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(54) Title: SOLUBLE POLYPEPTIDE

(57) Abstract: A polypeptide comprising an N-terminal truncated form of 2-OG dependent oxygenase, which retains hydroxylase activity.



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SOLUBLE POLYPEPTIDE

Field of the Invention

The present invention relates to 2-oxoglutarate oxygenases, and in particular,
5 to HIF prolyl-hydroxylases, in soluble and active form. The hydroxylases are useful
in assays to identify inhibitors and in structural studies.

Background to the Invention

In animals, cellular and physiological responses to reduced oxygen
10 concentration (hypoxia) are mediated by an α/β -heterodimeric transcription factor,
hypoxia-inducible factor (HIF). Under hypoxic conditions, HIF binds to response
elements linked to an array of genes associated with the hypoxic response. In humans
there are probably several hundred of these genes including those encoding for
proteins associated with metabolism (glycolysis), angiogenesis (vascular endothelial
15 growth factor, VEGF) and erythropoiesis (erythropoietin). The concentration of the
HIF- β subunit is independent of oxygen levels, whilst levels of HIF- α are low under
normal oxygen concentrations (normoxia), but rise in response to hypoxia.

Recent studies have identified the mechanisms by which the hypoxic
response is regulated by the availability of molecular oxygen. Post-translational
20 hydroxylation of either of two conserved proline residues in HIF- α , present in
independent oxygen dependent degradation domains, is sufficient to promote binding
of HIF- α to the von Hippel Lindau protein (pVHL) (Jaakkola *et al.*, Science 2001,
292, 468; Ivan *et al.*, Science 2001, 292, 464). The latter acts as a targeting protein
for a ubiquitin E3 ligase complex that enables proteasome mediated destruction of
25 HIF- α . The absolute dependence of HIF- α prolyl-4-hydroxylation on molecular
oxygen provides a direct link between dioxygen availability and levels of HIF- α
(Bruick and McKnight, Science 2001, 294, 1337; Epstein *et al.*, Cell 2001, 107, 43;
McNeil *et al.*, Bioorganic & Medicinal Chem. Letters 2002, 12, 1547). In a separate
oxygen dependent mechanism, the transcriptional activity of human HIF is even
30 more directly inhibited by hydroxylation at the *pro-S* β -position of Asn-803 in the C-
terminal transactivation domain (CAD) of HIF- α .

There are three human HIF- α (1-3) isoforms; all of which have been shown to
be substrates for one or more of the HIF hydroxylase, although HIF-3 α does not

contain a conserved asparaginyl residue in its CAD. The hydroxylases comprise three prolyl-4-hydroxylases (PHD1-3) (Bruick and McKnight *supra*, Epstein *et al. supra* and Ivan *et al.*, PNAS 2002, 99, 13459) and an asparaginyl hydroxylase, factor inhibiting HIF (FIH) (Hewitson *et al.*, J. Biol. Chem. 2002, 277, 26351; Lando *et al.*, 5 Genes & Development 2002, 16, 1466; Mahon *et al.*, Genes & Development 2001, 15, 2675). Inhibition of the HIF hydroxylases is of current interest from the perspective of upregulating hypoxically driven transcripts for the treatment of ischemia and anaemia. Sequence analyses coupled to crystallographic data for FIH, indicate that all of the HIF hydroxylases belong to the family of ferrous iron and 2-oxoglutarate (2-OG) dependent oxygenases. Spectroscopic and crystallographic 10 analyses have revealed that many of these enzymes, including FIH, bind their iron cofactor *via* a conserved 2-histidinyl-1-aspartatyl/glutamatyl triad of residues.

Although recombinant FIH and fragments of HIF- α have been efficiently produced in a bacterial host, studies on HIF have been hampered by problems 15 encountered in obtaining purified recombinant PHDs in highly active forms. In particular, PHDs expressed in *E. coli* are completely or essentially completely inactive and attempts to affinity purify tagged PHDs have not been successful (Hirsila *et al.*, J. Biol. Chem. 278, 30772). Similar problems have also been observed with the recombinant expression of other 2-oxoglutarate (2-OG) dependent 20 oxygenases. For example, collagen prolyl hydroxylase (CPH) has been expressed from chick embryos and can be prepared using a baculovirus system but *E. coli* expression of CPH does not appear to be possible.

Summary of the Invention

25 The present inventors have found that it is possible to produce a truncated functional 2-oxoglutarate (2-OG) dependent oxygenase in recombinant expression systems. In particular, the inventors have demonstrated expression of a truncated form of the gene encoding human prolyl-hydroxylase domain 2 (PHD2) in a bacterial expression system, where production of active PHD2 has previously proved difficult. 30 The inventors found that functional expression could be achieved by truncating PHD2, more particularly by incorporating an N-terminal truncation into the recombinant PHD2. The inventors have also successfully purified the truncated

PHD2 in large quantities. The truncated PHD2 may also be freeze-thawed without any loss of activity.

Accordingly, the present invention provides a polypeptide comprising an N-terminal truncated form of a 2-oxoglutarate dependent oxygenase, which retains hydroxylase activity. This 2-oxoglutarate dependent oxygenase maybe a HIF prolyl-hydroxylase, preferably PHD2. A preferred N-terminal truncated form of PHD2 comprises amino acids 181 to 426 of SEQ ID NO: 3.

The invention also provides:

- an expression vector comprising a polynucleotide of the invention operably linked to a promoter sequence;
- a host cell comprising a polynucleotide or vector according to the invention;
- a method of producing a polypeptide according to the invention comprising introducing a polynucleotide according to the invention into a host cell and expressing the polypeptide encoded by the polynucleotide;
- an assay for hydroxylase activity comprising obtaining a polypeptide according to the invention, contacting said polypeptide with a substrate of a 2-OG dependent oxygenase and monitoring for hydroxylation of the substrate; and
- an assay for identifying modulators of hydroxylase activity comprising contacting a polypeptide according to the invention with a substrate of a 2-OG dependent oxygenase in the presence of a test agent under conditions suitable for hydroxylase activity and monitoring for hydroxylation of the substrate, thereby determining whether said test agent modulates hydroxylase activity.

Brief Description of the Figures

Figure 1 is an alignment of the amino acid sequences of PHD1, PHD2, PHD3 and DAOCS, aligned using ClustalW. The N-terminus of the truncated PHD2 (residue 181) is indicated with an arrow, and was based upon homology with DAOCS and other 2-OG enzymes. Stars mark every 20 residues. Shading represents sequence conservation: black highlights 100% conservation (or equivalent residues), dark grey 80% conservation and light grey 60% conservation.

Figure 2 shows sections of the ^1H - ^{13}C HSQC spectra of i) the synthetic HIF₅₅₆₋₅₇₄ fragment (grey) and the synthetic HIF₅₅₆₋₅₇₄ fragment *trans*-hydroxylated at

the γ -position of Pro-564 (black), and ii) the synthetic HIF₅₅₆₋₅₇₄ fragment after incubation with hnt-PHD2. The bottom left hand box in each of (i) and (ii) boxes enclose the resonance from the proton on the 4-position (γ -proton) of the proline ring. It can be seen that incubation of the synthetic HIF₅₅₆₋₅₇₄ fragment with hnt-PHD2 results in the same spectrum as the synthetic HIF₅₅₆₋₅₇₄ fragment hydroxylated at the γ -position of Pro-564. The right hand box in each of (i) and (ii) boxes enclose the resonance from the δ -proton, which shifts on hydroxylation of the γ -position, and the top left hand box encloses the resonance from the α -proton.

Figure 3 depicts the effect of various N-oxalyl amino acid inhibitors on the activity of PHD2 in both Tris and HEPES buffers.

Figure 4 shows EPR spectra of nt-PHD2 preparations a) 1.8 mM nt-PHD2. b) 1.8 mM 2-OG added. c) 3.6 mM 2-OG added. d) 3.6 mM 2-OG and 1.8 mM HIF₅₅₆₋₅₇₄ substrate added. e) 3.6 mM 2-OG, 1.8 mM HIF₅₅₆₋₅₇₄ substrate, and 1.8 mM ascorbate added. f) 3.6 mM 2-OG, 1.8 mM HIF₅₅₆₋₅₇₄ substrate, and 3.6 mM ascorbate added.

Figure 5 shows soft ionisation MS analyses of nt-PHD2 with assignments: (A) nt-PHD2 as purified, (ii) nt-PHD2 with addition of 10mM 2-OG. Apo-PHD2 m/z = 2806.2; PHD2.Fe(II) m/z = 2811.8; PHD2.Fe(II).2-OG m/z = 2826.4.

Brief Description of the Sequences

SEQ ID NO:1 is the nucleotide and amino acid sequence of PHD2.

SEQ ID NO:2 is the amino acid sequence of PHD2.

SEQ ID NO:3 is the amino acid sequence of a truncated form of PHD2 which retains prolyl hydroxylase activity.

SEQ ID NO: 4 is the amino acid sequence of a truncated form of PHD2 modified to include a thrombin cleavage site which retains prolyl hydroxylase activity.

Detailed Description of the Invention

The present inventors tried a number of standard approaches, such as the use of GST tags, maltose binding proteins tags and intein tags, to overcome the problems encountered while trying to obtain soluble, active recombinant proteins in purified form from *E. coli*. None of these standard approaches have been successful.

However, the present inventors have now found that it is possible to express a 2-OG dependent oxygenase, and in particular human prolyl-hydroxylase domain 2 (PHD2) in soluble and highly active form, even in bacterial expression systems where production of soluble PHD2 has previously proved difficult. The inventors have also
5 succeeded in purifying the soluble PHD2 on a large scale enabling further characterization of this enzyme.

The 2-OG dependent oxygenase which is truncated in the present invention is one which, when the full-length enzyme is expressed in recombinant expression systems such as *E. coli*, forms insoluble aggregates and is not present, or is present
10 only in negligible amounts, in the soluble fraction. The 2-OG oxygenase may be a HIF hydroxylase, such as a HIF prolyl hydroxylase or may be another prolyl-hydroxylase. The prolyl hydroxylase may be collagen prolyl hydroxylase (CPH). The 2-OG dependent oxygenase may be a member of the JmjC family of proteins. The JmjC oxygenase may be one involved in regulation of chromatin structure and
15 hence transcriptional control. The 2-OG oxygenase is preferably not FIH or a human AlkB homologue (ABH).

The soluble form of the 2-OG dependent oxygenase is a truncated form of the 2-OG dependent oxygenase. The truncated forms of 2-OG dependent oxygenases, such as PHD2, of the invention preferably have a N-terminal truncation, but may also
20 have a C-terminal truncation. The truncated 2-OG dependent oxygenase of the invention has an improved solubility compared to the full length oxygenase, in particular when expressed in a bacterial expression system, in particular such as an *E. coli* expression system.

The failure of PHDs to bind affinity columns (Hirsila *et al.*, J. Biol. Chem.
25 278, 30772) indicates that the proteins are misfolded. Expression of soluble full length PHD2 in recombinant expression systems is thought to be problematic because the full length polypeptide chain is thought to be folded incorrectly and is sequestered in insoluble aggregates within inclusion bodies. In cells used to express recombinant truncated PHD2 of the invention, the truncated PHD polypeptide folds
30 to form a functional enzyme, although some of the truncated PHD polypeptide molecules may misfold resulting in the formation of insoluble aggregates as well as in the formation of soluble protein.

A soluble and active form of a 2-OG dependent oxygenase is a form which is present in the soluble fraction of the cells producing the 2-OG dependent oxygenase recombinant enzyme. The soluble fraction may be obtained by any suitable method and suitable methods are well known in the art. For example, the cells may be lysed
5 in an extraction buffer, typically after centrifuging the cells to remove the cell culture medium. The extraction buffer may comprise detergents, such as Triton X-100 and/or SDS (typically 1%), and/or lysozyme. The extraction buffer may be suitable for sonication of the cells. After lysis, the cells may be centrifuged. After centrifugation, the supernatant represents the soluble fraction. The concentration of
10 proteins present in the soluble fraction depends on the quantity of extraction buffer used. The truncated 2-OG oxygenase is present in the soluble fraction in an amount sufficient for the truncated enzyme to be purified. This can be determined by SDS PAGE. If it is possible to detect the truncated enzyme by SDS PAGE, there is sufficient enzyme present for purification.

15 The truncated 2-OG dependent oxygenase of the invention generally has an improved solubility in extraction buffer compared to the corresponding full-length enzyme. At least 50%, for example, at least 60%, at least 70%, at least 80% or at least 90% of the truncated 2-OG dependent oxygenase is found in the soluble fraction.

20 Typically, the truncated 2-OG dependent oxygenase of the invention represents between about 2% and about 20%, for example about 5%, of the total soluble protein present in the soluble fraction. This may be determined by SDS PAGE or other visualisation methods.

25 Polypeptides of the invention which comprise affinity tags typically also show improved binding to affinity columns compared to tagged versions of the corresponding full length 2-OG dependent oxygenase. Typically, at least 50%, for example, at least 60%, at least 70%, at least 80% or at least 90% of the truncated enzyme present in the cell extract exposed to the affinity column is bound to the affinity column after washing.

30 The truncated 2-OG dependent oxygenase polypeptides of the invention are generally more stable than the corresponding full length 2-OG dependent oxygenase polypeptides. Full length 2-OG dependent oxygenases, for example PHDs, degrade over time whilst the polypeptides of the invention may be frozen, for example at

from about -20°C to about -80°C, for example for up to a year and may be thawed without loss of activity. Activity may be monitored using any of the 2-OG dependent oxygenase assays described herein.

Truncated 2-OG dependent oxygenases of the invention, such as truncated
5 PHD2 may be purified either in the presence or absence of salt. Typically some salt, for example 0.5M NaCl, is present in the buffers and wash solutions used during the purification procedure. However, it is not necessary to add any additional salt or detergent, for example Triton X-100 to keep the truncated 2-OG dependent
oxygenase in solution. This is in contrast to full length 2-OG dependent oxygenases
10 such as PHDs which precipitate and hence lose activity even when additional salt or detergent is added.

Truncated 2-OG dependent oxygenase polypeptides of the invention may be purified by standard techniques known in the art. For example, where the polypeptide comprises a his tag, it may be purified using a his-binding resin by
15 following the manufacturer's instructions (e.g. Novagen). The purification procedure may comprise the following steps. The cells expressing a recombinant polypeptide of the invention may be pelleted and resuspended in a suitable buffer and then sonicated to break up the cells. The cell debris is separated from the soluble material by centrifugation and the soluble fraction is loaded on a his-bind
20 column. After washing the column with binding buffer and wash buffer, the bound protein is eluted from the column using elution buffer. The binding, wash and elution buffers each typically comprise 0.5M NaCl. It is not necessary to add additional salt. The eluted protein is then concentrated and incubated with thrombin (typically at a concentration of 1Umg⁻¹ at 4°C for 16 hours). The digested proteins
25 are separated using a gel filtration column and the truncated 2-OG dependent oxygenase eluted from the column is generally at least 90%, or at least 95% pure. It is preferable to de-salt the purified protein for use in the various assays described herein. The truncated 2-OG dependent oxygenases of the invention are extremely stable and so can be de-salted without this causing precipitation.

30 The invention thus provides a polypeptide comprising a truncated form of a 2-OG dependent oxygenase, preferably a HIF prolyl-hydroxylase, which retains hydroxylase activity. The HIF prolyl hydroxylase may be PHD1, PHD2 or PHD3. Preferably, the HIF prolyl hydroxylase is PHD2.

A polypeptide of the invention typically has a truncation of at least 50 amino acids from the N-terminus, more preferably between 100 to 230 amino acids truncation, more preferably from about 150 to 200 amino acids truncation. In a particularly preferred embodiment, the truncation in PHD2 is from 170 to 190 N-terminal amino acids, more preferably 175 to 185 N-terminal amino acids. A PHD2 fragment lacking the N-terminal 180 amino acids is particularly preferred. The sequence of this fragment is shown in SEQ ID NO: 3. The truncated 2-OG dependent oxygenase may comprise non-naturally derived amino acids. For example, amino acids may be inserted to incorporate a thrombin cleavage site, or other cleavage site, into the truncated PHD2 polypeptide. The preferred truncated PHD2 described in the Examples includes three additional amino acids, MAS, between residues 3 and 4 of SEQ ID NO: 3 which result in the introduction of a thrombin cleavage site. The sequence of this preferred PHD2 polypeptide is shown in SEQ ID NO: 4.

A polypeptide of the invention may comprise a C-terminal truncation, typically in addition to an N-terminal truncation, although the 2-OG dependent oxygenase may be truncated only at the N-terminus. The C-terminal truncation may be a deletion of up to 30 amino acids, for example of up to 25, 20, 15, 10 or 5 amino acids. Preferred PHD2 polypeptides with a C-terminal truncation comprise amino acids 181-142, 181-140, 181-418, 181-414, 181-410, 181-406 or 181-402 of SEQ ID NO: 2. Each of these polypeptides may include the additional amino acids, MAS, between amino acids 183 and 184 of SEQ ID NO: 2 to incorporate a thrombin cleavage site.

A polypeptide of the invention retains hydroxylase activity, typically prolyl-hydroxylase activity. The truncated PHD polypeptide of the invention preferably retains 2-OG dependent oxygenase activity.

The hydroxylase activity of a truncated polypeptide, and in particular a truncated PHD2 polypeptide, of the invention may also be iron-dependent. In an assay to determine hydroxylase activity of a truncated PHD2 polypeptide, iron and/or 2-OG may be added. It is not, however, essential that these components are added to the assay because PHD2 has a high affinity for iron and 2-OG and so these cofactors may be associated with the recombinant PHD2 polypeptide.

The amino acid sequence of PHD2 is shown in SEQ ID NO: 2. The PHD2 used in a method of the invention comprises a truncated form of the sequence shown in SEQ ID NO: 2 or variant thereof having prolyl hydroxylase activity.

The variant of PHD2 retains 2-OG-dependent oxygenase activity. The 2-OG-
5 dependent oxygenase activity of the variant may be enhanced or reduced compared to PHD2 having the sequence shown in SEQ ID NO:2. The variant of PHD2 typically shares at least 80%, at least 90% or at least 95% sequence identity with the truncated SEQ ID NO: 2, for example a variant may have at least 80%, at least 90% or at least 95% sequence identity with SEQ ID NO: 3 over the entire length of SEQ
10 ID NO: 3.

Sequence identity may be calculated using any suitable algorithm. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395). The PILEUP and BLAST algorithms can be
15 used to calculate homology or line up sequences (such as identifying equivalent or corresponding sequences (typically on their default settings), for example as described in Altschul S. F. (1993) *J Mol Evol* 36:290-300; Altschul, S, F *et al* (1990) *J Mol Biol* 215:403-10.

Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20
20 or 30 substitutions. Conservative substitutions may be made, for example according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Variant polypeptides within the scope of the invention may be generated by any suitable method, for example by gene shuffling (molecular breeding) techniques.

5 The oxygenase and substrate protein for use in an assay of the invention may be chemically modified, e.g. post-translationally modified. For example, they may be glycosylated or comprise modified amino acid residues. They may also be modified by the addition of histidine residues (typically six), or other sequence tag such as a maltose binding protein tag or intein tag, to assist their purification or by the addition
10 of a nuclear localisation sequence to promote translocation to the nucleus or by post translational modification including hydroxylation or phosphorylation. Polypeptides of the invention may be GST fusion polypeptides.

The polypeptides of the invention may be present in a substantially isolated form. They may be mixed with carriers or diluents which will not interfere with their
15 intended use and still be regarded as substantially isolated. They may also be in a substantially purified form, in which case they will generally comprise at least 90%, e.g. at least 95%, 98% or 99%, of the proteins, polynucleotides, cells or dry mass of the preparation.

The polypeptides of the invention may be used in assays for 2-OG dependent
20 oxygenase activity, for example to identify modulators, preferably inhibitors of hydroxylase activity. The inhibitors may be selective inhibitors. The polypeptides of the invention may also be used in structural analyses such as crystallography.

Polypeptides, Vectors and Host Cells

The invention also provides polynucleotides encoding polypeptides of the invention. The polynucleotide may be a fragment or variant of the coding region of the nucleotide sequence shown in SEQ ID NO: 1 which lacks one or more nucleotide
5 from the 5' end of the coding region, for example which lacks at least 150 nucleotides, for example between 300 and 690, between 450 and 600 nucleotides, more preferably 510 to 570, still more preferably 525 to 555, most preferably 540 nucleotides. The variant typically has at least 70%, 80%, 90%, 95%, 98% or 99% sequence identity to the coding region of the nucleotide sequence of SEQ ID NO: 1
10 over a region of at least 588 contiguous nucleotides, preferably 588 contiguous nucleotides at the 3' end of the coding region shown in SEQ ID NO: 1. Sequence identity may be determined by any suitable method, for example, as described above.

The polynucleotide is typically included in an expression vector. Such expression vectors are routinely constructed in the art and may for example involve
15 the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary and which are positioned in the correct orientation in order to allow full protein expression. Suitable vectors would be very readily apparent to those of skill in the art. Promoter sequences may be inducible or constitutive promoters depending on
20 the selected assay format. The promoter may be tissue specific. Thus the coding sequence in the vector is operably linked to such elements so that they provide for expression of the coding sequence (typically in a cell). The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner.

25 The vector may be, for example, a plasmid, virus or baculovirus vector. The vector is typically adapted to be used in a bacterial cell, such as *E. coli*. The vector may have an origin of replication. The vector may comprise one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a resistance gene for a fungal vector. Vectors may be used to transfect or
30 transform a host cell, for example, a bacterial host cell or fungal host cell. The bacterial host cell is preferably a strain of *E. coli*, for example BL21 (DE3).

The invention also provides a host cell comprising a polynucleotide or vector of the invention. The host cell may be a bacterial cell, such as *E. coli* (for example,

strain BL21 (DE3)), a fungal cell, a yeast cell, an insect cell or a mammalian cell. The host cell is typically in culture (*in vitro*). Preferably, the host cell is a bacterium, more preferably an *E. coli* cell.

Also provided by the invention is a method of producing a truncated 2-OG dependent oxygenase polypeptide, such as a PHD2 polypeptide, comprising
5 introducing a polynucleotide or vector according to the invention into a host cell and expressing the polypeptide encoded by the polynucleotide. The method may further comprise extracting the soluble fraction comprising the polypeptide from the cell culture. The polypeptide may be further purified from the soluble fraction, for
10 example by affinity purification, such as via an affinity tag fused to the truncated 2-OG dependent oxygenase.

Methods for introducing polypeptides and vectors into host cells are well known in the art, and include electroporation and heat shock techniques. Expression of the truncated polypeptide may then be achieved by culturing the host cells at a
15 suitable temperature. Conditions for achieving expression are commonplace in the art. For example, where the host cells are bacteria, such as *E. coli*, the cells may be cultured in 2TY medium. The culture temperature may be 37°C. IPTG may be added to the culture medium, either throughout the period of incubation (or growth period) or in the final stages of the incubation period.

20

Substrate

Any suitable substrate may be used in an assay of the invention. Substrates of 2-OG dependent oxygenase are well known in the art. The substrate may be a naturally occurring protein or a recombinant or synthetic protein. Fragments and
25 variants of substrate proteins which include the site of hydroxylation by a HIF hydroxylase, preferably a HIF prolyl hydroxylase such as PHD2, may be used as substrates in the assay of the invention. The substrate is preferably a HIF polypeptide. Other suitable substrates include ankyrin repeat proteins, such as p105, IκB-α, FEM-1, Bcl-3, P19-INK4d, GABPβ, Tankyrase, Gankyrin, Myotrophin,
30 M110 and FGIF and fragments and variants of any of these proteins.

In preferred embodiments of the invention, the HIF polypeptide is a HIF-1α, HIF-2α, or HIF-3α subunit protein or fragment of either or peptide analogue of the subunit or fragment. The fragment preferably comprises at least 15, for example at

least 17, 19, 20, 25 or 30 amino acids. Longer HIF fragments are preferred.

Preferably, such HIF polypeptides, fragments or peptide analogues incorporate a proline residue equivalent to Pro 402 and/or a proline residue equivalent to Pro 564 defined with reference to HIF-1 α . The proline equivalent to Pro 402 and/or Pro 564 of HIF-1 α may be determined by aligning the HIF variant, fragment or analogue to the sequence of HIF-1 α to obtain the best sequence alignment and identifying thereby the proline equivalent to Pro 402 and/or Pro 564.

Preferred HIF-1 α fragments include a polypeptide comprising or consisting of residues 786-826 of HIF-1 α , which is a substrate of FIH, or of residues 556-575 or 530-698 of HIF-1 α , which are substrates of PHDs.

A HIF polypeptide may be of eukaryotic origin, in particular a human or other mammalian, HIF- α subunit protein or fragment thereof. Alternatively, the polypeptide may be of *C. elegans* origin.

A number of HIF- α subunit proteins have been cloned. These include HIF-1 α , the sequence of which is available as Genbank accession number U22431, HIF-2 α , available as Genbank accession number U81984 and HIF-3 α , available as Genbank accession numbers AC007193 and AC079154. These are all human HIF- α subunit proteins and all may be used in the invention. HIF- α subunit proteins from other species, including murine HIF-1 α (accession numbers AF003695, U59496 and X95580), rat HIF-1 α (accession number Y09507), murine HIF-2 α (accession numbers U81983 and D89787) and murine HIF-3 α (accession number AF060194) may also be used in the invention.

One HIF- α protein of particular interest is the *C. elegans* HIF- α subunit protein. The *C. elegans* system may be used in assays of the present invention.

Variants of the above HIF- α subunits may be used, such as synthetic variants which have at least 45% amino acid identity to a naturally occurring HIF- α subunit (particularly to a human HIF- α subunit such as, for example HIF-1 α), preferably at least 50%, 60%, 70%, 80%, 90%, 95% or 98% identity. Such variants may include substitutions or modifications as described above with respect to HIF hydroxylases. Amino acid activity may also be calculated as described above with reference to HIF hydroxylases.

HIF fragments may also include non-peptidyl functionalities and may be optimised for assay purposes such that the level of identity is lowered. Such functionalities may be covalently bound such as sugars or non-covalently bound such as metal ions.

5 The polypeptides of the invention may be used to detect novel substrates of a 2-OG dependent oxygenase. In such an assay a test substrate is used and the detection of hydroxylase activity indicates that hydroxylation of the test substrate has occurred and that the test substrate is a substrate of the 2-OG dependent oxygenase.

10 *Assay Methods*

 The polypeptides of the invention may be used in an assay for hydroxylase activity, such as prolyl-hydroxylase activity, and hence in assays for identifying an agent which modulates hydroxylation of HIF or other substrates. The method comprises contacting a polypeptide of the invention and a test substance, such as a
15 potential inhibitor, in the presence of a substrate under conditions in which hydroxylation occurs in the absence of the test substance and determining the extent of hydroxylation of the substrate. Alternatively, the assay may be used to detect substances that increase the activity of the 2-OG dependent oxygenase by assaying for increases in activity.

20 Such assays of the present invention may be used to identify inhibitors of hydroxylase activity and are thus preferably carried out under conditions under which hydroxylation would take place in the absence of the test substance. In the alternative, the assays may be used to look for promoters of hydroxylase activity, for example, by looking for increased hydroxylation of substrates compared to assays
25 carried out in the absence of a test substance. The assays may also be carried out under conditions in which hydroxylation is reduced or absent, such as under hypoxic conditions, and the presence of or increased hydroxylation could be monitored under such conditions.

 The assays of the invention may also be used to identify inhibitors or
30 activators which are specific for one or more HIF prolyl hydroxylase and which do not have activity or are less active with other hydroxylases, for example, such as HIF asparagine hydroxylases or other prolyl hydroxylases. Conversely, the assays of the

invention may be used to identify inhibitors or activators specific for one or more 2-OG dependent oxygenase which do not inhibit HIF-prolyl hydroxylases.

The assays of the invention may also be used to identify inhibitors or activators which are specific for HIF prolyl hydroxylase activity at a particular
5 substrate or prolyl residue within a substrate.

In medicinal applications, for example, it is advantageous to modulate hydroxylase activity of a single enzyme or group of enzymes. Assays of the invention may therefore be use to identify agents which selectively modulate activity of a first 2-OG dependent oxygenase relative to a second 2-OG dependent
10 oxygenase.

For example, it is recognised that in some circumstances it is advantageous to selectively inhibit PHD2, but not FIH or one of the HIF prolyl hydroxylase isoforms such as PHD1 and PHD3. Alternatively, it may be wished to identify an inhibitor of PHD1, PHD3 and/or FIH but not of PHD2. For example, N-oxalyl-D-phenylalanine
15 (NOFD) is a selective inhibitor of FIH that has been identified by a method of the invention.

The invention provides for the use of such selective inhibitors in the manufacture of a medicament for the treatment of a condition associated with altered, i.e. enhanced or reduced, 2-OG dependent oxygenase activity, such as HIF
20 hydroxylase activity.

Activities of different enzymes may be compared to detect inhibitors that are selective for a particular HIF hydroxylase or group of HIF hydroxylases compared to the other 2-OG oxygenases including but not limited to AlkB, ABH1, ABH2, ABH3, procollagen prolyl and lysyl hydroxylases, Mina53, the phosphatidylserine receptor
25 and 2-OG oxygenases that have been characterized as JmjC proteins according to the SMART database.

It is also possible, using the method of the invention to identify selective inhibitors when the substrate of one or more of the enzymes being tested is unknown. In this embodiment, generally it will be one or more of the enzymes that it is wished
30 not to inhibit that has an unknown substrate. The effect of a test agent on activity of an oxygenase may be determined in the absence of a substrate by determining whether or not the test agent affects, for example inhibits or stimulates, the rate of turnover of 2-OG by the oxygenase.

Methods for monitoring modulation

The precise format of any of the screening or assay methods of the present invention may be varied by those of skill in the art using routine skill and knowledge. The skilled person is well aware of the need to additionally employ appropriate controlled experiments. The assays of the present invention may involve monitoring for hydroxylation of a suitable substrate, monitoring for the utilisation of substrates and co-substrates, monitoring for the production of the expected products between the enzyme and its substrate. Assay methods of the present invention may also involve screening for the direct interaction between components in the system. Alternatively, assays may be carried out which monitor for downstream effects mediated by HIF such as HIF mediated transcription using suitable reporter constructs or by monitoring for the upregulation of genes or alterations in the expression patterns of genes known to be regulated directly or indirectly by HIF.

Various methods for determining hydroxylation are known in the art. Any suitable method may be used for determining activity of the 2-OG dependent oxygenase such as by substrate or co-substrate utilization, product appearance such as peptide hydroxylation or down-stream effects mediated by hydroxylated or non-hydroxylated products.

The substrate, enzyme and potential inhibitor compound may be incubated together under conditions which, in the absence of inhibitor provide for hydroxylation of the substrate, and the effect of the inhibitor may be determined by determining hydroxylation of the substrate. This may be accomplished by any suitable means. Small polypeptide substrates may be recovered and subjected to physical analysis, such as mass spectrometry or chromatography, or to functional analysis. Such methods are known as such in the art and may be practiced using routine skill and knowledge. Determination may be quantitative or qualitative. In both cases, but particularly in the latter, qualitative determination may be carried out in comparison to a suitable control, e.g. a substrate incubated without the potential inhibitor.

In alternative embodiments, reporter constructs may be provided in which promoters mediated by HIF are provided operably linked to a reporter gene. Any suitable reporter gene could be used, such as for example enzymes which may then

be used in colorimetric, fluorometric, fluorescence resonance or spectrometric assays.

In the assay methods described herein, typically the 2-OG dependent oxygenase and the substrate of the oxygenase are contacted in the presence of a co-substrate, such as 2-oxoglutarate (2-OG) and/or dioxygen. Hydroxylase activity may be determined by determining the turnover of the co-substrate. This may be achieved by determining the presence and/or amount of reaction products, such as hydroxylated substrate or succinic acid. The amount of product may be determined relative to the amount of substrate. Typically, in such embodiments the substrate may be a HIF- α polypeptide and, for example, the product measured may be hydroxylated HIF- α polypeptide. For example, the extent of hydroxylation may be determined by measuring the amount of hydroxylated HIF- α polypeptide, succinate or carbon dioxide generated in the reaction, or by measuring the depletion of 2-OG or dioxygen. Methods for monitoring each of these are known in the scientific literature.

HIF- α asparagine and prolyl hydroxylase activity may be determined by determining the turnover of said 2-OG to succinate and CO₂, as described in Myllyharju J. *et al* EMBO J. 16 (6): 1173-1180 (1991) or as in Cunliffe C.J. *et al* Biochem. J. 240 617-619 (1986), or other suitable assays for CO₂, bicarbonate or succinate production.

Unused 2-OG may be derivatised by chemical reagents, exemplified by but not limited to hydrazine derivatives and *ortho*-phenylene diamine derivatives, to give indicative chromophores or fluorophores that can be quantified and used to indicate the extent of hydroxylation of the test polypeptide. Dissolved oxygen electrodes, exemplified by but not limited to a "Clarke-type" electrode or an electrode that uses fluorescence quenching, may be used to follow the consumption of oxygen in an assay mixture, which can then be used to indicate the extent of hydroxylation of the test polypeptide in an analogous manner to the above.

The fluorescent product of the reaction of *ortho*-phenylenediamine (OPD) with the α -ketoacid motif of 2-OG is 3-(2-Carboxyethyl)-2(1*H*)-quinoxalinone. This fluorescent product can be readily detected by standard equipment such as that manufactured by for example Molecular Devices, Tecan, BMG Labtechnologies,

Jasco and Perkin Elmer and there is extensive precedent demonstrating that the production of fluorescent products can be used in high-throughput screens.

The fluorescent product is generally detected with the excitation filter set as from about 300nm to about 400nm, preferably from about 335 to about 345 nm, most preferably at about 340nm. The emission filter is generally at from about 400 to about 450nm, preferably from about 415 to about 425nm, most preferably at about 420nm.

This assay procedure lends itself to high-throughput formats, such as multi-well plate formats e.g. 96-, 384-, or 1536-well plate formats.

Further, the nature of the fluorescent product can be tuned by modifying the nature of the derivatisation reagent used. For example, the sensitivity of the method may be increased by using either 1,2-dimethoxy-4,5-diaminobenzene, or 1,2-methylenedioxy-4,5-diaminobenzene.

The precise format of any of the screening or assay methods of the present invention may be varied by those of skill in the art using routine skill and knowledge. The skilled person is well aware of the need to additionally employ appropriate control experiments. Activity is measured by derivatisation of 2-OG with OPD or other aromatic diamines, such as 1,2-dimethoxy-4,5-diaminobenzene or 1,2-methylenedioxy-4,5-diaminobenzene, such that the derivative gives improved sensitivity compared to use of OPD (Mühling *et al.* Journal of Chromatography B (2003) 383-392, Nakamura *et al.* Chem. Pharm Bull. (1987) 687-692).

The assay is carried out under conditions suitable for hydroxylation of the substrate by the hydroxylase. Accordingly, 2-OG is present in the assay. The assay mixture may also contain iron, preferably ferrous iron.

Other components may be added to the assay mixture. For example, a reducing agent such as ascorbate, dithiothrietol (DDT), β -mercaptoethanol or N-acetylcysteine may be added to the assay to help maintain enzyme structure and/or catalase may be added to destroy any H_2O_2 that might be produced. However, the assay will work in the absence of a reducing agent or catalase.

The assay is typically carried out at a temperature of from about 25°C to about 40°C, for example at a temperature of from about 30°C to about 39°C, or from about 35°C to about 38°C or about 37°C. The pH of the assay mixture is typically between about pH 7 to about pH 9, for example from about pH 7.5 to about pH 8.

Suitable buffers, such as Tris or HEPES, may be used to maintain the pH of the assay mixture.

Typically, the assay is carried out under normoxic conditions. The assay may also be carried out under conditions in which hydroxylation is reduced or absent, such as under hypoxic conditions, in order to detect modulation of oxygenase activity by an agent which enhances hydroxylation.

Alternatively, the end-point determination may be based on conversion of HIF- α or peptide fragments (including synthetic and recombinant peptides) derived from HIF- α into detectable products. Peptides may be modified to facilitate the assays so that they can be rapidly carried out and may be suitable for high throughput screening.

For example, reverse phase HPLC (C-4 octadecylsilane column), as exemplified herein, may be used to separate starting synthetic peptide substrates for HIF hydroxylase from the hydroxylated products. The latter typically have a shorter retention time in the column. Modifications of this assay or alternative assays for HIF hydroxylase activity may employ, for example, mass spectrometric, spectroscopic, and/or fluorescence techniques as are well known in the art (Masimirembwa C. *et al* Combinatorial Chemistry & High Throughput Screening (2001) 4 (3) 245-263, Owicki J. (2000) J. Biomol. Screen. 5 (5) 297-305, Gershkovich A *et al* (1996) J. Biochem. & Biophys. Meths. 33 (3) 135-162, Kraaft G. *et al* (1994) Meths. Enzymol. 241 70-86). Fluorescent techniques may employ versions of the substrate modified in such as way as to carry out or optimise spectroscopic or fluorescence assays.

Binding of a molecule which discriminates between the hydroxylated and non-hydroxylated form of a HIF- α polypeptide or other substrate may be assessed using any technique available to those skilled in the art, which may involve determination of the presence of a suitable label.

Assay methods of the present invention may also take the form of an *in vivo* assay. The *in vivo* assay may be performed in a cell line such as a yeast or bacterial strain in which the relevant polypeptides or peptides are expressed from one or more vectors introduced into the cell.

Test Compounds

Agents which may be screened using the assay methods described herein may be natural or synthetic chemical compounds used in drug screening programmes.

Extracts of plants, microbes or other organisms which contain several characterised

5 or uncharacterised components may also be used.

Combinatorial library technology (including solid phase synthesis and parallel synthesis methodologies) provides an efficient way of testing a potentially vast number of different substances for ability to modulate an interaction. Such libraries and their use are known in the art, for all manner of natural products, small
10 molecules and peptides, among others. The use of peptide libraries may be preferred in certain circumstances.

Potential inhibitor compounds (i.e. antagonists) may be polypeptides, small molecules such as molecules from commercially available combinatorial libraries, or the like. Small molecule compounds which may be used include 2-OG analogues, or
15 substrate analogues, which inhibit the action of the enzyme. Small molecule compounds that may be used include all known 2-OG oxygenase inhibitors such as those known to inhibit HIF hydroxylases (see for example WO02/074981 and WO03/080566) and procollagen prolyl hydroxylases.

Potential promoting agents may be screened from a wide variety of sources,
20 particularly from libraries of small compounds which are commercially available. Oxygen-containing compounds may be included in candidate compounds to be screened, for example 2-OG analogues.

A test compound which increases, potentiates, stimulates, disrupts, reduces, interferes with or wholly or partially abolishes hydroxylation of the substrate and
25 which may thereby modulate activity, may be identified and/or obtained using the assay methods described herein.

Agents which increase or potentiate hydroxylation (i.e. agonists), may be identified and/or obtained under conditions which, in the absence of a positively-testing agent, limit or prevent hydroxylation. Such agents may be used to potentiate,
30 increase, enhance or stimulate the activity of a HIF hydroxylase.

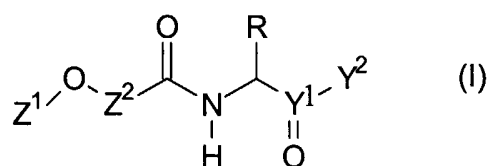
In various aspects, the present invention provides an agent or compound identified by a screening method of the invention to be a modulator of 2-OG

oxygenase activity e.g. a substance which inhibits or reduces, increases or potentiates the activity of a HIF hydroxylase.

Following identification of a modulator, the substance may be purified and/or investigated further (e.g. modified) and/or manufactured. A modulator may be used to obtain peptidyl or non-peptidyl mimetics, e.g. by methods well known to those skilled in the art and discussed herein. A modulator may be modified, for example to increase selectively, as described herein. It may be used in a therapeutic context as discussed below.

For therapeutic treatment, the compound may be used in combination with any other active substance, e.g. for anti-tumour therapy another anti-tumour compound or therapy, such as radiotherapy or chemotherapy.

Suitable test compounds are disclosed in WO03/080566 and WO02/074981. Other suitable test compounds include compounds of formula (I):



wherein

- Y² is selected from -OR' and -NR'R'' wherein R' is hydrogen, or unsubstituted C₁₋₄ alkyl and R'' is hydrogen, hydroxy or unsubstituted C₁₋₄ alkyl;
- Y¹ is selected from -C-, -S- and -S(O)-;
- Z² is selected from -C(O)- and -NR''- wherein R'' is selected from hydrogen, hydroxy or unsubstituted C₁₋₄ alkyl;
- Z¹ is selected from hydrogen and unsubstituted C₁₋₄ alkyl; and
- R is a side chain of a naturally occurring amino acid.

Preferably Y¹ is -C- and Y² is -OH or -NH₂. Most preferably Y¹ is -C- and Y² is -OH.

Preferably Z² is -C(O)- or -NR''- wherein R'' is hydrogen, methyl or ethyl. More preferably Z² is -C(O)- or -NH-. Preferably Z¹ is hydrogen, methyl or ethyl, more preferably hydrogen. Most preferably Z² is -C(O)- and Z¹ is hydrogen, methyl or ethyl.

Preferably R is a side chain of alanine, valine, leucine or phenylalanine.

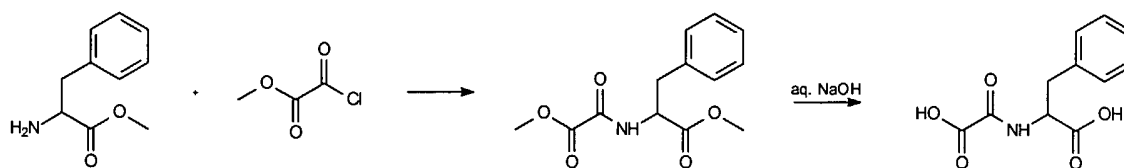
Preferably R is a side chain of valine, leucine or phenylalanine. More preferably R is a side chain of phenylalanine, i.e. $-\text{CH}_2\text{Ph}$.

The L-stereoisomers of these compounds are typically better inhibitors of
5 PHD2 than the D-stereoisomers (Figure 3). The D-stereoisomers of compounds of formula (I) are examples of compounds that exhibit selective inhibitory activity for one 2-OG dependent oxygenase. More particularly, these D-stereoisomers inhibit FIH activity, but not PHD2 activity.

The compounds which are acids can be present in the form of salts, such as
10 sodium salts. The compounds may also be present in the form of derivatives such as the dimethyl ester, diethyl ester, monoethyl ester or di- or mono-amide. In certain instances these derivatives may be preferred, for example when inhibition of the enzyme within a cell of an organism is required.

An exemplary synthetic scheme used to obtain test compounds of formula (I)
15 is shown below in Scheme 1. Here an amino acid is reacted with an oxalyl chloride in order to produce a compound of formula (I). In this scheme the amino acid used is phenylalanine, although it will be apparent that the same general reaction will occur with other amino acids. The first reaction yields a protected compound of the invention (the dimethyl ester form). The diacid form is easily generated through
20 reaction with aqueous sodium hydroxide.

Scheme 1:



Compounds in which X is $-\text{O}-$ or $-\text{S}-$ or Z is other than $-\text{CO}-\text{CO}-\text{OH}$ may by
25 synthesised as described in Mole *et al.* (2003) *Bioorg. Med. Chem. Lett.* **13**, 2677-2680 and Cunliffe *et al.* *J. Med. Chem.* (1992) **35** 2652-2658.

Therapeutic Applications

A compound, substance or agent which is found to have the ability to affect the hydroxylase activity of a 2-OG dependent oxygenase has therapeutic and other potential in a number of contexts, as discussed. For therapeutic treatment, such a
5 compound may be used in combination with any other active substance, e.g. for anti-tumour therapy with another anti-tumour compound or therapy, such as radiotherapy or chemotherapy.

An agent identified using one or more primary screens (e.g. in a cell-free system) as having ability to modulate hydroxylase activity may be assessed further
10 using one or more secondary screens.

Generally, an agent, compound or substance which is a modulator according to the present invention is provided in an isolated and/or purified form, i.e. substantially pure. This may include being in a composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more
15 preferably at least about 98%. Any such composition may, however, include inert carrier materials or other pharmaceutically and physiologically acceptable excipients, such as those required for correct delivery, release and/or stabilisation of the active agent. As noted below, a composition according to the present invention may include in addition to an modulator compound as disclosed, one or more other molecules of
20 therapeutic use, such as an anti-tumour agent.

Products obtained by assays of the invention

The invention further provides compounds obtained by assay methods of the present invention, and compositions comprising said compounds, such as
25 pharmaceutical compositions wherein the compound is in a mixture with a pharmaceutically acceptable carrier or diluent. The carrier may be liquid, e.g. saline, ethanol, glycerol and mixtures thereof, or solid, e.g. in the form of a tablet, or in a semi-solid form such as a gel formulated as a depot formulation or in a transdermally administrable vehicle, such as a transdermal patch.

30 HIF hydroxylases catalyse reactions involved in the medicinally important hypoxic response process.

Reduced dioxygen concentration in the tissues of multicellular organism triggers the hypoxic response which attempts to restore normoxia by improving the

supply of oxygen. The response involves an array of genes including erythropoietin and vascular endothelial growth factor and is mediated by a specific α,β -heterodimeric transcription factor, hypoxia-inducible factor (HIF), the α -subunit of which is upregulated under hypoxic conditions. Since the genes involved in the hypoxic response include those involved in angiogenesis, modulation of the hypoxic response is of interest from the perspectives of developing new therapies for both cancer and cardiovascular disease.

Both the levels and activity of HIF- α are regulated by HIF hydroxylases. Under normoxic conditions, two different but related dioxygenases, prolyl hydroxylase domain (PHD also known as EGLN and HPH) enzymes and factor inhibiting hypoxia-inducible factor (FIH) inhibit hypoxic responses by catalysing the post-translational hydroxylation of HIF- α .

Both HIF-1 α and HIF-2 α contain a central oxygen dependent degradation domain (ODDD). Pro-402 and Pro-564 (in HIF-1 α) are situated in two sub-domains of the ODDD. Hydroxylation of these residues, by PHD isoforms, enables binding of HIF- α to the pVHL-Elongin B/C (VBC) complex which recruits an E3 ubiquitin ligase, mediating ubiquitination of HIF- α and its consequent proteasomal destruction. The prolyl residues forms part of a conserved LXXLAP motif and modification of either residue can independently promote degradation.

Upregulation of HIF is involved in the development of tumours associated with defects in the von Hippel/Lindau tumour suppressor protein, pVHL. The binding of hydroxylated HIF- α to pVHL relies upon two hydrogen bonds involving the alcohol of the *trans*-4-hydroxylated prolyl residue in the former to the side-chains of a serinyl and histidinyl residues in pVHL.

Under hypoxic conditions, cytoplasmic HIF- α translocates into the nucleus and binds to HIF- β , forming the active heterodimer which then acts in conjunction with nuclear coactivators, including p300. Hydroxylation by FIH at the β -carbon of a conserved asparagine residue (Asn-803 in HIF-1 α) in the C-terminal activation domain (CAD) of HIF prevents binding of HIF to the CH1 domain of p300, a nuclear co-activator protein involved in transcription. Modulators of HIF hydroxylases are thus useful in the treatment or prevention of cancer.

The invention further provides a method of treatment which includes administering to a patient an agent which interferes with HIF hydroxylase activity. Such agents may include inhibitors of HIF hydroxylase activity.

5 The therapeutic/prophylactic purpose may be related to the treatment of a condition associated with reduced or suboptimal or increased HIF hydroxylase levels or activity, or conditions in which have normal HIF hydroxylase levels, but where an modulation in activity such as an increase or decrease in HIF hydroxylase activity is desirable such as:

- 10 (i) ischaemic conditions, for example organ ischaemia, including coronary, cerebrovascular and peripheral vascular insufficiency. The therapy may be applied in two ways; following declared tissue damage, e.g. myocardial infarction (in order to limit tissue damage), or prophylactically to prevent ischaemia, e.g. promotion of coronary collaterals in the treatment of angina;
- (ii) cancer; HIF- α is commonly up-regulated in tumour cells and has 15 major effects on tumour growth and angiogenesis;
- (iii) inflammatory disorders;
- (iv) immune disorders such as diabetes; and
- (v) anaemia.

20 A therapeutically effective amount of an agent is typically administered to a subject in need thereof. A therapeutically effective is an amount which ameliorates the symptoms of the condition or lessens the suffering caused to the subject by the condition.

Pharmaceutical Compositions

25 In various further aspects, the present invention thus provides a pharmaceutical composition, medicament, drug or other composition for such a purpose, the composition comprising one or more agents, compounds or substances as described herein, including inhibitors of 2-OG dependent oxygenase activity, the use of such a composition in a method of medical treatment, a method comprising 30 administration of such a composition to a patient, e.g. for treatment (which may include preventative treatment) of a medical condition as described above, use of such an agent compound or substance in the manufacture of a composition, medicament or drug for administration for any such purpose, e.g. for treatment of a

condition as described herein, and a method of making a pharmaceutical composition comprising admixing such an agent, compound or substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

In one embodiment the method for providing a pharmaceutical composition
5 may typically comprise:

- (a) identifying an agent by an assay method of the invention; and
- (b) formulating the agent thus identified with a pharmaceutically acceptable excipient.

The pharmaceutical compositions of the invention may comprise an agent,
10 polypeptide, polynucleotide, vector or antibody according to the invention and a pharmaceutically acceptable excipient.

The agent may be used as sole active agent or in combination with one another or with any other active substance, e.g. for anti-tumour therapy another anti-tumour compound or therapy, such as radiotherapy or chemotherapy.

15 Whatever the agent used in a method of medical treatment of the present invention, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend
20 on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

An agent or composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition
25 to be treated, e.g. as described above.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. In particular they may include a
30 pharmaceutically acceptable excipient. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil.

5 Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability.

10 Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Liposomes, particularly cationic liposomes, may be used in carrier

15 formulations. Examples of techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

The substance or composition may be administered in a localised manner to a particular site or may be delivered in a manner in which it targets particular cells or tissues, for example using intra-arterial stent based delivery.

20 Targeting therapies may be used to deliver the active substance more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons, for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

25 In a further embodiment the invention provides for the use of an agent of the invention in the manufacture of a medicament for the treatment of a condition associated with increased or decreased 2-OG dependent oxygenase levels or activity. The condition may, for example, be selected from the group consisting of ischaemia, cancer, and inflammatory and immune disorders.

30 All the documents cited herein are incorporated herein by reference.

The following Examples illustrate the invention.

Examples

5 **Example 1: Production of truncated PHD2**

Comparison of the PHD2 sequence with other 2-OG oxygenases (Figure 1), including deacetoxycephalosporin C synthase, led to the cloning of an N-terminally truncated form of PHD2 (residues 181-426 having the first 180 amino acids (including those containing a MYND finger motif) removed), which maintained, at least, its double stranded β -helix 'core'. Thus the enzyme, after removal of the hexa-His affinity tag, has a N-terminus of GSHMAS-from the vector construct and the N-terminus from PHD2 PNGQTKPLP. The enzyme was expressed from the pET28a(+) vector (Novagen), with the gene cloned between NheI and ManHI restriction sites. Growth and induction were using standard techniques, in that the pET28a(+) vector was transformed into BL21(DE3) *Escherichia coli* cells, and grown in 2TY medium at 37°C until the absorbance of the solution of 600nm was 0.8. IPTG was added to a final concentration of 0.5mM and growth was continued for three hours. This N-terminally hexa-His tagged truncated PHD2 (hnt-PHD2) was expressed as ca. 5% of total soluble protein in *Escherichia coli* BL21(DE3). Affinity purification using Novagen His-Bind resin was as per the manufacturers instructions. Purification by Ni-affinity and His tag cleavage followed by gel-filtration chromatography gave protein of > 90% purity by SDS-PAGE analysis.

Example 2: Detection of prolyl-hydroxylase activity

The assay of PHD2 activity was carried out by mixing 1mM DTT (Melford Laboratories), 0.6mg/ml catalase (Sigma), 2-OG, substrate (HIF-1 α 556-574) and 50mM HEPES pH 7.0 or Tris/HCl pH 7.5 to a final volume of 88 μ l warming to 37°C for 5 minutes in a water bath. Simultaneously, the enzyme and iron (iron ammonium sulphate (Sigma) prepared as 500mM stock in 20mM HCl, and diluted with water) were mixed at room temperature for 3 minutes. Reaction was initiated by addition of 12 μ l of enzyme/iron mix to the substrate/cofactor mix. The reaction was stopped by addition of 200 μ l 0.5M HCl; derivatisation was then achieved by the addition of 100 μ l 10mg/ml OPD (bought from Acros Organics and recrystallised from heptane

and petroleum ether (120-140)) in 0.5M HCl, and heating for 10 minutes at 95°C in a heating block. After centrifugation at top speed in a bench microfuge for 5 minutes, the supernatant (50µl) was made basic by the addition of 30µl 1.25M NaOH and the fluorescence was measured on a Novostar (BMG Labtechnologies Ltd.) with the
5 excitation filter at 340nm and the emission filter at 420nm.

Scanning emission and excitation spectra were recorded on a Perkin Elmer LK-50B spectrometer.

In the presence of Fe(II) and 2-OG, a specific activity of 2 (± 0.7) mol 2-OG/min/mol PHD2 was obtained for a 19 residue HIF fragment (HIF₅₅₆₋₅₇₄) using a
10 fluorescence-based assay for 2-OG consumption. This value is similar to that reported for full length PHD2 produced in a baculovirus expression system (approximately 4 mol/mol/min), but lower than the value calculated for endogenous PHD2 in a mammalian crude cell extract (MDA-MB-435 cells, 20 mol/mol/min) (Tuckerman *et al.*, FEBS Lett, 2004, 576, 145). The differences may be due to the
15 absence of activating/stabilising factors, or inactivation in the purified PHD2, or a relative lack of product inhibition in crude extracts.

Example 3: Characterisation of PHDs

The characterisation of the regio-selectivity of the PHDs, *i.e.* that they are
20 (*trans*)-prolyl-4-hydroxylases, has relied upon MS and amino acid analyses. Since both 4-hydroxylation and 3-hydroxylation of prolyl residues is known (Kivirikko and Pihlajaniemi, Adv. Enzymol. Relat. Areas Mol. Biol., 1998, **72**, 325; Clifton *et al.*, European Journal of Biochemistry, 2001, **268**, 6625) we wished to confirm the assignments.

25 The availability of large enough quantities (ca. 30mg from 20g of cells) of sufficiently active recombinant hnt-PHD2 enabled the hydroxylation of the HIF₅₅₆₋₅₇₄ peptide, on a sufficient scale (ca. 400µg) to allow its characterisation by ¹H NMR analysis.

To produce HIF₅₅₆₋₅₇₄ that had been hydroxylated by hnt-PHD2, the following
30 reaction was repeated five times. Incubation of 1m DTT (Melford Labs.), 0.6mg/ml catalase (Sigma), 5mM 2-oxoglutarate (Sigma), 4.4mM synthetic HIF₅₅₆₋₅₇₄ fragment, 400µM hnt-PHD2, 1mM iron(II) ammonium sulphate hexahydrate in a final volume of 100µl 50mM HEPES pH7.0 was carried out at 37°C for 30minutes.

The reaction was stopped with an equal volume of methanol added at 4°C.

Precipitated protein was removed by centrifugation and purification of the resulting peptide was carried out as described (McNeill *et al.*, Biochem. J., 2002, 367, 571) on a Jupiter C4 column (250mm x 10mm) in a gradient of acetonitrile in 0.1%

5 trifluoroacetic acid.

In the NMR analysis, assignment of resonances in all peptides was achieved by HSQC and TOCSY experiments and is shown in Table 1. The expected large shifts in the resonances of both the proline γ -proton (3.55ppm) and the proline γ -carbon (45.1ppm) on hydroxylation is accompanied by a shift in the resonance of
10 both the flanking β - and δ - carbons (7.5 and 7.2ppm respectively).

Table 1. Assignment of resonances in synthetic HIF peptides

Position on proline ring	Synthetic HIF ₅₅₆₋₅₇₄		Synthetic HIF ₅₅₆₋₅₇₄ (<i>trans</i> -4-hydroxylated at Pro-564)	
	δ_H	δ_C	δ_H	δ_C
α	4.41	61.4	4.52	60.1
β	1.87, 2.26	30.3	1.87, 2.26	37.8
γ	1.99 (2H)	25.6	4.54	70.7
δ	3.64, 3.75	49.0	3.78 (2H)	56.2

15 For the synthetic HIF₅₅₆₋₅₇₄ fragment (Peptide Protein Research Ltd., Fareham, UK.) and the synthetic hydroxylated HIF₅₅₆₋₅₇₄ fragment (Biopeptide Co. LLC, San Diego, USA), 1.5mg of peptide was dissolved in 300 μ l 90% D₂O/ 10% H₂O + 0.1% 1,4-dioxane, and spectra were recorded on a Bruker DRX500 with a 5mm TBI probe (¹H{¹³C,BB}) at 298K. For the synthetic HIF₅₅₆₋₅₇₄ fragment after
20 incubation with hnt-PHD2, approximately 100 μ g of hplc purified peptide was dissolved in 7 μ l 90% H₂O/ 10% D₂O and the spectrum was recorded on a Bruker AV600 with a 1mm TXI microprobe (¹H{¹³C, ¹⁵N}) at 295K. Reference 1,4-dioxane can be seen in Figure 2(i) at δ_H 3.74ppm, δ_C 68.0ppm. The resonance in Figure 2(i) at δ_H 3.7ppm, δ_C 53.5ppm is an unidentified impurity present in both synthetic samples.
25 Resonances in Figure 2(ii) at δ_H 3.7ppm and δ_C 60.0ppm and 71.0ppm are impurities from the enzyme incubation.

Thus, the comparison with a synthetic standard of HIF₅₅₆₋₅₇₄ peptide, with *trans*-4-hydroxyproline (2*S*, 4*R*) at the position corresponding to the conserved proline in human HIF- α , unequivocally demonstrated that the PHDs catalyse 4-hydroxylation of prolyl residues.

5

Example 4: Synthesis of Inhibitors

The following procedure (Cunliffe *et al.* J. Med. Chem. (1992) **35** 2652-2658) was used to make the diester of NOFD (N-oxalyl-D-phenylalanine).

To a stirred solution of 10 mmol of phenylalanine methyl ester in 10 ml of
10 toluene 10 mmol (1.23 g) methyl oxalylchloride was added and heated to reflux until no further HCl gas evolved (usually 4-6h). The solvent was then evaporated *in vacuo* and the product purified by column chromatography.

δ_H (300 MHz, CDCl₃) : 3.1 [t, CHCH₂Ar], 3.7-3.8 [2xs, OCH₃], 4.85 [q, NHCHCO₂Me], 7-7.25 [br m, CH₂C₆H₅], 7.45 [br s, NH]. δ_C (75 MHz, CDCl₃) :
15 38.0 [t, CH₂Ar], 53.0 [q, CH₂CH(CO₂Me)NH], 54.0-54.1 [2xs, OCH₃], 127.8-135.5 [4xs, C₆H₅], 156.1-171.1 [3xs, CO].

In order to obtain the diacid N-oxalyl derivative of the dimethylester derivative of N-oxalylphenylalanine, the following procedure was used. The ester compound was treated for 60 minutes with a sufficient amount of 2 N aqueous
20 NaOH solution ensuring 1.1 equivalents of sodium hydroxide for the sum of the ester functions to be cleaved in the compound. The reaction was percolated through a column of "Amberlite IR 120 H" ion exchange resin (previously washed with water to about pH 4) and eluted with water until the pH raised to 4 again. The water was evaporated *in vacuo* and the residue dried under vacuum.

25 δ_H (300 MHz, D₂O) : 3.0-3.2 [n, CHCH₂Ar], 4.63 [q, NHCHCO₂Me], 7-7.25 [br m, CH₂C₆H₅]. δ_C (75 MHz, D₂O) : 36.5 [t, CH₂Ar], 54.6 [q, CH₂CH(CO₂Me)NH], 127.5-136.5 [4xs, C₆H₅], 160.0-174.2 [3xs, CO].

Example 5: Activity and Inhibition of truncated PHD2

30 The effect of N-oxalyl amino acid inhibitors (1mM) on the activity of PHD2 in both Tris and HEPES buffers is shown in Figure 3.

Example 6: Requirement for cofactors

In the course of LC-MS analyses on hnt-PHD2 we unexpectedly observed that the highly purified enzyme appeared to catalyse hydroxylation of the HIF₅₅₆₋₅₇₄ peptide without the addition of ferrous iron or 2-OG. To eliminate the possibility that the Ni-affinity column was causing the co-purification of metal or cosubstrate the N-terminally truncated form of PHD2 was expressed in BL21(DE3) *E. coli* cells and purified using a cation exchange column. His tag cleavage followed by gel-filtration chromatography gave protein of > 95% purity by SDS-PAGE analysis (nt-PHD2). LC/MS/MS analysis of HIF₅₅₆₋₅₇₄ following incubation with nt-PHD2, but without the addition of 2-OG, revealed a small amount of oxidised product with chromatographic and spectroscopic properties identical to authentic hydroxyprolyl HIF₅₅₆₋₅₇₄, and that produced by the addition of 2-OG. Crucially, this enzymatic product exhibited an LC retention time and mass spectrum different from those of the two methionine-sulfoxidyl peptide contaminants, visible in solution before and after incubation.

Atomic absorption spectroscopy analyses on the purified nt-PHD2, using a Thermo Electron Corporation, Atomscan16 spectrometer, with nt-PHD2 samples at 7.7µM in 50mM Tris-HCl pH 7.5, indicated that nt-PHD2 contains iron together with low levels of copper. A more accurate determination of the relative amounts of different metals was obtained by micro-PIXE (microbeam particle induced X-ray emission) analysis). This technique enables the simultaneous detection of many elements, and in the case of proteins their quantification by comparison with the number of sulphur atoms calculated from the protein sequence. This analysis revealed that the major metal present in the nt-PHD2 was iron at a ratio of approximately 0.34 iron atoms per protein molecule. Notably, and unexpectedly, zinc was also found to be present at approximately the same ratio of ions per protein molecule.

On exhaustive aerobic dialysis (PHD2 solution (0.5ml, 4.7mg/ml) was dialysed at 4°C into 5L of dialysis buffer for 5 days with one change of buffer of both hnt-PHD2 (into 50mM Tris pH 7.0) and nt-PHD2 (into 50mM HEPES pH 7.0)), the specific activity (incubation at 37°C for 12 minutes) was reduced to approximately 0.2 mol 2-OG/min/mol. However, addition of ascorbate restored the ability of both preparations to catalyse the release of CO₂ from 2-OG, indicating that iron was

probably retained during the dialysis process, in agreement with a low binding constant for Fe(II), but that slow oxidation of Fe(II) to Fe(III) can occur.

EPR analyses (continuous-wave EPR spectra of nt-PHD2 preparations were obtained at a temperature of 50 K and a frequency of 9.757 GHz with a modulation amplitude of 0.5 mT and a microwave power of 2 mW in a Bruker E580 spectrometer) on the nt-PHD2 revealed the presence of signals for Fe(III) ($g = 4.3$) (as seen in other 2-OG oxygenases (de Jong, *Biochim. Biophys. Acta.*, 1982, **704**, 326)) and, probably, type 2 Cu(II) ($g_{\parallel} = 2.285$), as seen in Figure 4a. However, quantitative comparison with standard solutions showed that < 2% of the protein was bound to these ions, leading to the conclusion that the bulk of the metal present was EPR silent. Although the interpretations should be regarded as preliminary, the EPR analyses lead us to conclude that the predominant ion present in the nt-PHD2 is the low-spin form of Fe(II). Since the analyses and purification of nt-PHD2 were carried out under aerobic conditions, it is notable that little ferric iron was observed bound to the nt-PHD2. Addition of 2-OG led to an increase in the Fe(III) EPR signal and a change in hyperfine resolution and in the $g_{\parallel}$ value of the Cu(II) signal to $g_{\parallel} = 2.346$ (Figure 4). The change caused by 2-OG to the Cu(II) signal indicates that the Cu(II) binds to the active site (since if it was bound elsewhere it would not be expected to bind 2-OG). The increase in the Fe(III) signal in the absence of HIF₅₅₆₋₅₇₄, i.e., uncoupled turnover in the presence of 2-OG could be caused by turnover of 2-OG, as preceded for other 2-OG oxygenases. However the change in signal was not strong so, consistent with solution analyses, this indicates that reaction of PHD2 does not proceed rapidly in the presence of 2-OG and ferrous iron alone. A much more significant increase in the Fe(III) signal was observed in the presence of 2-OG and HIF₅₅₆₋₅₇₄ substrate (Figure 4); this was reduced by the addition of ascorbate, consistent with its proposed role in completing uncoupled reaction cycles. As before, the increased Fe(III) signal could be caused by partial uncoupled turnover due to the use of a non-optimal unnatural substrate. Precedent for this proposal comes from studies on other 2-OG oxygenases where both mutations and use of non-optimal substrates have led to uncoupling of 2-OG and prime substrate oxidation.

Electrospray ionisation MS analyses were then carried out with the aim of identifying small organic molecules bound to PHD2. Electrospray ionisation mass spectrometry (ESI-MS) was conducted on a Micromass (now Waters) Q-TOFmicro

quadrupole-time of flight mass spectrometer. For LC/ESI-MS (and LC/ESI-MS/MS) the instrument was coupled to an Agilent 1100 capillary LC system equipped with a Phenomenex Jupiter C4 (15 cm x 500 μ M) column using a gradient of water (0.1 % formic acid) and acetonitrile (0.1 % formic acid) as the mobile phase. For soft ionisation ESI-MS the standard Micromass source was replaced with an Advion BioSciences NanoMateTM chip-based nano-ESI source. Protein samples were sprayed from 10 mM NH₄OAc (pH 7) using a chip nozzle voltage of 1.66 kV, and cone voltages of 20, 80 and 170 V (80 V being the standard value used). Collisional cooling of ions was achieved by partially closing a valve on the rotary vacuum pump, leading to an increased pressure in the intermediate vacuum region of the mass spectrometer. CsI was used for calibration.

Under denaturing conditions the mass of nt-PHD2 was close to that predicted (28052 Da compared with a calculated value of 28058 Da). However, under soft ionisation conditions (spraying from NH₄OAc pH7, with collisional cooling and using chip-based nano-ESI) several ions were observed. The degree of non-covalent complex stability in ESI-MS is a charge state dependent phenomenon, so for clarity and consistency we focused on the $[M+10H]^{10+}$ charge state, the most abundant in the spectrum. At a cone voltage of 80V, ions corresponding to both apo nt-PHD2 (m/z 2806.2) and nt-PHD2 plus *ca.* 56 Da (m/z 2811.8, Figure 5A) were observed. Following from the spectroscopic analyses the latter was assigned as being due to Fe(II) (at least predominantly, as it was not possible to resolve, e.g. Fe- from Cu-protein complexes with the mass spectrometer used). Careful examination of the spectrum also revealed a signal at m/z 2826.4, an ion consistent with the nt-PHD2.Fe(II).2-OG complex. Moreover, addition of 2-OG (10 μ M) to the protein solution led to a clear increase in the intensity of this signal (Figure 5B). Further evidence for the identity m/z 2826.4 was provided by MS/MS analysis. Both with and without addition of 2-OG, the ion fragmented with loss of 146 amu to give m/z 2811.8. Together these data strongly suggest co-purification of Fe(II) and 2-OG with nt-PHD2. We estimate that 50 % of the protein contains metal and *ca.* 5-10% 2-OG. Interestingly, no signal was observed for the nt-PHD2-OG complex without iron, indicating that co-ordination to the metal is a key interaction in 2-OG binding.

Analyses using soft ionisation ESI-MS indicated that the binding constants for Fe(II) and 2-OG (in the presence of Fe(II)) with nt-PHD2 are $\ll 1$ μ M and < 2 μ M

respectively. The binding constant for 2-OG is significantly lower than the previously reported K_m values for PHDs 1, 2 and 3 (at 60 μM , 60 μM and 55 μM respectively). The K_m values may thus not accurately reflect binding constants. Further they were obtained using soluble extract of cells expressing PHD2 and may
5 thus be complicated by endogenous sources of 2-OG and Fe(II).

Our results demonstrate that PHD2 has a high affinity for Fe(II) and 2-OG. Since no binding of 2-OG was observed in the absence of Fe(II) they support a mechanism in which 2-OG binding succeeds that of Fe(II). The high affinity of PHD2 for Fe(II) and 2-OG may reflect the pivotal role of HIF hydroxylases in
10 hypoxic signaling, i.e. in the presence of sufficient concentrations of Fe(II) and 2-OG the enzyme is maintained in a form 'primed' for catalysis.

CLAIMS

- 5 1. A polypeptide comprising an N-terminal truncated form of a 2-oxoglutarate dependent oxygenase, which retains hydroxylase activity.
2. A polypeptide according to claim 1, wherein the 2-oxoglutarate dependent oxygenase is a HIF prolyl-hydroxylase.
- 10 3. A polypeptide according to claim 2, wherein the HIF prolyl-hydroxylase is PHD2.
4. A polypeptide according to claim 3, which comprises:
- 15 (a) a polypeptide of SEQ ID NO: 2 having an N-terminal truncation of at least 50 amino acids; or
- (b) a variant thereof which comprises a polypeptide having at least 80% homology to the polypeptide of (a), which variant has prolyl-hydroxylase activity.
- 20 5. A polypeptide according to claim 4, which comprises:
- (a) a polypeptide of SEQ ID NO: 2 having an N-terminal truncation of between 100 up to 230 amino acids; or
- (b) a variant thereof which comprises a polypeptide having at least 80%
- 25 homology to the polypeptide of (a), which variant has prolyl-hydroxylase activity.
6. A polypeptide according to any one of claims 3 to 5 comprising amino acids 181 to 426 of PHD2 (SEQ ID NO: 3).
- 30 7. A polypeptide according to any one of the preceding claims further comprising a truncation of up to 30 amino acids at the C-terminus.

8. A polynucleotide encoding a polypeptide according to any one of claims 1 to 7.
9. An expression vector comprising a polynucleotide according to claim 8
5 operably linked to a promoter sequence.
10. A host cell comprising a polynucleotide according to claim 8 or a vector according to claim 9.
- 10 11. A method of producing a polypeptide according to any one of claims 1 to 7 comprising introducing a polynucleotide according to claim 8 into a host cell and expressing the polypeptide encoded by the polynucleotide.
12. A method according to claim 9 wherein said host cell is a bacterial cell.
15
13. A method according to claim 12 wherein said host cell is *E.coli*.
14. An assay for hydroxylase activity comprising obtaining a polypeptide according to any one of claims 1 to 7;
20 contacting said polypeptide with a substrate of a 2-OG dependent oxygenase; and
monitoring for hydroxylation of the substrate.
15. An assay according to claim 14, wherein hydroxylation is monitored using a
25 derivatisation reagent capable of forming a fluorescent product with 2-oxoglutarate.
16. An assay for identifying modulators of hydroxylase activity comprising:
contacting a polypeptide according to any one of claims 1 to 7 with a
substrate of a 2-OG dependent oxygenase in the presence of a test agent under
30 conditions suitable for hydroxylase activity; and
monitoring for hydroxylation of the substrate, thereby determining whether
said test agent modulates hydroxylase activity.

Figure 1

```

PHD1 : ----MDSECDQPPLSQALP--QUPGSSSEPEPEPGRARMGVESYLPCPLLPSYHCPGVPSSEASQSDTP----RATAT : 69
PHD2 : MANDSGGGGSEPSERDRQYCELCCKMENLRCRCSRSSFYCKEHQRQDWKKHKLVCQSGALQHGVPHQHSQNP : 79
PHD3 : ----- : -
DAOCS : ----- : -

PHD1 : *STTASPLRDGFGGQDGGELRPLQSEGAANLVTKGCQRLAAQGARPEAEKRWAEEDGGDAPSPSKRPWARQENQE---A : 144
PHD2 : *PAAVPPPRAGAREPRKAAARRDNASGDAAKGVKAKPPADP-AAAASECRAAAGGQGSAAVAEEAEPEGKEEPPARSSLFQ : 157
PHD3 : ----- : -
DAOCS : ----- : -

      ↓
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PHD2 : *EKANLYPPSNTIPQDALSPGGGLRRNGQTKP---HPALN-LALEYVMPCHMKHGLC-FVDDFLQKETGQQIGDEVRAEH : 230
PHD3 : -----MPEGHIMR---EDLEN-LALEYVMPCHHEVGFVYEDMFLGEVVGDCVLERVVKQLHC : 52
DAOCS : -----MDTVPTFSLAELQGLHQDEFRRCLRDKGEFYETDCGLTDTEKSAKDEVIDFF : 55

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PHD2 : DTGKFTDQQLVSQKSDSSKDEFG-----DKIEMI-----EGKEPGCETIGLEMSSDDPIRH : 282
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DAOCS : E-HGSEAEKRAVTSVPPTMRREGFTGUESESTAQITNTGSYSDYSMCYSAGTADNLFPSGDFERIWTQYFDRQYTASRA : 132

PHD1 : *VDAVIRHCAQRLGSYVIN--GRTKAMVA--C--YPS-----NGLQVVRHMDNPHGDRGCTCIKYLNQNWVW : 321
PHD2 : C-----NKKCGSY-----KINGRTKAMVACVPE-----NGLQVVRHMDNPHGDRGCTCIKYLNQNWVW : 337
PHD3 : C-----BSRLQKY-----YVKERSKAMVACVPE-----NGLQVVRHMDNPHGDRGCTCIKYLNQNWVW : 159
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PHD3 : *VHGG--ILRIIEEGHNSFIADVEPIEDRLLEF---WSDRR---NPHEVQ---PAYATRYATVWVYF---DADE : 217
DAOCS : CANGFVSLQAEVGG--AEIDLPYRFDVILVCGAIALTVTGQVQKAPRHVAAPRRDQLAGSEPTSSVFLRPNADFTF : 273

PHD1 : *RAAAKDKYQLASGQKGVQVPVSQPPTPT----- : 407
PHD2 : *RARKVKVLTGEGVRVELNKPSSVQKDYF----- : 426
PHD3 : *RAEAKKERNLTKTESALTED----- : 239
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```

Figure 3

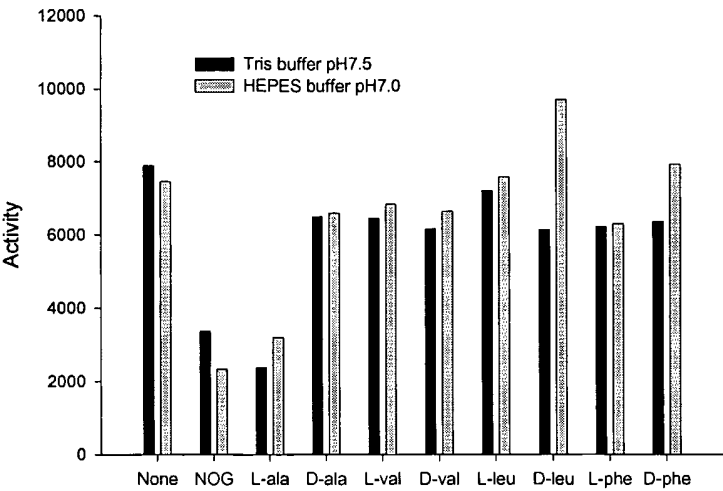
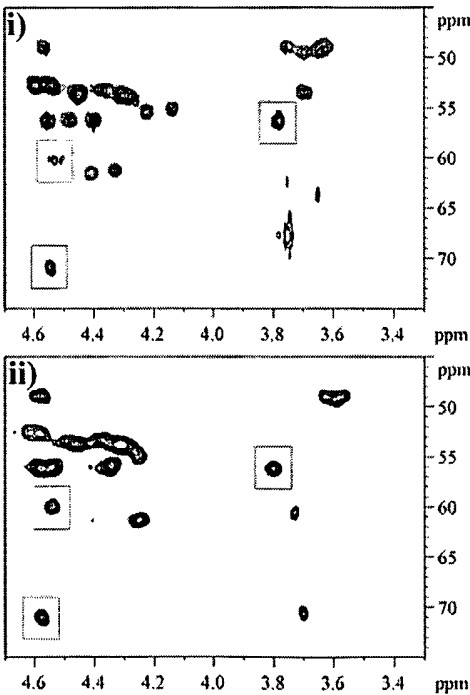
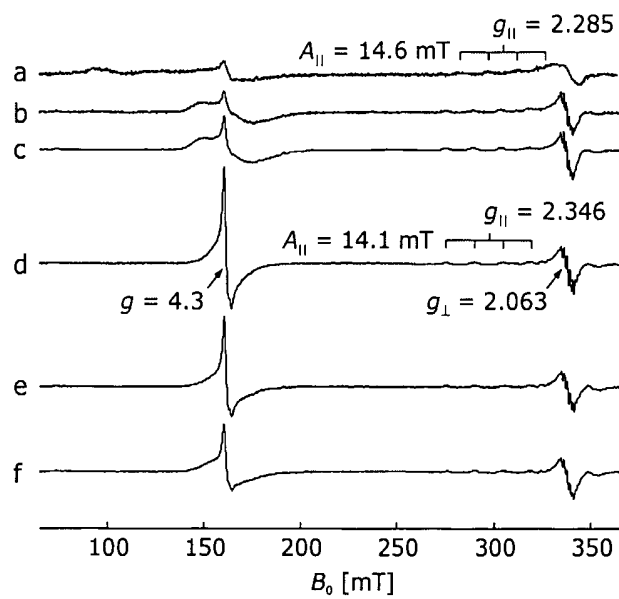
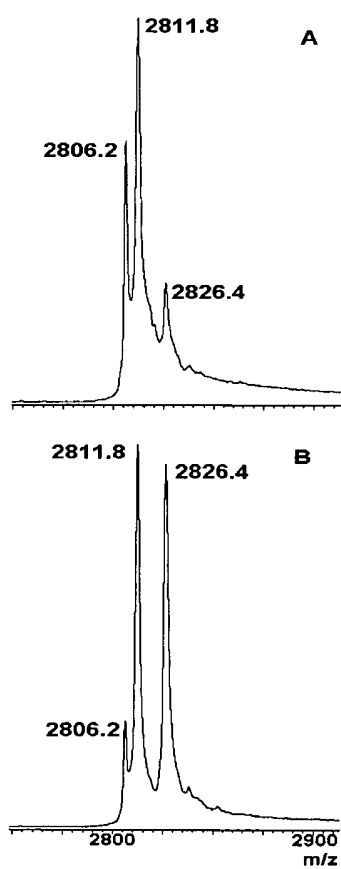


Figure 2



3/3

Figure 4**Figure 5**

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INTERNATIONAL SEARCH REPORT

Inte Application No
PCT/GB2005/002929

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/02 C12Q1/26 C07K14/47

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)

IPC 7 C12N

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 practical, search terms used)

EPO-Internal, Sequence Search, EMBASE, BIOSIS, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/028663 A (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 10 April 2003 (2003-04-10) Whole Document. In particular Table 1 (p.4). Note: PHD2=HPH2. -----	1-16
X	WO 02/074981 A (ISIS INNOVATION LTD; MAXWELL, PATRICK, HENRY; PUGH, CHRISTOPHER, WILLI) 26 September 2002 (2002-09-26) Whole Document; In particular truncated PHDs (p.8-12), N-terminal truncated EGL-9 (p.140), N-terminal truncated SM-20 (p.143-145). ----- -/--	1-16



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

23 September 2005

Date of mailing of the international search report

30/09/2005

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Roscoe, R

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/002929

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAYLOR M S: "Characterization and comparative analysis of the EGLN gene family" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, ELSEVIER SCIENCE PUBLISHERS, BARKING, GB, vol. 275, no. 1, 5 September 2001 (2001-09-05), pages 125-132, XP004307119 ISSN: 0378-1119 the whole document -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB2005/002929

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
WO 03028663	A	10-04-2003	US	6566088 B1	20-05-2003
WO 02074981	A	26-09-2002	EP	1379630 A2	14-01-2004
			JP	2004524848 T	19-08-2004
			US	2004146964 A1	29-07-2004