BIOTECH AUSTRALIA

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NOTICE OF ENTITLEMENT

We, BIOTECH AUSTRALIA PTY LIMITED, of 28 Barcoo Street, Roseville, New South Wales, 2069, Australia, the applicants in respect of an application for a patent for an invention entitled "Vitamin B_{12} Mediated Oral Delivery Systems for GCSF and EPO" filed under Application No. 67904/94, state the following:

The person nominated for the grant of the patent has, for the following reason, gained entitlement from the actual inventor(s):

The inventors made the invention for and on behalf of the nominated persons during the course of their normal employment.

The person nominated for the grant of the patent is the applicant of the provisional application(s) listed in the Declaration under Article 8 of the PCT.

Signed: ..... Date: 17 February 1998
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VITAMIN B-12 MEDIATED ORAL DELIVERY SYSTEMS FOR GCSF AND EPO

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(57) Claim

1. A complex between GCSF and a carrier comprising vitamin B_{12} or a vitamin B_{12} analogue wherein said GCSF and said carrier are covalently linked through a diradical spacer, said complex being capable of binding to intrinsic factor with high affinity with maintenance of GCSF bioactivity.

2. A complex according to claim 1 wherein said carrier is covalently linked to GCSF through: a disulphide bond with Cys-17 of GCSF; an amide linkage; an acyl hydrazide linkage; an imine linkage; or a hydrazone linkage.

3. A complex according to claim 1 , of the general formula:

$$V-X-A-Y-Z$$

wherein,

V is vitamin B_{12} or a vitamin B_{12} analogue, or derivative, bonded to X either through a carboxylate group pendant to the corrin nucleus of VB_{12} or through the central cobalt atom or to a functional group introduced onto the VB_{12} molecule,

X is selected from: -NHNH-, -NH-, -O-, S-, -SS- or $-CH_2-$, and

A is an optionally substituted, saturated or unsaturated, branched or linear, C_{1-50} alkylene, cycloalkylene or aromatic group, optionally with one or more carbons within the linear chain being replaced with N, O or S, and wherein the optional substituents

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are selected from carbonyl, carboxy, hydroxy, amino and other groups, and Y is the covalent linkage between A and Z where Y is selected from -NHCO-, -CONH-, -CONHNHCO-, -N=N-, -N=CH-, $-NHCH_2-$, $-NHN=CH-$, $-NHNHCH_2$, -SS-, $-SCH_2-$, $-CH_2S-$, -NHCRNH-, [R is O, S or NH_2], -COO-, -OCO-, and , Z is GCSF.

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(54) Title: VITAMIN B_{12} MEDIATED ORAL DELIVERY SYSTEMS FOR GCSF AND EPO

(57) Abstract

The invention describes complexes between VB_{12} analogues and either GCSF or EPO that retain both significant affinity for intrinsic factor (IF) in the VB_{12} portion of the complex and significant bioactivity of the GCSF or EPO portion of the complex. The invention also concerns a process for the synthesis of these complexes. This is achieved at least in part, by using a spacer compound, which is linked covalently between the VB_{12} portion and the GCSF or EPO. The complexes preferably have the formula V-X-A-Y-Z wherein V is vitamin B_{12} or a vitamin B_{12} analogue, or derivative, bonded to X either through a carboxylate group pendant to the corrin nucleus of VB_{12} or through the central cobalt atom or to a function group introduced onto the VB_{12} molecule, X is selected from: -NHNH-, -NH-, -O-, -S-, -SS- or $-CH_2-$, and A is an optionally substituted, saturated or unsaturated, branched or linear, $C_{1\text{-}50}$alkylene, cycloalkylene or aromatic group, optionally with one or more carbons within the linear chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups, and Y is the covalent linkage between A and Z where Y is selected from -NHCO-, -CONH-, -CONHNHCO-, -N=N-, -N=CH-, $-NHCH_2-$, -NHN=CH-, $-NHNHCH_2-$, -SS-, $-SCH_2-$, $-CH_2S-$, -NHCRNH-, -COO-, -OCO-, and R is O, S or NH_2, and Z is GCSF or EPO. The invention also describes reagents that can be used as probes for the detection of buried thiol groups of a protein or peptide, said reagent comprising a complex of either vitamin B_{12} (or an analogue thereof) or more generally of any instrumentally or visually detectable label, covalently linked to a diradical spacer, said spacer having a terminal reactive group capable of forming a disulphide bond with a free thiol in said protein or peptides.

VITAMIN B_{12} MEDIATED ORAL DELIVERY SYSTEMS FOR GCSF AND EPO

BACKGROUND OF THE INVENTION

The present invention relates to the oral delivery of the therapeutic substances granulocyte-colony stimulating factor (GCSF) and erythropoietin (EPO) by administration of a complex comprising these substances linked to vitamin B_{12} (VB_{12}) or an analogue thereof. More particularly, the invention relates to methods for the synthesis of these complexes and to methods for the amplification of the amount of GCSF or EPO delivered per VB_{12} carrier molecule.

An oral delivery system is known, because of recent work undertaken by one of the current inventors, which is described in PCT Application WO87/02251 (PCT/AU86/0299), whereby an active substance linked to at least one carrier molecule, which is VB_{12} or an analogue thereof, can use the natural VB_{12} uptake system mediated by the binding of VB_{12} to intrinsic factor (IF) to transport the resultant complex from the intestinal lumen into the circulation. Once delivered into serum or the lymphatic drainage system the complex substantially retains the bioactivity of the native active substance.

In common with virtually all proteins, peptides and other large bioactive molecules there is currently no method for the oral delivery of either GCSF or EPO. The oral route of administration is the most preferable means of delivering a pharmaceutically active agent, and as such there is a large and valuable market for any process which permits the oral delivery of either of these proteins to humans. Such a process would be available by the formation of a complex between VB_{12} and GCSF or EPO.

SUMMARY OF THE INVENTION

The present invention relates in one aspect to complexes between VB_{12} analogues and either GCSF or EPO that retain both significant affinity for intrinsic factor (IF) in the VB_{12}

portion of the complex and significant bioactivity of the GCSF or EPO portion of the complex. The invention also concerns a process for the synthesis of these complexes. This may be achieved at least in part, by using a spacer compound, which is linked covalantly between the VB_{12} portion and the GCSF or EPO.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the invention provides a complex which comprises at least one active substance linked to at least one carrier molecule, which is VB_{12} or an analogue of VB_{12} wherein the ability of the carrier to undergo the binding reactions necessary for uptake and transport of VB_{12} in a vertebrate host and the activity of the active substance are substantially maintained. This occurs by providing a complex between GCSF and a carrier selected from vitamin B_{12} or a vitamin B_{12} analogue wherein the GCSF and the carrier are covalently linked through a diradical spacer, the complex being capable of binding to intrinsic factor with high affinity with maintenance of GCSF bioactivity.

The complex preferably has the general form V–X–A–Y–Z, where V is the carrier molecule VB_{12} or an analogue (including derivatives) thereof that retains IF affinity, Z is the active substance selected from EPO or GCSF, A is a spacer arm of variable composition and length, X is the functional group through which V is attached to A, and Y is the functional group through which Z is attached to A. The nature of functional group X, its site of attachment to V and the nature of spacer arm A are chosen to maximise the IF affinity of the complex. The nature of functional group Y, its site of attachment to Z and the nature of spacer arm A are chosen to maintain substantially the bioactivity of Z.

Preferably X is selected from: -NHNH-, -NH-, -O-, S-, -SS- or $-CH_2-$. A is preferably an optionally substituted, saturated or unsaturated, branched or linear, C_{1-40} alkylene, cycloalkylene or aromatic group, optionally with one or more carbons within the linear chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups. Y is preferably a covalent linkage between spacer chain A and protein Z, where Y is selected from -NHCO-, -

CONH-, -CONHNHCO-, -N=N, -N=CH-, $NHCH_2$, -NHN=CH-, $-NHNHCH_2$, -SS-,-SCH_2-, $-CH_2S$-, -NHCRNH- [where R = O, S or NH_2], -COO-, -OCO-, and Z is GCSF or EPO.

In the context of the present invention, the term "active substance" (ie Z) includes all, part, an analogue, homologue, derivative or combination thereof of either granulocyte colony stimulating factor (GCSF) or erythropoietin (EPO).

The carrier is VB_{12} or VB_{12} analogue. The VB_{12} analogues include any variant or derivative of VB_{12} (cyanocobalamin) which possesses binding activity to intrinsic factor. Preferred analogues of VB_{12} also include aquocobalamin, adenosylcobalamin, methylcobalamin, hydroxycobalamin, cyanocobalamin, carbanalide, and 5-methoxybenzylcyanocobalamin ([(5-MeO)CN-Cbl] as well as the desdimethyl, monoethylamide and the methylamide analogues of all of the above. Other analogues include all alkyl cobalamins in which the alkyl chain is linked to the corrin nucleus by a direct CoC covalent bond. Other analogues include chlorocobalamin, sulfitocobalamin, nitrocobalamin, thiocyanatocobalamin, benzimidazolecyanocobalamin derivatives such as the: 5,6-dichlorobenzimidazole, 5-hydroxybenzimidazole, trimethylbenzimidazole, as well as adenosylcyanocobalamin [(Ade)CN-Cbl], cobalamin lactone, cobalamin lactam and the anilide, ethylamide, monocarboxylic and dicarboxylic acid derivatives of VB_{12} or its analogues.

Preferred derivatives of VB_{12} also include the mono-, di- and tricarboxylic acid derivatives or the propionamide derivatives of VB_{12}. Carriers may also include analogues of VB_{12} in which the cobalt is replaced by zinc or nickel. The corrin ring of VB_{12} or its analogues may also be substituted with any substituent which does not effect its binding to IF and such derivatives of VB_{12} or its analogues are part of this invention. Other derivatives of VB_{12} or its analogues which have a functional group which is able to react with the spacer compound are also part of the invention.

It is preferred that the complex may comprise GCSF linked through a disulphide bond to

a (dithiopyridyl propionamido) dodecylamino [DTP-dodecylamino] derivative of VB_{12}.

Another preferred embodiment of the invention provides a process for the production of a complex comprising GCSF linked through a disulphide bond to a (dithiopyridyl propionamido) dodecylsuberylhexylamino derivative of VB_{12}, (a long-chain analogue of the DTP-dodecylamino VB_{12}) which displays higher affinity for intrinsic factor.

Another preferred embodiment of the invention provides a process for the production of a complex comprising GCSF linked through a disulphide bond to an (dithiopyridyl propionamido) dodecylcarboxamidomethyl derivative of VB_{12}, in which the spacer is linked to the VB_{12} through an axial CoC bond.

Another embodiment of the invention provides a process for the production of a complex comprising at least one active substance linked to at least one carrier molecule through a spacer, the carrier molecule being VB_{12} or an analogue thereof, wherein the ability of the carrier to undergo the binding reactions necessary for uptake and transport of VB_{12} in a vertebrate host and the activity of the active substance are substantially maintained, the process comprising one or more of the following steps:

a) reacting together the active substance and the carrier and the spacer compound to form the complex;

b) reacting the active substance with the spacer, and then reacting the product with the carrier to form the complex;

c) reacting the carrier with the spacer, and then reacting the product with the active substance to form the complex;

d) following method of (a), (b) or (c) but with the additional step of having chemically modified the carrier and/or the active substance in a previous step to provide a functional group on the carrier and/or active substance which will react with the spacer compound; or

e) following the method of (a), (b), (c) or (d) but with the additional step of reacting the active substance or the carrier with a polymeric support, before carrying out the

further reactions. Preferably the polymeric support is bonded both to the carrier (or spacer-carrier), and also to the active substance or (spacer-active substance).

For example, it is possible to form these complexes using the "e" mono -acid of "e"VB_{12} by: (i) preparing the mono-acid derivative of VB_{12} by mild acid hydrolysis of cyanocobalamin and purifying the initial hydrolysate; (ii) modifying the e-mono-acid to give a terminal functional group attached to the eVB_{12} nucleus through a spacer arm; (iii) coupling the functionalised eVB_{12} derivative to carboxylate, amine, thiol, hydroxyl, phenol, aldehyde or ketone groups or other suitable functional groups present initially or introduced chemically on the active substance. The spacer compound can be selected to have suitable functional groups at either end of its backbone, or else these functional groups can be introduced, if necessary by normal chemical synthetic reactions.

The invention also involves the modification of a polymeric support to introduce functional groups capable of reacting either directly with the spacer compound or with the spacer linked with the active substance. The resulting polymer-active substance intermediate ideally contains many molecules of the active substance, and this intermediate is suitable for coupling to the carrier to give a complex capable of amplified delivery of the active substance.

The invention also concerns a general method for the modification of unreactive thiols in peptides and proteins, particularly those not normally exposed to reagents dissolved in aqueous solvents because they are buried in hydrophobic regions of the protein. These modified peptides and proteins can then be labelled, if desired.

The invention also involves a reagent , of the general formula:

$$V'-X-A-Y'$$

wherein, V' is vitamin B_{12} or a vitamin B_{12} analogue, bonded to X either through a carboxylate group pendant to the corrin nucleus of VB_{12} or through the central cobalt atom or to a functional group introduced onto the VB_{12} molecule, or V' is a label, where "label"

refers to any substance that is detectable by visual or instrumental methods. Labels can include chromogens, catalysts, fluorescent compounds, chemiluminescent compounds, radioactive isotopes, colloidal metal and non-metallic particles, dye particles, enzymes or substrates, antibodies or antigens, biotin, avidin or streptavidin, latex particles, liposomes or other vesicles containing signal producing substances, and the like.

X is selected from -NHCO-, -CONH-, -CONHNHCO-, -N=N-, -N=CH-, -$NHCH_2$-, -NHN=CH-, -$NHNHCH_2$, -SS-, -SCH_2-, CH_2S-, -NHCRNH-, [R is O, S or NH_2], -COO-, -OCO-, and A is an optionally substituted, saturated or unsaturated, branched or linear, C_{1-50} alkylene, cycloalkylene or aromatic group, optinally with one or more carbons within the chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups, and Y' is a functional group capable of reacting with thiols to give a stable covalent linkage, including iodoacetyl, bromoacetyl, chloroacetyl, maleimido, 3-carboxy-4-nitrophenyldithio or 2-pyridyldithio, and similar groups.

The complexes described herein may be formulated into pharmaceutical or veterinarially acceptable compositions utilising carriers and/or excipients as are well known in the art. Examples of suitable dosage forms which may be used in this invention are described for example in *Remington's Pharmaceutical Sciences (Mack Publishing Company, 10th Edition, which is incorported herein by reference)*. Compositions may be in the form of a capsule, tablet, slow-release dosage form, elixir, gel, paste, enterically coated dosage form, or any other suitable dosage form as is well known in the art.

Complexes and compositions according to this invention may be administered to a human or animal subject in need of treatment with GCSF or EPO. Modes of administration are not critical to this invention and include parenteral (intraveneous, intrasmuscular, or intraorgan injection), oral, transdermal, vaginal, anal, or other administration routes as are well known in the art. A therapeutically effective amount of a complex or compound according to this invention is that which provides treatment of a particular disease state.

What constitutes an effective amount will depend upon the nature of the disease being treated, the consulting physician or veterinary surgeon judgement, and other factors such as the age, weight and or sex of the subject. By way of example only, an effective amount of a complex composition according to the invention may comprise from one nanogram to 10 grams of a complex in accordance with the invention.

This invention also relates to the use of complexes described herein for the manufacture of medicament, and for the treatment of conditions responsive to GCSF or EPO.

BEST METHOD OF CARRYING OUT THE INVENTION

EXAMPLES

Materials: VB_{12} was obtained from Rousell-Uclaf. GCSF and EPO were obtained from Amgen. 1-Ethyl-3-(dimethylaminopropyl) carbodiimide.HCl (EDAC.HCl) was obtained from Biorad. N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and succinimidyl, 6-[3-(2-pyridyldithio) propionamido] hexanoate (LC-SPDP) were obtained from Pierce Chemical Co. All other reagents were obtained from Fluka.

Example 1: VB_{12} -GCSF complexes

Three classes of VB_{12}-GCSF complexes were prepared:

a) conjugated via an amide linkage, formed by carbodiimide (EDAC) mediated coupling of an amino terminal eVB_{12} derivative to the C-terminus of GCSF or the carboxylate side chains of GCSF.

b) conjugated via a disulphide linkage formed by a thiol insertion reaction of the free thiol at Cys-17 of GCSF into the disulphide bond present in the (dithiopyridylpropionamido) terminal derivatives of eVB_{12}.

c) conjugated via an acyl hydrazide linkage, formed by EDAC-mediated coupling of a hydrazido-terminal eVB_{12} derivative to the C-terminus of GCSF or the carboxylate side chains of GCSF.

1.1 Production and purification of the "e" isomer of monocarboxy-VB_{12}.

The "e" isomer of monocarboxy vitamin B_{12}, formerly named the d isomer, but reassigned by Anton and co-workers (1980: J. Am. Chem. Soc. 102:2215) as the e isomer, was separated from the b and d isomers formed during acid hydrolysis of cyanocobalamin by a combination of Dowex 1X2 chromatography and semi-preparative C-18 RP-HPLC developed with a gradient of acetonitrile in 0.1% TFA.

1.2 Production of amino-derivatives of eVB_{12}

A number of amino-derivatives of eVB_{12} were prepared by reacting the e isomer with:

i) 1,2-diaminoethane

ii) 1,6-diaminohexane

iii) 1,12-diaminododecane

iv) 1,3-diamino-2-hydroxypropane

v) 1,6-diamino-3,4-dithiahexane (a.k.a. cystamine)

All reactions were performed at pH 6.5 using a twenty fold molar excess of the diamine over e isomer and a twenty fold molar excess of EDAC. In a typical reaction 135 mg of eVB_{12} was dissolved in distilled water (6 ml) to which was added 1.2 ml of 1.0 M diamine, pH 6.5. Dry EDAC (270 mg) was then added and the reaction mixture was left overnight at room temperature.

All amino derivatives were purified by reverse phase chromatography on a semi-preparative C-4 column using a 5-100% acetonitrile gradient in 0.1% TFA. Eluted material was further purified by S-Sepharose chromatography. The amino-derivative was eluted with 0.1 M HCl, followed by extraction into phenol, and back-extraction into water after the addition of dichloromethane to the phenol phase. The amino-eVB_{12} derivatives were then recovered from the water phase by lyophilization.

1.3 Conjugation of 2-aminoethyl-eVB_{12} to GCSF

A solution of 2-aminoethyl-eVB_{12} (26.5 mg, 18 μmol) in 2 ml of GCSF (6 mg/ml, 0.63 μmol) was cooled to 4 C. An aliquot of freshly prepared EDAC solution (100 mg/ml, 120

μl, 63 μmol) was added. After 24 h at 4 C a second aliquot of freshly prepared EDAC solution was added. The reaction was allowed to proceed for a total of 48 h at 4 C, after which the unreacted 2-aminoethyl-eVB$_{12}$ was separated from the conjug e and aggregate by chromatography on Sephadex G-50 in 2.5% acetic acid.

1.4 Conjugation of cystaminyl-eVB$_{12}$ to GCSF

A solution of cystaminyl-eVB$_{12}$ hydrochloride (30 mg, 20 μmol) in 2.5 ml of GCSF (6 mg/ml, 0.80 μmol) at 4 C was treated with an aliquot of an aqueous EDAC solution (20 mg/ml, 75 μl, 8 μmol). Further aliquots of freshly prepared EDAC solution (20 mg/ml, 75 μl, 8 μmol) were added after 4.5 h, 7 h and 24 h. The reaction was allowed to proceed for a total of 48 h at 48 C, after which time the unreacted amino-eVB$_{12}$ was separated from the conjugate by chromatography on Sephadex G-50 in 2.5% acetic acid.

1.5 Preparation of 3-(2-pyridyldithio) propionamido derivatives of aminoethyl-eVB$_{12}$

The dithiopyridyl derivatives of amino-eVB$_{12}$ were prepared by reacting SPDP with:

i) 2-aminoethyl-eVB$_{12}$

ii) 6-aminohexyl-eVB$_{12}$

iii) 12-aminododecyl-eVB$_{12}$

In a typical reaction the terminal amino-eVB$_{12}$ was dissolved at 50 mg/ml in 0.1 M PO_4buffer, pH 7.5, containing 0.1 M NaCl. SPDP was dissolved at 50 mg/ml in acetone and 800 μl of the solution was added to the amino-eVB$_{12}$. After reaction overnight at room temperature the DTP-amino-eVB$_{12}$ was purified by RP-HPLC on a semi-prep C4 column, and then lyophilized.

1.6 Conjugation of DTP-amino-VB$_{12}$ to GCSF

In initial conversations with AMGEN it was revealed that they had found that it was impossible to modify the free cysteine in undenatured GCSF with standard thiol modifying agents. Initial experiments with DTP-aminoethyl-eVB$_{12}$ showed that it was possible to achieve some 20% substitution of GCSF with the VB$_{12}$ in the absence of guanidine; this

level rose to >80% in the presence of 4 M guanidine. It was therefore decided that it might be possible to access the free thiol with DTP-amino-eVB$_{12}$ in the absence of guanidine if a longer spacer was used for the conjugation.

In a second series of experiments GCSF was reacted with DTP-aminoethyl-, DTP-aminohexyl- and DTP aminododecyl-eVB$_{12}$ in the presence or absence of 4 M guanidine in 0.1 M sodium acetate buffer, pH 4.0.

The degree of substitution of GCSF by various DTP-amino-eVB$_{12}$ -spacer complexes is shown in the following table:

TABLE 1

Spacer	Guanidine	+ Guanidine
DTP-aminoethyl-	37.5%	89.3%
DTP-aminohexyl-	45.5%	95.2%
DTP-aminododecyl-	100.0%	100.0%

From Table 1 it can be seen that by switching to the longer dodecyl-spacer it was possible to conjugate to the buried thiol in GCSF without the use of guanidine.

The initial attempts to conjugate to the free thiol group in GCSF using the pyridyldithiopropionamido-aminoethyl derivative of eVB$_{12}$ resulted in a small degree of conjugation, of around 20-40 percent in the absence of guanidine. The addition of 4 M guanidine (final concentration) raised the conjugation efficiency to over 80%. Preparation of a longer, more hydrophobic derivative of eVB$_{12}$, pyridyldithiopropionamido-dodecyl eVB$_{12}$ resulted in 100% substitution of GCSF after 24 h at 4 C, without the need for the addition of guanidine. The use of the thiol interchange chemistry in this reaction proved advantageous as the eVB$_{12}$ conjugation was surprisingly successful at pH's which minimised the extent to which GCSF undergoes spontaneous aggregation. Chromatography of the conjugated material resulted in base-line separation of conjugate from free eVB$_{12}$.

This type of approach can be more generally applied for the development of reagents capable of detecting, quantifying and/or modifying thiol groups in proteins and peptides which were

hitherto regarded as chemically unreactive.

1.7 Scale up conjugation of DTP-aminododecyl-eVB$_{12}$ to GCSF

Following the initial success with the conjugation of the DTP-dodecyl-spacer, the reaction was scaled up as follows:

To 2.5 ml of GCSF (6 mg/ml; 15 mg) was added 1.6 ml of DTP-aminododecyl-eVB$_{12}$ (10 mg/ml in 2.5% acetic acid). The reaction was allowed to proceed for 48 h at 4 C, after which the unreacted eVB$_{12}$ was separated from the conjugate by chromatography on Sephadex G-25 in 2.5% acetic acid. Fractions containing GCSF were pooled, concentrated in an AMICON positive pressure cell using a YM 10 membrane and dialysed for 72 h against sterile distilled water.

1.8 Preparation of DTP-dodecylsuberlhexyl-eVB$_{12}$ reagent.

Although the DTP-dodecyl-eVB$_{12}$ reagent reacts efficiently with GCSF to give a stable, well characterised complex, the material had a low IF affinity (~2-3% of native eVB$_{12}$). A complex with increased IF affinity was prepared by synthesising an extended spacer analogue of the DTP-dodecylamino-eVB$_{12}$. The synthesis of this analogue uses the same SPDP chemistry to conjugate through the cysteine of GCSF, however a longer spacer arm is attached to the eVB$_{12}$. As anticipated, this resulted in the formation of a conjugate whose IF affinity is significantly greater than that of the conjugate prepared by the reaction of GCSF with DTP-dodecyl-eVB$_{12}$ (see Table 2).

This spacer was prepared by sequential reaction of the e-carboxylate of eVB$_{12}$ with:

i) 1,6-diaminohexane and EDAC (to give 6-aminohexyl eVB_{12}).

ii) disuccinimidylsuberate (DSS) (to give monosuccinimidylsuberylhexyl-eVB_{12}).

iii) 1,12-diaminododecane (to give 12-aminododecylsuberylhexyl-eVB_{12}).

That is:

$eVB_{12}CO_2H \rightarrow eVB_{12}CONH(CH)_6NH2 \rightarrow eVB_{12}CONH(CH_2)_6NHCO(CH_2)COOSu$

$\rightarrow eVB_{12}CONH(CH_2)_6NHCO(CH_2)_6CONH(CH_2)_{12}NH_2$

The resultant spacer, which is more than twice the length of aminododecyl-eVB_{12}, was derivatized at the terminal amino group with SPDP and coupled to GCSF by means of the protocol dsecribed in the following section.

1.9 Conjugation of DTP-dodecylsuberylhexyl-eVB_{12} to GCSF

A solution of the DTP-dodecylsuberlhexyl-eVB_{12} (9 mg, 7 μmol) was taken up in acetic acid (100 μl) and diluted to 1 ml with water. This solution was added to 5 ml of GCSF solution (4 mg/ml, 0.5 μmol) cooled to 4 C. The reaction mixture was left for 144 h at 4 C, then worked up using the standard protocol.

1.10 Production of hydrazide derivatives of eVB_{12} carboxylate

Two hydrazide derivatives of eVB_{12} carboxylate were prepared for conjugation to carboxyl groups of GCSF by reaction with EDAC. The two hydrazide derivatives used, and their (shorthand) chemical structure, are:

a) hydrazido-eVB_{12} (= eVB_{12}-$CONHNH_2$)

(b) adipyl-hydrazido-eVB_{12} (= eVB_{12}-$CONHNHCO(CH_2)_4CONHNH_2$)

1.10i Hydrazido-eVB_{12}

This reagent was prepared by a two step synthesis involving the coupling of tert butyl carbazate to eVB_{12} carboxylate and subsequent removal of the tBoc group to generate the

free hydrazide.

$$eVB_{12}CO_2H \xrightarrow[\text{EDAC}]{H_2NNHCO_2{}^tBu} eVB_{12}CONHNHCO_2{}^tBu \xrightarrow{10\%\ TFA/H_2O} eVB_{12}CONHNH_2$$

1.10ii Adipyl-hydrazido-eVB_{12}

This reagent was readily prepared in one step from eVB_{12} carboxylate by the addition of EDAC to a mixture of the acid and a 20-fold excess of adipylhydrazide.

$$eVB_{12}CO_2H \xrightarrow[\text{EDAC}]{\text{20eq. adipyl hydrazide}} eVB_{12}CONHNHCO(CH_2)_4CONHNH_2$$

EDAC-mediated coupling of hydrazido-eVB_{12} analogues to the carboxylate side-chains of GCSF proceeded more readily, and required significantly lower amounts of eVB_{12} derivative and EDAC, than the corresponding conjugations of amino eVB_{12} derivatives to GCSF. This is readily explainable in terms of the relative basicity of hydrazides (pKa 2.6) in comparison with amines (pKa 8-9). Thus at the pH at which the GCSF coupling takes place (4-5) a hydrazido eVB_{12} derivative will be primarily in the reactive, non-protonated form while an amino-eVB_{12} derivative will be primarily in the non-reactive, protonated form.

The coupling reaction used is:

$$eVB_{12}\text{-[spacer]-}CONHNH_2 + HO_2C\text{-}GCSF \xrightarrow{EDAC} eVB_{12}\text{-[spacer]-CONHNHCO-GCSF}$$

1.11 Conjugation of hydrazido-eVB_{12} to GCSF

A solution of hydrazido eVB_{12} (8.9 mg, 6.5 μmol) in 5 ml of GCSF solution (4 mg/ml, 1.05 μmol) was cooled to 4 C and an aliquot of EDAC solution (50 mg/ml, 40 μl, 10 μmol) was added. After 5 h an identical aliquot of fresh EDAC solution was added and the reaction mixture was left overnight at 4 C. Conjugate was removed from unreacted eVB_{12} and other reagents by chromatography on Sephadex G-50 in 2.5% acetic acid.

1.12 Conjugation of adipylhydrazido-eVB_{12} to GCSF

A solution of adipylhydrazido eVB_{12} (10 mg, 6.6 μmol) in 3 ml of GCSF solution (4 mg/ml, 0.64 μmol) was cooled to 4 C and an aliquot of EDAC solution (50 mg/ml, 40 μl, 10 μmol) was added. After 5 h a 20 μl aliquot of fresh EDAC solution (50 mg/ml, 20 μml, 5 μmol) was added and the reaction mixture was left overnight at 4 C. Conjugate was removed from unreacted eVB_{12} and other reagents by chromatography on Sephadex G-50 in 2.5% acetic acid.

1.13 Preparation of poly-GCSF-HPMA-eVB_{12} complex.

Two N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers can be synthesized as polymer backbones for the incorporation and derivatization with GCSF and eVB_{12}. A non-biodegradable polymer backbone (HPMA-GG) can be synthesized by the free radical copolymerization of HPMA with N-methacryloylglycylglycine p-nitrophenyl ester. A biodegradable polymer (HPMA-FALG) can be synthesized by the free radical copolymerization of HPMA with N-methacryloylglycylphenylalanylleucylglycine p-nitrophenol ester by the method of Rejmanova and coworkers [Rejmanova, P, Obereigner, B and Kopecek, J, 1981 Makromol Chem 182:1899-1915]. In order to incorporate GCSF and eVB_{12} onto the polymers, the polymers are initially reacted with a ten molar excess of a mixture of aminododecyl-eVB_{12} and dithiopyridyldodecylsuberyl-hexylamine (1:10 mole:mole) overnight. Unreacted nitrophenyl esters are subjected to aminolysis by the addition of 1-amino-2-propanol. The modified polymers are purified by chromatography on Sepharose 6B. A solution of the dithiopyridyldodecylsuberylhexyl modified eVB_{12}-substituted polymers is dissolved in 2.5% acetic acid and reacted with GCSF. The reaction mixture is left for 144 h at 4 C, after which the GCSFeVB_{12}-substituted polymers can be

purified by chromatography on Sepharose 6B.

Part 2: eVB_{12}-EPO complexes

Three classes of eVB_{12}-EPO complexes were prepared:

(a) Conjugated via an amide linkage, formed by EDAC-mediated coupling of an amino eVB_{12} derivative to the C-terminus of EPO, the carboxylate side chains of the Asp/Glu residues of EPO or the carboxylate groups of the sialic acid residues of the carbohydrate portion of EPO.

(b) Conjugated via an acyl hydrazide linkage, formed by EDAC-mediated coupling of a hydrazido-eVB_{12} derivative to the carboxylate side chains of the Asp/Glu residues of EPO or the carboxylate groups of the sialic acid residues of the carbohydrate portion of EPO.

(c) Conjugated via a hydrazone linkage between a hydrazido-eVB_{12} derivative and an aldehyde group generated by periodate oxidation of the carbohydrate residues of EPO.

2.1 Conjugation of 2-aminoethyl-eVB_{12} to EPO

A mixture of 2-aminoethyl-eVB_{12} (8 mg, 5.7 μmol) and EPO (27 mg/ml, 200 μl, 0.18 μmol) was cooled to 4 C and an aliquot of EDAC solution (10 mg/ml, 100 μl, 5 μmol) was added. The reaction mixture was left for 64 h at 4 C and finally purified by size-exclusion chromatography on a Superdex-75 column. Elution with a buffer consisting of Tris (pH 7.5, 10 mM; NaCl, 100 mM) afforded the purified EPO-eVB_{12} complex.

2.2 Conjugation of cystaminyl-eVB_{12} to EPO

A mixture of 6-amino-3,4-dithiahexylamide-eVB_{12} (12 mg, 8.1 μmol) and EPO (13.5 mg/ml, 500 μl, 0.23 μmol) was cooled to 4 C and an aliquot of EDAC solution (10 mg/ml, 250 μl, 13 μmol) was added. The reaction mixture was left for 48 h at 4 C and was finally purified by size-exclusion chromatography on a Sephadex G-75 column. Elution with a buffer consisting of Tris (pH 7.5, 10 mM)/NaCl (100 mM) afforded the purified EPO-eVB_{12} complex .

2.3 EDAC-mediated conjugation of hydrazido-eVB_{12} to EPO

A solution of hydrazido VB_{12} (10 mg, 7.3 μmol) in 7 ml of EPO solution (2.6 mg/ml, 0.6 μmol) was cooled to 4 C and an aliquot of EDAC solution (20 mg/ml, 50 μl, 5 μmol) was added. After 5 h a second aliquot of fresh EDAC solution (10 mg/ml, 25 μl, 1.3 μmol) was added and the reaction mixture was left overnight at 4 C. The conjugate was purified by size-exclusion chromatography on a G-50 column. Elution with a buffer consisting of Tris (pH 7.5, 10 mM; NaCl, 100 mM) afforded the purified EPO-eVB_{12} complex .

2.4 EDAC-mediated conjugation of adipyl-hydrazido-eVB_{12} to EPO

A solution of adipyl-hydrazido-eVB_{12} (11 mg, 7.3 μmol) in 4 ml of EPO solution (2.6 mg/ml, 0.35 μmol) was cooled to 4 C and an aliquot of EDAC solution (10 mg/ml, 100 μl, 5 μmol) was added. The reaction mixture was left overnight at 4 C. The conjugate was purified by size-exclusion chromatography on a G-50 column. Elution with a buffer consisting of Tris (pH 7.5, 10 mM)/NaCl (100 mM) afforded the purified EPO-eVB_{12} complex .

2.5 Periodate mediated conjugation of hydrazido-eVB_{12} to EPO

An aqueous solution of EPO (6.4 mg/ml, 1.56 ml, 0.33 μmol) was cooled to 4 C and freshly prepared sodium periodate solution (25 mM, 360 μl) was added. The solution was left to stir gently for fifteen minutes at 4 C and the excess periodate was quenched by the addition of ethylene glycol (5 μl). The solution was dialyzed overnight against 3 l of pH 5.6, 10 mM NaOAc buffer. A solution of hydrazido-eVB_{12} (7.5 mg, 5 μmol) in distilled water (200 μl) was added to the oxidised EPO solution and the reaction mixture was left for 7 h at 4 C. The conjugate was separated from unreacted hydrazide by SEC on G-75 Sephadex. Elution with a buffer consisting of Tris (pH 7.5/10 mMl)/NaCl (100 mM) afforded the purified EPO-eVB_{12} complex .

2.6 Preparation of poly-EPO-HPMA-eVB_{12}

Two N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers can be synthesized as polymer backbones for the incorporation and derivatization with EPO and VB_{12}. A

nonbiodegradable polymer backbone (HPMA-GG) is synthesized by the free radical copolymerization of HPMA with N-methacryloylglycylglycine p-nitropheny ester. A biodegradable polymer (HPMA-GFLAG) is synthesized by the free radical copolymerization of HPMA with N-methacryloylglycyl-phenylalanylleucylglycine p-nitrophenol ester by the method of Rejmanova and coworkers [Rejmanova, P, Obereigner, B and Kopecek J, 1981 Makromol Chem 182:1899-1915]. In order to incorporate EPO and eVB_{12} onto the polymers, the polymers are reacted with a ten molar excess of a mixture of aminododecyl-eVB_{12} and 6-(3-acylhydrazidylpropionamido)-1-amino-3,4-dithiahexane (1:10 mole:mole) overnight. Unreacted nitrophenyl esters are subjected to aminolysis by the addition of 1-amino-2-propanol. The modified polymers are purified by chromatography on Sepharose 6B. EPO is covalently linked to the 6-(3-acylhydrazidylpropionamido)-1-amino-3,4-dithiahexane modified eVB_{12}-substituted polymers by the addition of EDAC. The reaction mixture is left for 18 h at 4 C, afterwhich the EPO-eVB_{12}-substituted polymers are purified by chromatography on Sepharose 6B.

Example 3:

The diradical spacer may be selected with a suitable length and functionality to optimize the intrinsic factor (IF) affinity of the resulting GCSF or EPO eVB_{12} complex. As examples of this, the following table shows the result of altering the length and functionality of the spacer for various complexes and VB_{12} derivatives.

The IF affinity of the various eVB_{12} analogues and complexes referred to in the Examples are shown in Table 2. In particular, there is significant increase in IF affinity between the GCSF-dithiopropionamido[12-aminododecylamido]-eVB_{12} (preparated in section 1.7) and the GCSF- dithiopropionamido[12-aminododecyl-subeyl-hexylamido]-eVB_{12} (prepared in section 1.9). This increase in IF affinity presumably results from the increase in length of the spacer arm in the latter complex.

Table 2

Relative Intrinsic Factor affinity of VB_{12} analogues and complexes

eVB_{12} Analogue or complex	IF Affinity
eVB_{12}carboxylate	35%
2-aminoethylamido-eVB_{12}	48%
6-aminohexylamido-eVB_{12}	91%
12-aminododecylamido-eVB_{12}	74%
12-aminododecyl-suberyl-hexylamido-eVB_{12}	82%
dithiopropionamido[2-aminoethylamido]-eVB_{12}	6%
dithiopropionamido[6-aminohexylamido]-eVB_{12}	7%
dithiopropionamido[6-aminohexylamido]-eVB_{12}	11%
GCSF-dithiopropionamido[12-aminododecylamido]-eVB12	3%
GCSF-diothiopropionamido12-aminododecyl-suberyl-hexylamido-eVB_{12}	28%
hydrazidyl-eVB_{12}	100%
adipyldihydrazidyl-eVB_{12}	44%
GCSF-acylhydrazidyl-eVB_{12}	10%
GCSF-acyl[adipyldihydrazidyl]-eVB_{12}	7%
EPO-acylhydrazidyl-eVB_{12}	6%
EPO-acyl[adipyldihydrazidyl]-eVB_{12}	11%

IF Affinity is expressed as a percentage of the affinity of the eVB_{12} derivative relative to native, unmodified VB_{12}.

THE CLAIMS:

1. A complex between GCSF and a carrier comprising vitamin B_{12} or a vitamin B_{12} analogue wherein said GCSF and said carrier are covalently linked through a diradical spacer, said complex being capable of binding to intrinsic factor with high affinity with maintenance of GCSF bioactivity.

2. A complex according to claim 1 wherein said carrier is covalently linked to GCSF through: a disulphide bond with Cys-17 of GCSF; an amide linkage; an acyl hydrazide linkage; an imine linkage; or a hydrazone linkage.

3. A complex according to claim 1 , of the general formula:

$$V-X-A-Y-Z$$

wherein,

V is vitamin B_{12} or a vitamin B_{12} analogue, or derivative, bonded to X either through a carboxylate group pendant to the corrin nucleus of VB_{12} or through the central cobalt atom or to a functional group introduced onto the VB_{12} molecule,

X is selected from: -NHNH-, -NH-, -O-, S-, -SS- or $-CH_2-$, and

A is an optionally substituted, saturated or unsaturated, branched or linear, C_{1-50} alkylene, cycloalkylene or aromatic group, optionally with one or more carbons within the linear chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups, and

Y is the covalent linkage between A and Z where Y is selected from -NHCO-,

-CONH-, -CONHNHCO-, -N=N-, -N=CH-, $-NHCH_2-$, -NHN=CH-, $-NHNHCH_2$, -SS-, $-SCH_2-$, $-CH_2S-$, -NHCRNH-, [R is O, S or NH_2], -COO-, -OCO-, and , Z is GCSF.

4. A complex according to claim 1 wherein said vitamin B_{12} analogue is selected from cyano-cobalamin (CN-Cbl), aquocobalamin, adenosylcobalamin, methylcobalamin,

hydroxy-cobalamin, (5-methoxybenzyl)cyanocobalamin [(5-MeOF -N-Cbl]; the desdimethyl, monoethylamide and the methylamide analogues of these; alkyl cobalamins in which the alkyl chain is linked to the corrin nucleus by a direct Co C covalent bond; chlorocobalamin, sulfitocobalamin, nitrocobalamin, thiocyanatocobalamin, benzimidazole derivatives including 5,6-dichlorobenzimidazole, 5-hydroxybenzimidazole, trimethylbenzimidazole; adenosylcyanocobalamin [(Ade)CN-Cbl], cobalamin lactone, cobalamin lactam; the anilide, ethylamide, monocarboxylic and dicarboxylic acid derivatives of VB_{12} or its analogues; the mono-, di- and tricarboxylic acid derivatives of VB_{12}; and analogues of VB_{12} in which the cobalt is replaced by zinc or nickel.

5. A complex according to claim 1 wherein said vitamin B_{12} analogue is an analogue where the corrin ring is substituted with a substituent which does not affect binding to intrinsic factor.

6. A complex according to claim 1 wherein said GCSF is covalently bound to a pharmaceutically acceptable polymer.

7. The complex according to claim 6 wherein the polymer is selected from dextran, inulin, cellulose, starch and derivatives thereof, chondroitan sulfate, poly[N-α-(2-hydroxypropyl)-methacrylamide] and derivatives thereof, styrene-maleic anhydride copolymer, divinylether-maleic anhydride copolymer, polylysine, poly(glutamic acid), poly(hydroxypropyl glutamine), poly(lactic acid), water soluble polyurethanes formed by covalent linkage of PEG with lysine or other amino acids and branched chain polypeptides.

8. A complex according to claim 6 wherein said pharmaceutically acceptable polymer is biodegradeable within the human or animal body.

9. A process for the production of a complex between GCSF and a carrier selected from

vitamin B_{12} or a vitamin B_{12} analogue which comprises: reacting one or both of said carrier and GCSF with a diradical spacer having terminal reactive groups to form a carrier/linker and/or GCSF linker intermediate, and thereafter reacting together the respective components to give a complex between GCSF and said carrier wherein the components are covalently linked through said spacer.

10. A process according to claim 9 wherein GCSF and/or said carrier are modified prior to complex formation to provide at least one functional group capable of forming a chemical linkage.

11. A process according to claim 9 wherein said carrier is covalently linked to GCSF through a disulphide linkage with Cys-17 of GCSF, an amide linkage, an acyl hydrazide, an imine linkage, or a hydrazone linkage.

12. A complex between EPO and a carrier comprising vitamin B_{12} or a vitamin B_{12} analogue wherein said EPO and said carrier are covalently linked through a diradical spacer, said complex being capable of binding to intrinsic factor with high affinity with maintenance of EPO bioactivity.

13. A complex according to claim 12 wherein said carrier is covalently linked to EPO through: an amide linkage; an acyl hydrazide linkage; an imine linkage; or a hydrazone linkage.

14. A complex according to claim 12 of the formula:

$$V-X-A-Y-Z$$

wherein,

V is vitamin B_{12} or a vitamin B_{12} analogue, or deriviative, bonded to X either through a carboxylate group pendant to the corrin nucleus of VB_{12} or through the central cobalt atom or to a functional group introduced onto the VB_{12} molecule,

X is selected from: -NHNH-, -NH-, -O-, S-, -SS- or $-CH_2-$, and A is an optionally

substituted, saturated or unsaturated, branched or linear, C_{1-50} alkylene, cycloalkylene or aromatic group, optionally with one or more carbons within the linear chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups, and

Y is the covalent linkage between A and Z where Y is selected from -NHCO-, -CONH-, -CONHNHCO-, -N=N-, -N=CH-, $-NHCH_2-$, -NHN=CH-, $-NHNHCH_2$, -SS-, $-SCH_2-$, $-CH_2S-$, -NHCRNH-, [R is O, S or NH_2], -COO-, -OCO-, and Z is EPO.

15. A complex according to claim 12 wherein said vitamin B_{12} analogue is selected from cyano-cobalamin (CN-Cbl), aquocobalamin, adenosylcobalamin, methylcobalamin, hydroxy-cobalamin, 5-methoxybenzyl(cyano)cobalamin [(5-MeOBz)CN-Cbl]; the desdimethyl, monoethylamide and the methylamide analogues of these; alkyl cobalamins in which the alkyl chain is linked to the corrin nucleus by a direct Co C covalent bond; chlorocobalamin, sulfitocobalamin, nitrocobalamin, thiocyanatocobalamin, benzimidazole(cyano)cobalamin derivatives including 5,6-dichlorobenzimidazole, 5-hydroxybenzimidazole, trimethylbenzimidazole; adenosylcyanocobalamin [(Ade)CN-Cbl], cobalamin lactone, cobalamin lactam; the anilide, ethylamide, monocarboxylic and dicarboxylic acid derivatives of VB_{12} or its analogues; the mono-, di- and tricarboxylic acid derivatives or the propionamide derivatives of eVB_{12}; and analogues of VB_{12} in which the cobalt is replaced by zinc or nickel.

16. A complex according to claim 12 wherein said vitamin B_{12} analogue is an analogue where the corrin ring is substituted with a substituent which does not affect binding to intrinsic factor.

17. A complex according to claim 12 wherein said EPO is covalently bound to a pharmaceutically acceptable polymer.

AMENDED CLAIMS
[received by the International Bureau on 7 November 1994 (07.11.94); original claims 23-27 amended; remaining claims unchanged (2 pages)]

18. The complex according to claim 17 wherein the polymer selected from dextran, inulin, cellulose, starch and derivatives thereof, chondroitan sulfate, poly[N-α-(2-hydroxypropyl)-methacrylamide] and derivatives thereof, styrene-maleic anhydride copolymer, divinylether-maleic anhydride copolymer, polylysine, poly(glutamic acid), poly(hydroxypropyl glutamine), poly(lactic acid), water soluble polyurethanes formed by covalent linkage of PEG with lysine or other amino acids and branched chain polypeptides.

19. A complex according to claim 17 wherein said pharmaceutically acceptable polymer is biodegradeable within the human or animal body.

20. A process for the production of a complex between EPO and a carrier selected from vitamin B_{12} or a vitamin B_{12} analogue which comprises: reacting one or both of said carrier and EPO with a diaradical spacer having terminal reactive groups to form a carrier/linker and/or EPO linker intermediate, and thereafter reacting together the respective components to give a complex between EPO and said carrier wherein the components are covalently linked through a said spacer.

21. A process according to claim 20 wherein EPO and/or said carrier are modified prior to derivatization to provide at least one functional group capable of forming a chemical linkage.

22. A process according to claim 20 wherein said carrier is covalently linked to EPO through: an amide linkage, an acyl hydrazide, an imine linkage, or a hydrazone linkage.

23. A method for the detection of buried thiol groups of a protein or peptide, said method comprising the use of a reagent such as a complex of either vitamin B_{12} (or an analogue thereof) or more generally of a label detectable by visual or instrumental methods which is covalently linked to a diradical spacer, said spacer having a terminal reactive group capable of forming a

covalent bond with a free thiol in said protein or peptides.

24. The method according to claim 23, wherein said spacer has a backbone of from 1-50 atoms.

25. The method according to claim 23 wherein said reagent is of the general formula:

V'—X—A—Y'

wherein,

V' is vitamin B_{12} or a vitamin B_{12} analogue, bonded to X either through a carboxylate group pendant to the corrin nucleus of VB_{12} or through the central cobalt atom or to a functional group introduced onto the VB_{12} molecule, or V' is a label detectable by visual or instrumental methods, and

X is selected from: -NHCO-, -CONH-, -CONHNHCO-, -N=N-, -N=CH-, $-NHCH_2-$, -NHN=CH-, $-NHNHCH_2-$, -SS-, $-SCH_2-$, $-CH_2S-$, -NHCRNH-, [R is O, S or NH_2], -COO-, -OCO-, and

A is an optionally substituted, saturated or unsaturated, branched or linear, C_{1-50} alkylene, cycloalkylene or aromatic group, optionally with one or more carbons within the chain being replaced with N, O or S, and wherein the optional substituents are selected from carbonyl, carboxy, hydroxy, amino and other groups, and

Y' is a functional group capable of reacting with thiols to give a stable covalent linkage.

26. The method according to claim 25 wherein Y' is selected from iodoacetyl, bromoacetyl, chloroacetyl, maleimido, 3-carboxy-4-nitrophenyldithio or 2-pyrididyldithio.

27. The method according to claim 23 wherein said label is selected from chromagens, catalysts, fluorescent compounds, chemiluminescent compounds, radioactive isotopes, colloidal metal and non-metallic particles, dye particles, enzymes or substrates, antibodies or antigens, biotin, avidin or streptavidin, latex particles, liposomes or other vesicles containing signal producing substances.

28. A pharmaceutical composition which comprises a complex according to claims 1 to 8 or 12 to 19 optionally in association with a pharmaceutically acceptable carrier or excipient.

29. A method for treating disease in a patient responsive to therapy with GCSF or EPO which comprises administering to said patient a therapetucally effective amount of a complex according to any one of claims 1 to 8 or 12 to 19, or a composition according to claim 28.

30. Use of a complex according to any one of claims 1 to 8 or 12 through 19 for the manufcture of a medicament

31. Use of a complex according to any one of claims 1 to 8 or 12 through 19 for the manufacture of a medicament.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 94/00274

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[5] A61K 31/68, 37/02, 47/48

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 37/02: GRANULOCYTE COLONY STIMULATING FACTOR OR GCSF ERYTHROPOIETIN OR EPO

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)
WPAT, CASM
JAPIO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	WO 87/02251 (BIOTECHNOLOGY AUSTRALIA PTY LTD) 23 April 1987 (23.04.87)	23
X A	DE,A, 2546476 (ISRAEL, MURRAY) 21 April 1977 (21.04.77)	23 1-22, 24-43
Y	WO 93/25221 (ALKERMES CONTROLLED THERAPEUTICS, INC) 23 December 1993 (23.12.93)	17, 18
Y	US 5206219 (APPLIED ANALYTICAL INDUSTRIES, INC) 27 April 1993 (24.04.93)	17, 18

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 August 1994 (23.08.94)	6 Sept 1994 (06.09.94)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer J. HANSON Telephone No. (06) 2832263

Form PCT/ISA/210 (continuation of first sheet (2)) (July 1992) cophin

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	8702251	AU	65289/86	CA	1330791	EP	220030
DE	2546464	DE	2546474				
WO	9325221	AU	46308/93				
US	5206219	US	5206219				

END OF ANNEX