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(54) Title: ERYTHROPOIETIN COMPOSITIONS FOR ORAL ADMINISTRATION

(57) Abstract: The present invention relates to compositions comprising erythropoietin, one or more absorption enhancers and/or one or more bioadhesive polymers for oral administration.



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Erythropoietin compositions for oral administration

Abstract

- 5 The present invention relates to compositions comprising erythropoietin, one or more absorption enhancers and/or one or more bioadhesive polymers for oral administration.

Background state of the art

- 10 Erythropoietin (EPO) is a glycoprotein hormone, which is mainly produced in the kidney, but also to a certain extent in the liver. It controls erythropoiesis (red blood cell production) and acts as a cytokine for erythrocyte (red blood cell) precursors in the bone marrow. EPO exerts its biological effect of stimulating the proliferation and differentiation of erythroid progenitors by binding to specific cell-surface receptors, i.e., erythropoietin receptors (EPO-Rs) that are most abundant on erythroid progenitors located primarily in the bone marrow. EPO-Rs are also located in non-
- 15 hematopoietic tissues where they mediate other biological functions.

EPO increases hemoglobin serum levels thus reducing the need for blood transfusion. It is used in the treatment of anemia, for example resulting from chronic kidney disease and myelodysplasia, from cancer therapy (for example chemotherapy and/or radiation), as well as from other critical illnesses (for example heart failure).

- 20 There are currently no oral formulations of EPO available. Major constraints in this regard are the poor absorption as well as the rapid and extensive degradation of EPO in the gastrointestinal tract. The poor absorption is mostly due to EPO's large molecular size and low lipophilicity, which limits its permeability across the intestinal epithelium.

- EPO can be produced biosynthetically using recombinant DNA technology. The recombinant
- 25 human EPO (rhEPO) dosage form currently available on the market is a ready for injection liquid vial (syringe), which is usually administered 2–3 times weekly. To achieve a therapeutic effect of parenterally administered EPO, cumulative doses are required that significantly exceed levels of endogenous EPO. These high serum levels result in prolonged circulation times of EPO and unspecific binding to non-targeted tissue, which may lead to undesired side effects. Patients
- 30 experienced greater risk for death and serious cardiovascular events when ESAs (Erythropoietin Stimulating Agents) were administered at doses to target higher versus lower hemoglobin levels. ESAs shortened overall survival and/or increased the risk of tumor progression or reoccurrence in some clinical trials in patients with breast, non-small cell lung, head and neck, lymphoid and cervical cancers. Severe side effects have been recently reported in chemotherapy and/or
- 35 radiation receiving cancer patients taking anti-anemia drugs. These side effects, i.e., increased risk of death and tumor growth, were attributed to the high serum levels of parenterally administered EPO, which enable EPO to act on other non-target (undesired) tissues.

The parenteral administration, as practiced with the current EPO dosage forms on the market, is also inconvenient for patients, especially in case of chronic therapies. Apart from the

inconvenience for the patients, parenteral delivery systems are also more expensive in terms of production and drug administration through health care professionals.

Therefore, there is a need to develop new and improved EPO delivery systems, which enable a low dose long-term therapy and the delivery of EPO into the systemic circulation in an adequate amount, not exceeding the amount necessary for therapeutic effects, so that the adverse effects related to high EPO levels following parenteral administration are reduced.

From the standpoint of patient compliance, especially in case of chronic therapies, the peroral route would be the most convenient. It is also contemplated that the peroral delivery of EPO would benefit from entering the systemic circulation via the portal vein, which would more closely resemble the physiological pathway of endogenous EPO that is produced in the liver.

Consequently, there is an ongoing need for an effective peroral delivery system for EPO, which avoids the known disadvantages associated with the parenteral EPO formulations as described above, and which enhances patient compliance.

The present invention overcomes the aforesaid problems by providing a pharmaceutical composition of EPO suitable for oral administration.

Figure Legends

Figure 1: Relative number of reticulocytes in rats after subcutaneous administration of EPO (Study code P44).

Figure 2: Relative number of reticulocytes (% erythrocytes) in rats after peroral administration of EPO- and mC6EPO-loaded nanoparticles (EPO-NPs and mC6EPO-NPs) and placebo (demi water) (Study code P51).

Figure 3: Relative number of reticulocytes in rats after intrajejunal administration of EPO formulation (EPO-loaded nanoparticles, NPs-EPO-D") and placebo (empty nanoparticles) (Study code P55).

Detailed description

The present invention provides pharmaceutical compositions comprising erythropoietin (EPO) as defined herein, one or more absorption enhancers selected from the group of "CYMALs" and "PMALs" as defined herein, and/or one or more bioadhesive polymers. Such pharmaceutical compositions may be obtained by mixing appropriate solutions of EPO and the one or more other components of the pharmaceutical composition at an appropriate pH, optionally followed by, for instance, freeze drying. The appropriate pH for preparing the inventive pharmaceutical compositions will generally be maintained in a buffered solution and adjusted to maintain the biological activity of the erythropoietin and to ensure good association of the different components.

EPO and EPO Conjugates

The term "EPO" as used herein refers to erythropoietin and may be a natural or recombinant EPO, recombinant human EPO (rhEPO), or any protein or peptide having *in vivo* biological activity of the EPO glycoprotein, e.g., a wild type or mutant EPO, an EPO peptidomimetic, an EPO fragment, or an EPO conjugate as provided herein. The species from which EPO is derived can be animal, mammal or human species. Human EPO is, however, preferred. Human EPO comprises the known human erythropoietin having 165 amino acids after posttranslational cleavage of the N-terminal signal peptide of 27 amino acids and the C-terminal arginine. It further comprises human EPO in its naturally glycosylated state, i.e., including all carbohydrate side chains. The abbreviation "P" as used herein stands for EPO as defined above.

EPO Conjugates

The invention provides novel EPO conjugates, wherein one or more lipophilic side chain(s) are attached to erythropoietin. The one or more lipophilic side chain(s) may be attached to the α -amino group of the N-terminal amino acid and/or to the ϵ -amino group of lysine residues present on the EPO amino-acid sequence, e.g., via an amide bond.

Each of the one or more lipophilic side chain(s) attached to the EPO comprises one or more moieties "B" and/or a branched or straight chain alkylene moiety $-(CH_2)_m-$. While each of the one or more lipophilic side chain(s) attached to the EPO may comprise up to 6 moieties "B", it is equally preferred that each of the one or more lipophilic side chain(s) attached to the EPO comprises only one moiety "B".

The moiety B is a bivalent aromatic moiety selected from arylene or heteroarylene. The term "arylene" refers to a bivalent aromatic moiety comprising 4, 5, 6 or 7 ring carbon atoms. The term "heteroarylene" refers to an arylene group comprising 1, 2, 3 or 4 hetero-ring atoms selected from oxygen, nitrogen, and sulfur. The arylene or heteroarylene moiety may, each and independently, be unsubstituted, or may be substituted with one or two substituents each independently selected from straight chain or branched C_{1-4} -alkyl, -OH, -SH, -NH₂, -CHO, and -COOH.

The alkylene moiety $-(CH_2)_m-$ is a branched or straight chain alkylene moiety having 4 to 20 carbon atoms, or from 5 to 11 carbon atoms, or having 5, 7, 9 or 11 carbon atoms (i.e., m is an integer from 4 to 20, or from 5 to 11, or m is 5, 7, 9 or 11).

The lipophilic side chain(s) may further comprise a polar end group Y and/or one or more linker(s) each independently selected from the group consisting of L and X.

If present, the linker L is selected from -NHCO-, -CONH-, -CH=N-, or -NHCH₂-; the linker X is selected from -CH₂NH-, -NHCO-, -CONH-, -COO-, -SS-, -S-CH₂- and -NN-; and the polar end group Y is a polar group selected from -COOH, -CHO and -COOCH₃. While it is preferred that a polar group Y is present in the EPO conjugates of the invention, it is equally preferred that no

polar group Y is present therein.

The number of lipophilic side chains attached to EPO ranges from 1 to 6, for example, 1, 2, 3, 4, 5 or 6 side chains may be present in the EPO conjugates according to the invention. If more than one lipophilic side chains are present in the EPO conjugates of the invention, e.g., if 2 or
 5 more side chains are present, these lipophilic side chains may either be the same or different. In one embodiment, the one or more lipophilic side chain(s) attached to the EPO comprise(s) one or more of a bivalent aromatic moiety **B**, wherein **B** is arylene. Said arylene moiety may have 4, 5, 6 or 7 ring carbon atoms. Said arylene moiety is either unsubstituted, or is substituted with one or two substituents each independently selected from -CH₃, -OH, -SH, -NH₂, -CHO, and
 10 -COOH. The substituent -CH₃ is preferred. Preferably, **B** is a phenylene, toluylene, or xylene moiety. More preferably, **B** is an *o*-, *m*- or *p*-phenylene moiety, most preferably a *p*-phenylene moiety.

In another embodiment, the one or more lipophilic side chain(s) attached to the EPO comprise(s) one or more of a bivalent aromatic moiety **B** and/or a branched or straight chain alkylene moiety
 15 -(CH₂)_m-, and further one or more linker(s) each independently selected from the group consisting of **L** and **X**, and/or a polar end group **Y**, wherein

L represents -NHCO-;

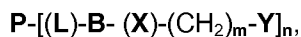
B represents an arylene group having 6 carbon atoms, and is preferably a *p*-phenylene group;

X represents -CH₂NH-;

20 **m** is an integer from 4 to 20, or from 5 to 11, or is 5, 7, 9 or 11;

Y is selected from -COOH, -CHO, and -COOCH₃; preferably **Y** is -COOH.

In one embodiment, the EPO conjugates of the present invention are represented by the following formula



25 wherein

P represents EPO;

L represents a covalent linkage between **P** and **B**, and is selected from -NHCO-, -NHCH₂-, more preferably **L** represents -NHCO-;

B represents a hetero- or homo-bifunctional linker, enabling attachment of the lipophilic moiety to
 30 the protein. For example, **B** represents a bivalent aromatic moiety, preferably an arylene or heteroarylene moiety with 4, 5, 6 or 7 ring carbon atoms, more preferably 6 carbon atoms, and optionally, one oxygen, nitrogen, or sulfur ring atom, which arylene or heteroarylene group may be unsubstituted or substituted with one or two groups selected from straight chain or branched C₁₋₄-alkyl, -OH, -SH, -NH₂, -CHO, -COOH. A preferred substituent for the arylene or
 35 heteroarylene group is methyl. Preferably, **B** represents a phenylene, toluylene, xylene or pyridylene group. More preferably, **B** is an *o*-, *m*- or *p*-phenylene group, most preferably a *p*-phenylene group;

X represents a covalent linkage between **B** and the lipophilic moiety, wherein **X** is either -CH₂NH-, -NHCO-, -CONH-, -COO-, -SS-, -S-CH₂- or -NN-, preferably **X** represents -CH₂NH-,

-NHCO-, or -CONH-, more preferably **X** represents -CH₂NH-;

(CH₂)_m represents a branched or straight chain alkylene moiety having 4 to 20 carbon atoms, or from 5 to 11 carbon atoms, or having 5, 7, 9 or 11 carbon atoms (i.e., **m** is an integer from 4 to 20, or from 5 to 11, or **m** is 5, 7, 9 or 11);

- 5 **Y** represents a polar group selected from -COOH, -CHO, and -COOCH₃. Preferably, **Y** is -COOH; and

n represents the number of lipophilic side chains attached to EPO, preferably attached via an amide bond to the α-amino group of the N-terminal amino acid and/or to the ε-amino group of lysine residues present on the EPO amino-acid sequence. **n** may be selected from 1 to 6.

- 10 Preferably **n** is 2 or 3. The values for **n** equal to 2 or 3 are preferred for fine tuning the lipophilicity.

In one embodiment, the EPO conjugate has the general formula

- 15 $P[(L)-B(X)-(CH_2)_m-Y]_n$,

wherein

P represents EPO;

L, **B**, **X**, **m** and **Y** are defined as in any one of the above embodiments, and

n represents the number of lipophilic side chains attached to EPO and ranges from 1 to 6. The

- 20 lipophilic side chain [(L)-B(X)-(CH₂)_m-Y] may be attached via an amide bond to the α-amino group of the N-terminal amino acid and/or to the ε-amino group of lysine residues present on the EPO amino-acid sequence.

In a specific embodiment, the EPO conjugates are represented by the following formulas:

- (a) rhEPO-[NHCO-p-phenylene-CH₂-NH-(CH₂)₅-COOH]_n,
 25 (b) rhEPO-[NHCO-p-phenylene-CH₂-NH-(CH₂)₇-COOH]_n,
 (c) rhEPO-[NHCO-p-phenylene-CH₂-NH-(CH₂)₉-COOH]_n, or
 (d) rhEPO-[NHCO-p-phenylene-CH₂-NH-(CH₂)₁₁-COOH]_n

wherein **n** independently represents 1, 2 or 3.

- 30 While not strictly mandatory, the sequence of the structural elements according to any of the above formulas is preferred as allows an attachment of the one or more lipophilic side chain(s) to the α-amino group of the N-terminal amino acid and/or to the ε-amino group of lysine residue present on the EPO amino-acid sequence, e.g., via an amide bond, and via an aromatic moiety **B** to the linker **X**. The partial group -(CH₂)_m-Y can be introduced, e.g., by a ω-amino carboxylic acid.

- 35 However, while it is preferred that the lipophilic side chains comprise the structural elements in the order given in the above formulas, it is equally preferred that the parameters as defined above (i.e., **L**, **B**, **X**, -(CH₂)_m-, **n** and **Y**) are independent of each other and can be selected and combined freely. If they occur repeatedly within one molecule, for example in the case that more

than one side chains are present per molecule erythropoietin, e.g., if **n** is 2 or more, the parameters may be different in each side chain. Additionally, the bivalent linker parameters **L**, **B** and **X** may be present in the orientation as depicted in the above formulas, but also in reversed orientation.

- 5 Further, the lipophilic side chain(s) of the EPO conjugates as defined above typically comprises one bivalent aromatic moiety **B**. However, more than one bivalent aromatic moieties **B** may be present as well, e.g., 2, 3, 4, 5 or 6 bivalent aromatic moieties **B** may be present. Moreover, the covalent linkage(s) **L** and/or **X** in the lipophilic side chain of the EPO conjugate as defined above may be present or omitted.
- 10 **P** as used in any of the above embodiments represents erythropoietin (EPO). The term erythropoietin, or abbreviated EPO, comprises any natural or recombinant EPO, recombinant human EPO (rhEPO), or any protein or peptide having in vivo biological activity of EPO glycoprotein, i.e. wild type or mutant EPO, EPO peptidomimetic, or EPO fragment. The species from which the EPO is derived comprise animal, mammal or human species, especially human
- 15 EPO.
Human EPO comprises the known human erythropoietin having 165 amino acids after posttranslational cleavage of the N-terminal signal peptide of 27 amino acids and the C-terminal arginine. It may further comprise the human erythropoietin in the naturally glycosylated state, i.e. including all carbohydrate side chains. In the EPO conjugates as defined above, **P** is preferably
- 20 a human recombinant EPO (rhEPO).
In the EPO conjugates of the present invention, increasing values for **m** (e.g., **m** = 5, 7, 9 or 11) correlate with an increase in lipophilicity of the EPO conjugates. Fine tuning of the lipophilicity of the EPO conjugates according to the present invention may further be accomplished by varying the number of the lipophilic side chains (**n**) per molecule of EPO (preferably, **n** = 1, 2 or 3).
- 25 The EPO conjugates according to the present invention possess many advantages. For example, the novel EPO conjugates are more lipophilic, less susceptible to enzymatic degradation and retain the *in vivo* biological activity of native or endogenous EPO of causing bone marrow cells to increase the production of reticulocytes (red blood cell progenitors) and red blood cells. In comparison with unmodified EPO, the present EPO conjugates have an increased
- 30 permeability across the intestinal membrane. Therefore, the present EPO conjugates are particularly suited for peroral administration and/or for the development of advanced oral drug delivery systems.

Preparation of the EPO conjugates

The present invention further provides a process for the production of the EPO conjugates according to the present invention.

- 5 The sequence of structural elements of the lipophilic side chain as defined above might be selected freely. A skilled artisan can produce any lipophilic side chain as defined herein by means of generally known methods, and further convert a suitable starting material into an EPO conjugate according to the invention. Particular embodiments will be described in the following.
- 10 The process for the preparation of the EPO conjugates, wherein the one or more lipophilic side chain(s) are attached to 1 to 6 α -amino group(s) of the N-terminal amino acid and/or to the ϵ -amino group(s) of lysine residue present on the EPO provided herein, generally comprises four steps: (1) modification (conversion) of a primary amine of the EPO into an amid linker and simultaneous introduction of an aldehyde group, (2) conjugation to an amino fatty acid and
- 15 reductive amination, (3) gel filtration, (4) concentration and sterile filtration.

- The first reaction step involves protein modification, wherein one or more bifunctional linker moieties **B** are attached to 1 to 6 α -amino group(s) of the N-terminal amino acid and/or to the ϵ -amino group(s) of lysine residue present on the EPO amino-acid sequence via reaction of EPO with a reagent comprising the aromatic moiety **B** substituted with an N-hydroxysuccinimidyl ester functional group (also referred to as NHS activated ester of **B**), and an aldehyde functional
- 20 group. **B** has the same meaning as defined in any of the above embodiments. The N-hydroxysuccinimidyl ester reacts in an acylation reaction with the amino group of the EPO in that the N-hydroxysuccinimide serves as leaving group and the carbonyl group of the former NHS ester forms an amide bond with the amino group of the protein. The intermediate product of this
- 25 step can be formulated as:



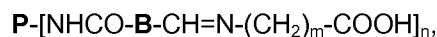
- wherein the parameters **P** and **B** have the same meaning as defined in any of the above embodiments. Preferably, the aromatic moiety **B** is a phenylene moiety. In this case, the specific reagent employed in this reaction step is N-hydroxysuccinimidyl 4-formylbenzoate (SFB).
- 30 While the respective reagent may be used in equimolar amounts, it is preferred that the reagent is used in excess amounts over EPO. The molar excess ratio of linker /EPO is from 1 to 100, or from 6 to 36, preferably 6 molecules of linker per molecule EPO. Specifically, the molar excess ratio of SFB/EPO is from 6 to 36. Preferably 6 molecules SFB per molecule EPO are used.

- 35 In a next step, the aldehyde function is condensed with the amino function of a ω -amino carboxylic acid to the corresponding Schiff's base. The ω -amino carboxylic acids may comprise 4 to 20 methylene groups: $\text{HOOC-(CH}_2\text{)}_{4-20}\text{-NH}_2$, preferably from 4 to 12 methylene groups. Preferred are ω -amino carboxylic acids having 5, 7, 9 or 11 methylene groups between the carboxylic acid group and the amino group, so that the total number of carbon atoms is 6, 8, 10

or 12, respectively, i.e. $\text{HOOC}-(\text{CH}_2)_5-\text{NH}_2$, $\text{HOOC}-(\text{CH}_2)_7-\text{NH}_2$, $\text{HOOC}-(\text{CH}_2)_9-\text{NH}_2$, and $\text{HOOC}-(\text{CH}_2)_{11}-\text{NH}_2$.

While the ω -amino carboxylic acid may be used in equimolar amounts, it is preferred that the ω -amino carboxylic acid is used in excess amounts over EPO. For example, the molar excess
 5 ratio of SFB/EPO may be from 30 to 120, or from 30 to 90. Preferably, 60 molecules of ω -amino carboxylic acid per molecule EPO are used.

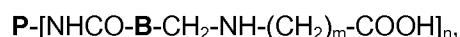
The intermediate product of this step can be formulated as:



10 wherein the parameters **P** and **B** have the same meaning as defined in any of the above embodiments.

The subsequent synthesis step comprises the reduction of the Schiff base to the corresponding amino function, i.e. $-\text{CH}=\text{N}-$ into $-\text{CH}_2-\text{NH}-$, by a reducing agent known to the skilled artisan. For example, complex alkali borohydrides such as sodium cyanoborohydride ($\text{Na}[\text{CN}-\text{BH}_3]$) may be

15 used. The final product can be formulated as:



wherein the parameters **P** and **B** have the same meaning as defined in any of the above embodiments.

The Schiff base intermediate may be isolated before performing the reduction step. It is,
 20 however, not necessary to isolate the Schiff base intermediate. Rather, the Schiff base intermediate can be processed further directly into the final product (i.e., reduction to the final EPO conjugate as defined above). Hence, the condensation and reducing reactions can be effected in a "one pot" manner.

The final reaction mixture can be purified by separating by-products, such as non-reacted EPO,
 25 e.g., rhEPO, or excess reagents such as SFB and ω -aminocarboxylic acid, from the desired conjugated EPO product. Suitable purification techniques are known to one skilled in the art, such as column chromatography, or size exclusion chromatography with a gel ("gel filtration"). For example, a Superdex75 PrepGrade (XK 26/60) column from Sigma/Aldrich may be used for purification by gel filtration. The fractions containing the desired conjugated EPO can be
 30 collected, concentrated, for example by ultrafiltration, and filtrated with a sterile filter into a sterile state.

Absorption enhancers

One group of preferred absorption enhancers to be used in the pharmaceutical compositions of
 35 the invention are cycloalkylglycosides, particularly cycloalkylmaltosides, referred to herein as "CYMALs". They can be regarded as non-toxic and safe due to their metabolism into CO_2 and water through the corresponding sugar and fatty acid metabolic pathways. These chemically synthesized molecules are composed of a sugar, typically a disaccharide. The carbohydrate component is linked by an O-glycosidic bond at the "free end" of maltose (carbon atom

number 1) to an alkyl chain, wherein the alkyl chain comprises at the most distal position of the alkyl chain, the so called ω -position, a cycloalkyl group. Independently of each other, the preferred disaccharide is maltose; the preferred alkyl chain comprises 1 to 7 methylene groups, i.e. $(\text{CH}_2)_{1-7}$, and the cycloalkyl rest at the ω -position is a cyclohexyl group. Thus, the general

5 formula for these compounds reads: ω -cycloalkyl-1-(C_{1-7} -alkyl)- β -D-maltosides, or more specifically ω -cyclohexyl-1-(CH_2) $_{1-7}$ - β -D-maltosides (= CYMAL®). Specific examples of these cyclohexyl alkyl maltosides (= CYMAL®) are 1-cyclohexyl-1-methyl- β -D-maltoside (= CYMAL®-1), 2-cyclohexyl-1-ethyl- β -D-maltoside (= CYMAL®-2), 3-cyclohexyl-1-propyl- β -D-maltoside (= CYMAL®-3), 4-cyclohexyl-1-butyl- β -D-maltoside (= CYMAL®-4), 5-cyclohexyl-1-pentyl- β -D-

10 maltoside (= CYMAL®-5), 6-cyclohexyl-1-hexyl- β -D-maltoside (= CYMAL®-6), 7-cyclohexyl-1-heptyl- β -D-maltoside (= CYMAL®-7). Preferred "CYMALs" are, however, CYMAL®-5, CYMAL®-6 and CYMAL®-7.

A further group of preferred absorption enhancers are amphiphilic polymers referred to herein as "PMALs" having a highly charged cationic and anionic backbone to which an aliphatic tail is

15 attached. The expression "PMAL" describes alternant polymers prepared by the monomers of maleic acid anhydride and an α -olefine having 6, 8, 10, 12, 14, 16 or 18 carbon atoms, respectively (i.e., "poly(maleic acid anhydride-alt- α -olefine)"), which are grafted with dimethylamino propylamine side chains. They may be prepared by reaction of poly(maleic acid anhydride-alt- α -olefine) with 3-dimethylamino propylamine, whereby the anhydride group reacts

20 with the primary amino function of the 3-dimethylamino propylamine to form an amide bond. By this way, the "non-reacting" or leaving carboxylic acid function of the original anhydride function is present as a free carboxylic acid, and simultaneously, the basic dimethylamino function is present in the side chain. Thus, a zwitterionic or ampholytic polymer ("amphipol") is obtained. Specific examples of PMALs are alternant polymers of maleic acid anhydride and α -olefine

25 having 6 to 18 carbon atoms, substituted with 3-dimethylamino propylamine (=PMAL®) such as the poly(maleic acid anhydride-alt-1-octene) 3-dimethylamino propylamine derivative (=PMAL-C6®); poly(maleic acid anhydride-alt-1-decene) 3-dimethylamino propylamine derivative (=PMAL-C8®); poly(maleic acid anhydride-alt-1-dodecene) 3-dimethylamino propylamine derivative (=PMAL-C10®); poly(maleic acid anhydride-alt-1-tetradecene) 3-dimethylamino

30 propylamine derivative (=PMAL-C12®); poly(maleic acid anhydride-alt-1-hexadecene) 3-dimethylamino propylamine derivative (=PMAL-C14®); and poly(maleic acid anhydride-alt-1-octadecene) 3-dimethylamino propylamine derivative (=PMAL-C16®). Preferably, poly(maleic acid anhydride-alt-1-dodecene) 3-dimethylamino propylamine derivative (=PMAL-C10®) is used.

Bioadhesive polymers

Suitable bioadhesive polymers to be used in the pharmaceutical compositions of the invention are selected from polyacrylic acid, polymethyl vinyl ether/maleic acid anhydride, acid-resistant enteric polymers, basic and acidic polysaccharides and polyesters. Other polymers include cationic natural polymers such as chitosan and its derivatives (e.g. trimethylchitosan, PEG-chitosan), gelatin type A etc. and anionic polysaccharides, glycosaminoglycans, polyaminoacids and various copolymers (e.g. alginic acid or its salt, hyaluronic acid or its salts, chondroitin sulfate, polyglutamic acid, poly(lactide-co-aspartic acid) etc.). Preferred bioadhesive polymers are chitosan (CS) and its derivatives, e.g. trimethylchitosan (TMC), alginic acid, its derivatives and salts, as well as combinations thereof.

The aforementioned bioadhesive polymers have the advantage that they are generally non-toxic and not irritating to the mucosa and do not require organic solvents, which would not be desirable from the point of protein stability and adverse effects on the patient. They may be added to the pharmaceutical compositions of the invention to form nanoparticles providing partial protection against protein degradation, and to enhance the mucoadhesiveness to localize the delivery system closer to the intestinal wall.

CS and its derivatives such as TMC have a dual effect, and also act as absorption enhancing agents as a part of the polymer carrier. Hence, if CS, or TMC, or a combination thereof, is present in the pharmaceutical compositions according to the invention, one or more additional absorption enhancing agent (e.g., as defined above) may be present; it is, however, equally preferred that no other absorption enhancing agents are comprised in the pharmaceutical compositions of the present invention in this case.

25

Nanoparticles

Nanoparticle formation is based on self-association of the ingredients in aqueous media under mild stirring conditions. The formation of nanoparticles or polyelectrolyte complexes can be obtained or assisted by ionotropic gelation and/or polyelectrolyte complexation using counter ions and/or polymers, for example polyphosphates such as tripolyphosphate (tpp) or polyionic polymers. This process does not require the use of organic solvents, toxic ingredients or covalent crosslinking agents and also does not employ harsh processing conditions. Therefore, the biological activity of the sensitive protein drug is preserved. The method also does not face technical and economical hurdles, therefore it is suitable for industrial scale production.

35

In one embodiment, the pharmaceutical composition comprises EPO as defined herein, one or more absorption enhancers selected from the group consisting of 1-cyclohexyl-1-methyl- β -D-maltoside (= CYMAL®-1), 2-cyclohexyl-1-ethyl- β -D-maltoside (= CYMAL®-2), 3-cyclohexyl-1-propyl- β -D-maltoside (= CYMAL®-3), 4-cyclohexyl-1-butyl- β -D-maltoside (= CYMAL®-4), 5-

- cyclohexyl-1-pentyl- β -D-maltoside (= CYMAL®-5), 6-cyclohexyl-1-hexyl- β -D-maltoside (= CYMAL®-6), 7-cyclohexyl-1-heptyl- β -D-maltoside (= CYMAL®-7), poly(maleic acid anhydride-alt-1-octene) 3-dimethylamino propylamine derivative (=PMAL-C6®), poly(maleic acid anhydride-alt-1-decene) 3-dimethylamino propylamine derivative (=PMAL-C8®), poly(maleic acid anhydride-alt-1-dodecene) 3-dimethylamino propylamine derivative (=PMAL-C10®),
 5 poly(maleic acid anhydride-alt-1-tetradecene) 3-dimethylamino propylamine derivative (=PMAL-C12®), poly(maleic acid anhydride-alt-1-hexadecene) 3-dimethylamino propylamine derivative (=PMAL-C14®), and poly(maleic acid anhydride-alt-1-octadecene) 3-dimethylamino propylamine derivative (=PMAL-C16®); and/or one or more bioadhesive polymers.
- 10 In one embodiment, the pharmaceutical composition comprises EPO as defined herein, and one or more absorption enhancers selected from the group consisting of CYMAL®-5, CYMAL®-6 and CYMAL®-7. Preferably, the absorption enhancer is CYMAL®-7. A bioadhesive polymer may further be present in the pharmaceutical compositions according to this embodiment. Preferably, the bioadhesive polymer is either chitosan (CS), or trimethylchitosan (TMC), or a combination
 15 thereof. More preferably, the bioadhesive polymer is chitosan (CS).

- In one embodiment of the invention, the pharmaceutical composition comprises EPO as defined herein, and one or more absorption enhancers selected from the group of amphiphilic polymers selected from poly(maleic acid anhydride-alt-1-octene) 3-dimethylamino propylamine derivative
 20 (=PMAL-C6®); poly(maleic acid anhydride-alt-1-decene) 3-dimethylamino propylamine derivative (=PMAL-C8®); poly(maleic acid anhydride-alt-1-dodecene) 3-dimethylamino propylamine derivative (=PMAL-C10®); poly(maleic acid anhydride-alt-1-tetradecene) 3-dimethylamino propylamine derivative (=PMAL-C12®); poly(maleic acid anhydride-alt-1-hexadecene) 3-dimethylamino propylamine derivative (=PMAL-C14®); and poly(maleic acid
 25 anhydride-alt-1-octadecene) 3-dimethylamino propylamine derivative (=PMAL-C16®). Preferably, the absorption enhancer is PMAL-C10®. In accordance with this embodiment of the invention the pharmaceutical composition is present in the form of a nanocomplex of EPO as defined herein and the one or more amphiphilic polymers. These nanocomplexes have a size in the range of from about 10 nm to about 300 nm, preferably from about 10 nm to about 100 nm,
 30 most preferably from about 10 nm to about 30 nm with a low polydispersity. These nanocomplexes are self-assembling and tend to favour complexes of about 20 nm. A bioadhesive polymer may further be present in the pharmaceutical compositions according to this embodiment. Preferably, the bioadhesive polymer is either chitosan (CS), or trimethylchitosan (TMC), or a combination thereof. More preferably, the bioadhesive polymer is a
 35 combination of chitosan (CS) and trimethylchitosan (TMC). If a combination of chitosan (CS) and trimethylchitosan (TMC) is used, CS and TMC are used at a ratio CS to TMC of, e.g., 1 to 0.2. According to a further alternative embodiment of the invention, the pharmaceutical composition comprises EPO as defined herein, and a bioadhesive polymer. Preferably, the bioadhesive polymer is chitosan (CS) or trimethylchitosan (TMC), or a combination thereof. Preferably, a

combination of CS and TMC is used. In such combinations, the ratio of CS/TMC is between 1:0.01 to 1:10, e.g., 1:0.1, or 1:0.2, or 1:0.3, or 1:0.4, or 1:0.5, or 1:1, or 1:3. In accordance with this embodiment, the pharmaceutical composition is present in the form of nanoparticles, obtained by a process of particle formation of CS or TMC, or of TMC and CS, together with EPO
5 as defined herein. These nanoparticles have a uniform particle size in the range of from 100 nm to 400 nm and also a low polydispersity. This embodiment is particularly advantageous, because the presence of CS and/or TMC has a dual effect in that these components act as bioadhesive polymers and also promote the absorption of EPO and EPO conjugates through the mucosal barrier. Therefore, the pharmaceutical compositions according to this embodiment of the
10 invention may comprise an additional absorption enhancing agent as defined herein, however, it is equally preferred that the pharmaceutical compositions according to this embodiment of the invention do not comprise an additional absorption enhancing agent.

The pharmaceutical compositions according to the invention may further comprise
15 triphenylphosphate (Tpp), which is beneficial for nanoparticle formation.

The pharmaceutical compositions of the invention are in the form of nanoparticles/nanocomplexes. It is preferred that the particle size of the nanoparticles/nanocomplexes is less than 4000 nm, or less than 1000 nm, or less than 500 nm,
20 or less than 400 nm, allowing the skilled person to select the particle size to fulfil the needs and ease of preparation for instance. For example, smaller particle sizes may be preferred for a good distribution of the drug in the gastrointestinal tract and adhesion to the mucosa without an excess concentration at a specific location. For a suitable composition with improved muco-adhesiveness, the nanoparticle size should be less than about 4000 nanometers, preferably less
25 than about 1000 nanometers, more preferably less than about 500 nanometers, and most preferably less than about 400 nanometers. Nanoparticles from 300 to 400 nanometers are particularly preferred. However, larger particle sizes may be preferred when a higher drug load is desired or required.

The nanosize pharmaceutical compositions of EPO provided herein have high oral bioavailability and efficacy. A particular advantage of the oral pharmaceutical compositions provided herein lays
30 in the closer resemblance to endogenous EPO entering the systemic circulation via the portal vein. Endogenous EPO is produced by the peritubular capillary endothelial cells in the kidney and liver. The liver accounts for 10-20% of EPO production and the liver can naturally regulate EPO plasma levels. Enteral absorption of EPO results in better regulation of hormone levels by
35 feed back regulation mechanisms in the liver, thereby preventing peak concentrations of the drug that are responsible for the undesired side effects. This regulation is not efficient upon peripheral administration of EPO. Thus, the pharmaceutical compositions of the present invention achieve plasma concentrations of EPO, which are lower than in case of parenteral administration, but higher as in untreated case. Hence, one of the benefits of the pharmaceutical

compositions of the invention is the reduction of severe side effects in comparison to parenterally administered EPO of the current commercial preparation.

Medical Use

- 5 The invention further provides the pharmaceutical compositions as defined herein for use in the treatment of anemia, for example resulting from chronic kidney disease and myelodysplasia, from the treatment of cancer, for example by chemotherapy and/or radiation, and from other critical illnesses, for example heart failure. The pharmaceutical compositions according to the present invention are further intended for use in the treatment of a disorder related to a reduction
10 of red blood cells, particularly for use in the treatment of a disorder related to a reduction of red blood cells, wherein the risk of side effects caused by abnormally high EPO levels is increased.

Test Methods

15 Caco-2 cell cultures, TEER and permeability measurements

- Human colon carcinoma cell line Caco-2, cultured on permeable supports, were used to determine the permeability coefficient of EPO or its conjugate in different formulations (i.e., in nanocomplexes and nanoparticles). Caco-2 cells were seeded on tissue-culture-treated
20 polyester filters (growth area 1.12 cm², membrane pore size 0.4 µm) in Transwell 12 wells per plates (Costar 3460) at a seeding density of 100.000 cells/well. DMEM (SIGMA, D5921; Dulbecco's modified Eagle's medium) supplemented with 10% HIFBS (heat inactivated fetal bovine serum), 1% glutamine and 1% antibiotic-antimycotic solution was used as culture medium, and added to both the donor and acceptor compartments. The medium was replaced
25 every 48 hours. The cultures were kept in an atmosphere of 95% air and 5% CO₂ at 37°C. Transport experiments were performed 21 days after the seeding.

- TEER values (transepithelial electrical resistance) of the Caco-2 cell monolayers were monitored with a Millicell RTM-Electrical Resistance System (Millipore Corp.) connected to a pair of
30 chopstick electrodes. To initiate the transport experiments, the culture media in the donor and acceptor compartments were aspirated and the cells were rinsed twice with pre-warmed transport media (DMEM). The cells were incubated for 3 hours with 0.5 ml transport media containing testing formulation. At predetermined time intervals (30, 60, 120, and 180 min) samples were taken from the acceptor chambers for protein analysis (ELISA) and replaced with
35 fresh DMEM buffer. Subsequently, testing formulations were carefully removed and cells were washed and replaced with fresh culture media for another 24 hours. The TEER value, which is a good indicator of Caco-2 cell monolayer integrity, was measured before and after the transport experiment (3h), and also after 24h in the culture media to study the reversibility of the effect of the testing formulations on Caco-2 cell monolayer. The apparent permeability coefficient, P_{app}

(cm/s) was calculated according to the equation (Eq.1):

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} , \quad \text{Equation 1}$$

- 5 where “dQ/dt” is the amount of compound transported within a given time period, “A” is the surface area of the insert; “C₀” is the initial concentration compound on the donor side. The permeability coefficient was calculated only for those testing molecules where the TEER value of the cells was not affected, or showed a gradual recovery over time to the initial TEER value.

10

In vivo PD studies

Evaluation of the pharmacodynamic response of EPO or mC6-EPO; bioavailability or pharmacological availability

- 15 *In vivo* pharmacodynamics studies were performed with series of nanoparticle formulations prepared with EPO or mC6EPO. Young adult female rats (200-225 g), Wist from Harlan, Italy, were treated according to the principles of Convention ETS 123 (The convention for the protection of animals used for experimental and other scientific purposes) and Directive 86/609/EEC (Council directive on the protection of animals used for experimental and other scientific purposes). They were housed under good hygienic conditions with food and drink *ad libitum*. Animal conditions were certified by a veterinarian. Only those animals in acceptable health conditions were used for the test. In addition, animals with hematological abnormalities were also excluded from the study.
- 20 Test items and placebo (under EXAMPLE 5 and EXAMPLE 6) were administered perorally or intrajejunally only once (at day 0 of the study). Blood samples were taken every second day throughout the 14 day lasting study. Blood samples were collected from the terminal vein (40 µl) into the pipette and immediately diluted with Cellpack reagent, Sysmex (160 µl, 1/5, v/v) in Eppendorf 0.5 ml tubes. Hematological parameters were evaluated with a hematological analyzer Sysmex XT-2000iV according to the Sysmex manufacturer's instructions.
- 25 **Evaluated parameters by hematological analyzer Sysmex XT-2000iV.**
- 30

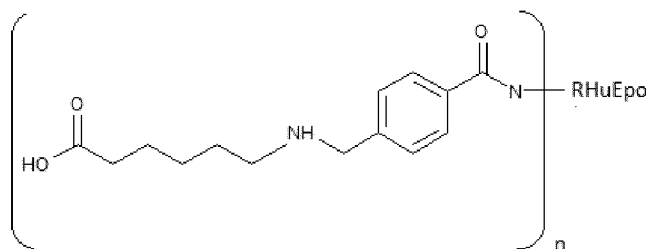
Parameters	Methodology
WBC (White Blood Cell count)	fluorescence flow cytometry
RBC (Red Blood Cell count)	DC sheath flow cytometry
Hgb (Hemoglobin concentration)	cyanide-free SLS method
Hct (Hematocrit)	DC sheath flow cytometry
MCV (Mean Corpuscular Volume)	calculation
MCH (Mean Corpuscular Hemoglobin)	calculation

MCHC (Mean Corpuscular Hemoglobin Concentration)	calculation
PLT (Platelet count)	DC sheath flow cytometry
RDW (Red Blood Cell Distribution Width)	calculation
Ret (Reticulocytes)	fluorescence flow cytometry

- The most important parameter, reticulocytes (Ret), was compared between formulations and placebo. Body weight and clinical signs were also observed before and after the application and daily during experiment. At the end of experiments all animals was humanly sacrificed under
- 5 CO₂ and anesthesia. Tissues and organs were macroscopically observed for potential abnormalities.

EXAMPLE 1:

Synthesis of an EPO conjugate: mC6-EPO



10

- Recombinant human EPO – rhEPO at concentration 2.5 mg/ml (15.6 ml, 1.28 μmol) was used in conjugation reactions. SFB was dissolved in 100% dimethylsulfoxide (DMSO) to a concentration of 2.0 mg/ml. All reactions were performed at pH 7.0 in phosphate buffer (150 mM NaCl, 50 mM Na phosphate) using thirty fold molar excess of the N-hydroxysuccinimidyl 4-formylbenzoate
- 15 (SFB) over rhEPO. The concentration of DMSO in protein reaction mixture was 3.5 %. The reaction mixture was stirred slowly at 60 rpm for 16 hours at 20 °C.

- In the second step conjugation with amino-fatty 6-aminocaproic acid was performed in the presence of sodium cyanborohydride (20mN NaCNBH₃). A sixty fold molar excess of 6-aminocaproic acid over rhEPO was used. The reaction mixture was stirred at 60 rpm for
- 20 overnight incubation at 20°C.

The conjugate was separated from unreacted rhEPO, SFB and 6-aminocaproic acid by gel filtration on Superdex75 PrepGrade (XK 26/60) column. Elution (4.5 ml/min) was done with

phosphate buffer (150 mM NaCl, 50 mM Na phosphate) containing 20 % glycerol. Fractions containing product were collected, concentrated and sterile filtrated. Purified EPO conjugate, mC6-EPO, was additionally analyzed by SDS-PAGE and RP-HPLC.

5 EXAMPLE 2:

Nanoparticles associating EPO or mC6EPO and cycloalkylmaltosides (CYMAL)

Nanoparticles (NPs) were obtained following a three step process. Firstly, buffer solution of EPO or mC6EPO (2.5 mg/ml in 0.4ml phosphate buffer pH 7 (50 mM Na-phosphate; 150 mM NaCl; pH=7.0)) was added in a dropwise manner into aqueous solution of permeation enhancer CYMAL-7® (6-cyclohexyl-1-heptyl-maltoside; 4.8 mg/ml, 0.313 ml) under gentle stirring (15 min) at room temperature. The mixture was then slowly added into a chitosan solution (CS, 4.8 mg/ml in 0.25 % acetic acid, pH 4.8, 1.042 ml). After gentle stirring for 20 min, a tripolyphosphate (tpp, 3 mg/ml, 0.25ml) was added to form nanoparticles of uniform size around 200-300 nm with low polydispersity (Pdl 0.2-0.3) measured by Zetasizer NanoZS, Malvern Instruments. Final dispersion was stirred for additional 30 min for complete nanoparticle recovery and showed characteristic Tyndal effect.

An additional formulation was prepared where EPO was slowly added into the polymer phase (chitosan solution) already containing CYMAL (Table 1, NPs: EPO+(CYMAL+CS)-tpp).

20

Permeability on Caco-2 cell monolayer

For permeability assay using Caco-2 cell monolayer, all samples, presented in Table 1, were diluted with cell culture medium (2.5x), yielding final testing composition as presented in Tables 1 and 2. Concentration of tpp is not included in the table.

Results showed that the addition of CYMAL and the increase in protein lipophilicity promoted the protein transport across Caco-2 monolayer (Table 1 and 2). Moreover, their formulation into chitosan nanoparticles by a mild method of ionotropic gelation exerted even much more pronounced increase in the apparent permeability coefficient (P_{app}) EPO. All cases with increased P_{app} EPO were characterized by the drop in transepithelial electrical resistance (TEER) of Caco-2 cells, that was recovered gradually in case of the formulation of protein with CYMAL and almost completely for the nanoparticle formulations.

Table 1. Composition of testing formulations of EPO with CYMAL, and their formulation in chitosan nanoparticles, TEER value after 3h of treatment and 24h after the experiment (to monitor Caco-2 cells recovery), and calculated permeability for EPO. Enhancement ratio of P_{app} EPO is also presented for the formulations relative to free EPO. In one nanoparticle formulation EPO was combined with CYMAL and then formulated in NPs, and in the other NP-formulation EPO was added to the polymer phase already containing CYMAL.

Sample	Conc. (mg/ml)	TEER 3h (%)	TEER 24h (%)	P _{app} EPO (cm/s)	ER
EPO	0.2	100	100	3.4E-10	
EPO+CYMAL-7	0.2+0.3	90	50*	8.07E-08	237
NPs:(EPO+CYMAL)+CS-tpp	(0.2+0.3)+1	15	90	3.54E-07	1040
NPs:EPO+(CYMAL+CS)-tpp	0.2+(0.3+1)	20	90	1.55E-07	450

*poor cell recovery

Table 2. Composition of testing formulations of mC6EPO with CYMAL, and their

- 5 **formulation in chitosan nanoparticles**, TEER value after 3h of treatment and 24h after the experiment (to monitor Caco-2 cells recovery), and calculated permeability for mC6EPO. Enhancement ratio of P_{app} EPO is also presented for the formulations relative to free EPO.

Sample	Conc. (mg/ml)	TEER 3h (%)	TEER 24h (%)	P _{app} EPO/ mC6EPO (cm/s)	ER
EPO	0.2	100	100	3.4E-10	
mC6EPO	0.2	70	100	1.33E-08	39
mC6EPO+CYMAL	0.2+0.3	6	40*	1.68E-07	494
NPs:(mC6EPO+CYMAL)+ CS-tpp	(0.2+0.3)+1	15	80	2.81E-07	826

*poor cell recovery

10

EXAMPLE 3:

Nanoparticles containing EPO or mC6EPO and TMC as a part of the carrier material and enhancer

- 15 Nanoparticles (NPs) were obtained by dropwise addition of EPO or mC6EPO (2.5 mg/ml, 0.3 ml) into the solution of chitosan and trimethylchitosan (TMC) (total polymer concentration was 4 mg/ml in 0.25% acetic acid, pH 4.8, 0.938 ml, where CS/TMC ratio was 0.5/0.5 and 0.25/0.75; and 4.6 mg/ml in 0.25% acetic acid, pH 4.8, 0.897 ml, where CS/TMC ratio was 1/0.1). After gentle stirring for 20 min, a tripolyphosphate (tpp, 2.6 mg/ml, 0.26 ml or 0.3 ml) was slowly
- 20 added to form nanoparticles of uniform particle size around 200 nm and low polydispersity (Pdl 0.35) measured by Zetasizer NanoZS, Malvern Instruments. Final dispersion was stirred for additional 30 min for complete nanoparticle recovery.

25

Permeability on Caco-2 cell monolayer

For permeability assay using Caco-2 cell monolayer, all samples, presented in Table 3, were diluted with cell culture medium (2.5x), yielding final testing composition as presented in Table 3.

5 Concentration of tpp is not included in the table.

Results showed that all nanoparticle formulation increased P_{app} EPO relative to free EPO although not containing absorption enhancer (Table 3). TMC included in the carrier polymer (chitosan) might therefore aid the protein transport across epithelial barrier.

10 **Table 3. Composition of testing formulations of EPO in chitosan nanoparticles associating trimethylchitosan (TMC)**, TEER value after 3h of treatment and 24h after the experiment (to monitor Caco-2 cells recovery), and calculated permeability for EPO. Enhancement ratio of P_{app} EPO is also presented for the formulations relative to free EPO.

Sample	Conc. (mg/ml)	TEER 3h (%)	TEER 24h (%)	P_{app} EPO/mC6EPO (cm/s)	ER
EPO	0.2	100	100	3.4E-10	
NPs:EPO+(CS/TMC)- tpp	(0.2)+0.5/0.5	23	70	1.71E-08	50
NPs:EPO+(CS/TMC)- tpp	(0.2)+ 0.25/0.75	20	60	5.54E-08	160
NPs:EPO+(CS/TMC)- tpp	(0.2)+ 1/0.1	70	100	2.68E-09	8
NPs:mC6EPO+(CS/T MC)-tpp	(0.2)+ 1/0.1	40	100	1.28E-08	38

15

EXAMPLE 4:

Nanocomplexes of EPO-PMAL or mC6EPO-PMAL, and their formulation in nanoparticles

20

Nanocomplexes were obtained upon dropwise addition of EPO or mC6EPO (2.5 mg/ml, 0.6 ml) using a pipette into an aqueous solution of PMAL-C10 (PMAL, [Poly(maleic anhydride-alt-1-dodecene) substituted with 3-(Dimethylamino) propylamine], 10% stock solution suitably diluted to final concentration, see Table 4 and 5) under gentle stirring at room temperature. Complex formation was verified by DLS measurements (Zetasizer NanoZS, Malvern Instruments), according to size, Pdi, zeta potential and scattering intensity of the new formation.

25

EPO, having size about 7 nm, and PMAL, having polymodal and polydispersed distribution,

formed a complex with size about 15 nm and low polydispersity index 0.2. Zeta potential (pH 7.0) of EPO, PMAL and EPO-PMAL complex were -10, -52mV and +13 mV, respectively. The scattering intensity of EPO (218 kcps) and PMAL solution (216 kcps) markedly increased on their addition (EPO-PMAL 6,600 kcps) giving evidence of the complex formation.

- 5 Nanoparticles containing EPO-PMAL or mC6EPO-PMAL assembly were obtained further by adding the nanocomplex solution slowly into a chitosan/trimethylchitosan solution (4.8/0.96 mg/ml in 0.25% acetic acid, pH 4.8, 1.56 ml). After gentle stirring for 20 min, a tripolyphosphate (3 mg/ml, 0.449 ml) was added to form nanoparticles of uniform size distribution (particle size 200-250 nm) and low polydispersity (Pdl 0.3) measured by Zetasizer NanoZS, Malvern
10 Instruments. Final dispersion was stirred for additional 30 min for complete nanoparticle recovery.

Permeability on Caco-2 monolayer

- 15 For permeability assay using Caco-2 monolayer, all samples were diluted with cell culture medium (2.5x), yielding final testing composition as presented in Tables 4 and 5. Concentration of tpp is not included in the table.

Results showed that EPO-PMAL or mC6EPO-PMAL assembly and their formulation in nanoparticles promoted the protein transport across Caco-2 monolayer (Table 4 and 5).

- 20 **Table 4. Composition of testing formulations of EPO-PMAL assembly and their formulation in chitosan/trimethylchitosan nanoparticles**, TEER value after 3h of treatment and 24h after the experiment (to monitor Caco-2 cells recovery), and calculated permeability for EPO. Enhancement ratio of P_{app} EPO is also presented for the formulations relative to free EPO.

Sample	Conc. (mg/ml)	TEER 3 h (%)	TEER 24 h (%)	P_{app} EPO (cm/s)	ER
EPO	0.2	100	100	3.4E-10	
EPO-PMAL	0.2+2	82	100	1.25E-09	4
EPO-PMAL	0.2+5	80	100	3.01E-09	9
NPs:(EPO-PMAL)+CS/TMC-tpp	(0.2+4)+1/0.2	15	50*	3.83E-08	113

- 25 *poor cell recovery

- Table 5. Composition of testing formulations of mC6EPO-PMAL assembly and their formulation in chitosan/trimethylchitosan nanoparticles**, TEER value after 3h of treatment and 24h after the experiment (to monitor Caco-2 cells recovery), and calculated permeability for mC6EPO. Enhancement ratio of P_{app} EPO is also presented for the formulations relative to free EPO.
- 30

Sample	Conc. (mg/ml)	TEER 3h (%)	TEER 24h (%)	P _{app} EPO/ mC6EPO (cm/s)	ER
EPO	0.2	100	100	3.4E-10	
mC6EPO	0.2	70	100	1.33E-08	39
mC6EPO-PMAL	0.2+1	76	100	1.91E-09	6
NPs:(mC6EPO- PMAL)+CS/TMC-tpp	(0.2+4)+1/0.2	15	40*	7.84E-08	230

*poor cell recovery

5 EXAMPLE 5:

In vivo PD study after peroral administration EPO or mC6EPO-formulation

Placebo and EPO- or mC6EPO-formulations (test items) were administered by a single gavage administration. UV sterilized Latex Free syringe with gavage needle was used for p.o. application. Placebo and test items were administered once, i.e. on day 0.

- 10 All animals were starved for 18 hours before the application and 15-30 min after the application. For that time the cage floor was changed with the metal grid to prevent coprophagia. After that period the animals were transposed into the normal cages with sawdust bedding and the food was offered as usual (ad libitum). The water was available ad libitum all the time. Dosing of animals and blood sampling schedule are presented in table 6.

15

Table 6: Dosing and blood sampling schedule

Groups		Sampling time (days)	No. of animals	No. of samples
1	Test item 1 80 µg EPO/rat	-4, -1 (before starvation), 2, 4, 6, 8, 10 and 14	6	48
2	Test item 2 80 µg mC6EPO/rat		6	48
3	Placebo		6	48
together		8 time points	18	144

- 20 Test items were prepared as freeze-dried products which were rehydrated in water before the application and prepared as a final dispersion for oral application. Test item 1 labeled **Lio-EPO-A** contained nanoparticles composed of EPO, CYMAL7, and chitosan /tripolyphosphate. Initial loading of EPO was 2.5% and 1.5x mass excess of CYMAL7 relative to EPO was used. Test item 2 labeled **Lio-mC6EPO-B** contained nanoparticles composed of mC6EPO, CYMAL7, and chitosan / tripolyphosphate with initial loading of mC6EPO 5% and 13x mass excess of CYMAL7 relative to mC6EPO. In Placebo group animals received demi water.

Data evaluation

The most responsive hematological parameter was the number of reticulocytes determined at individual time points in the blood samples. Response curves were generated by plotting
 5 reticulocyte count against time for individual animal in each group. Individual response was evaluated by calculating AUC (area under the curve) taking zero time point (or -1 day) and day six time point as a baseline values.

Bioavailability (BA) from PD response of perorally administered EPO or mC6EPO formulations
 10 was calculated relative to the average AUC values obtained in the same way for subcutaneously administered EPO or mC6EPO (0.8 µg/rat) using the equation:

$$BA_{p.o.} \% = \frac{AUC_{p.o.(-1 \rightarrow 6)}}{avgAUC_{s.c.(0 \rightarrow 6)}} \times \frac{Dose_{s.c.}}{Dose_{p.o.}} = \frac{AUC_{p.o.(-1 \rightarrow 6)}}{avgAUC_{s.c.(0 \rightarrow 6)}} \times \frac{Dose_{s.c.}}{100 \cdot Dose_{s.c.}} \times 100$$

15 $BA_{p.o.}$ is referred to pharmacological availability calculated from response curves of subcutaneously and preorally administered EPO or mC6EPO formulations. Results are presented in table 7 and figures 1 and 2.

20 **Table 7: AUC values for the number of reticulocytes after subcutaneous and peroral application of different EPO or mC6EPO-formulations.**

Applicatio n	s.c.		per oral		
	Reference		Test item 1	Test item 2	Placebo
Individual animal per group	EPO s.c. (Study code P44)	mC6EPO s.c. (Study code P44)	Lio-EPO-A p.o. (Study code P51)	Lio-mC6EPO-B''' p.o. (Study code P51)	Demi water p.o. (Study code P51)
1	10302	9504	13096.5	1187	307.5
			BA 1.2%	BA 0.1%	No response
2	12186	8198	6639.5	-1274,5	n.a.
			BA 0.6%	No response	n.a.
3	12422	9066	3257.5	9367	-936
			BA 0.3%	BA 1.1%	No response
4	9632	8492	-565.5	-1680	-2145.5
			No response	No response	No response
5	/	/	-1412	567	-3119

			No response	No response	No response
6	/	/	-693.5	141	-3054
			No response	No response	No response
AVG	11135	8815			
STD	1380	583			

In the groups receiving Test item 1 and Test item 2 individual animals showed an increase in the number of reticulocytes on the 4th day after application, which is expected physiological response of erythropoietin drug (Figure 1). Three out of six animals responded in the group receiving Test item 1, two out of six animals responded in the animal group receiving Test item 2, while no animals responded in the placebo group. Bioavailability (BA) of nanoparticle EPO formulations calculated from PD responses was close to 1% for individual animals after peroral application.

10 **EXAMPLE 6: In vivo PD study after intrajejunal administration EPO-formulation**

The test item and placebo were administered by a single intrajejunal (i.j.) administration. UV sterilized Latex Free syringe with 23G needle were used for i.j. application. The test item and placebo were administered once, on day 0.

All animals had restricted access to food 16 hours before application with total withdrawal of food at least 2 hours before application. For that time the cage floor was changed with the metal grid to prevent coprophagia. The animals were transposed into the normal cages with sawdust bedding after the application and the food was offered ad libitum 2 hours after the application. The water was available ad libitum all the time.

Rats were anaesthetized with intraperitoneal injection of a mixture of ketamine, xylazine and saline (5:4:5 v/v/v; 0.7 ml/rat, 20 % w/v solution). Once anaesthetized, the incision site at abdominal region (region xiphoidea) was shaved and disinfected with an iodine solution. The abdomen was entered through a midline incision, and the jejunum was located. UV sterilized Latex Free syringe with 23G needle was introduced into the jejunum and the substance was applied. The injection site was gently pressured after application to stop any leaking or bleeding. The jejunum was carefully returned to its normal position within the abdominal cavity. The abdominal muscles were closed with a 6-0 absorbable suture (Safire, Braun, Germany) and the abdominal skin was closed with the wound clips (Autoclip[®] Wound Closing System, Braintree Scientific, USA).

After surgery, the rats were caged individually and placed in a warm room ($T = 26 \pm 1^{\circ}\text{C}$) for 24 h. The incision site was sprayed with Bivacyn (Lek Pharmaceuticals d.d., Slovenia). Each rat received 0.03 ml of antibiotic Trioxyl (Univet Ltd, Ireland, 150 mg/ml) intramuscularly. Dosing of animals and blood sampling schedule are presented in table 8.

Table 8: Dosing and blood sampling schedule

Groups		Sampling time	No. of animals	No. of samples
1	NPs-EPO-D''' 80 µg EPO/rat	before application-0-2- 4-6-8-10-13 days	6	48
2	Empty NPs		4	24

EPO-loaded nanoparticles (NPs-EPO-D''') and empty nanoparticles were administered as freshly prepared nanoparticle dispersion. EPO-loaded nanoparticles, labeled **NPs-EPO-D'''** contained nanoparticles composed of EPO, CYMAL7, and chitosan /tripolyphosphate with initial loading of EPO 15% and 13x mass excess of CYMAL7 relative to EPO. Empty nanoparticles had the same composition but without the protein.

Data evaluation

- 10 The most responsive hematological parameter was the number of reticulocytes determined at individual time points in the blood samples. Response curves were generated by plotting reticulocyte count against time for individual animal in each group. Individual response was evaluated by calculating AUC (area under the curve) taking zero time point and day six time point as a baseline values.
- 15 Bioavailability (BA) from PD response of intrajejunally administered EPO formulation was calculated relative to the average AUC values obtained in the same way for subcutaneously administered EPO (0.8 µg EPO/rat) using the equation:

$$BA_{i,j} \% = \frac{AUC_{i,j,(0 \rightarrow 6)}}{avgAUC_{s.c.(0 \rightarrow 6)}} \times \frac{Dose_{s.c.}}{Dose_{i,j.}} = \frac{AUC_{i,j,(0 \rightarrow 6)}}{avgAUC_{s.c.(0 \rightarrow 6)}} \times \frac{Dose_{s.c.}}{100 \cdot Dose_{s.c.}} \times 100$$

- 20 $BA_{i,j.}$ is referred to pharmacological availability calculated from response curves of subcutaneously and intrajejunally administered EPO or mC6EPO formulations. Results are presented in table 9 and figure 3.

Table 9: AUC values for the relative number of reticulocytes (% of erythrocytes) after subcutaneous and intrajejunal administration of EPO-formulation.

Application	s.c.	Intrajejunal application	
		EPO-loaded NPs	Placebo
Individual animal per group	EPO s.c. (Study code P44)	EPO-NPs-D''' i.j. (Study code P55)	Empty NPs i.j. (Study code P55)
1	1438	220	-208
		BA 0.2%	No response
2	1518	-164	-24
		No response	No response
3	1650	1048	2
		BA 0.7%	No response
4	1520	16	-262
		No response	No response
5	/	-124	n.a
		No response	n.a.
6	/	1116	n.a.
		BA 0.73%	n.a.
AVG	1531		
STD	87		

- 5 In the animal group receiving EPO-loaded nanoparticles individual animals showed an increase in the number of reticulocytes on the 4th day after application, which is expected physiological response of erythropoietin drug (Figure 1). Two out of six animals responded in the group receiving EPO-NPs, while no response was observed for animals receiving empty nanoparticles. Bioavailability (BA) of EPO-NP formulation calculated from PD responses was 0.7% for
- 10 individual animals after intrajejunal application.

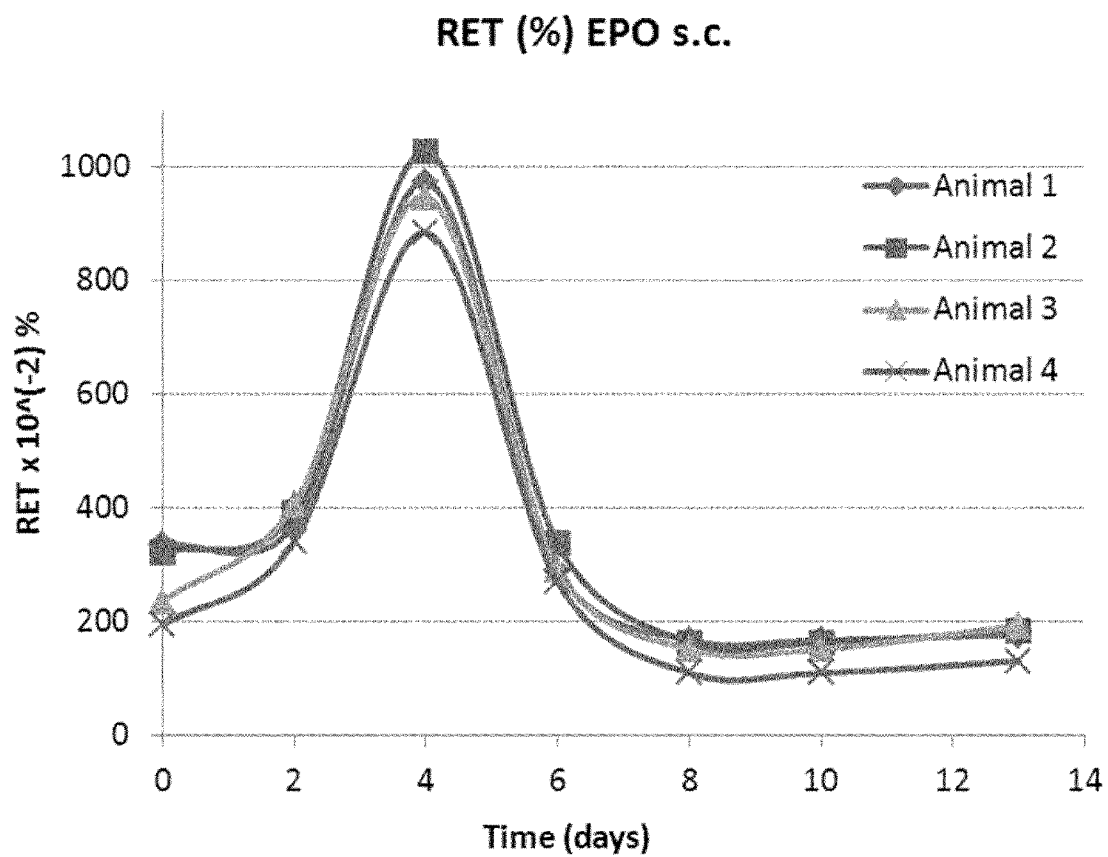
Claims

1. Pharmaceutical composition comprising erythropoietin (EPO), one or more absorption enhancers selected from the group consisting of alkyl glycosides, cycloalkyl glycosides, cycloalkyl-alkyl-maltosides, and poly(maleic acid anhydride-alt-C₈₋₁₈- α -olefine) 3-dimethylamino propylamine derivatives; and/or one or more bioadhesive polymers.
2. The pharmaceutical composition of claim 1, wherein the one or more absorption enhancer is selected from the group consisting of 1-cyclohexyl-1-methyl- β -D-maltoside (= CYMAL®-1), 2-cyclohexyl-1-ethyl- β -D-maltoside (= CYMAL®-2), 3-cyclohexyl-1-propyl- β -D-maltoside (= CYMAL®-3), 4-cyclohexyl-1-butyl- β -D-maltoside (= CYMAL®-4), 5-cyclohexyl-1-pentyl- β -D-maltoside (= CYMAL®-5), 6-cyclohexyl-1-hexyl- β -D-maltoside (= CYMAL®-6), 7-cyclohexyl-1-heptyl- β -D-maltoside (= CYMAL®-7), poly(maleic acid anhydride-alt-1-octene) 3-dimethylamino propylamine derivative (=PMAL-C6®), poly(maleic acid anhydride-alt-1-decene) 3-dimethylamino propylamine derivative (=PMAL-C8®), poly(maleic acid anhydride-alt-1-dodecene) 3-dimethylamino propylamine derivative (=PMAL-C10®), poly(maleic acid anhydride-alt-1-tetradecene) 3-dimethylamino propylamine derivative (=PMAL-C12®), poly(maleic acid anhydride-alt-1-hexadecene) 3-dimethylamino propylamine derivative (=PMAL-C14®) and poly(maleic acid anhydride-alt-1-octadecene) 3-dimethylamino propylamine derivative (=PMAL-C16®).
3. The pharmaceutical composition of claims 1 or 2, wherein the one or more absorption enhancer is selected from the group consisting of 5-cyclohexyl-1-pentyl- β -D-maltoside (= CYMAL®-5), 6-cyclohexyl-1-hexyl- β -D-maltoside (= CYMAL®-6), 7-cyclohexyl-1-heptyl- β -D-maltoside (= CYMAL®-7) and poly(maleic acid anhydride-alt-1-dodecene) 3-dimethylamino propylamine derivative (=PMAL-C10®).
4. The pharmaceutical composition according to any one of the preceding claims, wherein the absorption enhancer is 7-cyclohexyl-1-heptyl- β -D-maltoside (CYMAL®-7).
5. The pharmaceutical composition according to any one of claims 1 to 3, wherein the absorption enhancer is poly(maleic acid anhydride-alt-1-dodecene) 3-dimethylamino propylamine derivative (=PMAL-C10®).
6. The pharmaceutical composition according to any one of the preceding claims, wherein the bioadhesive polymer is chitosan (CS), or trimethylchitosan (TMC), or a combination thereof.

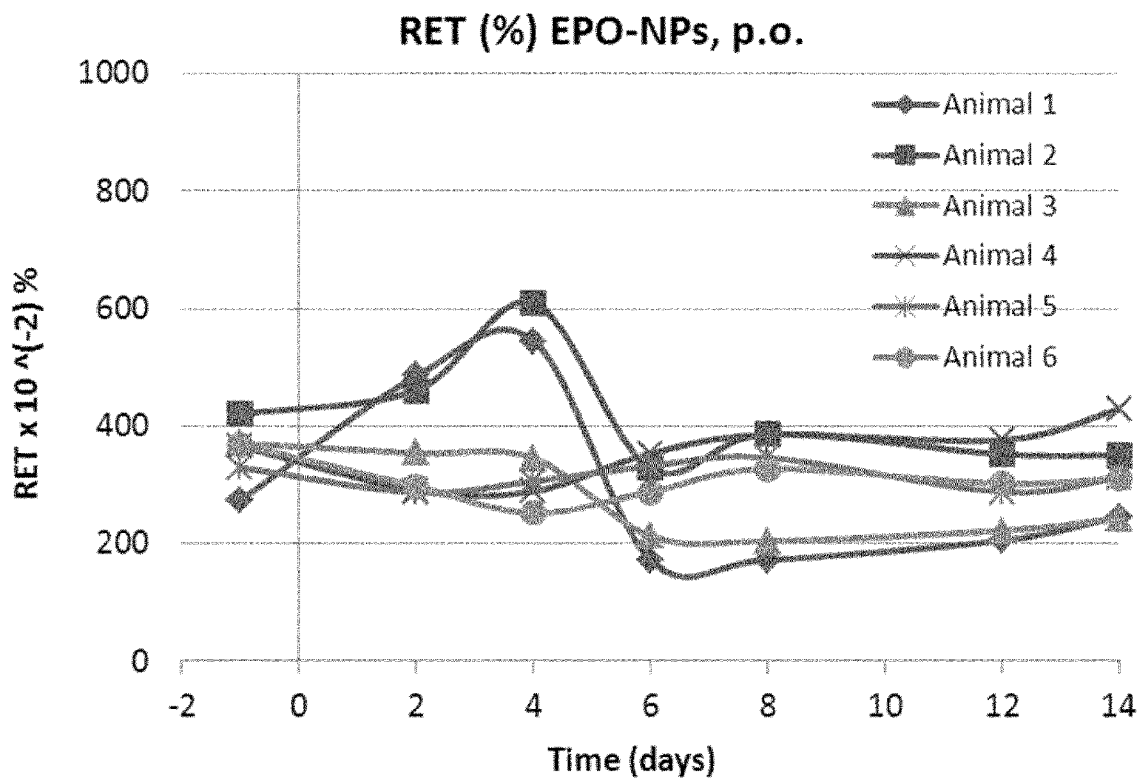
7. The pharmaceutical composition of claim 1, comprising erythropoietin (EPO) and a bioadhesive polymer selected from chitosan (CS), or trimethylchitosan (TMC), or a combination thereof.
- 5 8. The pharmaceutical composition according to any one of the preceding claims, further comprising tripolyphosphate.
9. The pharmaceutical composition according to any one of the preceding claims, wherein the pharmaceutical composition is present in the form of nanoparticles or
10 nanocomplexes.
10. The pharmaceutical composition according to claim 9, wherein the size of the nanoparticles or nanocomplexes is less than about 4000 nm, or less than about 1000 nm, or less than about 500 nm, or less than about 400 nm.
- 15 11. The pharmaceutical composition according to any one of the preceding claims, wherein the erythropoietin (EPO) is selected from a natural or recombinant EPO, a recombinant human EPO (rhEPO); a wild type or mutant EPO, an EPO peptidomimetic, or an EPO fragment having *in vivo* biological activity of the EPO glycoprotein; or an EPO conjugate.
- 20 12. The pharmaceutical composition according to claim 11, wherein the EPO conjugate has the general formula
- P[(L)-B-(X)-(CH₂)_m-Y]_n**
 wherein
 25 **P** represents erythropoietin (EPO),
L is -NHCO-;
B is a p-phenylene group;
X is -CH₂NH-;
m is an integer from 4 to 20, or from 5 to 11, or **m** is 5, 7, 9 or 11;
 30 **Y** is selected from -COOH, -CHO, and -COOCH₃; and
n is an integer from 1 to 6, or **n** is 1, 2 or 3.
13. The pharmaceutical composition according to claim 12, wherein the EPO conjugate has the formula
 35 (a) rhEPO-[NHCO-p-phenylene-CH₂-NH-(CH₂)₅-COOH]_n;
 (b) rhEPO-[NHCO-p-phenylene-CH₂-NH-(CH₂)₇-COOH]_n;
 (c) rhEPO-[NHCO-p-phenylene-CH₂-NH-(CH₂)₉-COOH]_n; or
 (d) rhEPO-[NHCO-p-phenylene-CH₂-NH-(CH₂)₁₁-COOH]_n;
 wherein **n** independently represents 1, 2 or 3.

14. The pharmaceutical composition of claims 12 or 13, wherein the lipophilic side chain $[(L)-B(X)-(CH_2)_m-Y]_n$ is attached to P *via* an amide bond to the α -amino group of the N-terminal amino acid and/or to the ϵ -amino group of lysine residues present in the EPO amino-acid sequence.
15. The pharmaceutical composition according to any one of the preceding claims for use in therapy or diagnosis.
16. The pharmaceutical composition according to any one of claims 1 to 14 for use in the treatment of a disorder related to a reduction of red blood cells.
17. Use of the pharmaceutical composition according to any one of claims 1 to 14 for the preparation of a medicament for oral administration.
18. A process for preparing a pharmaceutical composition according to any one of claims 1 to 14, comprising the steps of (a) adding a solution of EPO to a first solution comprising one or more absorption enhancers or one or more bioadhesive polymer to give a mixture, (b) optionally adding a solution of tripolyphosphate; and (c) isolating the resulting nanoparticles.
19. The process of claim 18, wherein the first solution comprises one or more absorption enhancers, and wherein step (a) further comprises adding said mixture to a solution comprising chitosan, or trimethylchitosan, or a combination thereof.
20. The process of claim 18, wherein the first solution comprises the one or more bioadhesive polymers.

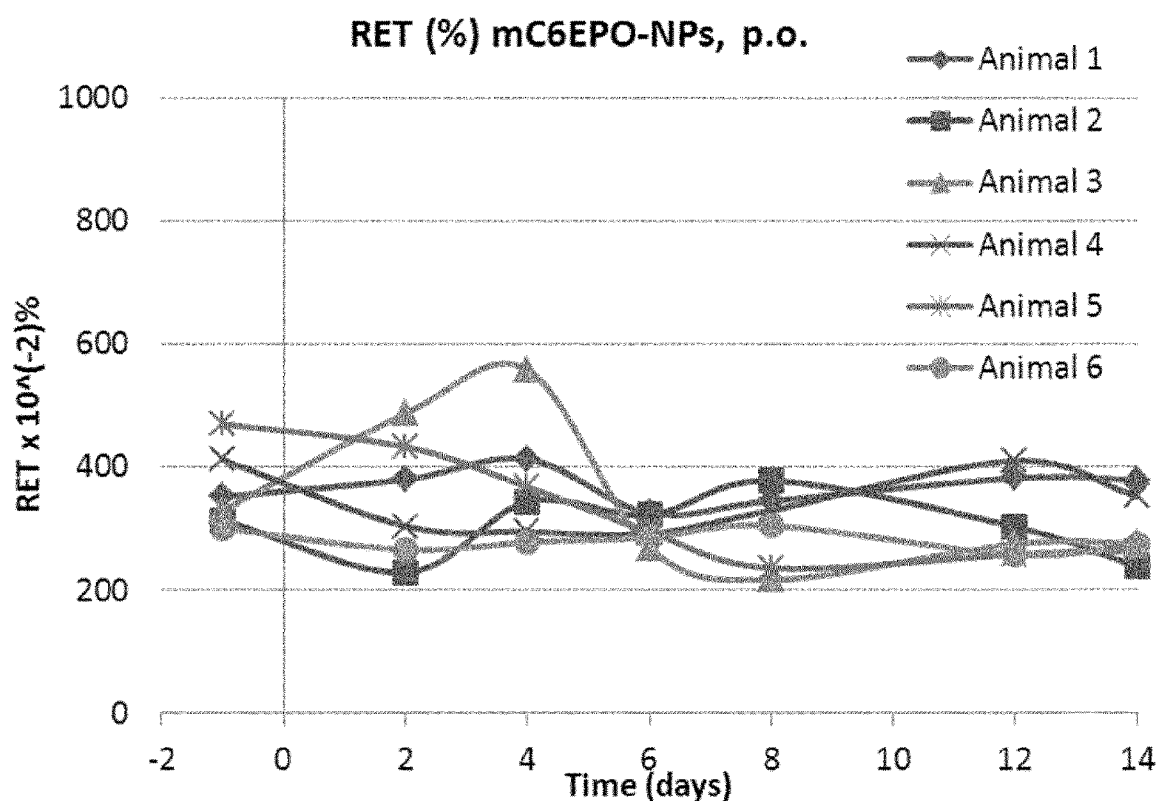
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*Figure 1*

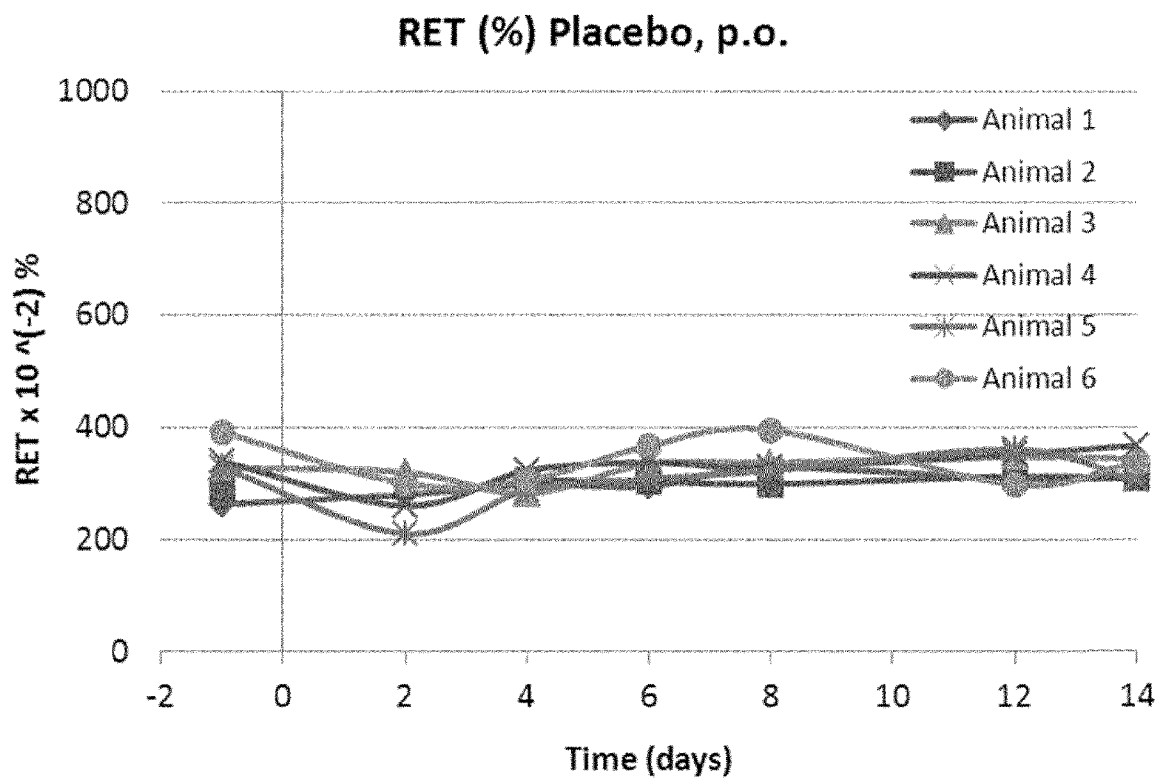
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*Figure 2*

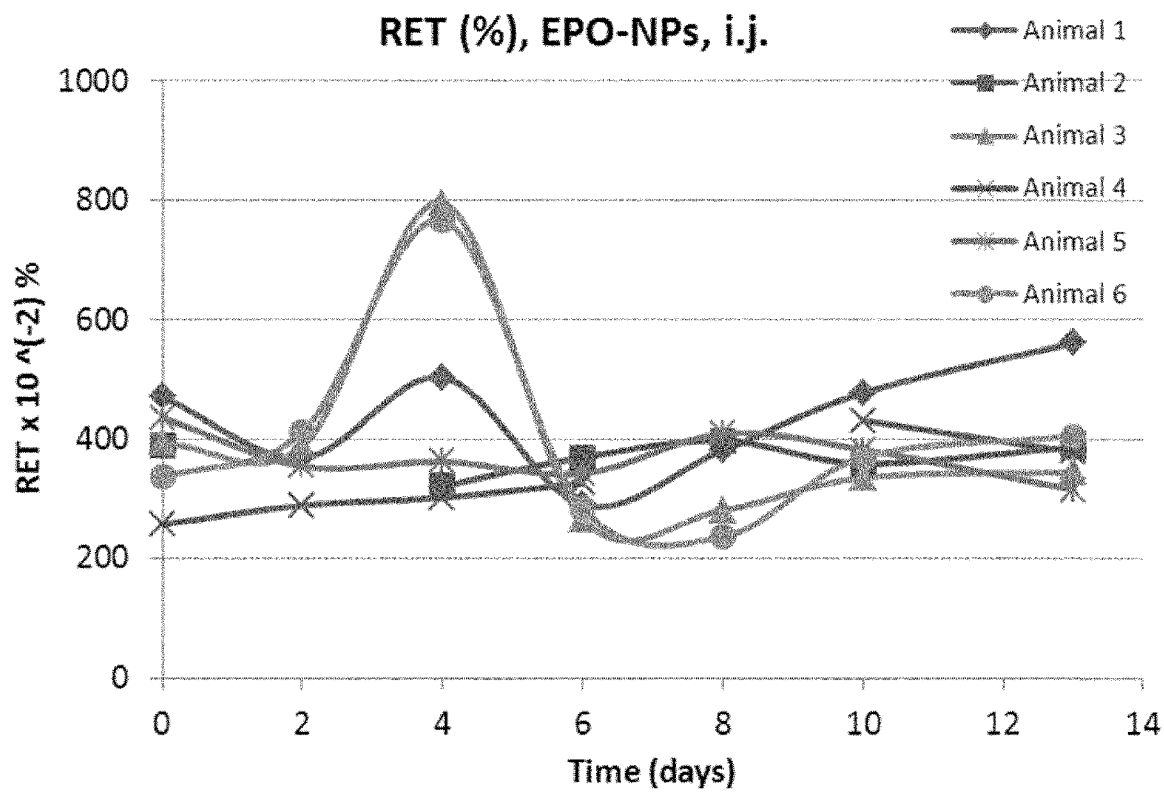
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*Figure 2 continued*

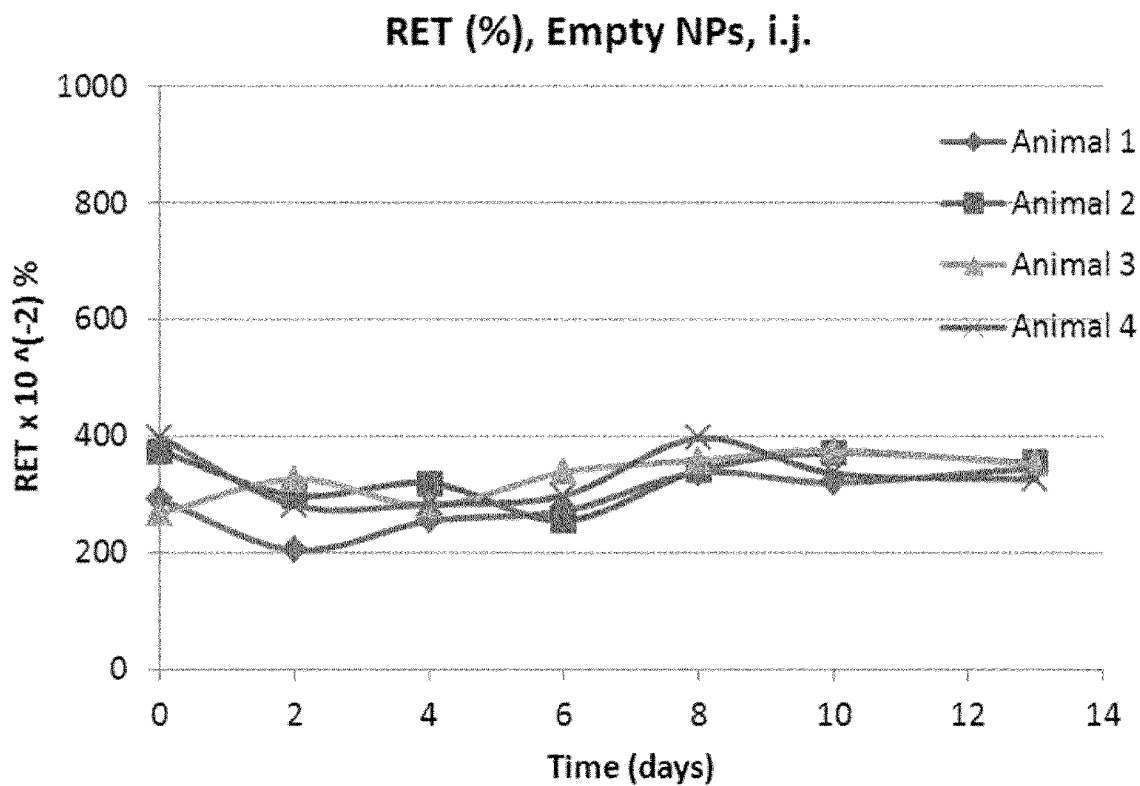
4/6

*Figure 2 continued*

5/6

*Figure 3*

6/6

*Figure 3 continued*

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/069183

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K9/00 A61K38/18 A61K47/48 A61K9/51
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	----- N. VENKATESAN ET AL: "Pharmacokinetic and pharmacodynamic studies following oral administration of erythropoietin mucoadhesive tablets to beagle dogs", INTERNATIONAL JOURNAL OF PHARMACEUTICS, vol. 310, no. 1-2, March 2006 (2006-03), pages 46-52, XP055093315, ISSN: 0378-5173, DOI: 10.1016/j.ijpharm.2005.11.014 the whole document ----- -/-	1,15-17



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

30 September 2014

Date of mailing of the international search report

09/10/2014

Name and mailing address of the ISA/

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Authorized officer

Palma, Vera

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/069183

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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Y	BONNIE M. GORZELLE ET AL: "Amphipols Can Support the Activity of a Membrane Enzyme", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 124, no. 39, October 2002 (2002-10), pages 11594-11595, XP055093761, ISSN: 0002-7863, DOI: 10.1021/ja027051b the whole document -----	2,3,5
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International application No

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