

Carolyn C. Cairns
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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. EL904321098US

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, CA	
<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		<input type="text"/>		<input type="checkbox"/> Place Customer Number Bar Code Label here	
OR					
<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		31		<input type="checkbox"/> CD(s), Number	
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets		8		<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					
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number.		50-0811		80.00	
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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

Christopher Turner

TYPED or PRINTED NAME Christopher Turner, Ph.D.

TELEPHONE 650-866-7200

Date 5 June 2002

REGISTRATION NO
(if appropriate)
Docket Number:

45,167

FP0603.1 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. 1013, pg 315

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PTO/SB/17 (10-01)
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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.	FP0603.1 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
Independent Claims	-20** =	X	0
Multiple Dependent Claims	-3** =	X	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, Ph.D.
Signature: [Signature]

Registration No. 45,167
(Attorney/Agent)

Complete (if applicable)

Telephone: 650-866-7200
Date: 5 June 2002

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IPR2016-01315
GSK Ex. 1013, pg 316

METHODS FOR TREATING ERYTHROPOIETIN-ASSOCIATED CONDITIONS

FIELD OF THE INVENTION

5 The present invention relates to methods for increasing endogenous erythropoietin levels *in vitro* and *in vivo*. 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 such as anemia due to, e.g., chronic kidney failure, HIV infection, 10 chemotherapy, surgery, and the like; and neurological disorders, e.g., stroke, trauma, epilepsy, and neurodegenerative disease.

BACKGROUND OF THE INVENTION

Erythropoietin (EPO), a naturally occurring hormone in the human body, is normally secreted by the 15 kidneys in response to hypoxia (lack of oxygen in the blood) and stimulates the production of red blood cells, which carry oxygen throughout the body. Anemia is a condition in which the blood is deficient in red blood cells (erythrocytes), in hemoglobin, or 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 20 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.

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, due to accidents, surgery, or gastrointestinal bleeding 25 caused by 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 large intestine.

Various conditions can cause the destruction of erythrocytes (hemolysis), leading to anemia. For 30 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 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 35 chemical agents can also cause bone marrow destruction, producing aplastic anemia. Cancer patients

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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.

- 5 Erythropoietin is also produced by astrocytes and neurons in the central nervous system (CNS), and EPO and EPO receptors are expressed at capillaries of the brain-periphery interface. Furthermore, systemically administered EPO crosses the blood-brain barrier and reduces neuronal cell loss in response to cerebral and spinal chord ischemia, mechanical trauma, epilepsy, excitotoxins, and neuroinflammation. (Sakanaka (1998) Proc Natl Acad Sci U S A 95:4635-4640; Celik et al. (2002) Proc Natl Acad Sci USA 99:2258-
10 2263; Brines et al. (2000) Proc Natl Acad Sci USA 97:10526-10531; Calapai et al. (2000) Eur J Pharmacol 401:349-356; and Siren et al. (2001) Proc Natl Acad Sci USA 98:4044-404.)

- 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,
15 decreasing the need for blood transfusions. EPO is also used to treat anemia associated with HIV infection or azidothymidine (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
20 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, surgery, and
25 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, e.g., neuroprotective and erythropoietic effects of EPO to alleviate neurological and anemic conditions, respectively.

SUMMARY OF THE INVENTION

- 30 Described herein are methods of increasing endogenous erythropoietin levels in cell culture or in an animal, preferably wherein the animal is a human. In one embodiment, the methods of the invention increase synthesis of endogenous erythropoietin in tissues including, but not limited to, renal, hepatic, hematopoietic, and/or neural tissues. In another embodiment, the methods of the invention are used to prevent, pre-treat, or treat erythropoietin-associated conditions such as neurological disorders and anemia.

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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. Erythropoietin-associated conditions associated with neurological disorders include, but are not limited to, stroke, trauma, epilepsy, and neurodegenerative diseases. In one specific embodiment, the method is used to increase endogenous erythropoietin levels in patients prior to surgery requiring aortic clamping such as thorcoabdominal aortic surgery. In yet another embodiment, the method is used to increase endogenous erythropoietin produced by cells *in vitro*.

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 an erythropoietin-associated condition. 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

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

In another aspect, the invention provides compounds that increase endogenous erythropoietin produced by cells in culture and methods of using the compounds to produce erythropoietin using *in vitro* cell culture technologies. In one embodiment, the method comprises adding an effective amount of the compound or a pharmaceutically acceptable salt thereof to cells in culture under conditions suitable for production of erythropoietin, and collecting and purifying the erythropoietin produced thereby. Examples of cells that produce erythropoietin *in vitro* include hepatic cells such as Hep3B hepatocarcinoma cells.

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 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, 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-

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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-((1-bromo-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 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-((benzyloxy)-carbonyl)-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. In a specific embodiment, the compound is N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine.

In one aspect, a compound of the invention increases endogenous erythropoietin plasma levels by increasing synthesis of erythropoietin in tissues, such as renal, hepatic, hematopoietic, and/or neural tissues, *in vivo* or *in vitro*. 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 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 or to cultured cells and measuring erythropoietin levels in the blood or conditioned culture media, respectively. An increase in EPO in treated animals or cells relative to untreated controls 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., 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.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows erythropoietin induction *in vitro* in response to a compound of the invention. Cells in culture were treated with a compound of the invention, N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, at 0, 5, or 25 μ M. Cell types shown in the figure are human liver cells derived from a hepatocarcinoma (Hep3B) and fibroblasts derived from human foreskin (HFF).

Figures 2A, 2B, and 2C show erythropoietin induction and subsequent hematocrit increase in animals treated with a compound of the invention. Figure 2A shows expression of erythropoietin transcript in the liver and kidney of animals treated for 3 days with either a vehicle control (0 mg compound/kg body weight/day) or a compound of the invention, N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (100 mg compound/kg body weight/day). Figure 2B shows erythropoietin levels in plasma, and Figure 2C shows blood hematocrit, in blood samples collected 4 hours after final treatment from the same animals represented in Figure 2A.

Figures 3A, 3B, 3C, and 3D show changes in serum erythropoietin, blood hemoglobin level, and hematocrit in animals treated with varying doses of a compound of the invention, N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, for 2 weeks. Figures 3A and 3B show serum levels of erythropoietin in animals at days 3, 10 and 14. Figure 3C shows changes in blood hematocrit and Figure 3D shows changes in hemoglobin level over the course of the experiment.

Figures 4A, 4B, 4C, and 4D show changes in serum erythropoietin, circulating blood reticulocytes, blood hemoglobin level and hematocrit, respectively, in animals treated with variable dosing regimens of a compound of the invention, N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine.

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Figures 5A, 5B, and 5C show levels of blood urea nitrogen and serum creatinine, and change in body weight, respectively, in animals treated with a compound of the invention, N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, relative to untreated controls.

- 5 Figures 6A and 6B show changes in hematocrit and circulating blood reticulocytes in animals exposed to a single dose of cisplatin and subsequently treated with a compound of the invention, N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine.

DESCRIPTION OF THE INVENTION

- 10 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.

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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.

20

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 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.

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30

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

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- (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications); Maniatis, T. et al., eds. (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).

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, the normal ratio of the volume of packed red blood cells to the volume of whole blood as determined by centrifugation (hereinafter simply referred to as “hemacrit”) shows a 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 azidothymidine (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

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conditions include anemic conditions, such as those described above, wherein an increase in EPO plasma level would provide therapeutic benefit. Erythropoietin-associated conditions also include neurological disorders and/or injury, such as stroke, trauma, epilepsy, neurodegenerative disease and the like, wherein erythropoietin may provide a neuroprotective effect. Neurodegenerative diseases contemplated by the invention include Alzheimer's disease, Parkinson's disease, Huntington's disease, and the like.

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 human recombinant erythropoietin (Amgen, Inc., Thousand Oaks CA), ARANESP human recombinant erythropoietin (Amgen), and PROCRIT human 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 human HIF-1 α native sequence at L₃₉₇TLLAP and L₅₅₉EMLAP. For example, a HIF peptide for use in the screening assay of Example 7 may comprise [methoxycoumain]-DLDLEALAPYIPADDDFQL-amide. Additionally, a fragment of HIF α includes any fragment retaining at least one functional or structural characteristic of HIF α .

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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 terms “HIF prolyl hydroxylase” and “HIF PH” refer to any enzyme that is capable of hydroxylating a proline residue in the HIF protein. Preferably, the proline residue hydroxylated by HIF PH includes the proline found within the motif LXXLAP, e.g., as occurs in the human HIF-1 α native sequence at L₃₉₇TLLAP and L₅₅₉EMLAP. HIF PH includes members of the Egl-Nine (EGLN) gene family described by Taylor (2001, Gene 275:125-132), and characterized by Aravind and Koonin (2001, Genome Biol 2:RESEARCH0007), Epstein et al. (2001, Cell 107:43-54), and Bruick and McKnight (2001, Science 294:1337-1340). Examples of HIF PH enzymes include human SM-20 (EGLN1) (GenBank Accession No. AAG33965; Dupuy et al. (2000) Genomics 69:348-54), EGLN2 isoform 1 (GenBank Accession No. CAC42510; Taylor, *supra*), EGLN2 isoform 2 (GenBank Accession No. NP_060025), and EGLN3 (GenBank Accession No. CAC42511; Taylor, *supra*); mouse EGLN1 (GenBank Accession No. CAC42515), EGLN2 (GenBank Accession No. CAC42511), and EGLN3 (SM-20) (GenBank Accession No. CAC42517); and rat SM-20 (GenBank Accession No. AAA19321). Additionally, HIF PH may include *Caenorhabditis elegans* EGL-9 (GenBank Accession No. AAD56365) and *Drosophila melanogaster* CG1114 gene product (GenBank Accession No. AAF52050). HIF PH also includes any active fragment of the foregoing full-length proteins.

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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 increase the effect(s) 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(s) 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,
- 20 saliva, blood, urine, serum, plasma, vitreous, synovial fluid, cerebral spinal fluid, amniotic fluid, and 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 erythropoietin or HIF α or to fragments thereof, or suitable for screening for molecules that increase endogenous levels of erythropoietin or HIF α or to fragments thereof. Methods for obtaining such samples are within the level of skill in the art.

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INVENTION

The present invention provides methods of increasing endogenous erythropoietin (EPO) levels *in vivo*, e.g. in blood plasma, or *in vitro*, e.g. in cell culture conditioned medium. Methods of increasing endogenous EPO levels *in vivo* can be used to prevent, pre-treat, or treat EPO-associated conditions, e.g.,

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associated with 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 EPO levels in an HIV-infected anemic patient being treated with azidothymidine (zidovudine) or other reverse transcriptase inhibitors, to increase endogenous EPO levels in an anemic cancer patient receiving cyclic cisplatin- or non-cisplatin-containing chemotherapeutics, or to increase endogenous EPO levels in an anemic patient scheduled to undergo elective surgery. Methods of increasing endogenous EPO levels *in vivo* can also be used to prevent, pre-treat, or treat EPO-associated conditions associated with nerve damage or neural tissue degeneration including, but not limited to, stroke, trauma, epilepsy, spinal cord injury, and neurodegenerative disorders. Methods of increasing endogenous EPO levels *in vitro* can be used to produce EPO using cell culture technologies.

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 levels in media from cultured cells treated *in vitro* (Figure 1) and in blood plasma from animals treated *in vivo* (Figure 2B). The compounds of the invention significantly increase endogenous erythropoietin expression in, e.g., liver and kidney cells *in vivo* (see, e.g., Figure 2A). Although the invention is not to be limited by the method in which endogenous erythropoietin is induced, one potential mechanism by which the compounds increase synthesis of erythropoietin in tissues such as kidney, liver, and/or bone marrow is 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.

Further, the compounds of the invention increase the hematocrit and blood hemoglobin level in animals treated *in vivo*. (See, e.g., Figure 2C.) The increases in plasma EPO, hematocrit, and blood hemoglobin level in response to the compounds of the invention are dose-sensitive, as can be seen in Figures 3A and 3B, 3C, and 3D, respectively. Dosing regimes can also be established to produce a constant, controlled level of response to the compounds of the invention. (See, e.g., Figures 4A, 4C, and 4D). Doses of compound effective at increasing blood hemoglobin and hematocrit do not produce overt signs of toxicity as determined by changes in blood urea nitrogen (Figure 5A), serum creatinine (Figure 5B), or body weight (Figure 5C). Further, treatment with compounds of the invention can correct anemia, for example, induced by a toxic compound such as the chemotherapeutic agent cisplatin (Figure 6A).

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The increase in hematocrit and blood hemoglobin level in animals treated with compounds of the invention is preceded by an increase in the percentage of circulating immature red blood cells (reticulocytes) within the blood (Figure 4B). As such, the invention contemplates the use of the compounds of the invention in methods to increase reticulocyte levels in the blood of animals for production of cell-free reticulocyte lysates as described by, e.g., Pelham and Jackson (1976, Eur J Biochem 67:247-256). Circulating reticulocyte levels are increased in animals, e.g. rabbits, by treatment with compounds of the invention alone or in combination with another compound such as acetylphenylhydrazine. The blood is collected, and reticulocytes are pelleted by centrifugation and lysed with distilled water. Extracts can be further processed using any appropriate methodology. (See, e.g., Jackson and Hunt, 1983, Methods Enzymol 96:50-74).

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.

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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.

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.

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.

Additional compounds contemplated by the present invention include small molecule inhibitors of prolyl 4-hydroxylase described in Majamaa et al. (1985) Biochem J 229:127-133; Kivirikko and Myllyharju (1998)

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Matrix Biol 16:357-368; Bickel et al. (1998) Hepatology 28:404-411; Friedman et al. (2000) Proc Natl Acad Sci USA 97:4736-4741; and Franklin et al. (1991) Biochem Soc Trans. 19(4):812-815, all of which are incorporated by reference herein. An exemplary compound contained in Franklin et al. (*supra*) is N-((1-bromo-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine.

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Therefore, the invention provides methods for treating erythropoietin-associated conditions such as anemic and neurological 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. Additionally, the invention provides methods for producing endogenous erythropoietin using *in vitro* cell culture technologies. The invention also contemplates methods of increasing the number of circulating reticulocytes in animals for the production of cell-free reticulocyte lysates for *in vitro* messenger RNA translation.

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Methods of Using the Compounds to Prevent and Treat Erythropoietin-associated Conditions

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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.

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The present invention also provides methods of increasing endogenous erythropoietin levels to prevent, pre-treat, or treat erythropoietin-associated neurological disorders including, but not limited to, acute disorders such as stroke, trauma, epilepsy, and spinal cord injury, and chronic disorders such as neurodegenerative disease. The methods can also be used to treat neurological disorders associated with treatments including, but not limited to, surgery such as thoracoabdominal aortic surgery.

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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

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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. In another 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 a neurological disorder including, but not limited to, stroke, trauma, epilepsy, spinal cord injury, and neurodegenerative disease. 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 a patient scheduled to undergo surgery requiring aortic clamping such as thoracoabdominal aortic surgery.

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

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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.

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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*.)

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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.

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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.

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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' 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 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

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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 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, hydroxypropylmethylcellulose, 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 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. 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,

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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.

- 5 These typically contain a powder mix of the compound and a suitable powder base such as lactose or starch.

- 10 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.

- 15 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.

- 25 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.

- 30 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

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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.

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Dosages preferably fall within a range of circulating concentrations that includes the ED₅₀ 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 2.) 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,

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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.

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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).

10

Exemplary procedures that measure HIF α hydroxylation are described in Ivan et al. (*supra*) and Example 7. 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); for example, an exemplary substrate for use in the assay described in Example 7 is [methoxycoumain]-DLDLEALAPYIPADDDFQL-amide. 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.

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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.)

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Production of Endogenous Erythropoietin *in vitro*

The invention provides methods for producing endogenous erythropoietin using *in vitro* cell culture technologies. Cells derived from animal tissues, preferably human tissues, which are capable of expressing erythropoietin when stimulated by compounds of the invention can be cultured using any techniques defined in the art for the *in vitro* production of endogenous proteins. Cells contemplated by

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the invention include, but are not limited to, cells derived from hepatic, hematopoietic, renal, and neural tissues. An exemplary cell line for production of endogenous erythropoietin is Hep3B derived from hepatic tissue.

- 5 Cell culture techniques include any method that maintains cell viability and facilitates expression of endogenous proteins. Generally, cells are cultured in a growth medium optimized for cell growth, viability, and protein production. Cells may be in suspension or attached to a substrate, and medium may be supplied in batch feed or continuous flow-through regimens. Compounds of the invention are added to the culture medium at levels that stimulate erythropoietin production without compromising cell viability.
- 10 Erythropoietin produced by the cells is secreted into the culture medium. The medium is then collected and the erythropoietin is purified using methods known to those of skill in the art (see, e.g., Lai et al., United States Patent No. 4,667,016, issued May 19, 1987; and Egrie, United States Patent No. 4,558,006, issued December 10, 1985).
- 15 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

- 20 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
- 25 accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Example 1: Increase in Endogenous Erythropoietin Levels *in vitro*.

- Human cells derived from hepatocarcinoma (Hep3B) and foreskin fibroblast (HFF) tissues (see, e.g., American Type Culture Collection, Manassas VA) were separately seeded into 35 mm culture dishes and
- 30 grown at 37°C, 20% O₂, 5% CO₂ in media as follows: Hep3B in Minimal Essential Medium (MEM), Earle's balanced salt solution (Mediatech Inc., Herndon VA), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10% FBS; and HFF in Dulbecco's Modification of Eagle's Medium (DMEM), 10% FBS. When cell layers reached confluence, the media was replaced with OPTI-MEM media (Invitrogen Life Technologies, Carlsbad CA) and cell layers were incubated for approximately 24

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hours in 20% O₂, 5% CO₂ at 37°C. One of the following was then added to existing medium and incubation was continued overnight: 5 or 25 µM N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine; or 1% DMSO (negative control).

- 5 Following incubation, the conditioned medium was collected from cell cultures and analyzed for erythropoietin expression using a QUANTIKINE immunoassay (R&D Systems, Inc., Minneapolis MN) according to the manufacturer's instructions. As can be seen in Figure 1, liver-derived cells (Hep3B) show significant increase in expression of erythropoietin when treated with a compound of the invention. However, as expected, fibroblasts (HFF) do not show any erythropoietin expression when treated with the same concentration of compound. Thus, compounds of the invention increase erythropoietin expression *in vitro* in cells that normally produce erythropoietin in animals.

Example 2: Increase in Endogenous Erythropoietin Levels *in vivo*.

- Twelve Swiss Webster male mice (30-32 g) were obtained from Simonsen, Inc (Gilroy, CA) and treated by oral gavage two times per day for 2.5 days (5 doses) with a 4 ml/kg volume of either 0.5% carboxymethyl cellulose (CMC; Sigma-Aldrich, St. Louis MO) (0 mg/kg/day) or 2.5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (25 mg/ml in 0.5% CMC) (200 mg/kg/day). Four hours after the final dose, animals were anesthetized with isoflurane and two blood samples were collected from the abdominal vein. One blood sample was collected into a MICROTAINER serum separator tube (Becton-Dickinson, Franklin Lakes NJ), incubated at room temperature for 30 minutes, centrifuged at 8,000 rpm at 4°C for 10 min, and then the serum fraction was processed and analyzed for erythropoietin (EPO) expression using a QUANTIKINE immunoassay (R&D Systems) according to the manufacturer's instructions. The second blood sample was collected into a MICROTAINER EDTA-2K tube (Becton-Dickinson) for hematocrit analysis. Hematocrit was measured by drawing EDTA-blood into a 75 mm x 1.1-1.2 mm I.D. capillary tube (Chase Scientific Glass, Inc., Rockwood TN) to approximately ¾ length. One end of the tube was sealed with CRITOSEAL sealant (Sherwood Medical Company) and the tube was centrifuged in a J-503M MICROHEMATOCRIT centrifuge (Jorgensen Laboratories, Inc., Loveland CO) at 12,000 rpm for 5 min. Hematocrit was read against a reader card. The mice were then sacrificed and approximately 150 mg of liver and each kidney were isolated and stored in RNALATER solution (Ambion) at -20°C.

RNA isolation was carried out using the following protocol. Tissue slices were cut into small pieces, 1.75 ml of RLT lysis buffer (RNI ASY kit; Qiagen Inc., Valencia CA) was added, and the pieces were homogenized for about 20 seconds using a rotor-stator POLYTRON homogenizer (Kinematica, Inc.,

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Cincinnati OH). A 350 µl volume of homogenate was micro-centrifuged for 3 minutes to pellet insoluble material, the supernatant was transferred to a new tube and RNA was isolated using an RNEASY kit (Qiagen) according to the manufacturer's instructions. The RNA was eluted into 80µL of water and quantitated with RIBOGREEN reagent (Molecular Probes, Eugene OR). Genomic DNA was then removed from the RNA using a DNA-FREE kit (Ambion Inc., Austin TX) according to the manufacturer's instructions. The absorbance at 260 and 280 nm was measured to determine RNA purity and concentration.

cDNA synthesis was performed using 1µM random hexamer primers, 1µg of total RNA, and OMNISCRIPT reverse transcriptase (Qiagen), according to the manufacturer's instructions. Resulting cDNA was diluted 5-fold with water to give 100µL final volume. Analysis of the relative level of erythropoietin gene expression was performed by quantitative PCR using a FASTSTART DNA MASTER SYBR GREEN I kit (Roche) and gene-specific primers, using a LIGHTCYCLER system (Roche), according to manufacturer's instructions. Samples were heated to 94°C for 6 minutes and then cycled through 95°C for 15 seconds, 60°C for 5 seconds, and 72°C for 10 seconds for a total of 42 cycles. Erythropoietin-specific primers were as follows:

mEPO-R3	TTCTGGCCCCGAGGATGTCA
mEPO-F3	ACGAACCTTGCTCCCCGTCCTG

The relative level of 18S ribosomal RNA gene expression was measured as a control. Quantitative PCR was performed using a QUANTITECT SYBR GREEN PCR kit (Qiagen) and gene-specific primers, using a LIGHTCYCLER system (Roche), according to manufacturer's instructions. Samples were heated to 95°C for 15 minutes and then cycled through 94°C for 15 seconds, 60°C for 20 seconds, 72°C for 10 seconds for a total of 42 cycles. Ribosomal RNA-specific primers were as follows:

18S-rat-2B	TAGGCACGGCGACTACCATCGA
18S-rat-2A	CGGCGGCTTTGGTGACTCTAGAT

Each PCR run included a standard curve and water blank. In addition, a melt curve was run after completion of each PCR run to assess the specificity of the amplification. Erythropoietin gene expression was normalized relative to the expression level of 18S ribosomal RNA for that sample.

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As can be seen in Figure 2A, erythropoietin gene expression is induced in both the kidney and liver in treated animals relative to untreated controls. The liver shows an approximately 32-fold increase and the kidney shows an approximately 580-fold increase in EPO transcript level relative to the untreated liver and kidney, respectively (y-axis units are arbitrary). As can be seen in Figure 2B, the same animals show a significant increase in erythropoietin plasma level in the treated group relative to untreated controls. Further, as can be seen in Figure 2C, the increase in endogenous erythropoietin induced by the compound of the invention leads to a significant increase in hematocrit in the treated animals relative to untreated controls.

Example 3: Dose Response *in vivo*.

Experiment 1

Eighteen Sprague Dawley male rats (approx. 260 g) were obtained from Charles River Laboratories, Inc. and treated by oral gavage two times per day with a 2 ml/kg volume of either 0.5% carboxymethyl cellulose (CMC; Sigma-Aldrich, St. Louis MO) (0 mg/kg/day), 0.5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (5 mg/ml in 0.5% CMC) (20 mg/kg/day), 1.5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (15 mg/ml in 0.5% CMC) (60 mg/kg/day) or 5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (50 mg/ml in 0.5% CMC) (200 mg/kg/day). All groups were treated daily for 14 consecutive days except the 200 mg/kg/day group, which was treated for only the first seven consecutive days. Animals were monitored for change in body weight and signs of toxicity. Blood samples (0.6 ml) were collected on days 3, 7, 10, and 14 as follows: Animals were anesthetized with isoflurane and blood was collected from the tail vein into a MICROTAINER EDTA-2K tube (Becton-Dickinson). Blood samples were processed for erythropoietin levels and hematocrit as described in Example 2. Complete Blood Count (CBC) analysis, including blood hemoglobin level, reticulocyte number, and hematocrit, was performed by IDEXX veterinary service (W. Sacramento, CA).

As can be seen in Figure 3A, the compound significantly increased serum erythropoietin level within 3 days of treatment at the highest administered dose (200 mg/kg/day). Lower doses of compound may have also increased serum EPO levels, but the increase was not statistically significant (Figure 3B). However, as can be seen in Figures 3C and 3D, the compound produced a statistically significant increase in blood hematocrit and hemoglobin levels, respectively, at both the 60 and 200 mg/kg/day doses. Overall, the compound at 60 mg/kg/day produced a modest increase in serum erythropoietin and a significant increase in both hematocrit and hemoglobin levels over the 14-day period.

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Experiment 2

Twelve Sprague Dawley male rats (approx. 260 g) were obtained from Charles River Laboratories, Inc. and treated by oral gavage as follows: A) Four rats were dosed daily for days 1-7 with 0.5% carboxymethyl cellulose (CMC; Sigma-Aldrich, St. Louis MO) (0 mg/kg), were not treated for days 8-14, and then were dosed daily for days 15-19 with 0.5% CMC (0 mg/kg); B) four animals were treated on day 0 and day 3 with a total of 7 ml/kg/day of 5.0% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (50 mg/ml in 0.5% CMC) (350 mg/kg), were not treated days 8-14, and then were dosed daily for days 15-19 with 3% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (30 mg/ml in 0.5% CMC) (60 mg/kg/day); and C) four animals were treated daily for days 1-7 with a total volume of 4 ml/kg/day of 2.5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (25 mg/ml in 0.5% CMC) (100 mg/kg), were not treated days 8-14, and then were dosed daily for days 15-19 with 3% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (60 mg/kg/day). Thus, groups B and C received a total of 700 mg compound/kg body weight over the first 7 days, no treatment from days 8 to 14, and then 60 mg/kg/day for another 5 days. Animals were monitored for change in body weight and signs of toxicity. Blood samples (2 x 0.5 ml) were collected on days 1, 3, 7, 10, 17 and 21 as follows: Animals were anesthetized with isoflurane and 0.5 ml of blood was collected from the tail vein into each of two MICROTAINER EDTA-2K tubes (Becton-Dickinson). Blood samples were processed for erythropoietin levels, hemoglobin, and hematocrit as described above.

As can be seen in Figure 4A, the compound significantly increased serum erythropoietin level within 1 day of treatment. As can be seen in Figure 4B, the increase in serum EPO led to a subsequent increase in immature blood cells (reticulocytes), demonstrating that the compound stimulates new red blood cell formation. Additionally, as can be seen in Figures 4C and 4D, the compound increased blood hemoglobin levels and hematocrit, respectively. Further, the increase in hemoglobin and hematocrit could be maintained over an extended period of time with lower doses of the compound. These results demonstrate that a compound of the invention can produce a controlled increase in red blood cells and the animals remain responsive to the compound over an extended period of time.

Figure 5 shows that a concentration of compound necessary to produce an appreciable increase in hematocrit and hemoglobin level over an extended period of time produces no toxic affects. Figure 5A shows blood urea nitrogen (BUN) levels in treated animals similar to those seen in controls. Additionally, Figure 5B shows serum creatinine levels remain essentially unchanged in treated animals compared to untreated controls. Further, Figure 5C shows that animals treated with the compound show the same level of weight gain as the control animals.

Example 4: Treatment of Anemia Induced by Cisplatin.

The ability of a compound of the invention to treat anemia associated with post-ischemic acute renal failure was assayed using a procedure described by Vaziri et al. (1994, Am J Physiol 266(3 Pt 2):F360-6).

5 Fifteen Sprague Dawley male rats (280-300 g) were obtained from Charles River Laboratories. On day 0, rats were treated by intraperitoneal injection with a single dose of saline (control; n = 3) at 8 ml/kg, or cisplatin (CP; Bedford Laboratories, Bedford OH) at either 7 mg/kg (7 ml/kg; n = 6) or 10 mg/kg (10 ml/kg; n = 6). Blood samples (0.2 ml) were collected on days 5, 9, and 16 as follows: Animals were anesthetized with isoflurane and 0.2 ml of blood was collected from the tail vein into a MICROTAINER.

10 EDTA-2K tube (Becton-Dickinson). Blood samples were processed for hematocrit as described above to determine the degree of anemia produced in each animal.

Beginning on day 19, one half of each cisplatin-treated group ($n = 3 \times 2$) and all of the control animals were treated by oral gavage once per day for five consecutive days with a 2 ml/kg volume of 0.5% CMC (Sigma-Aldrich). The other half of each cisplatin-treated group ($n = 3 \times 2$) were treated by oral gavage once per day for five consecutive days with a 2 ml/kg volume of 2.5% N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine (25 mg/ml in 0.5% CMC). Blood samples (0.5 ml) were collected as described above immediately prior to treatment and 4 days after treatment initiation. Blood samples were analyzed for CBC and reticulocyte counts as described above. On day 9 after initiation of oral treatment, a blood sample (0.1 ml) was collected and processed for hematocrit as described above.

Figure 6A shows that treatment with 7 and 10 mg/kg CP reduced hematocrit by 14 and 22%, respectively, relative to controls by day 19. The compound of the invention, however, increased hematocrit in the CP-treated animals 4 days after initiating treatment, and hematocrits were significantly higher than non-
25 treated counterparts by day 9. As can be seen in Figure 6A, hematocrit levels in animals initially exposed to 7 mg/kg CP and subsequently treated with the compound of the invention were at or above normal control values by day 9. Figure 6B shows that the increase in hematocrit is due to the formation of new red blood cells, as the number of circulating reticulocytes is also increased in animals treated with the compound.

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Example 5: 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

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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 6: 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 7: 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 or purified HIF prolyl hydroxylase 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. Alternatively, the peptide is attached to methoxycoumarin, a pH insensitive fluorophore. The fluorophore may provide sensitivity and specificity to enhance detection in assays run with crude cell lysate. An exemplary HIF peptide for use in the screening assay may comprise [methoxycoumain]-DLDLEALAPYIPADDDFQL-amide. The non-hydroxylated and hydroxylated peptides are then separated by reverse-phase HPLC on a C18 column with UV detection at 214 nm.

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.

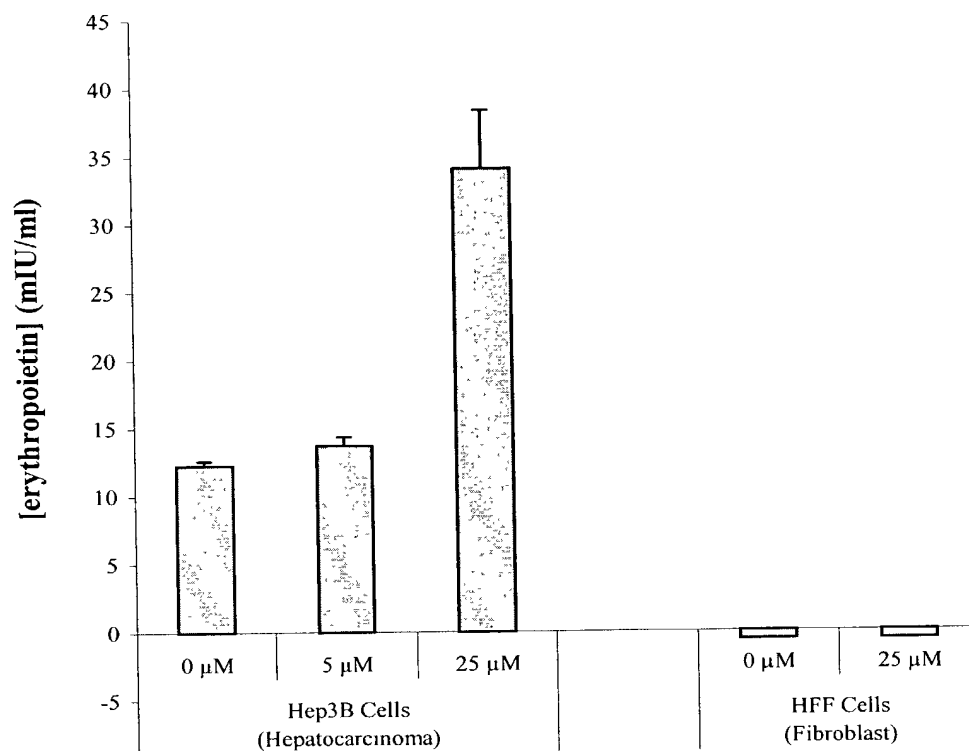
All references cited herein are hereby incorporated by reference in their entirety.

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ABSTRACT

5 The present invention relates to methods for treating erythropoietin-associated conditions by increasing endogenous erythropoietin levels *in vitro* and *in vivo*. 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 such as anemia due to, e.g., chronic kidney failure, HIV infection, chemotherapy, surgery, and the like; and neurological disorders, e.g., stroke, trauma, epilepsy, and neurodegenerative disease.

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[N-((1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine]

Figure 1

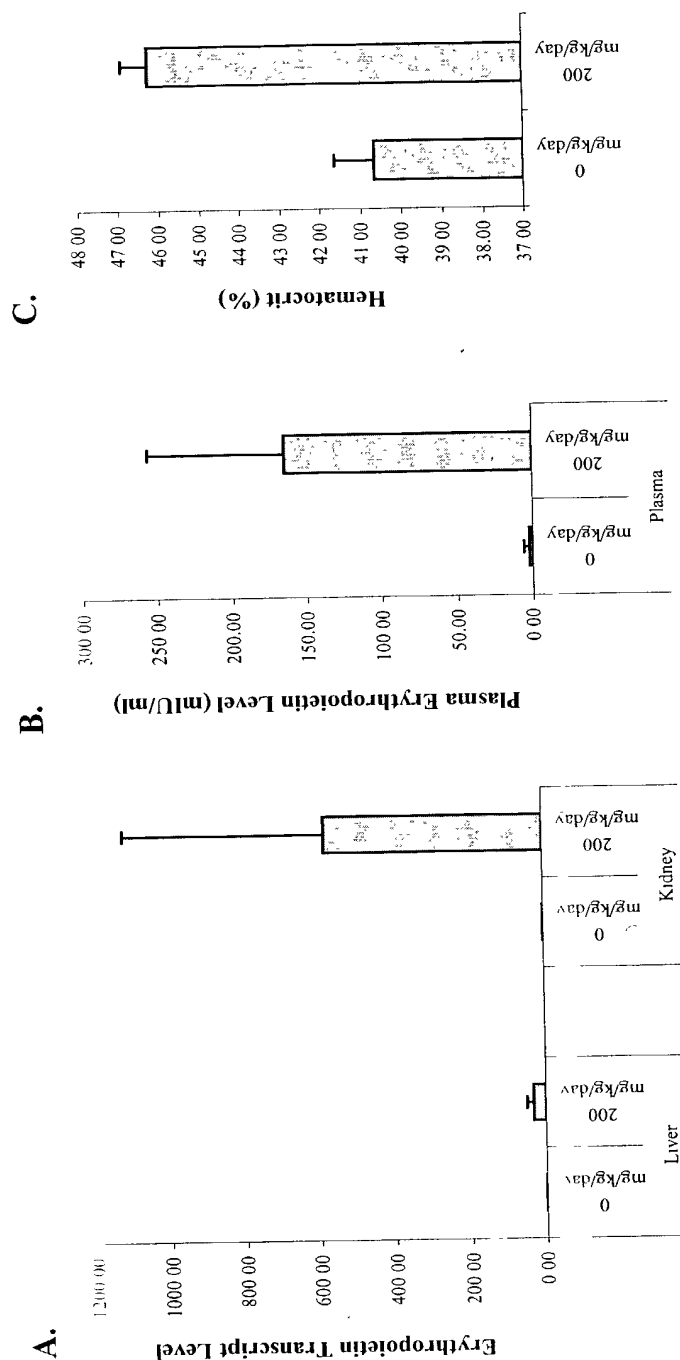
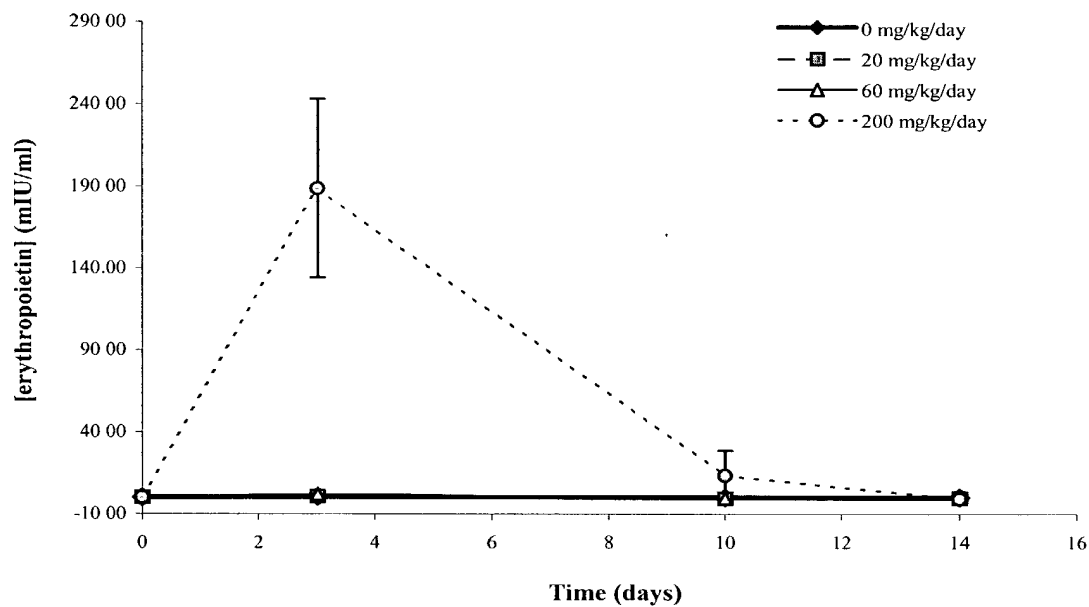


Figure 2

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A.



B.

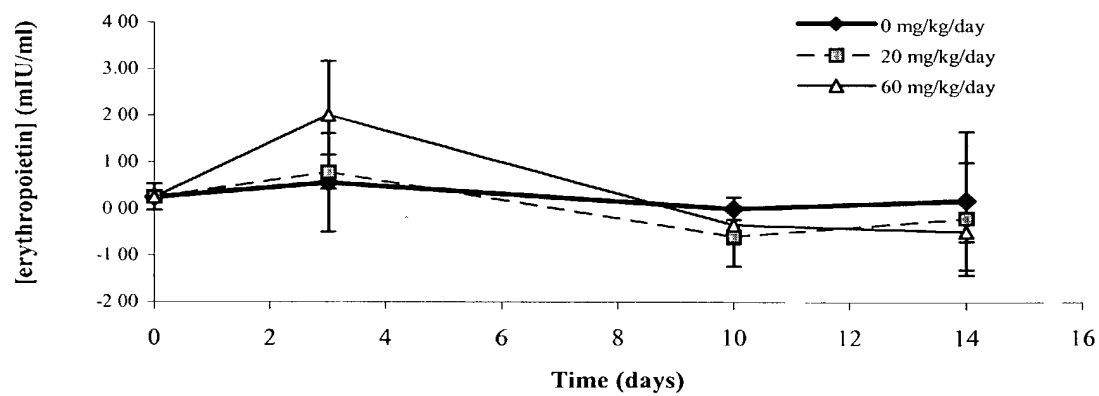
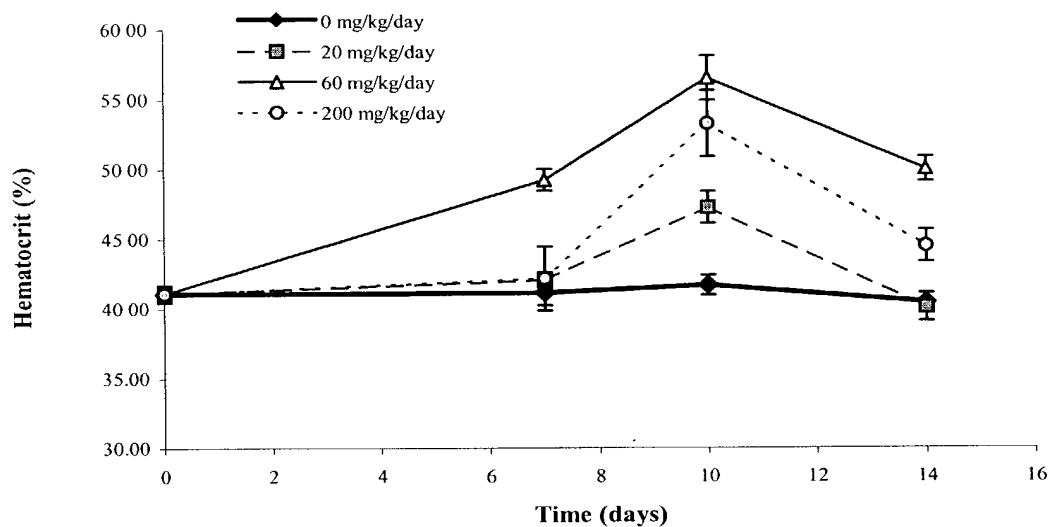


Figure 3

C.



D.

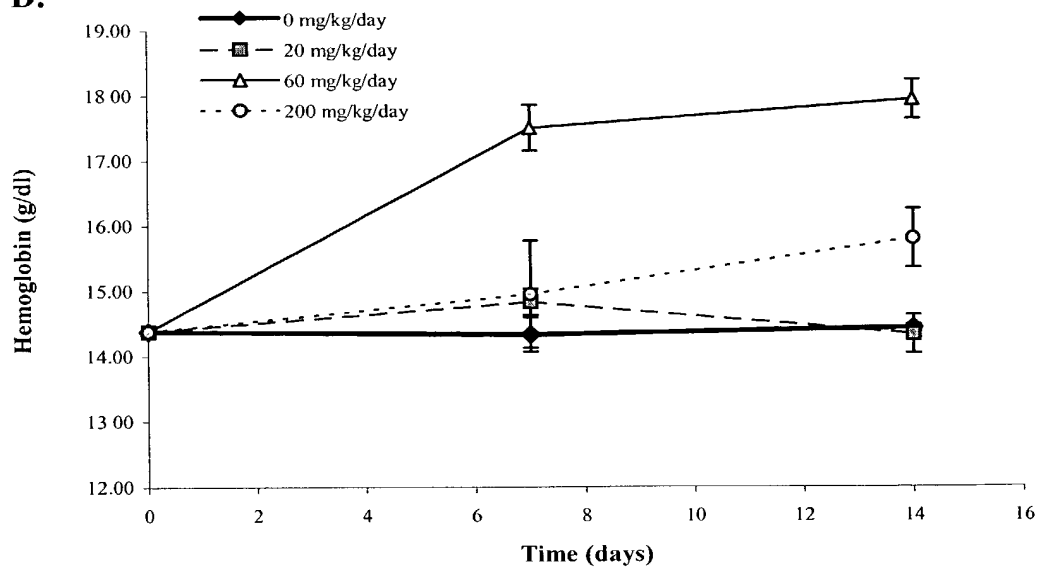


Figure 3 (con't)

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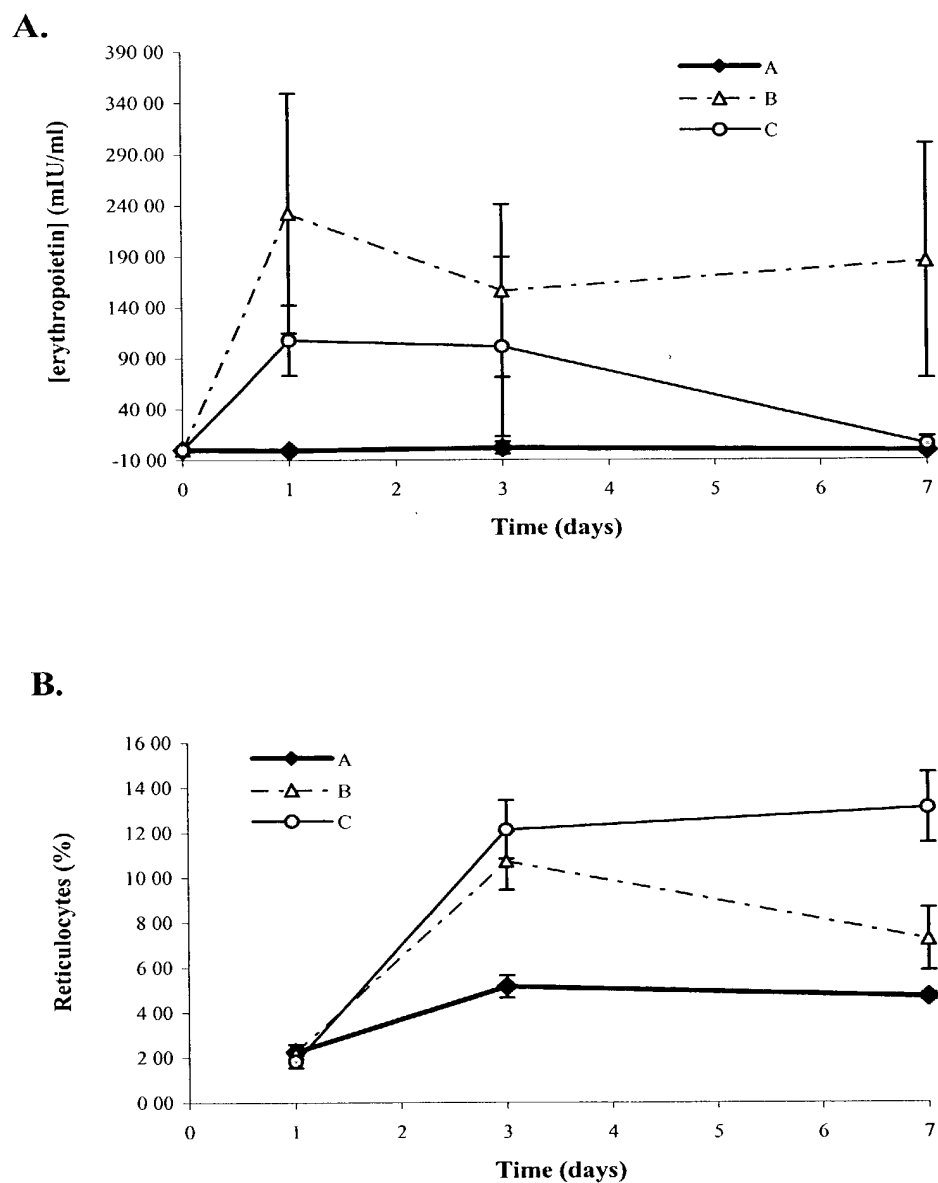
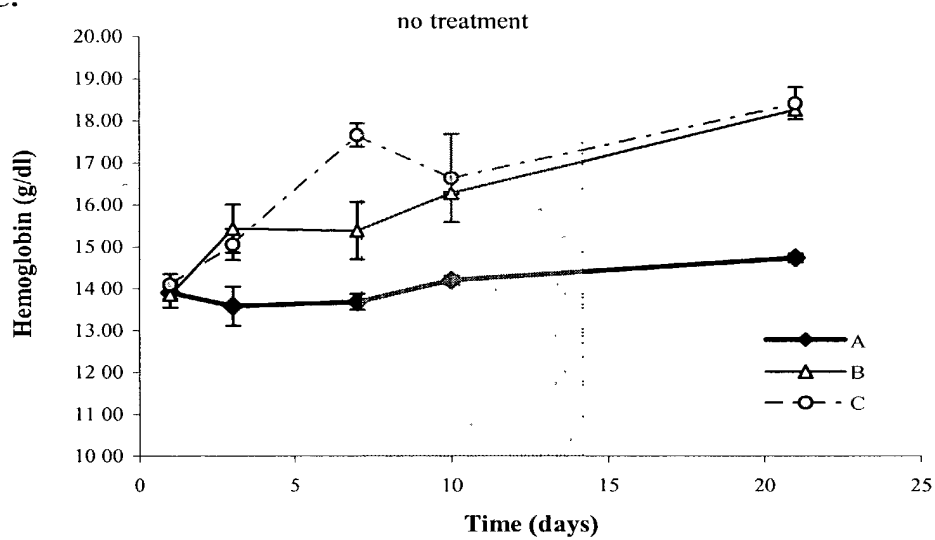


Figure 4

C.



D.

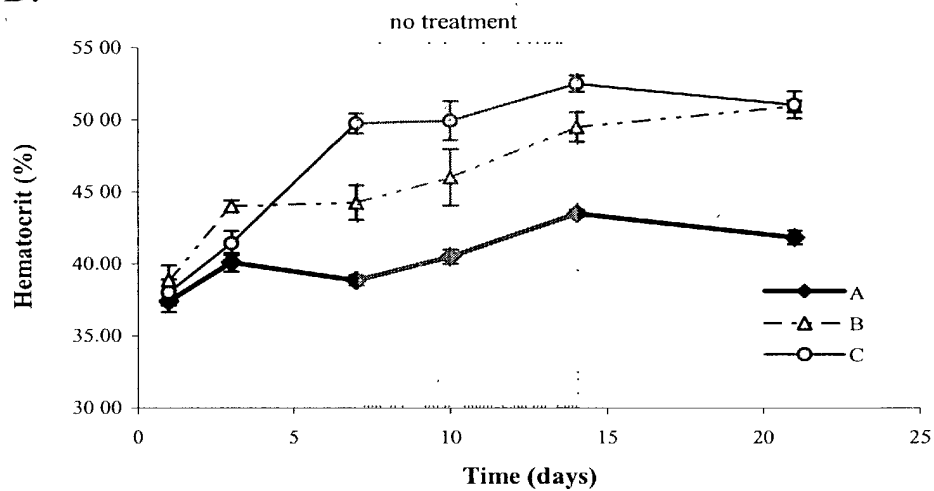


Figure 4 (con't)

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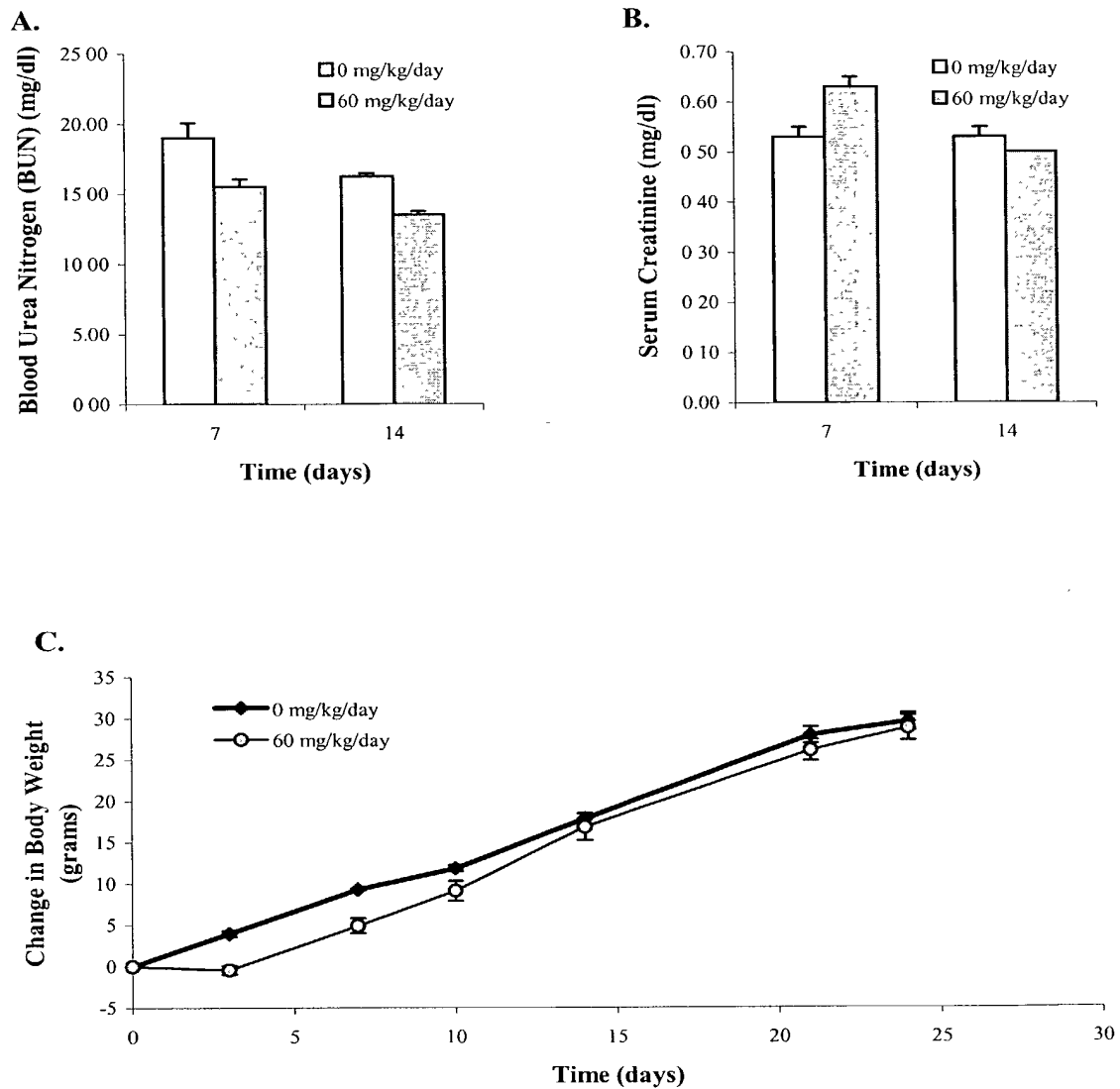


Figure 5

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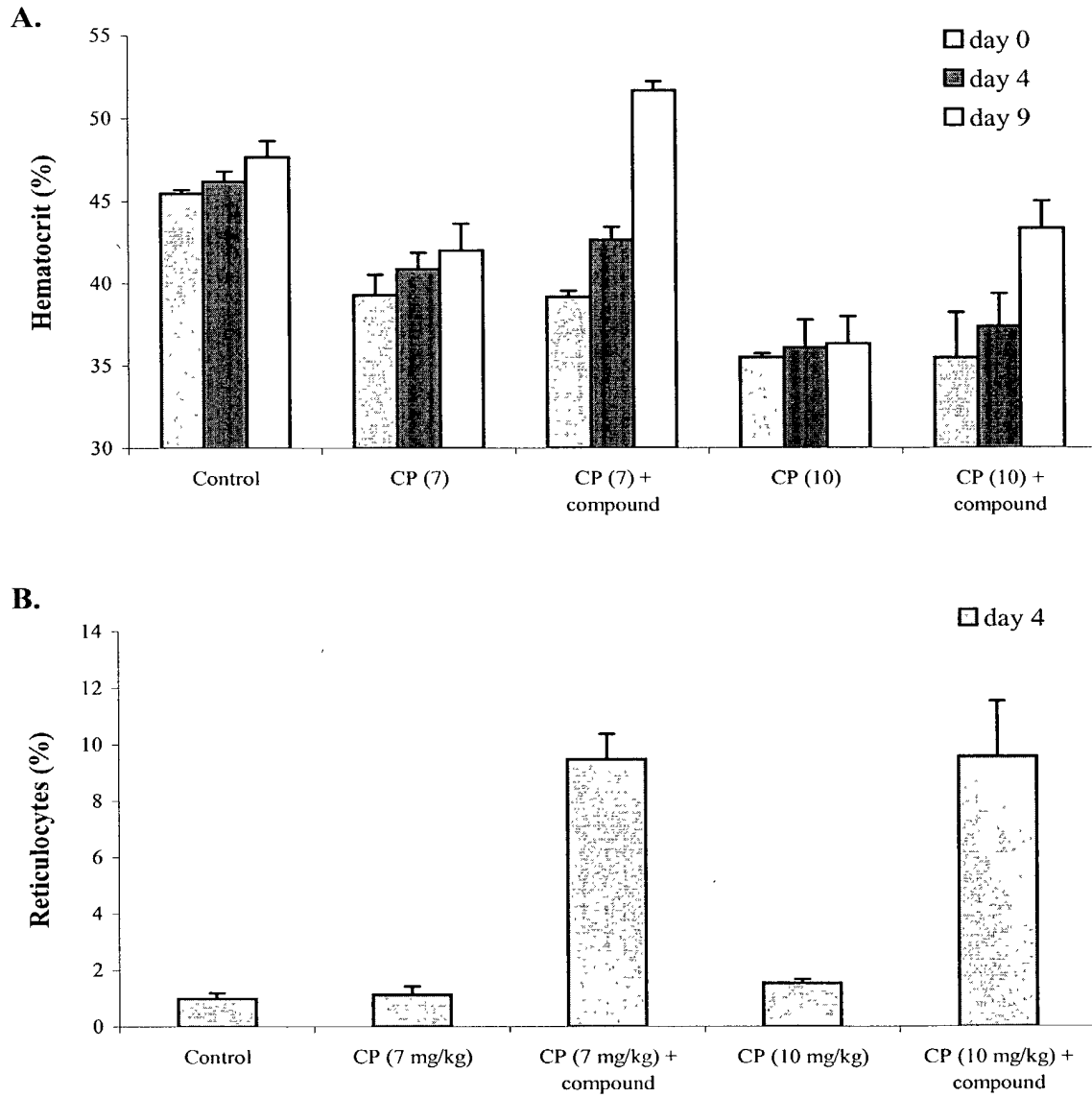


Figure 6