusion:

1. More than 1 prior chemotherapy regimen (including adjuvant therapy if given within the last 12 months); any chemotherapy/immunotherapy within 4 weeks

prior to study entry; cumulative anthracycline dose that would preclude 2 full-dose cycles of doxorubicin in this study.

2. Prior use of any nitrosoureas (BCNU, CCNU) or mitomycin-C within 6 weeks of study chemotherapy.
3. Cardiac history, signs or symptoms that, in the Investigator's opinion, preclude the use of an anthracycline-based chemotherapy regimen.
4. Prior surgery or radiation therapy within 2 weeks of study chemotherapy.
5. Prior wide field irradiation to the pelvis or to greater than 20% of the marrow-bearing areas, or bone marrow involvement.
6. Prior high-dose chemotherapy with hematopoietic stem cell transplant.
7. Prior use of myeloid (G-CSF or GM-CSF) growth factors within 4 weeks of study chemotherapy.
8. Prior use of erythropoietin within 4 weeks of study chemotherapy.
9. History of myeloid malignancy or myelodysplasia.
10. Known brain metastases unless adequately treated (surgery or radiotherapy), no evidence of progression with a minimum of 3 weeks observation and neurologically stable off anticonvulsants and steroids.
11. Known sickle cell disease.
12. Diagnosis of adult respiratory distress syndrome (ARDS).
13. Current infection requiring intravenous or oral antibiotics.
14. Known history of allergies to yeast-derived products.
15. Known hypersensitivity to E coli-derived proteins, pegfilgrastim, filgrastim, or any other component of pegfilgrastim (phase 2 only).
16. Pregnant female or nursing mother (over the course of the study, all females must practice a method of contraception with greater than 90% reliability, or be sterile or postmenopausal).
17. Known HIV positive or active hepatitis (patients with unknown status will not be tested).
18. Males who do not agree to use effective contraception throughout the study and for a period of 30 days after the last dose of study agent.

[0184] Subjects were removed from further treatment for the following reasons:

1. Disease progression
2. Unacceptable toxicities despite optimal treatment
3. Intercurrent illness at the investigator's discretion
4. Doxorubicin regimen – Maximum lifetime permissible cumulative dose reached (see eligibility criteria)
5. Withdrawal of consent
6. Non-compliance/Loss to follow-up
7. Pregnancy

[0185] If treatment with NEUG was stopped, subjects remained on study and were followed at least 30 days following the final dose of any study drug for scheduled safety and PK assessments.

3. Study agent

[0186] NEUG (recombinant human albumin-human granulocyte colony stimulating factor, rHSA-G-CSF), is a fusion protein with a molecular mass of approximately 85kDa connected in a single chain comprising residues 1-585 corresponding to the mature form of HSA and residues 586-759 corresponding to the mature form of human G-CSF. The therapeutic moiety of NEUG is recombinant human DNA-derived G-CSF.

[0187] NEUG was supplied as a sterile, lyophilized formulation in single-use Type 1 glass vials and stored at 2-8ºC. Upon reconstitution with 1.1 ml of sterile water for injection, each vial contained 15mg/ml (15mg/vial deliverable) NEUG in 10 mM sodium phosphate, 200mM mannitol, 60mM trehalose dehydrate, 0.01% (w/v) polysorbate 80, pH 7.2.

[0188] The composition of the NEUG drug product used in Phase I is presented in **FIG. 13.**

[0189] Commercially available Neulasta® (pegfilgrastim) was supplied in 0.6 ml prefilled syringes for subcutaneous injection. Each syringe contains 6 mg pegfilgrastim (based on protein weight), in a sterile, clear, colorless, preservative-free solution (pH 4.0) containing acetate (0.35 mg), sorbitol (30.0 mg), polysorbate 20 (0.02 mg), sodium (0.l02 mg) in water for injection. USP.

[0190] NEUG (50, 150, 300 or 450 μg) or Neulasta® (pegfilgrastim) (6 mg) was administered by subcutaneous administration.

4. Study characteristics

a. Study schedule and duration

[0191] This study was a first-in-man, multi-center, open-label non-controlled sequential dose escalation of a followed by a controlled, randomized trial conducted in 62 subjects with breast cancer scheduled to receive doxorubicin/docetaxel. The study consisted of 2 parts. Part A was a sequential dose escalation in 13 subjects, 4 dose cohorts (50, 150, 300, or 450 μg/kg) with 3 subjects in each of the 50, 150 and 450 μg/kg cohorts and 4 subjects in the 300 μg/kg cohort , to evaluate safety prior to the randomized, Part B of the trial.

[0192] In Part A, subjects received the first dose of NEUG at least 2 weeks prior to the start of chemotherapy (cycle 0) for an initial assessment of safety and effects on absolute neutrophil count ("ANC") in the absence of cytotoxic chemotherapy. After a minimum of 2 weeks follow-up, subjects received NEUG at the same dose following chemotherapy in cycles 1 and 2 if there were no dose-limiting adverse events considered related to NEUG in cycle 0 and the subject continued to meet all eligibility criteria.

[0193] In Part A, dose limiting toxicity (DLT) was defined as grade 2 or greater clinically significant adverse event(s) considered possibly, probably or definitely related to the study agent with the exception of grade 2 bone pain. Within each Part A cohort, the initial study drug administration to each subject entering the trial was separated by a minimum of 24 hours to monitor for acute adverse events.

[0194] The decision to escalate to the next dose level was based upon the review of the safety data for at least 7 days after the first dose administration of NEUG for all subjects in a given cohort. If none of the 3 subjects experienced a DLT, dose escalation continued with the enrollment of 3 subjects at the next dose level. If 1 of 3 subjects in a given cohort exhibited evidence of a DLT, another 3 subjects were recruited at that dose level for a total of 6 subjects per cohort. Dose escalation continued to occur if only 1 of 6 subjects experienced a DLT. If 2 of 6 subjects develop a DLT, dose escalation stopped and no further NEUG treatments were administered.

[0195] The remaining subjects completed their scheduled safety, pharmacokinetic and pharmacodynamic evaluations.

[0196] Following demonstration of safety in the initial Part A cohorts, Part B was performed. In Part B, subjects were randomized in a parallel fashion to 1 of 3 treatment

groups: NEUG 300 µg/kg (n = 20), NEUG 450 µg/kg (n = 21), or pegfilgrastim (n = 10) at the approved dose of 6 mg administered approximately 24 hours after study chemotherapy.

[0197] Tables 3 and 4 below summarize the disposition of the subjects in Phase I, Parts A and B. **FIG. 6** shows the chemotherapy cycles for Phase I study, Parts A and B.

Table 3: Disposition of Subjects in Phase I

Part A	**Dose**	**N (NEUG / pegfilgrastim)**
Sequential Dose Escalation	NEUG 50 µg/kg	3 / 0
	NEUG 150 µg/kg	3 / 0
	NEUG 300 µg/kg	4 / 0
	NEUG 450 µg/kg	3 / 0
Part B	**Dose**	**N (NEUG / pegfilgrastim)**
Parallel Randomization	NEUG 300 µg/kg	20 / 5
	NEUG 450 µg/kg	21 / 5

Table 4: Disposition of Subjects in Phase I

Treatment	**Part A (N)**	**Part B (N)**	**Total**
Neulasta® (pegfilgrastim)	0	10	10
NEUG 50	3	0	3
NEUG 150	3	0	3
NEUG 300	4	20	23
NEUG 450	3	21	24
Total	13	51	64

b. Concomitant therapy during Phase I, Parts A and B

Chemotherapy

[0198] The chemotherapy regimen for this trial consisted of doxorubicin 50 mg/m^2 and docetaxel 75 mg/m^2 administered sequentially by intravenous infusion on day 1 of treatment for up to two 21-day cycles.

[0199] Prior to receiving each cycle of therapy, subjects had to have an absolute neutrophil count (ANC) > 1.5 x 10^9/L and platelets > 100 x 10^9/L. Treatment could be delayed up to two weeks for hematologic recovery.

[0200] The combination of doxorubicin and docetaxel has been reported to have significant clinical activity in patients with breast cancer. However, the combination is highly myelosuppressive with higher rates of grade 3 or 4 neutropenia than other standard regimens.

[0201] Even with the addition of CSFs, the combination of doxorubicin and docetaxel has induced grade 4 neutropenia in 79% of patients and febrile neutropenia rates of 9-18%. This doxorubicin/docetaxel regimen has been used in studies of new agents to prevent neutropenia and its complications. Therefore, the combination of doxorubicin and docetaxel is an appropriate chemotherapy regimen to study the potential of a new agent like NEUG.

Doxorubicin

Pharmacologic Data

[0202] Doxorubicin hydrochloride is an anthracycline antibiotic obtained from streptomyces peucetius var caesius which inhibits DNA and DNA-dependent RNA synthesis, as well as protein synthesis. Doxorubicin is active in all phases of the cell cycle but maximally cytotoxic in S phase. Excretion of the drug is predominately by the liver; renal clearance is minor.

Pharmaceutical Data

[0203] The drug is marketed commercially in 10, 20 50, 100 or 200 mg vials. Lyophilized preparations may be reconstituted with sterile water for injection, dextrose 5% solution, or 0.9% saline for injection.

Side Effects and Toxicity

[0204] Myelosuppression, primarily leukopenia, with a nadir of approximately 10-14 days, and cardiotoxicity, including a rare, acute pericarditis- myocarditis syndrome and a delayed, cumulative dose related cardiomyopathy are the dose-limiting toxicities of doxorubicin. Marked alopecia and moderate nausea/vomiting are expected toxicities. Extravasation reactions producing local skin and tissue damage at the site of inadvertent extravasation, stomatitis, hyperpigmentation of the skin (particularly the nailbeds), and a "recall" phenomenon at sites of previous irradiation have been reported.

Docetaxel

Pharmacologic Data

[0205] Docetaxel is a semisynthetic taxoid that binds to free tubulin and promotes assembly of stable microtubules, interfering with mitosis and cell replication (cell cycle specific for M

phase). Docetaxel is extensively protein-bound, extensively metabolized in the liver, with fecal excretion of approximately 75% of the dose within 7 days.

Pharmaceutical Data

[0206] Docetaxel (Taxotere™, Sanofi Aventis) is provided in 80mg/2 mL or 20 mg/0.5 ml single-dose vials with an accompanying diluent (13% ethanol in Water for Injection) vial. Each ml of Taxotere contains 40 mg of docetaxel (anhydrous) and 1080 mg polysorbate 80.

Side Effects and Toxicity

[0207] Docetaxel should not be given to patients who have a history of severe hypersensitivity reactions to docetaxel or other drugs formulated with polysorbate 80 such as etoposide and vitamin E.

[0208] Patients who experience severe hypersensitivity reactions should not be rechallenged. Patients receiving docetaxel should be premedicated with corticosteroids as outlined below.

[0209] Mild to moderate liver impairment results in delayed metabolism by 27% and a 38% increase in systemic exposure (AUC). Docetaxel should not be given to patients with SGOT and/or SGPT > 1.5 times normal limits and alkaline phosphatase > 2.5 times normal limits. Fluid retention occurred in 17% (moderate) and 6% (severe retention) of patients in Phase III studies despite corticosteroid premedication. Severe neurosensory symptoms (paresthesia, dyesthesia, pain) have been observed.

[0210] Expected side effects include myelosuppression, primarily leukopenia, with a nadir of approximately 9 days with recovery by day 15-21. Alopecia, nail and cutaneous changes, stomatitis, myalgia/arthralgia, nausea/vomiting, and hypotension have been reported.

Chemotherapy Dosage, Administration and Dose Modifications

[0211] On day 1 of each treatment cycle, chemotherapy (doxorubicin followed by docetaxel) was administered.

[0212] Doxorubicin was administered at a dose of 50 mg/m^2 by IV bolus through the side arm of an infusing intravenous line or central venous catheter to avoid extravasation injury.

[0213] Docetaxel 75 mg/m^2 was diluted in 250 mL 0.9% saline or 5% dextrose solution and administered intravenously over approximately 1 hour via a polyethylene-lined infusion set. Vital signs were obtained immediately prior to and after the end of the docetaxel infusion.

[0214] Prior to receiving each cycle of therapy, subjects had to have an absolute neutrophil count (ANC) > 1500/mm^3 and platelets > 100,000/mm^3. Treatment could be delayed up to two weeks for hematologic recovery. A 25% dose reduction of chemotherapy doses was allowed for grade 3-4 non-hematologic toxicities, two grade 3-4 infectious episodes, or grade 4 thrombocytopenia.

[0215] Subjects experiencing severe hypersensitivity reactions or non-hematologic toxicities that preclude further cycles of chemotherapy were removed from study treatment but completed follow-up.

Chemotherapy Pre-medication

[0216] Oral (IV as needed) corticosteroids (such as dexamethasone 8mg BID) were administered for three days starting 1 day prior to docetaxel administration in order to reduce the incidence and severity of fluid retention and hypersensitivity reactions.

[0217] The use and selection of anti-emetic agents or other pre-medications (e.g. H_2 antagonists) was left to the discretion of the treating physician.

Prohibited Medications

[0218] Subjects should not have received any of the following medications and or procedures during this study and for the additional times specified below:

1. Other investigational agents within 30 days of initiating study agent and for the duration of the trial.
2. Subsequent cycles of chemotherapy should not be initiated until 14 days following dosing with NEUG.
3. Cytokines, other hematopoietic growth factors and prophylactic antibiotics for the duration of the trial unless prolonged or febrile neutropenia occurs. If the subject was treated with G-CSF at any time between the screening period and Day 0 they were not eligible to receive NEUG and were discontinued from the study.

Allowed Medications

[0219] Subjects were allowed to continue their baseline medications(s). The daily dose of each medication was maintained throughout the study if possible. If for any reason deemed necessary by the investigator, a subject required additional medication(s) or change of dose,

the medication(s), route of administration, and the indication for which it was given was recorded on the appropriate pages of the CRF.

Antibiotics

[0220] All subjects received prophylactic oral antibiotics (*e.g.* ciprofloxacin) following each course of chemotherapy to reduce the likelihood of infection. If febrile neutropenia or persistent severe neutropenia (ANC < 0.5×10^9/L for ≥ 5 days) occurred, the subject was considered a treatment failure, removed from the study, completed study follow-up and received all standard supportive care, including growth factor support at the Investigator's discretion.

[0221] Subjects who experienced severe hypersensitivity reactions or non-hematologic toxicities that precluded further cycles of chemotherapy were also removed from study treatment and completed follow-up.

c. Safety assessments

[0222] The safety of NEUG was assessed by evaluation of the type, frequency, and severity of adverse events ("AEs"), changes in clinical laboratory tests (hematology and clinical chemistry), immunogenicity, physical examinations, and the monitoring of vital signs over time. All AEs and laboratory toxicities were graded based on the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE Version 3.0, 12 December 2003). Adverse events (to include serious adverse events, "SAEs") were captured from the start of study drug administration through 30 days following the final dose of any study drug. Laboratory assessments were obtained as outlined in the Schedule of Assessments. In the event of any Grade 4 neutropenia toxicity, labs were obtained every day until ANC > 500. If the subject's next cycle of therapy was delayed (and after the last cycle of treatment), complete blood count (CBC) with differential was obtained at least twice weekly until ANC > 1500.

5. Results of Phase I, Parts A and B

a. General

Statistical Methods:

[0223] The data related to safety, pharmacokinetics (PK), pharmacodynamics (PD) and immunogenicity parameters were analyzed using descriptive statistical methods.

[0224] For frequency and severity of adverse events, and for laboratory toxicity grading, counts and rates are presented.

[0225] Efficacy analyses included the incidence and duration of grade 4 and grade 3-4 neutropenia, nadir ANC, time to nadir ANC, time to recovery (to ANC > 0.5 x 10^9/L and ANC > 1.0 x 10^9/L), and the incidence of febrile neutropenia.

[0226] No strict statistical power requirement was used to select the sample size for this study. A study with a power of 80% to demonstrate non-inferiority of NEUG to pegfilgrastim at a significance level of 5% was calculated to require approximately 37 subjects per treatment arm. As this was a phase 1/2a study conducted primarily for safety, it was determined that the required sample size to be powered for effect was larger than appropriate. As such, efficacy trends were evaluated.

Disposition/Demographics:

[0227] A total of 13 subjects were enrolled in the Part A, sequential dose escalation portion of the trial. A total of 51 subjects were enrolled in the Part B portion, and randomized to NEUG 300 µg/kg (n=20), NEUG 450 µg/kg (N=21), or pegfilgrastim 6 mg (n=10).

b. Study results

[0228] In initial dose-finding, in the absence of chemotherapy, NEUG was well tolerated and resulted in the expected rise in ANC, which peaked between days 2 and 4 and returned to normal by day 14 **(FIG. 22).**

[0229] In Part A, all three subjects in the 50 µg/kg NEUG dose group and 1 subject in the 450 µg/kg Neugranin dose group experienced febrile neutropenia or severe neutropenia lasting greater than 5 days. In Part B, one subject in the 300 µg/kg NEUG dose group and 2 subject in the 450 µg/kg NEUG dose group experienced febrile neutropenia or severe neutropenia lasting greater than 5 days. One subject in the pegfilgrastim group experienced febrile neutropenia or severe neutropenia lasting greater than 5 days.

c. Immunogenicity

[0230] Serum samples for antibodies to NEUG were obtained prior to dosing on Day 1 of every NEUG cycle and at the end of treatment visit (at least 15 days after the last dose) in

subjects receiving NEUG. If at any time during the study a subject developed a positive anti-NEUG antibody response, a repeat sample was obtained approximately 6 months after the final NEUG dose.

[0231] Testing was completed on all subjects through the end of treatment for both Part A and B. All samples were negative for antibodies to NEUG.

d. Adverse events

[0232] During Part A, dose-limiting toxicity (DLT) was defined as grade 2 or greater clinically significant adverse event(s), considered possibly, probably or definitely related to the study agent with the exception of grade 2 medullary bone pain.

[0233] No DLT was encountered in cycle 0 in any of the Part A cohorts. Only 2 adverse event were reported as related to NEUG administration: bone pain and exacerbation of pre-existing hypertension, the latter occurring 7 days after NEUG administration. Both events resolved without sequeale.

[0234] Thirty one of the 41 NEUG-treated subjects experienced at least 1 adverse event. The incidence of AEs among NEUG - and pegfilgrastim-treated subjects was comparable (75.6% and 70% respectively).

[0235] A summary of commonly reported adverse events (AEs greater than or equal to 5% of all subjects) for Part B is provided in Table 5.

Table 5: Summary of Treatment-Emergent Adverse Events in the Phase 1, Part B Population

Med DRA Preferred Term	NEUG 300 (N=20)	NEUG 450 (N=21)	Pegfilgrastim (N=10)
Related AE[1]:			
Bone Pain	1 (4.5%)	3 (14.3%)	0 (0%)
Unrelated AE[2]:			
Nausea	3 (15%)	3 (14.3%)	3 (30%)
Vomiting	1 (5%)	3 (14.3%)	3 (30%)
Diarrhea	1 (5%)	1 (4.8%)	1 (10%)
Stomatitis	0 (0%)	3 (14.3%)	0 (0%)

Table 5: Summary of Treatment-Emergent Adverse Events in the Phase 1, Part B Population

Fatigue	0 (0%)	0 (0%)	1 (10%)
Pharyngitis	2 (10%)	0 (0%)	1 (10%)
Alopecia	4 (20%)	7 (33%)	2 (20%)
Thrombocytopenia	0 (0%)	0 (0%)	1 (10%)
Headache	0 (0%)	1 (4.8%)	1 (10%)
Hypokalaemia	0 (0%)	2 (10%)	1 (10%)
Vitamin D Deficiency	3 (15%)	0 (0%)	1 (10%)
Hypertension	0 (0%)	1 (4.8%)	1 (10%)

[1] Related = considered possibly, probably or definitely related
[2]Unrelated = considered probably not related or not related

[0236] The most commonly reported adverse event considered related to NEUG was bone pain, a typical adverse reaction associated with all G-CSF products, which was reported in 5 patients 4 listed in the table above, plus one Part A subject receiving 450 µg/kg). In all cases, the bone pain was NCI-CTCAE grade 1-2 in intensity, transient in duration and resolved without sequelae. Grade 1 elevations in alkaline phosphatase and uric acid occurred following administration of NEUG in Cycle 0; these events were deemed to be not clinically significant by the Investigators and resolved without intervention. These are expected effects in patients receiving a G-CSF (e.g., Neulasta®).

[0237] Other commonly reported adverse events during chemotherapy cycles (nausea, vomiting, alopecia, stomatitis) were consistent with anticipated adverse events in patients receiving the doxorubicin/docetaxel regimen.

[0238] The majority of reported AEs were of NCI CTC Grade 1 or 2 severity. Four AEs were reported as serious adverse events. Two subjects, one receiving 150 µg/kg and one 450 µg/kg, experienced vomiting that caused hospitalization and one of these subjects experienced a second SAE in the following chemotherapy cycle; vomiting that was mild in intensity but caused or prolonged hospitalization. A third subject received 450 µg/kg was hospitalized for febrile neutropenia. The events were considered unrelated to NEUG.

e. Pharmacokinetics

[0239] All subjects receiving NEUG were sampled for serum NEUG concentrations over the course of the study. The drug was detected using a sandwich enzyme-linked

immunosorbent assay (ELISA) specific for NEUG. The serum drug concentration–time data was subjected to PK analysis using WinNonlin Enterprise Edition, Version 4.1 or higher, using noncompartmental or model-based analysis.

[0240] The following PK parameters were obtained: area under the curve ($AUC_{0-\infty}$), clearance (CL/F), volume of distribution (Vz/F), maximum concentration (Cmax), absorption half-life (t1/2, abs), elimination half-life (t1/2, elim), and mean residence time (MRT). Pharmacokinetic data were assessed for linearity across the dose range employed in the protocol.

[0241] Pharmacokinetic parameters from cycle 0 (pre-chemotherapy) are summarized in **Table 6** and the cycle 0 PK profile is illustrated in **FIG 3.**

Table 6 Neugranin Pharmacokinetics in Human Subjects (Phase 1 Cycle 0)

Parameter	**NEUG**	**NEUG**	**NEUG**
Dose (mcg/kg)	150 µg/kg	300 µg/kg	450 µg/kg
Number of Subjects	3	4	3
AUC (hr*ng/mL) (mean ± SD)	1758 ± 1675	3390 ± 2003	10131 ± 9563
$t_{1/2,term}$(hr) (mean ± SD)	14.4 ± 4.0	23.5 ± 10	29 ± 9.3
C_{max} (ng/mL) (mean ± SD)	72.7 ± 59.7	108.9 ± 50.5	294 ± 351
t_{max} (hr) (mean)	12	15	18

[0242] Drug exposure as measured by maximum serum NEUG concentration and area under the time-concentration curve increased in a dose-dependent manner. Serum concentrations for subjects in the initial 50 µg/kg dose cohort were consistently below the lower limit of quantization (6.3 ng/mL). Tmax was in the range of 6-24 hours for all doses from 150 through 450 µg/kg. Cmax ranged from 72.7 ± 59.7 (mean ± SD) ng/mL at a dose of 150 µg/kg to 294 ± 351 ng/mL, at a dose of 450 µg/kg. Correspondingly, $AUC_{0-\infty}$ ranged from 1758 ± 1675 ng/mL*hr at a dose of 150 mcg/kg to 10131 ± 9563 ng/mL*hr at a dose of 450 µg/kg. Cycle 1 ranges were similar. The mean elimination half-life of NEUG ranged from 14-30 hours.

[0243] As noted in "Study Characteristics" (section 4, above), subjects in Part A received the first dose of NEUG at least 2 weeks prior to the start of chemotherapy (cycle 0) for an

initial assessment of safety and effects on absolute neutrophil count ("ANC") in the absence of cytotoxic chemotherapy. After a minimum of 2 weeks follow-up, subjects received NEUG at the same dose following chemotherapy in cycles 1 and 2 if there were no dose-limiting adverse events considered related to NEUG in cycle 0 and the subject continued to meet all eligibility criteria. NEUG was administered 24 hours following chemotherapy administration. **FIG. 7** shows the ANC and WBC for subjects that received NEUG during cycles 1 and 2.

f. Pharmacodynamics and establishment of Part B dosages

[0244] Analysis of the data from Part A of Phase I of the study yielded the following observations:

1. NEUG induces a dose-dependent rise in WBC and ANC rise in Cycle 0 (prior to chemotherapy) (*see* cycle 0 data at **FIG. 7A**).
2. ANC increases in Cycle 0 were comparable to historical data for pegfilgrastim at equimolar doses
3. As anticipated, WBC and ANC drop following chemotherapy
4. Recovery from Nadir ANC appears dose related
5. ANC and WBC returned to normal by day 15

[0245] Based on these observations and demonstration of safety at all dose levels in Part A, the doses chosen for the Part B evaluation were 300 and 450 μg/kg. As described above, subjects were randomized to NEUG 300 μg/kg, NEUG 450 μg/kg, or pegfilgrastim at the approved fixed dose of 6 mg. Subjects received the NEUG or pegfilgrastim one day following doxorubicin/docetaxel (administered for 2 cycles, 21 days apart). Data for Part B includes the cycle 1 ANC profiles of the population. Results are summarized in Figure 2 and Table 5, below.

[0246] The incidence of grade 3 and 4 neutropenia, and the ANC profiles during Cycle 1 were determined in 48 of 51 treated subjects as show in Table 7, below. Note that 70-80% of patients treated with doxorubicin/docetaxel get Grade 4 neutropenia with durations average of 5 days in the absence of prophylactic G-CSF treatment.

Table 7: Incidence and duration of Grade 4 neutropenia in Phase I, Part B after cycle 1 of chemotherapy

Treatment	NEUG		Pegfilgrastim
Dose	300 µg/kg	450 µg/kg	6 mg
Number of subjects	20	21	10
Grade 4 Neutropenia	9	6	3
% grade 4 Neutropenia	45.0%	28.6%	30.0%
Mean (days)	1.1	1.0	0.7
SD (days)	1.33	1.67	1.16
Range (days)	0-4	0-5	0-3

[0247] Mean ANC curves for the treatment groups are presented in **FIG. 2**.

[0248] NEUG is effective for treating grade 3, grade 4 and febrile neutropenia. In the absence of G-CSF treatment for this chemotherapy regimen, the incidence of febrile neutropenia is about 40%. A dose-related elevation in ANC and a lower rate of neutropenia than is expected with doxorubicin/docetaxel were observed following administration of NEUG. There were no unexpected or serious adverse events attributed to NEUG.

[0249] The incidence of grade 3 and grade 4 neutropenia was higher in patients receiving 300 µg/kg NEUG than those receiving pegfilgrastim (Neulasta®) and the rate of return to normal ANC also appeared slower in patients who received 300 µg/kg NEUG than in those subjects who received pegfilgrastim. The ANC profiles in patients who received NEUG at 450µg/kg and those who received pegfilgrastim were similar, though the ANCs during recovery from neutropenia were generally lower in patients who received NEUG than in patients receiving pegfilgrastim. In summary, NEUG at these doses appears to provide similar effect as pegfilgrastim.

g. PK/PD profile, Phase I, Part B

[0250] The PK/PD profile from patients receiving 450 µg/kg NEUG one day after doxorubicin/docetaxel administration in cycle 1 of treatment for breast cancer is shown in **FIG. 4.** Cmax for NEUG is achieved within one day of administration and gradually falls to undetectable levels by day 10. Following administration of NEUG, the ANC rises to a peak by day 4 and then, as expected in patients receiving doxorubicin/docetaxel and G-CSF treatment, the ANCs fall to a nadir on day 8 and return to normal on day 10. By day 12, ANC values are in the normal range and NEUG is undetectable. Note that in patients who do not receive prophylactic G-CSF treatment, the duration of nadir ANC and time to reach

recovery ANC are much longer (e.g., 5-7 days). After a dose of 450 μg/kg, the NEUG median elimination half-life was approximately 30 hours, as compared to the 15-80 hours reported for a standard dose of pegfilgrastim. .

h. Additional differences between NEUG and pegfilgrastim

[0251] More detail of the differences between NEUG and pegfilgrastim at the tested doses in effectiveness in hastening the recovery form neutropenia is evident in comparison of the individuals ANC profiles in cycle 1 of treatment. The peak ANCs in all groups were very similar, nadir ANCs in subjects receiving NEUG at 300 μg/kg were lower than in subjects receiving NEUG at 450 μg/kg, and the ANC nadirs in subjects receiving pegfilgrastim were on average the highest. Recovery from nadir ANC to baseline occurred by day 14 in all treatment groups, but was slower for those receiving 300 μg/kg NEUG, than 450 μg/kg NEUG, and most rapid for subjects receiving pegfilgrastim.

[0252] Available published data for a pegfilgrastim trial with a similar prechemotherapy administration were compared to NEUG PK/PD data from patients who completed the Phase I through the scheduled cycle 0 (pre-chemotherapy). Results of this comparison were as follows:

1. Emax (maximum observed ANCs) at NEUG dose of 150 μg/kg matches the 30 μg/kg dose of pegfilgrastim in Cycle 0, a dose later demonstrated to be inferior for efficacy to the confirmed efficacious pegfilgrastim dose of 100 μg/kg.
2. Emax for 300 and 450 μg/kg Neugranin doses are more consistent with Cycle 0 levels for 100 μg/kg dose of pegfilgrastim.
3. At 300 and 450 μg/kg NEUG median Cmax and median Emax are nearly the same, thus Cmax continued to predict Emax.
4. ANC increases were comparable to published data for pegfilgrastim at equimolar doses.

[0253] As discussed above, PK/PD assessment in animals and in man was consistent with an estimate of NEUG and pegfilgrastim dose equivalence when dosed on an equimolar basis. In mice, equivalent AUC_{ANC} were achieved with a 7.7 fold higher dose thane pegfilgrastim. Because albumin contributes significantly to the molecular weight of NEUG, and Neulasta®

(pegfilgrastim) is dosed base on the weight of the rhG-CSF (not including the contribution of the polyethylene glycol in pegfilgrastim), a 4.5 fold greater dose of NEUG (based on weight) is predicted to be as effective as an equal dose of Neulasta® (pegfilgrastim). Efficacy data in animals were consistent with a 4.5-7.7 fold equivalence to pegfilgrastim (1 mg pegfilgrastim = 4.5-7.7 NEUG). Non-clinical safety and effect data are consistent with this dose estimate and when considered with available clinical data, form the basis for the doses elected for clinical evaluation.

i. Results of Phase I

[0254] The Results from the Phase I pharmacokinetic evaluation are as follows.

NEUG was detected in serum samples from all subjects treated with NEUG at doses of 150 μg/kg, 300 μg/kg and 450 μg/kg on Cycle 0 and Cycle 1.

In Cycle 1, NEUG was detected uptown 144 hours in most subjects (45/50 sampled) in the 150 mg/kg, 300 μg/kg and 450 μg/kg dose groups. Virtually no cycle to cycle drug accumulation was observed.

Drug exposure was higher in Cycle 1 and in Cycle 0 (pre-chemotherapy) with each dose group. The increased exposure to NEUG in Cycle 1 is likely due to the decreased number of neutrophils, which plan a role in the receptor-mediated clearance of G-CSF.

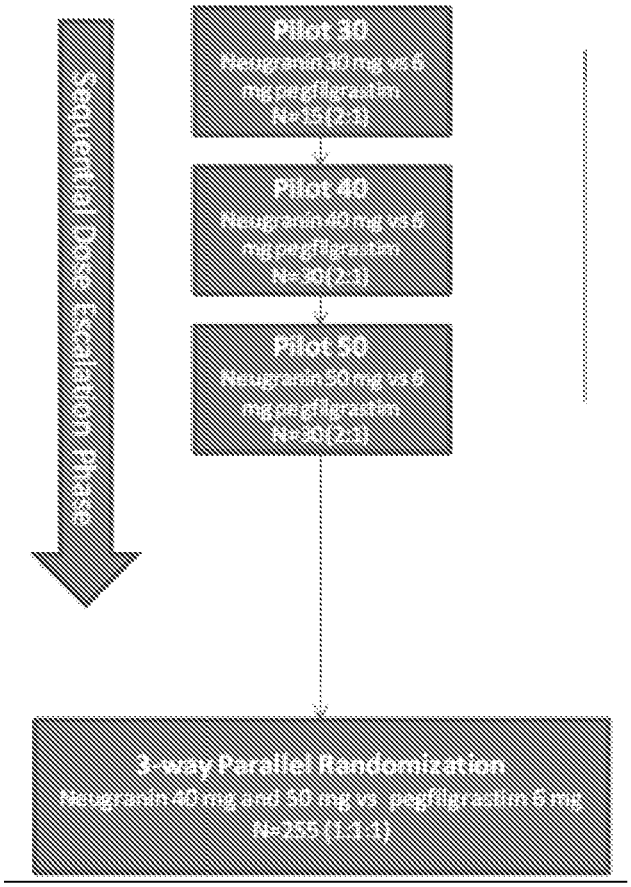
The median elimination half-life of NEUG in Cycle 1 was about 36 hours for dose group 300 μg/kg and 30 hour for dose group 450 μg/kg. The elimination half-life is reported to be 3-4 hours for filgrastim and 42-67.5 hours, depending on dose, for pegfilgrastim.

Statistically significant differences across doses were observed in the time to maximal serum concentration (t_{max}) and the absorption half-life ($t_{1/2,abs}$). Both of these parameters increased with increasing NEUG dose. No other dose normalized PK parameters showed statistically significant differences across doses.

Example 12: PHASE II

[0255] Phase II of the study was a controlled, randomized trial, conducted in 334 subjects with breast cancer who received up to 4 doses of doxorubicin/docetaxel. The study, was

conducted at 45 clinical sites, and consisted of a two-way randomized pilot phase to assess the safety and effect of subcutaneously administered NEUG versus pegfilgrastim, followed by a main phase in which subjects were randomized to pegfilgrastim and two, well-tolerated doses of NEUG (1:1:1) selected based on the pilot phase. The sample size for the main phase was powered to establish non-inferiority of NEUG to pegfilgrastim with regard to the primary endpoint, duration of sever (grade 4) neutropenia (DSN) during chemotherapy cycle 1. The study design is shown schematically below.



1. Objectives

[0256] The primary objectives of Phase II were to select doses of NEUG demonstrating a comparable effect to pegfilgrastim and to assess the duration of severe neutropenia (DSN) in cycle 1 of chemotherapy after treatment with NEUG. Secondary objectives were to assess the DSN in cycles 2-4, to assess the time to absolute neutrophil count recovery and rates of

febrile entroopenia in cycles 1-4; and to assess the safety, tolerability, pharmacokinetics (in cycle 1), and immunogenicity of NEUG.

2. Patient characteristics

[0257] For Phase II, patients were screened based on the following characteristics or parameters:

[0258] Inclusion:

1. Patients with histologically-confirmed breast cancer scheduled to receive doxorubicin 60 mg/m^2 and docetaxel 75 mg/m^2
2. 18 years of age or older
3. Adequate hematologic function:
4. ANC > $1500/mm^3$
5. Platelets > $100,000/mm^3$
6. Adequate hepatic and renal function:
7. Serum creatinine < 1.5 x upper limit normal
8. Total bilirubin within normal limits (WNL) for local laboratory
9. Serum transaminases (SGOT/SGPT) < 1.5 x upper limit normal
10. Alkaline phosphatase < 2.5 x upper limit normal
11. Eastern Cooperative Oncology Group ("ECOG") performance status 0 - 2
12. Eligible to receive doxorubicin based on a left ventricular ejection fraction (LVEF) within normal limits
13. Have the ability to understand the requirements of the study, provide written informed consent (including consent for use and disclosure of research-related health information) and comply with the study protocol procedures.

[0259] Exclusion:

1. More than 1 prior chemotherapy regimen (including adjuvant therapy if given within the last 12 months)
2. A cumulative anthracycline dose that would preclude 4 full-dose cycles of doxorubicin in this study
3. Prior chemotherapy/immunotherapy within 30 days prior of study chemotherapy (within 6 weeks of study chemotherapy for nitrosoureas (BCNU,

CCNU) or mitomycin-C)

4. Concomitant trastuzumab (Herceptin)
5. Received any investigational agent in the past 30 days
6. Cardiac history, signs or symptoms that, in the Investigator's opinion, preclude the use of an anthracycline-based chemotherapy regimen
7. Prior surgery within 2 weeks of study chemotherapy
8. Prior radiation therapy within 4 weeks of study chemotherapy (except spot irradiation for bone metastases)
9. Prior high-dose chemotherapy with hematopoietic stem cell transplant
10. Prior use of G-CSF, GM-CSF or erythropoietin within 4 weeks of study chemotherapy
11. Received systemic antibiotics within 72 hours of study chemotherapy
12. History of myeloid malignancy or myelodysplasia
13. Known brain metastases unless adequately treated (surgery or radiotherapy), no evidence of progression with a minimum of 3 weeks observation and neurologically stable off anticonvulsants and steroids.
14. Known sickle cell disease
15. Diagnosis of adult respiratory distress syndrome (ARDS)
16. Known history of allergies to yeast-derived products
17. Known hypersensitivity to E coli-derived proteins, pegfilgrastim, filgrastim, or any other component of pegfilgrastim
18. Pregnant female or nursing mother. (All females with an intact uterus must have a negative serum pregnancy test at screening. All non-sterile or non-postmenopausal females must practice a medically accepted method of contraception over the course of the study and for 30 days after the last dose of study agent.)
19. Males who do not agree to use effective contraception throughout the study and for a period of 30 days after the last dose of study agent
20. Known HIV positive or active hepatitis (Patients with unknown status will not be tested)

[0260] Subjects were removed from further treatment for the following reasons:

1. Disease progression
2. Unacceptable toxicities despite optimal treatment
3. Intercurrent illness at the investigator's discretion
4. Doxorubicin regimen – Maximum lifetime permissible cumulative dose reached (see eligibility criteria)
5. Withdrawal of consent
6. Non-compliance/Loss to follow-up
7. Pregnancy

[0261] If treatment with study drug was stopped, subjects remained on study were followed at least 30 days following the final dose of any study drug for scheduled safety and PK assessments.

3. Study agent

[0262] NEUG (recombinant human albumin- human granulocyte colony stimulating factor, rHSA-GCSF), is a fusion protein with a molecular mass of approximately 85kDa connected in a single chain comprising residues 1-585 corresponding to the mature form of HSA and residues 586-759 corresponding to the mature form of human G-CSF. The therapeutic moiety of NEUG is recombinant human DNA-derived G-CSF.

[0263] NEUG was supplied as a sterile, lyophilized formulation in single-use Type 1 glass vials and stored at 2-8ºC. Upon reconstitution with 1.0 ml of sterile water for injection, each vial contains 50 mg/ml (50 mg/vial deliverable) NEUG in 20 mM sodium phosphate, 180 mM, mannitol, 60mM trehalose dehydrate, 0.01% (w/v) polysorbate 80, pH 6.0. Note that NEUG is also be provided as a liquid, either in vials or in pre-filled syringes.

[0264] The composition of the drug product used in Phase II is shown in **FIG. 14.** Difference between the NEUG formulations used in Phase I and Phase II are shown below in Table 8.

Table 8: cGMP formulation comparison

Excipient Formulation Attribute	Phase I formulation	Phase II formulation	Rationale for change
API	15.0 mg/mL	50 mg/mL	Increased API concentration to reduce volume of injection

Table 8: cGMP formulation comparison			
Excipient Formulation Attribute	**Phase I formulation**	**Phase II formulation**	**Rationale for change**
Sodium Phosphate	10 mM	20 mM	Higher ionic strength reduces concentration dependent aggregation
Mannitol	200 mM	180 mM	Reduced to provide iso-osmotic solution
Trehalose dihydrate	60 mM	60 mM	Unchanged—acts as robust cryo/lyo protectant.
Polysorbate 80	0.01%	0.01%	Unchanged—inhibits nonspecific aggregation and adsorption
pH	7.2	6.0	Lower pH reduces concentration dependent aggregation

[0265] The formulation used in Phase I was quite stable, with a shelf-life of at least 2 years. Studies demonstrated that higher ionic strength and lower pH further stabilized the API at higher concentration (> 25 mg/mL) (data not shown). To this end, the Phase II formulation has a lower pH (6.0 vs 7.2) and higher phosphate concentration (20 vs. 10 mM). Forced degradation studies demonstrate that this formulation protects the drug substance in the liquid state from vigorous shaking, repeated freeze-thawing, and concentration induced aggregation. Freeze drying of the Phase II formulation also produces well-formed cakes.

[0266] Commercially available Neulasta® (pegfilgrastim) is supplied in 0.6 ml prefilled syringes for subcutaneous injection. Each syringe contains 6 mg pegfilgrastim (based on protein weight), in a sterile, clear, colorless, preservative-free solution (pH 4.0) containing acetate (0.35 mg), sorbitol (30.0 mg), polysorbate 20 (0.02 mg), sodium (0.l02 mg) in water for injection. USP.

[0267] NEUG (30, 40, 50, or 60 mg) or Neulasta® (pegfilgrastim) (6 mg) was administered by subcutaneous administration.

[0268] **Dose Rationale**

[0269] The data from Phase I demonstrated that doses of NEUG of 300 and 450 µg/kg were safe and well tolerated. Moreover, compared to the approved fixed doses of pegfilgrastim, both doses of NEUG resulted in similar effects on ANC profiles in breast cancer patients

receiving cytotoxic chemotherapy. The AUC for the ANC profiles serves as a single-point measure of effect. There was no statistically significant difference among these treatment groups in terms of AUC_{ANC}, however, the AUC for the 450 μg/kg group is slightly higher than that observed for the 300 μg/kg group and nearly identical to that observed for the pegfilgrastim group (**FIG. 23**). Based on available data, it was estimated that 300 μg/kg NEUG was less effective than pegfilgrastim and 450 μg/kg approximates a minimum necessary dose to provide equivalent effect to pegfilgrastim.

[0270] The intent of a fixed dose is to identify doses that will provide patients with a dose sufficient to provide efficacy and safety regardless of patient weight. Based on the results of Phase I, it as estimated that 450 μg/kg NEUG may be a minimum dose necessary to provide similar effect as pegfilgrastim, and > 300 μg/kg was set as the minimum dose for further evaluation in Phase II. To select fixed doses of NEUG, the patient population (breast cancer) for Phase II was modeled. Using 40-100 kg weight range, a 30 mg fixed dose provides the heaviest patient with a minimum dose (300 μg/kg or 0.3 mg/kg), while approximately 75% of patients receive at least the target dose, 450 mg/kg, at a fixed dose of 40 mg. Thus, the doses selected for evaluation in Phase II were 30 mg, 40 mg and 50 mg. These provide an average 70 kg patient with 0.42, 0.57 and 0.71 mg/kg doses, respectively.

[0271] The equivalent dose per kilogram based on the fixed doses evaluated in this trial is provided in Table 9.

Table 9: Equivalent dose per kilogram for the anticipated subject weight range						
	50 kg	60 kg	70 kg	80 kg	90 kg	100 kg
30 mg	0.600	0.500	0.429	0.375	0.333	0.300
40 mg	0.800	0.667	0.571	0.500	0.444	0.400
50 mg	1.000	0.833	0.714	0.625	0.556	0.500
60 mg	1.200	1.000	0.857	0.750	0.666	0.600

[0272] The nonclinical safety for NEUG provides additional support for the expectation of safety at these doses. Exposure in patients at these fixed doses (AUC and Cmax) is expected to be lower than exposure at well tolerated doses in monkeys. For example, Cmax and AUC in the monkey at the well-tolerated dose of 1 mg/kg was 12-fold higher than exposure in patients at 0.45 mg/kg suggesting a further margin of safety exists for higher dose evaluation in patients and in a repeat-dose toxicology study in monkey, doses up to and including 10 mg/kg were well tolerated. Doses of pegfilgrastim as high as 0.3 mg/kg have been demonstrated to be safe in patients.

4. Study characteristics

a. Study Schedule and Duration

[0273] This study was a controlled, randomized trial conducted in approximately 330 subjects with breast cancer scheduled to receive up to 4 doses of doxorubicin/docetaxel. The study, which was conducted at 45 clinical sites, consisted of two phases, a pilot phase and a main phase.

[0274] The pilot phase, Part A, consisted of a two-way randomized study to assess the safety and effect of NEUG versus pegfilgrastim, with sequential enrollment to the following doses: NEUG 30 mg (N=10) vs. pegfilgrastim (N=5); NEUG 40 mg (N=20) vs. pegfilgrastim (N=10), and NEUG 50 mg (N=20) vs. pegfilgrastim (N=10). In a further study, NEUG 60 mg (N=20) vs. pegfilgrastim (N=10) could also be tested. In the Part A pilot phase, subjects were randomized in a 2:1 ratio of NEUG to pegfilgrastim with a total of 10 subjects in the 30 mg cohort and 20 subjects for each of the other cohorts. NEUG or pegfilgrastim was administered to subjects 24 hours after the chemotherapy treatment in each cycle. Subjects were assigned to treatment groups using a stratified randomization for balance among

treatment groups based on weigh (<50 kg, ≥ 50 kg and < 80 kg, or ≥ 80 kg), prior chemotherapy exposure and global location.

[0275] Following the pilot phase, 255 subjects were randomized (1:1:1) to pegfilgrastim and the two well tolerated doses of NEUG with the more comparable effect to pegfilgrastim in the pilot phase (a 3-arm, balanced parallel-randomized phase). NEUG or pegfilgrastim was administered 24 hours after the chemotherapy treatments in each cycle. Subjects were assigned to treatment groups using a stratified randomization for balance among treatment groups based on weight (<50 kg, ≥ 50 kg and < 80kg, or ≥80 kg).

[0276] During the pilot phase, adverse events were reviewed on an ongoing basis. Escalation of the dose from 30 through 50 mg occurs unless the ongoing review of data suggested a safety concern. If the Cycle 1 ANC profile for Neugranin at 40 mg appeared inferior to the profile observed from pegfilgrastim patients and 50 mg of Neugranin is safe, then an additional arm may be randomized in a 2:1 ratio of Neugranin at 60 mg to pegfilgrastim with a total of 30 patients in the cohort.

[0277] Each dose level of NEUG is compared to pegfilgrastim for safety and efficacy. Table 10 summarize the patient allocation for Phase II, Part A and Part B.

Table 10: Allocation of Subjects in Phase II, Parts A and B

Phase	NEUG 30 mg	NEUG 40 mg	NEUG 50 mg	Pegfilgrastim 6 mg
Pilot 30	10	-	-	5
Pilot 40	-	20	-	10
Pilot 50	-	-	20	10
3-Arm Randomized	-	85	85	85
Total	10	105	105	110

Safety Evaluation:

[0278] The safety of NEUG was assessed by evaluation of the type, frequency, and severity of AEs, changes in clinical laboratory tests (hematology and clinical chemistry), immunogenicity, physical examinations, and the monitoring of vital signs over time. All AEs and laboratory toxicities were graded based on the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE Version 3.0, 12 December 2003).

[0279] Adverse events were captured from the start of study drug administration through 30 days following the final dose of any study drug. Serious adverse events (SAE) were captured from the time of consent through 30 days following the final dose of any study drug. Laboratory assessments were obtained as outlined in the Schedule of Assessments.

c. Concomitant therapy

Chemotherapy

[0280] The chemotherapy regimen for this trial consisted of doxorubicin 60 mg/m^2 and docetaxel 75 mg/m^2 administered sequentially by intravenous infusion on day 1 of treatment for up to four 21-day cycles.

[0281] Prior to receiving each cycle of therapy, subjects were required to have an absolute neutrophil count (ANC) > 1000/mm^3 and platelets > 100,000/mm^3. Treatment could be delayed up to two weeks for hematologic recovery. A 25% dose reduction of chemotherapy doses was allowed for grade 3-4 non-hematologic toxicities, two grade 3-4 infectious episodes, or grade 4 thrombocytopenia. The use of prophylactic antibiotics or other hematopoietic growth factors was prohibited during trial participation.

[0282] The combination of doxorubicin and docetaxel has been reported to have significant clinical activity in patients with breast cancer. However, the combination is highly myelosuppressive with higher rates of grade 3 or 4 neutropenia than other standard regimens.

[0283] Even with the addition of CSFs, the combination of doxorubicin and docetaxel has induced Grade 4 neutropenia in 79% of patients and febrile neutropenia rates of 9-18% . This doxorubicin/docetaxel regimen has been used in studies of new agents to prevent neutropenia and its complications. Therefore, the combination of doxorubicin and docetaxel is an appropriate chemotherapy regimen to study the potential of a new agent like NEUG.

Doxorubicin

Pharmacologic Data

[0284] Doxorubicin hydrochloride is an anthracycline antibiotic obtained from streptomyces peucetius var caesius which binds directly to DNA base pairs (intercalates) and inhibits DNA and DNA-dependent RNA synthesis, as well as protein synthesis. Doxorubicin is active in all phases of the cell cycle but maximally cytotoxic in S phase. Excretion of the drug is predominately by the liver; renal clearance is minor.

Pharmaceutical Data

[0285] The drug is marketed commercially in 10, 20 50, 100 or 200 mg vials. Lyophilized preparations may be reconstituted with sterile water for injection, dextrose 5% solution, or 0.9% saline for injection.

Side Effects and Toxicity

[0286] Myelosuppression, primarily leukopenia, with a nadir of approximately 10-14 days, and cardiotoxicity, including a rare, acute pericarditis-myocarditis syndrome and a delayed, cumulative dose related cardiomyopathy are the dose-limiting toxicities of doxorubicin.

[0287] Marked alopecia and moderate nausea/vomiting are expected toxicities. Extravasation reactions producing local skin and tissue damage at the site of inadvertent extravasation, stomatitis, hyperpigmentation of the skin (particularly the nailbeds), and a "recall" phenomenon at sites of previous irradiation have been reported.

Docetaxel

Pharmacologic Data

[0288] Docetaxel is a semisynthetic taxoid that binds to free tubulin and promotes assembly of stable microtubules, interfering with mitosis and cell replication (cell cycle specific for M phase). Docetaxel is extensively protein-bound, extensively metabolized in the liver, with fecal excretion of approximately 75% of the dose within 7 days.

Pharmaceutical Data

[0289] Docetaxel (Taxotere™, Sanofi Aventis) is provided in 80mg/2 mL or 20 mg/0.5 ml single-dose vials with an accompanying diluent (13% ethanol in Water for Injection) vial. Each ml of Taxotere contains 40 mg of docetaxel (anhydrous) and 1080 mg polysorbate 80.

Side Effects and Toxicity

[0290] Docetaxel should not be given to patients who have a history of severe hypersensitivity reactions to docetaxel or other drugs formulated with polysorbate 80 such as etoposide and vitamin E.

[0291] Patients who experience severe hypersensitivity reactions should not be rechallenged. All patients receiving docetaxel should be premedicated with corticosteroids as outlined below.

[0292] Mild to moderate liver impairment results in delayed metabolism by 27% and a 38% increase in systemic exposure (AUC). Docetaxel should not be given to patients with SGOT and/or SGPT > 1.5 times normal limits and alkaline phosphatase > 2.5 times normal limits. Fluid retention occurred in 17% (moderate) and 6% (severe retention) of patients in phase III studies despite corticosteroid premedication. Severe neurosensory symptoms (paresthesia, dyesthesia, pain) have been observed.

[0293] Expected side effects include myelosuppression, primarily leukopenia, with a nadir of approximately 9 days with recovery by day 15-21. Alopecia, nail and cutaneous changes, stomatitis, myalgia/arthralgia, nausea/vomiting, and hypotension have been reported.

Chemotherapy Dosage, Administration and Dose Modifications

[0294] On day 1 of each treatment cycle, chemotherapy (doxorubicin followed by docetaxel) was be administered.

[0295] Doxorubicin was administered at a dose of 60 mg/m^2 by IV bolus through the side arm of an infusing intravenous line or central venous catheter to avoid extravasation injury.

[0296] Docetaxel 75 mg/m^2 was diluted in 250 ml 0.9% saline or 5% dextrose solution and administered intravenously over approximately 1 hour via a polyethylene-lined infusion set. Vital signs were obtained immediately prior to and after the end of the docetaxel infusion.

[0297] Subjects experiencing severe hypersensitivity reactions or non-hematologic toxicities that preclude further cycles of chemotherapy were be removed from study treatment and complete follow-up.

Chemotherapy Pre-medication

[0298] Oral (IV as needed) corticosteroids (such as dexamethasone 8mg BID) was administered for three days starting 1 day prior to docetaxel administration in order to reduce the incidence and severity of fluid retention and hypersensitivity reactions.

[0299] The use and selection of anti-emetic agents or other pre-medications (*e.g.* H_2 antagonists) was left to the discretion of the treating physician.

Prohibited Medications

[0300] Subjects were not to receive any of the following medications and or procedures during this study and for the additional times specified below:

1. Systemic antibiotics within 72 hours of cycle 1 chemotherapy.
2. Other investigational agents within 30 days of initiating study agent and for the duration of the trial
3. Subsequent cycles of chemotherapy should not be initiated until 14 days following dosing with NEUG.
4. Cytokines, other hematopoietic growth factors and prophylactic antibiotics for the duration of the trial unless prolonged or febrile neutropenia occurs. If the subject is treated with G-CSF at any time between the screening period and Day 0 they will not be eligible to receive NEUG and will be discontinued from the study.

Allowed Medications

[0301] Subjects were allowed to continue their baseline medications(s). The daily dose of each medication was maintained throughout the study if possible. If for any reason deemed necessary by the investigator, a subject required additional medication(s) or change of dose, the medication(s), route of administration, and the indication for which it was given was be recorded.

[0302] Subjects experiencing severe hypersensitivity reactions or non-hematologic toxicities that preclude further cycles of chemotherapy were removed from study treatment and completed follow-up.

d. Pharmacokinetics

[0303] All subjects receiving NEUG were sampled for serum NEUG concentrations during cycle 1. The drug was detected using a sandwich enzyme-linked immunosorbent assay (ELISA) specific for NEUG. The serum drug concentration–time data was subjected to PK analysis using WinNonlin Enterprise Edition, Version 5.0 or higher, using noncompartmental or model-based analysis. The following PK parameters were determined: area under the

curve ($AUC_{0-\infty}$), clearance (CL/F), volume of distribution (Vz/F), maximum concentration (Cmax), absorption half-life (t1/2, abs), elimination half-life (t1/2, elim), and mean residence time (MRT).

e. Immunogenicity

[0304] Serum samples for antibodies to NEUG were obtained prior to dosing on Day 1 of every NEUG cycle and at the end of treatment visit (approximately 30 days after the last dose) in subjects receiving NEUG. If at any time during the study a subject developed a positive anti-NEUG antibody response, a repeat sample was obtained approximately 6 months after the final NEUG dose; if this sample was positive, a sample was obtained at 12 months. The protocol was later amended to require 6 and 12 month immunogenicity samples from all subjects.

5. Results

a. general

Statistical Methods

[0305] The sample size of about 85 subjects per arm in the main phase of this trial (Part B) was chosen to provide 91% power to establish non-inferiority of NEUG to pegfilgrastim with regard to the primary endpoint of mean duration of severe neutropenia (DSN) in cycle 1, with a non-inferiority margin of 1 day and an overall 1-sided significance level adjusted for multiple testing (by the Hochberg method) of 0.025. Sample sizes were calculated based on the normal approximation for two independent groups, an estimate of 1.6 days as the within-treatment standard deviation of cycle 1 DSN, and a maximum rate of 20% not evaluable for the primary endpoint of cycle 1 DSN.

[0306] Efficacy comparison was made between the two selected NEUG doses (either 40 mg and 50 mg) and pegfilgrastim, based on subjects in the 3-arm randomized phase (Part A).

[0307] Secondary efficacy analyses include the DSN in each of chemotherapy cycles 2 through 4, depth of ANC nadir in each of the cycles 1 through 4, rates of FN (defined as ANC $< 0.5 \times 10^9$/L with coincidental oral equivalent temperature > 38.2°C) by cycle and across all cycles, and times to ANC recovery to $>1.5 \times 10^9$/L in all cycles.

[0308] The data related to secondary efficacy analysis was analyzed using appropriate statistical methods. Safety, PK, and immunogenicity parameters were analyzed by descriptive statistical methods.

[0309] For frequency and severity of adverse events, and for laboratory toxicity grading, counts and rates are presented.

Efficacy Measures

[0310] Complete blood counts ("CBC") were obtained on day 1, 3 and daily from day 5 until ANC > 2.0 x 10^9/L after the nadir, then twice weekly, and at the end of treatment.

b. Efficacy of Phase II, Part A

[0311] Of the 78 subjects enrolled in the pilot phase of the study, 13 subjects did not complete the study, 3 (27.3%) treated with NEUG 30 mg, 3 (14.3%) treated with NEUG 40 mg, 3 (15.0%) treated with NEUG 50 mg, and 4 (15.4%) treated with pegfilgrastim. The most frequent reasons for early discontinuation were withdrawal of consent (7 subjects) and decision of the investigator (3 subjects). One NEUG 30 mg subject was withdrawn due to an adverse event (diabetic foot).

[0312] The incidence of severe neutropenia and the mean duration of severe neutropenia (DSN) were similar across treatment groups in each chemotherapy cycle; however, the time to ANC recovery and the incidence of febrile neutropenia suggested that NEUG 30 mg was not quite as effective as NEUG 40 mg, NEUG 50 mg, or pegfilgrastim.

[0313] During Cycle 1, the proportion of subjects experiencing febrile neutropenia was 20.0%, 9.5%, 10.0% and 8.0% for the NEUG 30 mg, 40 mg, 50 mg and pegfilgrastim group, respectively. Febrile neutropenia was observed for only three additional subjects during Cycles 2-4, one each in the NEUG 30 mg, NEUG 40 mg and pegfilgrastim groups. **FIG. 5** shows the ANC profile of a subset of patients receiving either NEUG 30 or pegfilgrastim and who later presented with grade 4 neutropenia.

[0314] In Cycle 1, the mean DSN was similar for NEUG 30 mg (0.9 days), NEUG 50 mg (1.1 days), and pegfilgrastim (0.9 days). Although the mean DSN was slightly longer for NEUG 40 mg (1.6 days) than the other three treatments, the differences among treatments were all less than 1 day, the criterion to consider the treatments equivalent in the main phase. The median DSN was 0 or 1 day in all four treatment groups.

[0315] Summary statistics for the incidence and duration of Grade 3 or 4 neutropenia followed a similar pattern, *i.e.*, the NEUG 30 mg, NEUG 50 mg, and pegfilgrastim groups had similar outcomes, while the incidence and duration of Grade 3 or 4 neutropenia were

slightly higher for the NEUG 40 mg group than for the other treatment groups. The number of subjects in the pilot phase (Part A) was fairly small, and the observed differences were not statistically significant. NEUG 40 mg and NEUG 50 mg were selected for further evaluation in Part B, the 3-arm randomized phase of the study.

c. Efficacy of Phase II, Part B

[0316] Of the 256 subjects enrolled in the main phase of the study, 18 subjects did not complete the study; 10 (11.6%) treated with NEUG 40 mg, 5 (6.0%) treated with NEUG 50 mg, and 3 (3.5%) treated with pegfilgrastim. The most frequent reasons for early discontinuation were withdrawal of consent (7 subjects) and AEs (4 subjects), including 2 deaths. The investigator considered all of these AEs to be not related to study medication or chemotherapy. In the main phase, 1 (1.2%) NEUG 40 mg subject was withdrawn before being treated with study drug.

[0317] The incidence and duration of severe neutropenia in Cycle 1 are summarized in Table 11.

Table 11: Phase II, Part B: Incidence and duration of severe neutropenia in Cycle 1

	Neugranin				
	40 mg (N=85)	**50 mg (N = 84)**	**All Neug. (N = 169)**	**Pegfil-grastim (N = 86)**	**95% CI 97.5% CI**
Incidence of severe neutropenia n (%) NEUG 50 mg - NEUG 40 mg NEUG 40 mg - Pegfilgrastim NEUG 50 mg - Pegfilgrastim	50 (58.8%)	55 (65.5%)	105 (62.1%)	50 (58.1%)	 (-7.94; 21.24) (-14.09; 15.45) (-7.23; 21.90)
Duration (days) of severe neutropenia n Mean (SD) Median Min/Max	 84 1.0 (1.09) 1 0/4	 84 1.3 (1.22) 1 0/5	 168 1.2 (1.16) 1 0/5	 86 1.2 (1.34) 1 0/5	
NEUG 50 mg - NEUG 40 mg NEUG 40 mg - Pegfilgrastim NEUG 50 mg - Pegfilgrastim					**95% CI** (-0.07; 0.58) (-0.57; 0.15) (-0.31; 0.41)

NEUG 50 mg - NEUG 40 mg NEUG 40 mg - Pegfilgrastim NEUG 50 mg - Pegfilgrastim					**97.5% CI** (-0.12; 0.63) (-0.62; 0.21) (-0.37; 0.46)

[0318] The incidence of severe neutropenia ranged from 58.1% in the pegfilgrastim group to 65.5% in the NEUG 50 mg group. The treatment effect was not statistically significant (p=0.559). The treatment groups were comparable for Cycle 1 DSN, with mean values of 1.0, 1.3, and 1.2 days for the NEUG 40 mg, NEUG 50 mg, and pegfilgrastim groups, respectively. The 95% and 97.5% two-sided confidence intervals for differences between NEUG and pegfilgrastim were strictly less than 1 day for both NEUG doses. This analysis established non-inferiority of NEUG to pegfilgrastim. Across treatment cycles, the incidences of severe neutropenia and Grade 3 or 4 neutropenia were lower in Cycles 2 – 4 than in Cycle 1. The mean DSN and mean duration of Grade 3 or 4 neutropenia were smaller in Cycles 2-4 than in Cycle 1. Within treatment cycles, the treatments were similar, and treatment effect was not significantly different for any of these parameters in any chemotherapy cycle.

[0319] The DSN were compared in patients grouped into weight quartiles to determine if the fixed doses of NEUG provided adequate support for patients of all weights. These results show that all weight groups were adequately supported, as there is no significant difference in the mean DSN among weight subgroups (Table 12).

TABLE 12: Cycle 1 duration of sever neutropenia (in days), by weight

		Baseline weight (kg)			
		40-62	**63-71**	**72-80**	**81-127**
Pegfilgrastim 6 mg	Mean (SD) N	1.1 (1.3) 16	1.3 (1.4) 21	1.5 (1.6) 26	1.0 (1.0) 23
Neugranin 40 mg	Mean (SD) N	1.0 (1.0) 22	1.0 (1.2) 21	0.9 (1.0) 21	1.4 (1.4) 21
Neugranin 50 mg	Mean (SD) N	1.3 (1.1) 15	1.0 (1.4) 26	1.4 (1.3) 20	1.5 (1.1) 23

[0320] Febrile neutropenia is summarized for all cycles in Table 13. During Cycle 1, the proportion of subjects experiencing febrile neutropenia was 2 subjects (3.5%), 5 subjects (6.0%), and 2 subjects (2.3%) in the NEUG 40 mg, NEUG 50 mg, and pegfilgrastim groups, respectively. Febrile neutropenia was observed for only three additional subjects during Cycles 2-4, 2 subjects in the NEUG 40 mg group and 1 subject in the pegfilgrastim group. The treatment effect was not statistically significant in any chemotherapy cycle.

Table 13: Incidence of febrile neutropenia in cycles 1-4					
Treatment	**Overall**	**Cycle 1**	**Cycle 2**	**Cycle 3**	**Cycle 4**
Neugranin 40 mg	4.7% (4/85)	3.5%	0.0%	2.4%	0.0%
Neugranin 50 mg	6.0% (5/85)	6.0%	0.0%	0.0%	0.0%
Pegfilgrastim	3.5% (3/86)	2.3%	0.0%	0.0%	1.2%

[0321] There were no significant differences between treatments for duration of severe neutropenia in cycles 2-4 (Table 14).

Table 14: Mean duration of severe neutropenia in cycles 2-4			
Treatment	**Cycle 2**	**Cycle 3**	**Cycle 4**
Neugranin 40 mg	0.5	0.4	0.4
Neugranin 50 mg	0.4	0.5	0.6
Pegfilgrastim	0.5	0.4	0.6

[0322] The mean time to ANC recovery (>1.5 x 109/L) was 2.0, 2.1, and 2.6 days for the Neugranin 40 mg, NEUG 50 mg, and pegfilgrastim groups, respectively (Table 15). There were no significant differences between treatment groups for the depth of ANC nadir or time to nadir.

Table 15: ANC nadir, time to ANC nadir and time to recovery						
	Neugranin					
Parameter	**40 mg (N=85)**	**50 mg (N = 84)**	**All Neug. N = 169)**	**Pegfil-grastim (N = 86)**	**95% CI**	**p-value**
Nadir ANC (10^9/L)						
n	85	84	169	86		0.423
Mean (SD)	0.7 (0.88)	0.6 (0.68)	0.6 (0.79)	0.7 (1.04)		
Median	0	0	0	0		
Min/Max	0/5	0/3	0/5	0/7		
Time (days) to Nadir ANC						
n	85	84	169	86		0.610
Mean (SD)	604 (1.38)	6.7 (2.62)	6.5 (2.09)	6.5 (2.05)		
Median	6	6	6	6		
Min/Max	5/18	5/20	5/20	4/17		
Time (days to ANC recover > 1500						0.005
N	71	73	144	72		

Table 15: ANC nadir, time to ANC nadir and time to recovery						
	Neugranin					
Parameter	**40 mg (N=85)**	**50 mg (N = 84)**	**All Neug. N = 169)**	**Pegfil-grastim (N = 86)**	**95% CI**	**p-value**
Mean (SD) Median Min/Max	2.0 (0.94) 2 1/6	2.1 (1.03 2 1/6	2.0 (0.98) 2 1/6	2.6 (1.23) 2 1/6		
Treatment comparisons NEUG 50 mg – NEUG 40 mg NEUG 40 mg – Pegfilgrastim NEUG 50 mg - Pegfilgrastim					(-0.31;0.39) (-0.88;-0.17) (-0.84; -0.13)	

d. Pharmacokinetics of Phase II, part B

[0323] Serum Neugranin concentrations were determined using a validated sandwich ELISA with a lower limit of quantification (LLQ) of 6.312 ng/mL. Pharmacokinetic parameters were calculated using noncompartmental modeling techniques, with the exception of the absorption half-life, which was determined using a first-order absorption, first-order elimination one-compartment model. Modeling was performed with WinNonlin Professional (version 5.0.1). Serum NEUG concentrations were determined in chemotherapy Cycle 1 in all subjects treated with NEUG in Phase II. In the Part A of Phase II, the median elimination half-life of NEUG was 33 hours in the 30 mg dose group, 46 hours in the 40 mg dose group, and 18 hours in the 50 mg dose group (Table 16). In Part B, the median elimination half-life of NEUG was 40 hours for 40 mg dose group, and 39 hours for the 50 mg dose group (Table 17). During the Part A, PK sampling was more frequent (pre-dose, 3h, 6h, 12h, 24h Day 3, Day 5-9, Day 11) than for Part B (pre-dose, Day 3, Day 5-8).

Table 16: Median elimination half-life by treatment, Phase II, Part A				
	NEUG 30 mg	**NEUG 40 mg**	**NEUG 50 mg**	**Pegfilgrastim 6 mg**
Number of subjects	10	20	20	26

Number of subjects evaluated for elimination half-life	3	12	16	19
Median half-life (hr)	33	46	18	40

Table 17: Median elimination half-life by treatment, Phase II, Part B			
	NEUG 40 mg	**NEUG 50 mg**	**Pegfilgrastim 6 mg**
Number of subjects	85	84	84
Number of subjects evaluated for elimination half-life	48	54	52
Median half-life (hr)	40	39	50

[0324] Serum pegfilgrastim concentrations were determined using a validated sandwich ELISA in chemotherapy Cycle 1 in all subjects treated with pegfilgrastim in Phase II. In Part A, the median elimination half-life of pegfilgrastim was about 40 hours. In Part B, the median elimination half-life of pegfilgrastim was about 50 hours. The elimination half-life is reported to be 3-4 hours for filgrastim and 42-67.5 hours (depending on dose) for pegfilgrastim.

e. Immunogenicity

[0325] Among the study participants, there was one confirmed anti-G-CSF/neoepitope antibody response in the Neugranin-treated subjects and one anti-G-CSF response in the pegfilgrastim-treated group, or 0.5% and 0.9%, respectively (Table 18). In both cases, the subjects had elevated non-specific binding in pre-dose samples.

Table 18: Summary of G-CSF specific treatment emergent immune responses to NEUG and Pegfilgrastim		
	NEUG Positive response/ number of subjects	**Pegfilgrastim Positive response/number of subjects**
Phase II, Part A (4 cycles maximum)	0/50	0/26
Phase II, Part B (4 cycles maximum)	1/169	1/86
Total number of subjects	1/219	1/112

[0326] After NEUG treatment, very low levels of confirmed positive antibodies were seen in the patient, with no apparent increase in the magnitude of the response after repeated doses (data not shown). In the pegfilgrastim-treated patient, an unusually high non-specific background binding was observed; however, only a transient confirmed antibody response was seen after Cycle 2 treatment (data not shown). No antibody response was neutralizing.

[0327] Anti-HSA antibodies were naturally occurring at a low level in this population, with 6.9% of the subjects testing positive for HSA antibodies in the pre-dose evaluation. Treatment emergent anti-HSA antibodies were observed in four NEUG-treated subjects, 1.8% (Table 19). All responses were transient and weak. Three responses emerged after the first treatment cycle and were undetectable after Cycles 2, 3 and 4. One response occurred after the third treatment but was undetectable at the 30 day follow-up after the 4th treatment (data not shown).

Table 19: Summary of HSA-specific treatment emergent immune responses to NEUG

	NEUG Positive response/ number of subjects
Phase II, Part A (4 cycles maximum)	0/50
Phase II, Part B (4 cycles maximum)	4/169
Total number of subjects	4/219

f. Treatment-emergent adverse events in Phase II, Part B

[0328] In Phase II, Part B, ≥90% of subjects in each treatment group experienced at least one treatment-emergent adverse event (TEAE), and the percent of subjects with at least one TEAE related to study medication ranged from 23.1% in the pegfilgrastim group to 35.0% in the Neugranin 50 mg group. The percent of subjects with at least one SAE was highest in the NEUG 30 mg group (30%), but was approximately 15% in the other three treatment groups. None of the SAEs were related to study medication. One patient (NEUG 30 mg) was withdrawn from the study due to diabetic foot, which was considered to be not related to study medication. In the Part B, all except 8 subjects (2 NEUG 40 mg, 3 NEUG 50 mg, 3 pegfilgrastim) had at least one TEAE. The percent of subjects with at least one TEAE related to study medication was 20.2% in the NEUG 50 mg group, 22.4% in the NEUG 40 mg group and 22.1% in subjects receiving pegfilgrastim. Two subjects (1 NEUG 40 mg, 1 pegfilgrastim) died during the study, and 6 – 8 subjects in each treatment group experienced at least one SAE. No deaths or SAEs were considered to be related to study medication.

[0329] The total number of TEAEs was similar across treatment groups in Part A, when sample size is taken into consideration for the NEUG 30 mg dose, and in Part B. In both Parts A and B, the percent of TEAEs with CTC Grade 3 or higher was similar for NEUG and pegfilgrastim as was the percent of TEAEs related to study medication.

g. Dose response

[0330] The results of Phase II demonstrated that both 40 and 50 mg fixed doses of NEUG provided equivalent safety and efficacy to 6 mg of pegfilgrastim in breast cancer subjects treated with myelotoxic chemotherapy. While the mean DSN for the 40 mg treatment group was slightly lower than the mean DSN of the 50 mg group, these differences were not statistically significant. A dose response was observed for AUC_{ANC} (Days 0-15 in cycle 1) both when weight-adjusted dose was considered and for fixed dose cohorts (**FIG. 24**). The AUC_{ANC} for the 30 mg cohort was slightly lower than that of pegfilgrastim, indicating that the 30 mg fixed dose was less effective in this study, whereas AUC_{ANC} for the 40 mg and the 50 mg cohorts were dose-related and higher (although not significantly) than the AUC_{ANC} for pegfilgrastim treated subjects. From the above analysis, a dose response is apparent when NEUG is administered on a weight adjusted basis (mg/kg). However, comparison of DSN in cycle 1 for Phase II, Part B suggested that patients of all weight quartiles were adequately

supported as DSN did not vary significantly among the treatment arms (40 and 50 mg NEUG and pegfilgrastim) nor with weight-adjusted dose (mg/kg). Further, there was no evidence that a fixed dose might result in an altered safety profile in lighter patients as the incidence and severity of related adverse events (bone pain in particular; data not shown) did not correlate with dose received per kilogram body weight, nor were they different from those with pegfilgrastim.

* * * *

[0331] It will be apparent to those skilled in the art that various modifications and variations can be made in the methods and compositions of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

WHAT IS CLAIMED IS:

1. A method of treating or preventing neutropenia in a human subject comprising administering to a human subject exhibiting neutropenia or at risk of developing neutropenia, recombinant human albumin-human granulocyte colony stimulating factor in an amount effective to treat the subject.

2. A method of treating or preventing leukopenia in a human subject comprising administering to a human subject exhibiting leukopenia or at risk of developing leukopenia, recombinant human albumin-human granulocyte colony stimulating factor in an amount effective to treat the subject.

3. The method according to claims 1 or 2 wherein the human subject is suffering from a non-myeloid malignancy and is receiving at least one myelosuppressive anti-cancer drug associated with a clinically significant incidence of febrile neutropenia.

4. A method of decreasing the incidence of infection, as manifested by febrile neutropenia, in a human subject with non-myeloid malignancies and receiving at least one myelosuppressive anti-cancer drug associated with a clinically significant incidence of febrile neutropenia, comprising administering to the subject recombinant human albumin-human granulocyte colony stimulating factor in an amount effective to treat the subject.

5. The method according to any one of claims 1-4, wherein:
   (a) grade 4 neutropenia in the subject is eliminated;
   (b) grade 4 neutropenia in the subject is reduced;
   (c) the duration of severe neutropenia is reduced in the subject;
   (d) the duration of grade 4 neutropenia in the subject is less than 5 days;
   (e) the duration of grade 3 neutropenia in the subject is eliminated;
   (f) the duration of grade 3 neutropenia in the subject is decreased; or
   (g) any combination thereof.

6. The method according to any one of claims 1-5, wherein administering recombinant human albumin-human granulocyte colony stimulating factor induces a rise in white blood cells (WBC).

7. The method according to any one of claims 1-6, wherein:
    (a) the number of neutrophils is increased in the subject;
    (b) a decrease in the number of neutrophils is inhibited in the subject;
    (c) the nadir absolute neutrophil count (ANC) is increased in the subject;
    (d) the recovery ANC is increased in the subject;
    (e) the time to ANC recovery is reduced in the subject; or
    (f) any combination thereof.

8. The method according to any one of claims 1-7, wherein the amount of recombinant human albumin-human granulocyte colony stimulating factor administered to the subject is selected from the group consisting of:
    (a) from about 50μg/kg to about 450 μg/kg;
    (b) about 50μg/kg;
    (c) about 150μg/kg;
    (d) about 300μg/kg;
    (e) about 450μg/kg;
    (f) from about 30 mg to about 60 mg;
    (g) about 30mg;
    (h) about 40mg;
    (i) about 50mg;
    (j) about 60mg; or
    (k) any combination thereof.

9. The method according to any one of claims 1-8, wherein neutropenia is selected from the group consisting of primary neutropenia, acute neutropenia, severe chronic neutropenia (SCN), severe congenital neutropenia (Kostmann's syndrome), severe infantile genetic agranulocytosis, benign neutropenia , cyclic neutropenia, chronic idiopathic neutropenia, secondary neutropenia, syndrome associated neutropenia, and immune-mediated neutropenia.

10. The method according to any one of claims 1-9, wherein neutropenia is caused or associated with radiation, alcoholism, drugs, allergic disorders, aplastic anemia,

autoimmune disease, T-γ lymphoproliferative disease (T-γ LPD), myelodysplasia, myelofibrosis, dysgammaglobulinemia, paroxysmal nocturnal hemoglobinuria, cancer, vitamin B_{12} deficiency , folate deficiency, viral infection, bacterial infection, spleen disorder, hemodialysis, or transplantation, leukemia, myeloma, lymphoma, metastatic solid tumors which infiltrate and replace the bone marrow, toxins, bone marrow failure, Schwachman-Diamond syndrome, cartilage-hair hypoplasia, dyskeratosis congenita, glycogen storage disease type IB, splenomegaly of any cause, intrinsic defects in myeloid cells or their precursors.

11. The method of claim 3, wherein recombinant human albumin-human granulocyte colony stimulating factor is administered at a time selected from the group consisting of:

(a) at least 12 hours after administration of the myelosuppressive anti-cancer drug;

(b) at least 18 hours after administration of the myelosuppressive anti-cancer drug;

(c) at least 24 hours after administration of the myelosuppressive anti-cancer drug.

12. The method of claim 11 wherein administering recombinant human albumin-human granulocyte colony stimulating factor prior to the myelosuppressive anti-cancer drug induces a rise in WBC.

13. The method according to any one of claims 11 or 12, wherein administering recombinant human albumin-human granulocyte colony stimulating factor prior to chemotherapy induces a rise in ANC.

14. The method according to any one of claims 3 or 11-13, wherein the non-myeloid malignancy comprises breast cancer.

15. The method according to any one of claims 3 or 11-14, wherein the myelosuppressive anticancer drugs comprise doxorubicin and docetaxel.

16. The method of claim 15 wherein about 50 mg/m^2 doxorubicin and about 75 mg/m^2 docetaxel are administered sequentially by intravenous infusion on the same day for at least one treatment cycle.

17. The method of claim 15 wherein about 60 mg/m^2 doxorubicin and about 75 mg/m^2 docetaxel are administered sequentially by intravenous infusion on the same day for at least one treatment cycle.

18. The method any one of claims 3 or 11-17, wherein ANC and WBC return to normal at a time period selected from the group consisting of:
    (a) by day 10 after chemotherapy;
    (b) by day 11 after chemotherapy;
    (c) by day 12 after chemotherapy;
    (d) by day 13 after chemotherapy:
    (e) by day 14 after chemotherapy; or
    (f) by day 15 after chemotherapy.

19. The method of any one of claim 3 or 11-18, wherein on day 14 after chemotherapy administration the rise in ANC in patients treated with recombinant human albumin-human granulocyte colony stimulating factor is lower than the rise in ANC in patients treated with an equivalent dose of pegfilgrastim.

20. The method according to any one of claims 1-19, wherein administering recombinant human albumin-human granulocyte colony stimulating factor induces a rise in lymphocytes, monocytes, eosinophils, basophils, or any combination thereof.

21. The method according to any one of claims 1-20, wherein the number of lymphocytes, monocytes, eosinophils, basophils or any combination thereof is increased in the subject.

22. The method according to any one of claims 1-21, wherein a decrease in the number of lymphocytes, monocytes, eosinophils, or basophils is inhibited in the subject.