

Carolyn C. Cain
Carolyn C. Cain

PTO/SB/16 (10-01)

Approved for use through 10/31/2002. OMB 0651-0032

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EL904321053 US

INVENTOR(S)					
Given Name (first and middle [if any])		Family Name or Surname		Residence (City and either State or Foreign Country)	
Thomas B.		Neff		Atherton, California	
<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
Methods For Treating Erythropoietin-Associated Conditions					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
<input type="checkbox"/> Customer Number				Place Customer Number Bar Code Label here	
OR Type Customer Number here					
<input checked="" type="checkbox"/> Firm or Individual Name		FibroGen, Inc.			
Address		Intellectual Property Department			
Address		225 Gateway Blvd.			
City		South San Francisco	State	CA	ZIP 94080
Country		US	Telephone	650-866-7200	Fax 650-866-7292
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification		Number of Pages	23	<input type="checkbox"/> CD(s), Number	
<input checked="" type="checkbox"/> Drawing(s)		Number of Sheets	1	<input type="checkbox"/> Other (specify)	
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76					
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.				FILING FEE AMOUNT (\$)	
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees				80.00	
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:		50-0811			
<input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.					
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input checked="" type="checkbox"/> No.					
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: _____					

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME Christopher Turner

TELEPHONE 650-866-7200

Date 16 Jan 2002 16 January 2002

REGISTRATION NO.
(if appropriate)
Docket Number:

45,167

FP0603 P

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

IPR2016-01315

GSK Ex. 1011, pg 248

Carolyn C. Cairns
Carolyn C. Cairns

PTO/SB/17 (10-01)
Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT (\$) 80

Complete if Known

Application Number	Not yet assigned
Filing Date	Concurrently herewith
First Named Inventor	Thomas B. Neff
Examiner Name	Not yet assigned
Group Art Unit	Not yet assigned
Attorney Docket No.	FP0603 P

METHOD OF PAYMENT

1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

Deposit Account Number 50-0811
Deposit Account Name FibroGen, Inc.

- ☒ Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17

- ☒ Applicant claims small entity status. See 37 CFR 1.27

2. ☐ Payment Enclosed:

☐ Check ☐ Credit card ☐ Money Order ☐ Other

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
101 740	201 370	Utility filing fee	
106 330	206 165	Design filing fee	
107 510	207 255	Plant filing fee	
108 740	208 370	Reissue filing fee	
114 160	214 80	Provisional filing fee	80

SUBTOTAL (1) (\$) 80

2. EXTRA CLAIM FEES

Total Claims	Extra Claims	Fee from below	Fee Paid
<input type="checkbox"/> -20** =	<input type="checkbox"/> X	<input type="checkbox"/>	0
<input type="checkbox"/> Independent Claims - 3** =	<input type="checkbox"/> X	<input type="checkbox"/>	0
<input type="checkbox"/> Multiple Dependent	<input type="checkbox"/>	<input type="checkbox"/>	0

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description
103 18	203 9	Claims in excess of 20
102 84	202 42	Independent claims in excess of 3
104 280	204 140	Multiple dependent claim, if not paid
109 84	209 42	** Reissue independent claims over original patent
110 18	210 9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) (\$) 0

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
105 130	205 65	Surcharge - late filing fee or oath	
127 50	227 25	Surcharge - late provisional filing fee or cover sheet	
139 130	139 130	Non-English specification	
147 2,520	147 2,520	For filing a request for ex parte reexamination	
112 920*	112 920*	Requesting publication of SIR prior to Examiner action	
113 1,840*	113 1,840*	Requesting publication of SIR after Examiner action	
115 110	215 55	Extension for reply within first month	
116 400	216 200	Extension for reply within second month	
117 920	217 460	Extension for reply within third month	
118 1,440	218 720	Extension for reply within fourth month	
128 1,960	228 980	Extension for reply within fifth month	
119 320	219 160	Notice of Appeal	
120 320	220 160	Filing a brief in support of an appeal	
121 280	221 140	Request for oral hearing	
138 1,510	138 1,510	Petition to institute a public use proceeding	
140 110	240 55	Petition to revive - unavoidable	
141 1,280	241 640	Petition to revive - unintentional	
142 1,280	242 640	Utility issue fee (or reissue)	
143 460	243 230	Design issue fee	
144 620	244 310	Plant issue fee	
122 130	122 130	Petitions to the Commissioner	
123 50	123 50	Processing fee under 37 CFR 1.17(q)	
126 180	126 180	Submission of Information Disclosure Stmt	
581 40	581 40	Recording each patent assignment per property (times number of properties)	
146 740	246 370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149 740	249 370	For each additional invention to be examined (37 CFR § 1.129(b))	
179 740	279 370	Request for Continued Examination (RCE)	
169 900	169 900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) (\$) 0

SUBMITTED BY

Name (Print/Type) Christopher Turner
Signature Christopher Turner

Registration No. 45,167
(Attorney/Agent)

16 January 2002

Complete (if applicable)

Telephone 650-866-7200

Date 16 January 2002

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231

METHODS FOR TREATING ERYTHROPOIETIN-ASSOCIATED CONDITIONS

FIELD OF THE INVENTION

5 The present invention relates to methods for treating erythropoietin-associated conditions by increasing endogenous erythropoietin levels in the blood. The present invention also relates to compounds including heterocyclic carbonyl glycines that can be used in the present methods. Methods of identifying compounds that increase endogenous erythropoietin levels are also provided. The methods and compounds are useful in preventing or treating erythropoietin-associated conditions, for example, anemia
10 due to chronic kidney failure, HIV infection, chemotherapy, surgery, and the like.

BACKGROUND OF THE INVENTION

Erythropoietin (EPO), is a naturally-occurring hormone in the human body. EPO is normally secreted by the kidneys in response to hypoxia (lack of oxygen in the blood) and stimulates the production of red
15 blood cells, which carry oxygen throughout the body. Synthetically produced EPO is currently administered to patients suffering from anemia due to chronic kidney failure, chemotherapy, and AIDS therapy.

Anemia is a condition in which the blood is deficient in erythrocytes (red blood cells), in hemoglobin, or
20 in total blood volume. All types of anemia are characterized by the blood's reduced capacity to carry oxygen, and thus are associated with similar signs and symptoms, including pallor of the skin and mucous membranes, weakness, dizziness, easy fatigability, and drowsiness, leading to a decrease in quality of life. Subjects with severe cases of anemia show difficulty in breathing, heart abnormalities, and digestive complaints.

25 The most common causes of anemia include deficiencies of iron, vitamin B₁₂, and folic acid. Anemia may also be caused by loss of blood, for example, through gastrointestinal bleeding due to medications such as aspirin and ibuprofen. Excessive blood loss can also be seen in women with heavy menstrual periods, and in people with stomach ulcers, duodenal ulcers, hemorrhoids, or cancer of the stomach or
30 large intestine.

Various conditions can cause the destruction of erythrocytes (hemolysis), leading to anemia. For example, allergic-type reactions to bacterial toxins and various chemical agents such as sulfonamides and benzene can cause hemolysis. Hemolytic anemia is often caused by chemical poisoning, infection, or
35 sickle-cell anemia. In addition, there are unusual situations in which the body produces antibodies against

its own erythrocytes, resulting in hemolysis. Any disease or injury to the bone marrow can cause anemia, since that tissue is the site of erythropoiesis, i.e. erythrocyte synthesis. Irradiation, disease, or various chemical agents can also cause bone marrow destruction, producing aplastic anemia. Cancer patients undergoing chemotherapy often have aplastic anemia. Anemia is also associated with renal dysfunction, the severity of the anemia correlating highly with the extent of the dysfunction. Most patients with renal failure undergoing dialysis suffer from chronic anemia.

In 1989, Amgen introduced a genetically engineered EPO for the treatment of anemia in chronic renal failure patients. Additionally, EPO is used in cancer patients undergoing radiation and/or chemotherapy, decreasing the need for blood transfusions. EPO is also used to treat anemia associated with HIV infection or AZT therapy. Although the market for EPO therapy is increasing, future sales are adversely affected by the high cost of the product. In addition, recombinant EPO therapy requires injection of EPO one to three times per week for up to twelve weeks, which limits self-administration and is inconvenient for the patient. Further, human serum EPO shows size heterogeneity due to extensive glycosylation, not seen in recombinant human EPO.

Due to deficiencies in current production and use of recombinant EPO, there remains a need for methods and compounds that are effective in the treatment of erythropoietin-associated conditions such as anemia, including anemia associated with diabetes, ulcers, kidney failure, cancer, infection, dialysis, and chemotherapy. Specifically, there is a need in the art for methods and compounds that increase the endogenous levels of erythropoietin in the body and thus stimulate erythropoiesis and alleviate anemic conditions.

SUMMARY OF THE INVENTION

Described herein are methods of increasing endogenous erythropoietin levels in the plasma, thereby increasing erythropoiesis. In one embodiment, the methods of the invention increase synthesis of erythropoietin in tissues including, but not limited to, kidney, liver, and/or bone marrow. In one embodiment, the methods of the invention are used to prevent, pre-treat, or treat erythropoietin-associated conditions such as anemia. Erythropoietin-associated conditions associated with anemia include, but are not limited to, polycystic kidney disease, chronic renal failure, diabetes, cancer, ulcers, and AIDS. In another embodiment, the methods of the invention are used to treat anemia associated with treatments including, but not limited to, radiation therapy, chemotherapy, kidney dialysis, or surgery. In one specific embodiment, the method is used to increase endogenous erythropoietin levels in an HIV-infected anemic patient being treated with zidovudine or other reverse transcriptase inhibitors. In another specific

embodiment, the method is used to increase endogenous erythropoietin levels in an anemic cancer patient receiving cyclic cisplatin- or non-cisplatin-containing chemotherapy. In yet another specific embodiment, the method is used to increase endogenous erythropoietin levels in an anemic patient scheduled to undergo elective, noncardiac, nonvascular surgery, thereby reducing the need for allogenic blood transfusions.

In another aspect, the invention provides compounds that increase endogenous erythropoietin plasma levels and methods of using the compounds to prevent, pre-treat, or treat erythropoietin-associated conditions such as anemia as described above. In one embodiment, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient is administered to a patient with a erythropoietin-associated conditions. In another embodiment, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient is administered to a patient with anemia due to a condition or disorder including, but not limited to, polycystic kidney disease, chronic renal failure, diabetes, cancer, ulcers, and AIDS. In another embodiment, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient is used to treat anemia associated with a treatment including, but not limited to, radiation therapy, chemotherapy, kidney dialysis, or surgery. In one specific embodiment, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient is administered to an HIV-infected patient being treated with zidovudine or other reverse transcriptase inhibitors. In another specific embodiment, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient is administered to an anemic cancer patient receiving cyclic cisplatin- or non-cisplatin-containing chemotherapy. In yet another specific embodiment, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient is administered to an anemic patient scheduled to undergo elective, noncardiac, nonvascular surgery to reduce the need for allogenic blood transfusions.

Preferred embodiments of the invention comprise methods using oral and transdermal delivery mechanisms. Such mechanisms could provide advantages over current therapies, e.g., in ease of administration, self-administration by patient, reduced cost, fewer physician visits, and reduced risks due to infection and immunogenic complications, such as the adverse reactions some patients develop in

response to dosing with recombinant EPO. In one preferred embodiment, the present methods involve oral administration of a compound that increases endogenous erythropoietin levels. Thus, the present invention also provides an oral formulation comprising a compound of the invention. In another preferred embodiment, the present methods involve transdermal administration of a compound that increases

5 endogenous erythropoietin levels. Thus, the present invention also provides a transdermal patch or pad comprising a compound of the invention.

In some embodiments, compounds used in the methods of the invention are heterocyclic carbonyl glycines selected from the group consisting of quinolines, isoquinolines, 3-methoxy pyridine carbonyl

10 glycines, 3-methoxy pyridine carbonyl glycine esters, 3-hydroxy pyridine carbonyl glycines, 3-hydroxypyridine carbonyl glycine esters, 5-sulfonamidocarbonyl pyridine carboxylates, and 5-sulfonamidocarbonyl-pyridine-carbonyl-glycine esters. In one embodiment, the compound is a quinoline selected from the group consisting of N-((6-(1-butyloxy)-3-hydroxyquinolin-2-yl)-carbonyl)-glycine; N-((6-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine; N-((3-hydroxy-6-(2-propyloxy)-quinolin-2-yl)-carbonyl)-glycine; and N-((7-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine. In another embodiment,

15 the compound is an isoquinoline selected from the group consisting of N-((1-chloro-4-hydroxy-7-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine; N-((1-chloro-4-hydroxy-6-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine; N-((1-chloro-4-hydroxy-7-methoxyisoquinolin-3-yl)-carbonyl)-glycine; N-((1-chloro-4-hydroxy-6-methoxy-isoquinolin-3-yl)-carbonyl)-glycine; N-((7-butyloxy)-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine; N-((7-benzyloxy-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine; N-((6-benzyloxy-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine; N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine; N-((8-chloro-4-hydroxy-isoquinolin-3-yl)-carbonyl)-glycine; and 1-Chloro-4-hydroxy-7-(2-propyloxy)-isoquinoline-3-carboxylic acid N-(2-hydroxyethyl)-amide. In yet another embodiment, the compound is a 3-methoxy pyridine carbonyl

20 glycines or an ester thereof selected from the group consisting of [(3-methoxy-pyridine-2-carbonyl)-amino]-acetic acid; 3-methoxypyridine-2-carboxylic acid N-(((hexadecyloxy)-carbonyl)-methyl)-amide hydrochloride; 3-methoxypyridine-2-carboxylic acid N-(((1-octyloxy)-carbonyl)-methyl)-amide; 3-methoxypyridine-2-carboxylic acid N-(((hexyloxy)-carbonyl)-methyl)-amide; 3-methoxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide; 3-methoxypyridine-2-carboxylic acid N-(((nonyloxy)-carbonyl)-methyl)-amide racemate; 3-methoxypyridine-2-carboxylic acid N-(((heptyloxy)-carbonyl)-methyl)-amide; 3-benzyloxypyridine-2-carboxylic acid N-(((octyloxy)-carbonyl)-methyl)-amide; 3-benzyloxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide; 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-((benzyloxycarbonyl)-methyl)-amide; 5-(((3-(1-Butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-

25

30

(((1-butyloxy)-carbonyl)-methyl)-amide; and 5-(((3-Lauryloxy)-propyl)amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-(((benzyloxy)-carbonyl)-methyl)-amide. In a specific embodiment, the compound is N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine.

5 In one aspect, a compound of the invention increases endogenous erythropoietin plasma levels by increasing synthesis of erythropoietin in tissues such as kidney, liver, and/or bone marrow. In one embodiment, the compound increases erythropoietin synthesis by inhibiting hydroxylation of the alpha subunit of hypoxia inducible factor (HIF α), thereby stabilizing HIF within a cell. In one embodiment, the agent inhibits hydroxylation of HIF α proline residues. In yet another embodiment, the agent inhibits
10 hydroxylation of the HIF α P₅₆₄ residue.

The present invention also provides methods for identifying compounds that increase endogenous erythropoietin plasma levels, the methods comprising administering a compound of interest to an animal and measuring erythropoietin levels in the blood. An increase in EPO in treated animals relative to
15 control animals is indicative of a compound that increases endogenous EPO. Alternatively, the methods identify compounds that indirectly increase synthesis of erythropoietin by stabilizing HIF α in cells.

The methods and compounds of the invention can be administered in combination with various other therapeutic approaches. In one embodiment, the compound is administered with an iron supplement, e.g.,
20 ferrous sulfate, vitamin B₁₂, and/or folic acid. In another embodiment, the compound is administered in conjunction with administration of exogenous erythropoietin, e.g., recombinant human erythropoietin.

These and other embodiments of the subject invention will readily occur to those of skill in the art in light of the disclosure herein.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows plasma erythropoietin levels in mice at 2, 4, and 20 hours following treatment. Group A represents mice treated with a vehicle control and group B represents mice treated with a compound of the invention, N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, at 100 mg compound/kg body
30 weight/dose.

DESCRIPTION OF THE INVENTION

Before the present compositions and methods are described, it is to be understood that the invention is not limited to the particular methodologies, protocols, cell lines, assays, and reagents described, as these may

vary. It is also to be understood that the terminology used herein is intended to describe particular embodiments of the present invention, and is in no way intended to limit the scope of the present invention as set forth in the appended claims.

5 It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural references unless context clearly dictates otherwise. Thus, for example, a reference to "a fragment" includes a plurality of such fragments, a reference to an "antibody" is a reference to one or more antibodies and to equivalents thereof known to those skilled in the art, and so forth.

10 Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications cited herein are incorporated herein by reference in their entirety for the purpose of
 15 describing and disclosing the methodologies, reagents, and tools reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

20 The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, cell biology, genetics, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Gennaro, A.R., ed. (1990) Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co.; Colowick, S. et al., eds., Methods In Enzymology, Academic Press, Inc.; Handbook of Experimental Immunology, Vols. I-IV (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications); Maniatis, T. et al., eds.
 25 (1989) Molecular Cloning: A Laboratory Manual, 2nd edition, Vols. I-III, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al., eds. (1999) Short Protocols in Molecular Biology, 4th edition, John Wiley & Sons; Ream et al., eds. (1998) Molecular Biology Techniques: An Intensive Laboratory Course, Academic Press); PCR (Introduction to Biotechniques Series), 2nd ed. (Newton & Graham eds., 1997, Springer Verlag).

30

DEFINITIONS

The term "anemia" refers to a reduction in the number of red blood cells and/or hemoglobin in blood relative to normal blood levels. Normal hemoglobin ranges between 14-18 g/dl in men and 12-16 g/dl in women. A hemoglobin level of 10-14 g/dl in men and 10-13 g/dl in women is a sign of moderate anemia,

while a value less than 10 g/dl is a sign of severe anemia in men and women. Alternatively, normal hematocrit levels range between 40-52% for men and 35-46% in women. Anemia is considered to be moderate when the hematocrit is between 35-40% in men and 30-35% in women, and severe when the hematocrit falls below 35% in men and 30% in women. Still further, normal plasma erythropoietin levels range from 0.01-0.03 mUnits/mL and increase up to 100- to 1000-fold during hypoxia or anemia. Thus, in patients with anemia associated with an endogenous erythropoietin level, e.g., at or below 500 mUnits/ml or less than 200 mUnits/ml, therapy to induce erythropoietin may be beneficial.

Anemia may arise due to conditions such as polycystic kidney disease, chronic renal failure, diabetes, cancer, surgery, bacterial infection, and AIDS. Anemia is also associated with blood loss due to, e.g., stomach ulcer, duodenal ulcer, hemorrhoids, cancer of the stomach or large intestine, or injury. Also, anemia is associated with radiation therapy, chemotherapy, and kidney dialysis. Specifically, anemia is associated with HIV-infected patients undergoing treatment with zidovudine or other reverse transcriptase inhibitors; and in cancer patients undergoing chemotherapy, e.g. with cyclic cisplatin- or non-cisplatin-containing chemotherapeutics. Anemia may also be associated with surgery.

The terms “disorders” and “diseases” and “conditions” are used inclusively and refer to any condition deviating from normal. The terms “anemic conditions” and “anemic disorders” refer to any condition, disease, or disorder that is associated with anemia. Such disorders include those disorders described above. The term “erythropoietin-associated conditions” is used inclusively and refers to any condition that is associated with normal or inappropriate modulation of erythropoietin. Erythropoietin-associated conditions include anemic conditions, including those described above, wherein an increase in EPO plasma level would provide therapeutic benefit.

The term “erythropoietin” refers to any recombinant or naturally occurring erythropoietin including human erythropoietin (GenBank Accession No. AAA52400; Lin et al. (1985) Proc Natl Acad Sci USA 82:7580-7584), EPOITIN recombinant erythropoietin (Amgen, Inc., Thousand Oaks CA), ARANESP recombinant erythropoietin (Amgen), and PROCRIT recombinant erythropoietin (Ortho Biotech Products, L.P., Raritan NJ).

The term “HIF α ” refers to the alpha subunit of hypoxia inducible factor protein. HIF α may be any human or other mammalian protein, or fragment thereof, including human HIF-1 α (Genbank Accession No. Q16665), HIF-2 α (Genbank Accession No. AAB41495), and HIF-3 α (Genbank Accession No. AAD22668); murine HIF-1 α (Genbank Accession No. Q61221), HIF-2 α (Genbank Accession No.

BAA20130 and AAB41496), and HIF-3 α (Genbank Accession No. AAC72734); rat HIF-1 α (Genbank Accession No. CAA70701), HIF-2 α (Genbank Accession No. CAB96612), and HIF-3 α (Genbank Accession No. CAB96611); and bovine HIF-1 α (Genbank Accession No. BAA78675). HIF α may also be any non-mammalian protein or fragment thereof, including *Xenopus laevis* HIF-1 α (Genbank Accession No. CAB96628), *Drosophila melanogaster* HIF-1 α (Genbank Accession No. JC4851), and chicken HIF-1 α (Genbank Accession No. BAA34234). HIF α gene sequences may also be obtained by routine cloning techniques, for example by using all or part of a HIF α gene sequence described above as a probe to recover and determine the sequence of a HIF α gene in another species.

Fragments of HIF α include the regions defined by human HIF-1 α from amino acid 401 to 603 (Huang et al., *supra*), amino acid 531 to 575 (Jiang et al. (1997) J Biol Chem 272:19253-19260), amino acid 556 to 575 (Tanimoto et al., *supra*), amino acid 557 to 571 (Srinivas et al. (1999) Biochem Biophys Res Commun 260:557-561), and amino acid 556 to 575 (Ivan and Kaelin (2001) Science 292:464-468). Further, a fragment of HIF α includes any fragment containing at least one occurrence of the motif LXXLAP, e.g., as occurs in the HIF α native sequence at L₃₉₇TLLAP and L₅₅₉EMLAP. Additionally, a fragment of HIF α includes any fragment retaining at least one functional or structural characteristic of HIF α .

The terms "amino acid sequence" or "polypeptide" as used herein, e.g., to refer to HIF α and fragments thereof, refer to an oligopeptide, peptide, or protein sequence, or to a fragment of any of these, and to naturally occurring or synthetic molecules. "Fragments" can refer to any portion of a sequence that retains at least one structural or functional characteristic of the protein. Immunogenic fragments or antigenic fragments refer to fragments of polypeptides, preferably, fragments of about five to fifteen amino acids in length, that retain at least one biological or immunological activity. Where "amino acid sequence" is recited to refer to the polypeptide sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms are not meant to limit the amino acid sequence to the complete native sequence associated with the recited protein molecule.

The term "related proteins" as used herein, for example, to refer to proteins related to HIF α prolyl hydroxylase, encompasses other 2-oxoglutarate dioxygenase enzymes, especially those family members that similarly require Fe²⁺, 2-oxoglutarate, and oxygen to maintain hydroxylase activity. Such enzymes may include, e.g., procollagen lysyl hydroxylase and procollagen prolyl 4-hydroxylase.

The term “agonist” refers to a molecule that increases or prolongs the duration of the effect of a particular molecule. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules that bind to and modulate the effects of the target molecule.

5 The term “antagonist” refers to a molecule which, when bound to a particular molecule, decreases the extent or duration of the effect of the biological or immunological activity. Antagonists may include proteins, nucleic acids, carbohydrates, antibodies, or any other molecules that decrease the effect of the target molecule.

10 The term “microarray” refers to any arrangement of nucleic acids, amino acids, antibodies, etc., on a substrate. The substrate can be any suitable support, e.g., beads, glass, paper, nitrocellulose, nylon, or any appropriate membrane, etc. A substrate can be any rigid or semi-rigid support including, but not limited to, membranes, filters, wafers, chips, slides, fibers, beads, including magnetic or nonmagnetic beads, gels, tubing, plates, polymers, microparticles, capillaries, etc. The substrate can provide a surface for coating
15 and/or can have a variety of surface forms, such as wells, pins, trenches, channels, and pores, to which the nucleic acids, amino acids, etc., may be bound.

The term “sample” is used herein in its broadest sense. Samples may be derived from any source, for example, from bodily fluids, secretions, tissues, cells, or cells in culture including, but not limited to, saliva, blood, urine, serum, plasma, vitreous, synovial fluid, cerebral spinal fluid, amniotic fluid, and
20 organ tissue (e.g., biopsied tissue); from chromosomes, organelles, or other membranes isolated from a cell; from genomic DNA, cDNA, RNA, mRNA, etc.; and from cleared cells or tissues, or blots or imprints from such cells or tissues. Samples may be derived from any source, such as, for example, a human subject, or a non-human mammalian subject, etc. Also contemplated are samples derived from
25 any animal model of disease. A sample can be in solution or can be, for example, fixed or bound to a substrate. A sample can refer to any material suitable for testing for the presence of HIF α or of fragments of HIF α or suitable for screening for molecules that bind to HIF α or to fragments thereof. Methods for obtaining such samples are within the level of skill in the art.

30 INVENTION

The present invention provides methods of increasing endogenous blood plasma erythropoietin (EPO) levels. The methods can be used to prevent, pre-treat, or treat erythropoietin-associated conditions such as anemia including, but are not limited to, polycystic kidney disease, chronic renal failure, diabetes, cancer, ulcers, and AIDS. The methods can also be used to treat anemia associated with radiation

therapy, chemotherapy, kidney dialysis, or surgery. For example, the methods can be used to increase endogenous erythropoietin levels in an HIV-infected anemic patient being treated with zidovudine or other reverse transcriptase inhibitors, to increase endogenous erythropoietin levels in an anemic cancer patient receiving cyclic cisplatin- or non-cisplatin-containing chemotherapy, or to increase endogenous erythropoietin levels in an anemic patient scheduled to undergo elective surgery.

The invention also provides compounds that can be used in the methods described above. The invention is based on the discovery that the compounds of the invention increase endogenous erythropoietin plasma levels as shown in Figure 1. Although the invention is not limited by the method in which the compound increases endogenous erythropoietin plasma levels, the compounds potentially increase synthesis of erythropoietin in tissues such as kidney, liver, and/or bone marrow by inhibiting hydroxylation of the alpha subunit of hypoxia inducible factor (HIF α), thereby stabilizing HIF within the cell. More specifically, the compounds inhibit hydroxylation of HIF α proline residues, e.g., the P₅₆₄ residue.

The compounds of the present invention encompass heterocyclic carbonyl glycines including quinolines, isoquinolines, and 3-methoxy pyridine carbonyl glycine esters.

U.S. Patent Nos. 5,719,164 and 5,726,305, incorporated by reference herein, describe quinolines. The compounds listed in the foregoing patents, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds contained therein are the quinolines N-((6-(1-butyloxy)-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, N-((6-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, N-((3-hydroxy-6-(2-propyloxy)-quinolin-2-yl)-carbonyl)-glycine, and N-((7-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine.

U.S. Patent No. 6,093,730, incorporated by reference herein, describes isoquinolines. The compounds listed in U.S. Patent No. 6,093,730, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds contained therein are N-((1-chloro-4-hydroxy-7-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-6-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-7-methoxyisoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-6-methoxyisoquinolin-3-yl)-carbonyl)-glycine, N-((7-butyloxy)-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((7-benzyloxy-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((6-benzyloxy-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((1-

chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((8-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, and 1-Chloro-4-hydroxy-7-(2-propyloxy)-isoquinoline-3-carboxylic acid N-(2-hydroxyethyl)-amide.

- 5 U.S. Patent No. 5,658,933, incorporated by reference herein, describes 3-methoxy pyridine carbonyl glycine esters. The compounds listed in U.S. Patent No. 5,658,933, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds contained therein are 3-methoxypyridine-2-carboxylic acid N-(((hexadecyloxy)-carbonyl)-methyl)-amide hydrochloride, 3-methoxypyridine-2-carboxylic acid N-(((1-octyloxy)-carbonyl)-methyl)-amide, 3-methoxypyridine-2-carboxylic acid N-(((hexyloxy)-carbonyl)-methyl)-amide, 3-methoxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide, 3-methoxypyridine-2-carboxylic acid N-(((nonyloxy)-carbonyl)-methyl)-amide racemate, 3-methoxypyridine-2-carboxylic acid N-(((heptyloxy)-carbonyl)-methyl)-amide, 3-benzyloxypyridine-2-carboxylic acid N-(((octyloxy)-carbonyl)-methyl)-amide, 3-benzyloxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide, 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-((benzyloxycarbonyl)-methyl)-amide, 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-(((1-butyloxy)-carbonyl)-methyl)-amide, and 5-(((3-lauryloxy)-propyl)amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-(((benzyloxy)-carbonyl)-methyl)-amide.

- 20 Additionally, European Patent No. EP0650961, incorporated by reference herein, describes 3-methoxy pyridine carbonyl glycines. The compounds listed in European Patent No. EP0650961, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. An exemplary compound contained therein is [(3-methoxy-pyridine-2-carbonyl)-amino]-acetic acid.

- 30 Additional compounds contemplated by the present invention are 3-hydroxypyridine carbonyl glycines described in U.S. Patent No. 5,620,995, incorporated by reference herein; 3-hydroxypyridine carbonyl glycine esters described in U.S. Patent No. 6,020,350, incorporated by reference herein; 5-sulfonamidocarbonyl pyridine carboxylates described in U.S. Patent No. 5,607,954, incorporated by reference herein; and 5-sulfonamidocarbonyl-pyridine-carbonyl-glycine esters described in U.S. Patent Nos. 5,610,172 and 5,620,996, incorporated by reference herein. The compounds listed in these patents, in particular, those compounds listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein.

Therefore, the invention provides methods for treating erythropoietin-associated conditions such as anemic conditions, in particular using the compounds described herein. Further, the invention provides methods for treating a patient having anemia due to therapies, e.g., individuals undergoing chemotherapy, dialysis, etc., using the compounds described herein.

Methods of Using the Compounds to Prevent and Treat Conditions Associated with Anemia

The present invention provides methods of increasing endogenous erythropoietin levels in the plasma, thereby increasing erythropoiesis. The methods can be used to prevent, pre-treat, or treat erythropoietin-associated conditions such as anemia. Conditions and disorders associated with anemia include, but are not limited to, polycystic kidney disease, chronic renal failure, diabetes, cancer, ulcers, and AIDS. The methods can also be used to treat anemia associated with treatments including, but not limited to, radiation therapy, chemotherapy, kidney dialysis, or surgery. Further, the methods of the invention may be used to increase endogenous erythropoietin levels in the plasma by increasing synthesis of erythropoietin in tissues including, but not limited to, kidney, liver, and bone marrow.

The invention provides compounds that can be used in the methods described above. For example, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient can be administered to a patient diagnosed with anemia. The anemia may be due to a condition or disorder including, but not limited to, polycystic kidney disease, chronic renal failure, diabetes, cancer, ulcers, and AIDS; or the anemia may be associated with a medical procedure including, but not limited to, radiation therapy, chemotherapy, kidney dialysis, or surgery. In a specific example, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient may be administered to an HIV-infected patient being treated with zidovudine or other reverse transcriptase inhibitors. In another specific example, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient may be administered to an anemic cancer patient receiving cyclic cisplatin- or non-cisplatin-containing chemotherapy. In yet another specific example, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof either alone or in combination with a pharmaceutically acceptable excipient may be administered to an anemic patient scheduled to undergo elective, noncardiac, nonvascular surgery to reduce the need for allogenic blood transfusions.

Preferred routes of administration include oral and transdermal delivery mechanisms. Such mechanisms benefit over current EPO replacement therapies in the ease of administration, self-administration by patient, reduced cost, fewer physician visits, and reduced risks due to infection and immunogenic complications, such as the adverse reactions some patients develop in response to dosing with recombinant EPO.

The methods and compounds of the invention can be administered in combination with various other therapeutic approaches. In one embodiment, the compound is administered with an iron supplement, e.g., ferrous sulfate, vitamin B₁₂, and/or folic acid. In another embodiment, the compound is administered in conjunction with administration of exogenous erythropoietin, e.g., recombinant human erythropoietin.

Pharmaceutical Formulations And Routes Of Administration

The compositions of the present invention can be delivered directly or in pharmaceutical compositions along with suitable carriers or excipients, as is well known in the art. Present methods of treatment can comprise administration of an effective amount of a compound of the invention to a subject having or at risk for anemia due to, e.g., chronic renal failure, diabetes, cancer, AIDS, radiation therapy, chemotherapy, kidney dialysis, or surgery. In a preferred embodiment, the subject is a mammalian subject, and in a most preferred embodiment, the subject is a human subject.

An effective amount of such agents can readily be determined by routine experimentation, as can the most effective and convenient route of administration and the most appropriate formulation. Various formulations and drug delivery systems are available in the art. (See, e.g., Gennaro, A.R., ed. (1995) Remington's Pharmaceutical Sciences, *supra*.)

Suitable routes of administration may, for example, include oral, rectal, transmucosal, nasal, or intestinal administration and parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. The agent or composition thereof may be administered in a local rather than a systemic manner. For example, a suitable agent can be delivered via injection or in a targeted drug delivery system, such as a depot or sustained release formulation.

The pharmaceutical compositions of the present invention may be manufactured by any of the methods well-known in the art, such as by conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. As noted above, the

compositions of the present invention can include one or more physiologically acceptable carriers such as excipients and auxiliaries that facilitate processing of active molecules into preparations for pharmaceutical use.

5 Proper formulation is dependent upon the route of administration chosen. For injection, for example, the composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal or nasal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. In a preferred embodiment of the present invention, the present
10 compounds are prepared in a formulation intended for oral administration. For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject. The compounds may also be formulated in rectal compositions such as
15 suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical preparations for oral use can be obtained as solid excipients, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to
20 obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof
25 such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures.
30 Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations for oral administration include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can

contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

In one embodiment, the compounds of the present invention can be administered transdermally, such as through a skin patch, or topically. In one aspect, the transdermal or topical formulations of the present invention can additionally comprise one or multiple penetration enhancers or other effectors, including agents that enhance migration of the delivered compound. Transdermal or topical administration could be preferred, for example, in situations in which location specific delivery is desired.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or any other suitable gas. In the case of a pressurized aerosol, the appropriate dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin, for use in an inhaler or insufflator may be formulated. These typically contain a powder mix of the compound and a suitable powder base such as lactose or starch.

Compositions formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion can be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Formulations for parenteral administration include aqueous solutions or other compositions in water-soluble form.

Suspensions of the active compounds may also be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil and synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively,

the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

As mentioned above, the compositions of the present invention may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the present compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Suitable carriers for the hydrophobic molecules of the invention are well known in the art and include co-solvent systems comprising, for example, benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The co-solvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system is effective in dissolving hydrophobic compounds and produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied. For example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80, the fraction size of polyethylene glycol may be varied, other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone, and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic molecules may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Liposomal delivery systems are discussed above in the context of gene-delivery systems. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using sustained-release systems, such as semi-permeable matrices of solid hydrophobic polymers containing the effective amount of the composition to be administered. Various sustained-release materials are established and available to those of skill in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

For any composition used in the present methods of treatment, a therapeutically effective dose can be estimated initially using a variety of techniques well known in the art. For example, in a cell culture assay, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture. Dosage ranges appropriate for human subjects can be determined, for example, using data obtained from cell culture assays and other animal studies.

A therapeutically effective dose of an agent refers to that amount of the agent that results in amelioration of symptoms or a prolongation of survival in a subject. Toxicity and therapeutic efficacy of such molecules can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, which can be expressed as the ratio LD_{50}/ED_{50} . Agents that exhibit high therapeutic indices are preferred.

Dosages preferably fall within a range of circulating concentrations that includes the ED_{50} with little or no toxicity. Dosages may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration, and dosage should be chosen, according to methods known in the art, in view of the specifics of a subject's condition.

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety that are sufficient to modulate endogenous erythropoietin plasma levels as desired, i.e. minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from, for example, *in vitro* data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Agents or compositions thereof should be administered using a regimen which maintains plasma levels above the MEC for about 10-90% of the duration of treatment, preferably about 30-90% of the duration of treatment, and most preferably between 50-90%. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of agent or composition administered will, of course, be dependent on a variety of factors, including the sex, age, and weight of the subject being treated, the severity of the affliction, the manner of administration, and the judgment of the prescribing physician.

The present compositions may, if desired, be presented in a pack or dispenser device containing one or more unit dosage forms containing the active ingredient. Such a pack or device may, for example,

comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of conditions, disorders, or diseases in which anemia is a major indication.

Compound Screening and Identification

The present invention further provides methods of screening for and identifying additional compounds that increase endogenous erythropoietin plasma levels. Various assays and screening techniques, including those described below, can be used to identify small molecules that increase the level of endogenous erythropoietin in the blood. One assay that is particularly useful involves treating animals with a compound of interest and measuring erythropoietin levels in plasma. (See, e.g., Example 1.) Assays will typically provide for detectable signals associated with the consumption of a reaction substrate or production of a reaction product. Detection can involve, for example, fluorophores, radioactive isotopes, enzyme conjugates, and other detectable labels well known in the art. The results may be qualitative or quantitative. Isolation of the reaction product may be facilitated by a label such as biotin or a histidine tag that allows purification from other reaction components via precipitation or affinity chromatography.

As one possible mechanism for increasing endogenous erythropoietin expression involves stabilization of HIF α within cells, assays used to identify small molecules that modulate (e.g., increase or decrease) the level or activity of HIF α would be applicable. Assays for HIF α hydroxylation may involve measuring hydroxylated proline or lysine residues in HIF α or a fragment thereof (see, e.g., Palmerini et al. (1985) J Chromatogr 339:285-292), or measuring formation of succinate from 2-oxoglutarate in the presence of enzyme and HIF α or a fragment thereof (see, e.g., Cunliffe et al. (1986) Biochem J 240:617-619). An exemplary procedure that measures HIF α hydroxylation is described in Ivan et al. (*supra*). An exemplary procedure that measures production of succinate from 2-oxoglutarate is described by Kaule and Gunzler (1990; Anal Biochem 184:291-297). Substrate molecules may include HIF α or a fragment thereof, e.g., HIF(556-575). Enzyme may include, e.g., HIF α prolyl hydroxylase (see, e.g., GenBank Accession No. AAG33965), obtained from any source. Enzyme may also be present in a crude cell lysate or in a partially purified form. Compounds that inhibit hydroxylation of HIF α may be identified by measuring and comparing enzyme activity in the absence and presence of the compound.

Additionally and in combination with the above methods, compounds can be identified by any of a variety of screening techniques known in the art. Such screening methods may allow for target polypeptides or the compounds to be free in solution, affixed to a solid support, borne on a cell surface, or located within a cell. For example, microarrays carrying test compounds can be prepared, used, and analyzed using methods available in the art. (See, e.g., Shalon, D. et al., (1995) PCT Application No. WO95/35505; Baldeschweiler et al., (1995) PCT Application No. WO95/251116; Brennan, T.M. et al., (1995) U.S. Patent No. 5,474,796; Heller, M.J. et al., (1997) U.S. Patent No. 5,605,662.)

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

EXAMPLES

The invention will be further understood by reference to the following examples, which are intended to be purely exemplary of the invention. These examples are provided solely to illustrate the claimed invention. The present invention is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only. Any methods that are functionally equivalent are within the scope of the invention. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Example 1: Increase in Endogenous Erythropoietin levels *in vivo*.

Twelve Swiss Webster male mice (30-32 g) were obtained from Charles River Laboratories, Inc (Wilmington MA) and treated by oral gavage two times per day for approximately 50 hours (5 doses) with a 2 ml/kg volume of either 0.5% carboxymethyl cellulose (CMC; Sigma-Aldrich, St. Louis MO) (group A) or 5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (50 mg/ml in 0.5% CMC) (group B). Two and five hours after the final dose, blood was collected from an orbital sinus into a heparinized tube and stored at -80°C. Twenty hours after the final dose, animals were subjected to a sub-lethal dose of CO₂ and blood was collected from the abdominal vein into a heparinized tube and stored at -80°C.

Plasma samples were processed and analyzed for erythropoietin (EPO) expression using a QUANTIKINE immunoassay (R&D Systems) according to the manufacturer's instructions. As can be seen in Figure 1, plasma from mice treated with the compound (group B) showed higher blood levels of EPO at 2 and 4 hours following the final dose relative to control animals (group A).

Example 2: Increase in Erythrocyte Count.

The ability of a compound of the invention to increase plasma erythrocyte levels is measured using a procedure described by Pitt et al., USPN 5,354,934. Briefly, rats are treated by oral gavage two times per day for five days with a 2 ml/kg volume of either 0.5% CMC (Sigma-Aldrich) (group A) or a compound of the invention, e.g., 5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (50 mg/ml in 0.5% CMC), (group B). On day 8 following the first oral treatment, blood is drawn from each of the experimental animals by cardiac puncture and the hematocrit is measured. Rats that receive the compound of the invention, e.g., N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, may exhibit up to 25% increase in hematocrit levels as compared to the vehicle-treated controls.

Example 3: Treatment of Hemolytic Anemia.

The ability of a compound of the invention to treat hemolytic anemia is assayed using a procedure described by Rencricca et al. (1970, Blood 36:764-71). Briefly, mice are treated by oral gavage two times per day for five days with a 2 ml/kg volume of either 0.5% CMC (Sigma-Aldrich) (group A) or a compound of the invention, e.g., 5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (50 mg/ml in 0.5% CMC), (group B). Beginning at day 3, mice are treated with three consecutive daily subcutaneous doses of either saline or 60 mg/kg phenylhydrazine (PHZ). On day 8, blood is drawn from each of the experimental animals by cardiac puncture and the hematocrit is measured. Mice that receive PHZ alone are expected to exhibit the lowest hematocrit levels (approximately 50% of control) four days after the first PHZ administration. Hematocrit levels return to normal in about eight days. Treatment with the compound of the invention, e.g., N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, is expected to minimize the drop in hematocrit levels in mice as compared to the vehicle-treated controls.

Alternatively, rats can be treated using a procedure described by Criswell et al. (2000, J Appl Toxicol 20:25-34). The procedure is essentially as described above for mice, except rats are given 50 mg/kg PHZ by intraperitoneal injection instead of by subcutaneous administration. Maximum decreases (68%) in red blood counts are expected on day three in PHZ-treated animals.

Example 4: Treatment of Anemia Associated with Renal Failure

The ability of a compound of the invention to treat anemia associated with post-ischemic acute renal failure is assayed using a procedure described by Tan et al. (1996, Kidney Int 50:1958-64). Briefly, rats are subjected to unilateral clamping of the left renal artery for one hour. The arterial clamp is then removed and the incisional wound is closed. Rats are treated by oral gavage two times per day with a 2

ml/kg volume of either 0.5% CMC (Sigma-Aldrich) (group A) or 5% a compound of the invention, e.g., N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (50 mg/ml in 0.5% CMC), (group B). At two hours, twenty four hours, and one week following release of the arterial clamp, blood is drawn for determination of the hematocrit. Hematocrit values in the rats treated with vehicle are expected to be about 85%, 91% and 93% of sham control rats at the respective time points.

Additionally, the ability of a compound of the invention to treat anemia associated with ischemic acute renal failure is assayed using a procedure described by Nemoto et al. (2001, *Kidney Int* 59:246-51).

Briefly, rats are treated by oral gavage two times per day with a 2 ml/kg volume of either 0.5% CMC (Sigma-Aldrich) (group A) or 5% a compound of the invention, e.g., N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (50 mg/ml in 0.5% CMC), (group B). Rats are subjected to clamping of the right kidney with a vascular clip with simultaneous nephrectomy of the left kidney. After each occlusion, the clip is released at either 30 (moderate) or 45 (severe) minutes, and reperfusion is observed.

Example 5: Treatment of Anemia Induced by Cisplatin.

The ability of a compound of the invention to treat anemia associated with post-ischemic acute renal failure is assayed using a procedure described by Vaziri et al. (1994, *Am J Physiol* 266(3 Pt 2):F360-6). Rats are treated with a single dose of 7 mg/kg cisplatin by intraperitoneal injection. Rats are then treated by oral gavage two times per day with a 2 ml/kg volume of either 0.5% CMC (Sigma-Aldrich) (group A) or a compound of the invention, e.g., 5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (50 mg/ml in 0.5% CMC), (group B).

Example 6: Screening Assay

Compounds that increase endogenous erythropoietin levels in the blood can be identified using the procedure described in Example 1. Additionally, compounds that inhibit HIF-specific prolyl hydroxylase activity and thereby stabilize HIF α will indirectly increase endogenous erythropoietin synthesis; such compounds can be identified and characterized using the following assay. A 50 μ l aliquot of a reaction mix containing 4 mg/ml BSA, 0.1 M Tris HCl (pH 7.2), 2 mM ascorbate, 80 μ M ferrous sulfate, 0.2 mM 2-oxoglutarate, 600 units/ml catalase, with or without 100 μ M HIF α peptide is mixed with 50 μ l HeLa cell extract and incubated 1.5 hours at 37°C. Following incubation, 50 μ l of streptavidin beads are added and the mixture is incubated for 1 hour with agitation at 4°C. The mixture is transferred to tubes and centrifuged at low speed to pellet the beads. The beads are washed three times with 0.5-1 ml 20 mM Tris HCl (pH 7.2). The peptide is then eluted from the beads with 5 μ l 2 mM biotin in 20 mM Tris HCl (pH 7.2) for 1 hour. The tubes are centrifuged to pellet the resin and 40-50 μ l of supernatant is removed and

an equal volume of acetonitrile is added. The non-hydroxylated and hydroxylated peptides are then separated by reverse-phase HPLC on a C18 column with UV detection at 214 nm.

- 5 Various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are hereby incorporated by reference in their entirety.

10

11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

ABSTRACT

The present invention relates to methods for treating erythropoietin-associated conditions by increasing endogenous erythropoietin levels in the blood. The present invention also relates to compounds including
5 heterocyclic carbonyl glycines that can be used in the present methods. Methods of identifying compounds that increase endogenous erythropoietin levels are also provided. The methods and compounds are useful in preventing or treating erythropoietin-associated conditions, for example, anemia due to chronic kidney failure, HIV infection, chemotherapy, surgery, and the like.

20160131 10:00:00

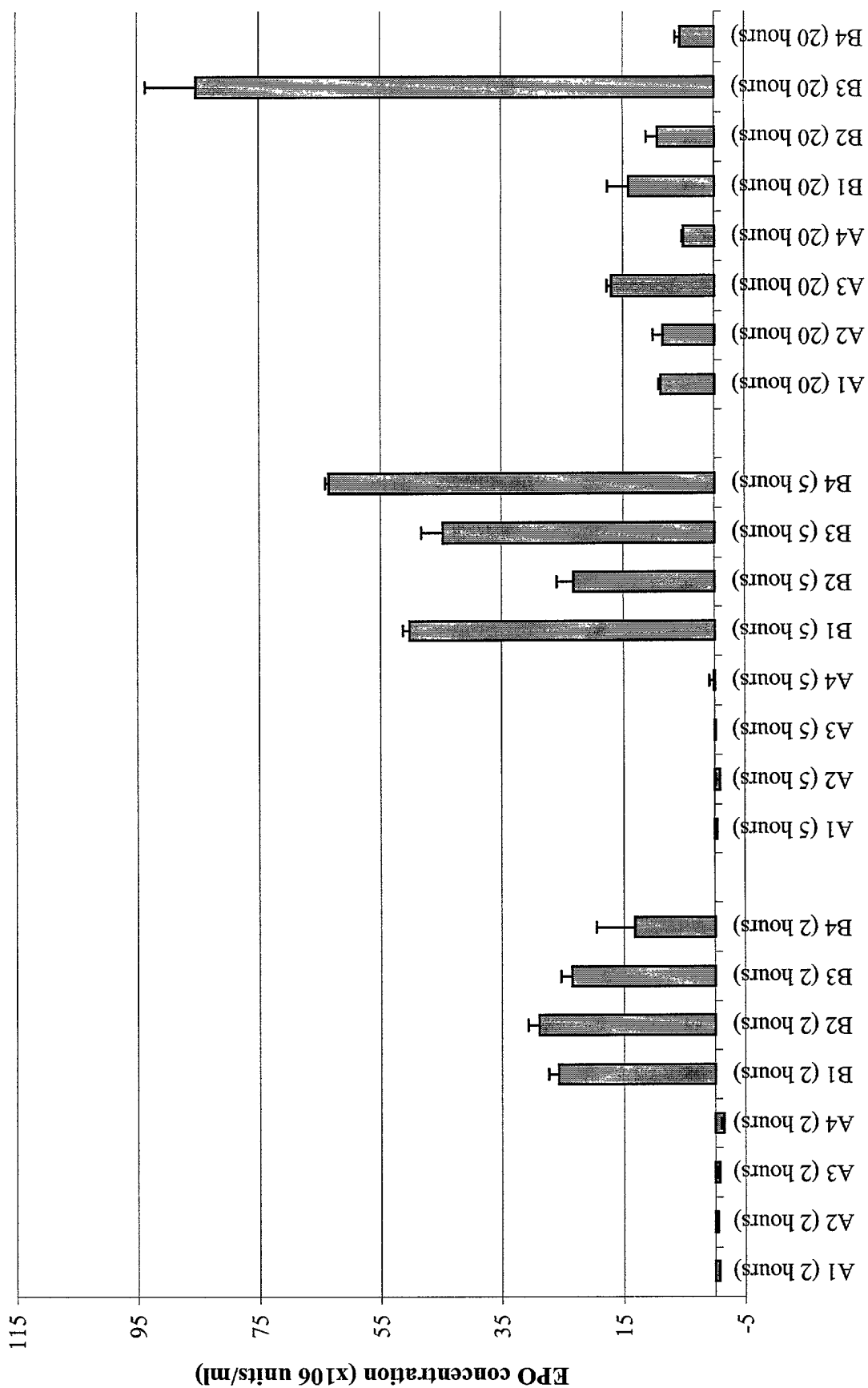


FIGURE 1