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(54) Title: STABILIZED PROLACTIN RECEPTOR ANTAGONISTS

(57) Abstract: The invention relates to novel prolactin receptor antagonist compounds, to methods of preparing said compounds, to pharmaceutical compositions comprising these compounds and to the use of the compounds for the treatment of diseases related to proliferative disorders.

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STABILIZED PROLACTIN RECEPTOR ANTAGONISTS

FIELD OF THE INVENTION

The invention relates to novel prolactin receptor antagonist compounds, to methods of preparing said compounds, to pharmaceutical compositions comprising these compounds
5 and to the use of the compounds for the treatment of diseases related to proliferative disorders.

BACKGROUND OF THE INVENTION

It is projected that 1.2 million new cases of breast cancer are diagnosed each year. In 2002 it was estimated that over 40 000 deaths occurred due to breast cancer in the US
10 alone.

Recent evidence suggests that prolactin may play a role as a growth promoting factor for cancer cells (Wennbo *et al.* J. of Clin. Invest. 1997, 100, 2744-2651; Liby, *et al.* Breast Cancer Research and Treatment, 2003, 79, 241-252; Clevenger *et al.* Endocrine Rev. 2003, 24, 1-27).

In vitro experiments have demonstrated that the prolactin receptor antagonist G129R-hPRL shows an additive effect on the inhibition of proliferation of T47D breast cancer cells when combined with tamoxifen (Chen *et al.* Clin. Cancer Res. 1999, 5, 3583). The same compound alone has shown *in vivo* inhibition of T47D tumour xenograft growth (Chen *et al.* Int. J. Oncology, 2002, 20, 813-818).
15

However, high levels of these prolactin receptor antagonists are necessary to obtain effects *in vivo* (Goffin *et al.* Endocrine Rev. 2005, 26, 400-422). It is therefore an object of the invention to provide a prolactin receptor antagonist with an improved affinity for the prolactin receptor in order to reduce the levels required to obtain effects *in vivo*.
20

SUMMARY OF THE INVENTION

The present invention provides a stabilised prolactin receptor antagonist comprising one or more engineered linkers, wherein each linker is formed between two amino acid residues in the prolactin receptor antagonist.
25

The present invention also provides a method of stabilising a prolactin receptor antagonist which comprises the steps of:

- 30 (a) substituting one or more pairs of existing amino acid residues within said antagonist for one or more pairs of cysteine residues; and

- (b) forming one or more disulfide bridges between said one or more pairs of cysteine residues.

The present invention also provides a method of treatment or prophylaxis of a proliferative disorder, which comprises administration of a prolactin receptor antagonist according to the invention.

The present invention also provides the use of a prolactin receptor antagonist in the manufacture of a medicament for the treatment or prophylaxis of a proliferative disorder.

The present invention also provides a pharmaceutical composition comprising a prolactin receptor antagonist for use in the treatment or prophylaxis of a proliferative disorder.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 demonstrates the results of the phospho-STAT3 ELISA described in the Examples. A is PRL(12-199) Q12S G129R, B is PRL(12-199) Q12S L32C I119C G129R, C is PRL(12-199) Q12S L95C E120C G129R, and D is PRL(12-199) Q12S S90C Y147C G129R.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a stabilised prolactin receptor antagonist comprising one or more engineered linkers, wherein each linker is formed between two amino acid residues in the prolactin receptor antagonist.

It will be appreciated that the one or more linkers are positioned to increase the stability of the antagonist. This improved stability may increase the affinity of the antagonist for the prolactin receptor and thus reduce the levels required to obtain the antagonistic effect *in vivo*. In one embodiment, the stabilised prolactin receptor antagonist comprises one linker.

Prolactin (PRL) is a single chain polypeptide of 199 amino acid residues with a molecular weight of about 24,000 Daltons. It is synthesised in the adenohypophysis (anterior pituitary gland), in the breast and in the decidua and has a structure similar to that of growth hormone (GH) and placental lactogen (PL). The molecule is folded due to the activity of three disulfide bonds. The sequence of human prolactin is given in SEQ ID No. 1, the sequence of human growth hormone is given in SEQ ID No. 2 and the sequence of human placental lactogen is given in SEQ ID No. 3.

Human prolactin (hPRL) has two separate and different binding sites (site 1 and site 2) that each interact with a prolactin receptor to form a 1:2 ligand-receptor complex. Proper

ligand-induced receptor dimerisation induces conformational changes in the receptors that bring about activation of the receptor associated Janus family of tyrosine kinases JAK2 or JAK1, which stimulate signal transducers and activators of transcription STAT5 or STAT3, respectively. Receptor activation also leads to the activation of Ras/Raf/MAPK kinase/Erk and phosphatidylinositol 3-kinase/Akt signalling pathways. It is primarily via these pathways that the receptors for these ligands induce cell differentiation, proliferation, and/or survival.

The binding of prolactin is reported to be sequential due to a difference in affinity between the two receptor-binding sites. Thus the higher affinity site (Site 1) interacts with the first receptor, which causes conformational changes in the ligand such that the lower affinity site (Site 2) can interact with the second receptor. This ligand-induced dimerisation of the receptors is essential for hPRL signal transduction.

The term "polypeptide" and "peptide" as used herein means a compound composed of at least five constituent amino acids connected by peptide bonds. The constituent amino acids may be from the group of the amino acids encoded by the genetic code and they may be natural amino acids which are not encoded by the genetic code, as well as synthetic amino acids. Natural amino acids which are not encoded by the genetic code are e.g. hydroxyproline, γ -carboxyglutamate, ornithine, phosphoserine, D-alanine and D-glutamine. Synthetic amino acids comprise amino acids manufactured by chemical synthesis, i.e. D-isomers of the amino acids encoded by the genetic code such as D-alanine and D-leucine, Aib (α -aminoisobutyric acid), Abu (α -aminobutyric acid), Tle (tert-butylglycine), β -alanine, 3-aminomethyl benzoic acid and anthranilic acid.

The term "analogue" as used herein referring to a polypeptide means a modified peptide wherein one or more amino acid residues of the peptide have been substituted by other amino acid residues and/or wherein one or more amino acid residues have been deleted from the peptide and or wherein one or more amino acid residues have been added to the peptide. Such addition or deletion of amino acid residues can take place at the N-terminal of the peptide and/or at the C-terminal of the peptide. All amino acids for which the optical isomer is not stated are to be understood to mean the L-isomer.

The term "prolactin analogue" as used herein refers to an analogue of prolactin, which has the capability of binding to the prolactin receptor. A simple system is used to describe analogues of prolactin. For example, G129R-PRL designates an analogue of prolactin formally derived from prolactin by substituting the naturally occurring amino acid residue Glycine (G) in position 129 with Arginine (R). PRL(9-199) and PRL(12-199) designates an analogue formally derived from PRL by removal of the first 8 or 11 amino acids of the chain.

In one embodiment, the prolactin analogue has an amino acid sequence having at least 80% identity to SEQ ID No. 1. In one embodiment, the prolactin analogue has an amino acid sequence having at least 85%, such as at least 90%, for instance at least 95%, such as for instance at least 99% identity to SEQ ID No. 1.

5 The term "growth hormone analogue" as used herein refers to an analogue of growth hormone, which has the capability of binding to the prolactin receptor. The same system is used to describe analogues of growth hormone as described for prolactin.

10 In one embodiment, the growth hormone analogue has an amino acid sequence having at least 80% identity to SEQ ID No. 2. In one embodiment, the growth hormone analogue has an amino acid sequence having at least 85%, such as at least 90%, for instance at least 95%, such as for instance at least 99% identity to SEQ ID No. 2.

 The term "placental lactogen analogue" as used herein refers to an analogue of placental lactogen, which has the capability of binding to the prolactin receptor. The same system is used to describe analogues of placental lactogen as described for prolactin.

15 In one embodiment, the placental lactogen analogue has an amino acid sequence having at least 80% identity to SEQ ID No. 3. In one embodiment, the placental lactogen analogue has an amino acid sequence having at least 85%, such as at least 90%, for instance at least 95%, such as for instance at least 99% identity to SEQ ID No. 3.

20 The term "identity" as known in the art, refers to a relationship between the sequences of two or more peptides, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between peptides, as determined by the number of matches between strings of two or more amino acid residues. "Identity" measures the percentage of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or
25 computer program (i.e., "algorithms"). Identity of related peptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G.,
30 eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math. 48, 1073 (1988).

35 Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity are described in publicly

available computer programs. Preferred computer program methods to determine identity between two sequences include the GCG program package, including GAP (Devereux et al., Nucl. Acid. Res. 12, 387 (1984); Genetics Computer Group, University of Wisconsin, Madison, Wis.), BLASTP, BLASTN, and FASTA (Altschul et al., J. Mol. Biol. 215, 403-410 (1990)). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (BLAST Manual, Altschul et al. NCB/NLM/NIH Bethesda, Md. 20894; Altschul et al., supra). The well known Smith Waterman algorithm may also be used to determine identity.

For example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, Wis.), two peptides for which the percentage sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3.times. the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually {fraction (1/10)} times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff et al., Atlas of Protein Sequence and Structure, vol. 5, supp.3 (1978) for the PAM 250 comparison matrix; Henikoff et al., Proc. Natl. Acad. Sci USA 89, 10915-10919 (1992) for the BLOSUM 62 comparison matrix) is also used by the algorithm.

Preferred parameters for a peptide sequence comparison include the following:
Algorithm: Needleman et al., J. Mol. Biol. 48, 443-453 (1970); Comparison matrix: BLOSUM 62 from Henikoff et al., PNAS USA 89, 10915-10919 (1992); Gap Penalty: 12, Gap Length Penalty: 4, Threshold of Similarity: 0.

The GAP program is useful with the above parameters. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps) using the GAP algorithm.

In one embodiment, the prolactin analogue has an amino acid sequence, which sequence is at least 80% similar to SEQ ID No. 1. In one embodiment, the prolactin analogue has an amino acid sequence, which sequence is at least 85%, such as at least 90%, for instance at least 95%, such as for instance at least 99% similar to SEQ ID No. 1.

In one embodiment, the growth hormone analogue has an amino acid sequence, which sequence is at least 80% similar to SEQ ID No. 2. In one embodiment, the growth hormone analogue has an amino acid sequence, which sequence is at least 85%, such as at

least 90%, for instance at least 95%, such as for instance at least 99% similar to SEQ ID No. 2.

In one embodiment, the placental lactogen analogue has an amino acid sequence, which sequence is at least 80% similar to SEQ ID No. 3. In one embodiment, the placental lactogen analogue has an amino acid sequence, which sequence is at least 85%, such as at least 90%, for instance at least 95%, such as for instance at least 99% similar to SEQ ID No. 3.

The term "similarity" is a concept related to identity, but in contrast to "identity", refers to a sequence relationship that includes both identical matches and conservative substitution matches. If two polypeptide sequences have, for example, (fraction (10/20)) identical amino acids, and the remainder are all non-conservative substitutions, then the percentage identity and similarity would both be 50%. If, in the same example, there are 5 more positions where there are conservative substitutions, then the percentage identity remains 50%, but the percentage similarity would be 75% ((fraction (15/20))). Therefore, in cases where there are conservative substitutions, the degree of similarity between two polypeptides will be higher than the percentage identity between those two polypeptides.

Conservative modifications of a peptide comprising a given amino acid sequence (and the corresponding modifications to the encoding nucleic acids) will produce peptides having functional and chemical characteristics similar to those of a peptide comprising the given amino acid sequence. In contrast, substantial modifications in the functional and/or chemical characteristics of such peptide as compared to an original peptide may be accomplished by selecting substitutions in the amino acid sequence that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a nonnative residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide may also be substituted with alanine, as has been previously described for "alanine scanning mutagenesis" (see, for example, MacLennan et al., *Acta Physiol. Scand. Suppl.* 643, 55-67 (1998); Sasaki et al., *Adv. Biophys.* 35, 1-24 (1998), which discuss alanine scanning mutagenesis).

Desired amino acid substitutions (whether conservative or non-conservative) may be determined by those skilled in the art at the time such substitutions are desired. For example, amino acid substitutions can be used to identify important residues of the peptides

according to the invention, or to increase or decrease the affinity of the peptides described herein for the receptor in addition to the already described mutations.

Naturally occurring residues may be divided into classes based on common side chain properties:

- 5 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
- 2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- 3) acidic: Asp, Glu;
- 4) basic: His, Lys, Arg;
- 5) residues that influence chain orientation: Gly, Pro; and
- 10 6) aromatic: Trp, Tyr, Phe.

In making such changes, the hydropathic index of amino acids may be considered.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine
15 (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is understood in the art. Kyte et al., J. Mol. Biol. 157, 105-131
20 (1982). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those that are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

25 It is also understood in the art that the substitution of like amino acids may be made effectively on the basis of hydrophilicity, particularly where the biologically functionally equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in the present case. The greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its
30 immunogenicity and antigenicity, i.e., with a biological property of the protein.

The following hydrophilicity values have been assigned to amino acid residues:

arginine (+3.0); lysine (+3.0); aspartate (+3.0 $\pm$ 1); glutamate (+3.0 $\pm$ 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 $\pm$ 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8);
35 isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). In making changes

based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those that are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred. One may also identify epitopes from primary amino acid sequences on the basis of hydrophilicity. These regions are also referred to as

5 "epitopic core regions".

The term prolactin receptor antagonist as used herein refers to a ligand having antagonistic activity at the prolactin receptor, causing it to act as an inhibitor of one or more cellular processes. The ligand may be prolactin, a prolactin analogue or another hormone or analogue with the same capability of binding to the prolactin receptor, e.g. growth hormone (GH) or a growth hormone analogue and placental lactogen (PL) or a placental lactogen analogue. Such prolactin antagonistic activity may be measured by Western blot analysis of the phosphorylation status of STAT5 as set out in Langenheim, J.F. *et al*, Mol Endocrinol, 2006; 20(39):661-674.

Without wishing to be bound by any one theory, it is believed that a prolactin receptor ligand, that comprises one or more mutations that affect the structural integrity of 'Site 2', will not trigger receptor dimerisation, and activate subsequent signal transduction, because it can only bind to one receptor. Thus, such a ligand does not activate the receptor and instead acts as prolactin receptor antagonist.

Six prolactin receptor antagonists are currently known in the literature (Goffin *et al*. Endocrine Rev. 2005, 26, 400-422):

- (a) G120R/K-hGH, a variant of human growth hormone;
- (b) G120R-hPL, a variant of human placental lactogen;
- (c) G129R-hPRL a full-length variant of human prolactin;
- (d) S179D-hPRL, a full-length variant of human prolactin;
- 25 (e) G129R-hPRL (10-199), a truncated variant of human prolactin; and
- (f) G129R-hPRL (15-199), a truncated variant of human prolactin.

The term "first prolactin receptor binding site" as used herein refers to the region of a ligand with a higher affinity site that interacts with the first prolactin receptor. This region of prolactin is well known to those skilled in the art and may be interchangeably known as "Site 1" (Langenheim, J.F. *et al*, Mol Endocrinol, 2006; 20(39):661-674).

The term "second prolactin receptor binding site" as used herein refers to the region of the ligand with a lower affinity site that interacts with the second prolactin receptor. Recently it has been shown that the interaction of Site 1 with the first receptor induces conformational changes in the ligand to create a functional Site 2. This region of prolactin is

well known to those skilled in the art and may be interchangeably known as "Site 2" (Langenheim, J.F. et al, Mol Endocrinol, 2006; 20(39):661-674).

By "engineered linker" is meant one or more chemical bonds, which may be covalent or non-covalent, that link amino acid residues in the prolactin receptor antagonist.

5 The one or more chemical bonds may be created using standard molecular biology techniques and are additional to those which exist in the parent polypeptide. The parent polypeptide should be understood as the polypeptide from which the stabilised prolactin receptor antagonist is derived, specifically human prolactin, human growth hormone or human placental lactogen.

10 In one embodiment, the engineered linker comprises a disulfide bridge between two cysteine residues. It will be appreciated that the amino acid residues are selected such that the disulfide bridge may form under the standard refolding conditions used for prolactin analogues, which are well known to those skilled in the art.

In one embodiment, the one or more linkers are positioned between amino acid residues that are selected using distance and geometry criteria described in Dombkowski A. A., Bioinformatics, 2003, 19, 1852-1853 and Petersen *et al.*, Protein Eng., 1999, 12(7): 535-548.

In one embodiment, the one or more linkers are positioned between a first amino acid residue and a second amino acid residue selected from, but not limited to, the amino acid residues exemplified in Table 1:

Table 1:

	First Amino Acid as defined by sequence alignment with SEQ ID No. 1.	Second Amino Acid as defined by sequence alignment with SEQ ID No. 1.
1.	1	135
2.	22	129
3.	23	186
4.	26	183
5.	32	119
6.	33	175
7.	33	176
8.	33	179

	First Amino Acid as defined by sequence alignment with SEQ ID No. 1.	Second Amino Acid as defined by sequence alignment with SEQ ID No. 1.
9.	36	115
10.	37	172
11.	45	151
12.	57	170
13.	59	148
14.	63	86
15.	66	71
16.	66	72
17.	77	137
18.	78	142
19.	78	138
20.	81	134
21.	82	143
22.	82	144
23.	85	144
24.	86	146
25.	88	127
26.	89	147
27.	90	147
28.	93	150
29.	95	120
30.	99	116
31.	102	113
32.	105	108
33.	138	141
34.	158	164
35.	160	193

It will be appreciated that an existing residue may be substituted for a cysteine residue in the regions or positions as defined hereinbefore in order to facilitate formation of the one or more engineered linkers as required. Insertions of amino acid residues in

peptides can be brought about by standard techniques known to persons skilled in the art, such as post-translational chemical modification or transgenetic techniques.

In one embodiment, one or more disulfide bridges may be formed by the substitution of cysteine residues in the positions selected from, but not limited to, L1C/S135C,

5 A22C/G129C, V23C/L186C, S26C/D183C, L32C/I119C, S33C/L175C, S33C/R176C, S33C/S179C, M36C/K115C, F37C/L172C, T45C/I51C, S57C/N170C, H59C/P148C, L63C/S86C, P66C/Q71C, P66C/A72C, Q77C/V137C, K78C/K142C, K78C/H138C, L81C/V134C, S82C/E143C, S82C/N144C, V85C/N144C, S86C/I146C, L88C/L127C, R89C/Y147C, S90C/Y147C, E93C/W150C, L95C/E120C, V99C/A116C, V102C/L113C,
10 M105C/A108C, H138C/T141C, M158C/R164C, and D160C/S193C.

In one embodiment, the one or more disulfide bridges may be formed by the substitution of cysteine residues in the positions selected from L32C/I119C, S90C/Y147C and L95C/E120C.

In one embodiment, the disulfide bridge may be formed by the substitution of
15 cysteine residues in the position S90C and Y147C.

It will also be appreciated that the prolactin receptor antagonist may only have one functional prolactin receptor binding site. In one embodiment, the linker may be positioned between amino acid residues within or adjacent to the first or second prolactin receptor binding site, such that after conjugation, the first or second binding site, respectively, is
20 disrupted. Alternatively, the prolactin receptor antagonist may comprise one or more mutations that disrupt the 'first' or 'second' binding site.

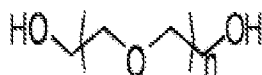
In one embodiment, at least one of the prolactin receptor antagonist may be truncated as compared to the parent polypeptide. The parent polypeptide should here be understood as the polypeptide from which the prolactin receptor antagonist is derived,
25 specifically human prolactin, human growth hormone or human placental lactogen. In one embodiment, at least one of said prolactin receptor binding monomers are PRL (10-199). In one embodiment, at least one of said prolactin receptor binding monomers are PRL (12-199). In one embodiment, at least one of said prolactin receptor binding monomers are PRL (15-199).

30 In one embodiment, the prolactin receptor antagonist may be modified further, for example, by pegylation as described in US 4,179,337 and US2004/0136952, which are incorporated herein by reference. Alternatively, the antagonist may be modified as described in WO 2005/070468, which is also incorporated herein by reference.

The term "PEG" is intended to indicate polyethylene glycol of a molecular weight
35 between approximately 100 and approximately 1,000,000 Da, including analogues thereof,

wherein for instance the terminal OH-group has been replaced by an alkoxy group, such as e.g. a methoxy group, an ethoxy group or a propoxy group. In particular, the PEG wherein the terminal –OH group has been replaced by methoxy is referred to as mPEG.

A PEG has the following structure:



5

PEGylation is the act of adding a PEG structure to another larger molecule, for example, a therapeutic protein (which is then referred to as PEGylated).

PEGylation has been used for the improvement of pharmacokinetic parameters (Pasut *et al.* Expert Opin. Ther. Patents 2004, 14, 859-894).

10

The production of polypeptides is well known in the art. For example, polypeptides may be produced by classical peptide synthesis, e.g. solid phase peptide synthesis using t-Boc or Fmoc chemistry or other well established techniques, see e.g. Green and Wuts, "Protective Groups in Organic Synthesis", John Wiley & Sons, 1999.

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The polypeptides may also be produced by a method which comprises culturing a host cell containing a DNA sequence encoding the polypeptide and capable of expressing the polypeptide in a suitable nutrient medium under conditions permitting the expression of the peptide. For polypeptides comprising non-natural amino acid residues, the recombinant cell should be modified such that the non-natural amino acids are incorporated into the polypeptide, for instance by use of tRNA mutants.

20

The polypeptides may also be produced using cell-free *in vitro* transcription/-translation systems. A polypeptide containing novel unnatural amino acids may also be produced using frameshift or nonsense suppression systems e.g. as described in J. Am. Chem. Soc. 125, 11782-11783 (2003), Science 301, 964-967 (2003), Science 292, 498-500 (2001), Science 303, 371-373 (2004) and references herein.

25

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The peptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration. For extra cellular products the proteinaceous components of the supernatant are isolated by filtration, column chromatography or precipitation, e.g. microfiltration, ultrafiltration, isoelectric precipitation, purification by a variety of

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chromatographic procedures, e.g. ion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography, affinity chromatography, or the like, dependent on the type of polypeptide in question. For intracellular or periplasmic products the cells isolated from the culture medium are disintegrated or permeabilised and extracted to recover the product polypeptide or precursor thereof.

The DNA sequence encoding the polypeptide may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the peptide by hybridisation using specific DNA or RNA probes in accordance with standard techniques (see, for example, Sambrook, J, Fritsch, EF and Maniatis, T, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989). The DNA sequence encoding the polypeptide may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, *Tetrahedron Letters* 22, 1859- 1869 (1981), or the method described by Matthes et al., *EMBO Journal* 3, 801 - 805 (1984). The DNA sequence may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki et al., *Science* 239, 487-491 (1988).

The DNA sequence may be inserted into any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector may be an expression vector in which the DNA sequence encoding the polypeptide is operably linked to additional segments required for transcription of the DNA, such as a promoter. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the peptide of the invention in a variety of host cells are well known in the art, cf. for instance Sambrook et al., *supra*.

The DNA sequence encoding the polypeptide may also, if necessary, be operably connected to a suitable terminator, polyadenylation signals, transcriptional enhancer sequences, and translational enhancer sequences. The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question.

The vector may also comprise a selectable marker, for instance a gene the product of which complements a defect in the host cell or one which confers resistance to a drug, for instance ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For large scale manufacture the selectable marker may for instance not be antibiotic resistance, e.g. antibiotic resistance genes in the vector may be excised when the vector is used for large scale manufacture. Methods for eliminating antibiotic resistance genes from vectors are known in the art, see e.g. US 6,358,705 which is incorporated herein by reference.

To direct a parent peptide of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the peptide in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that normally associated with the peptide or may be from a gene encoding another secreted protein.

The procedures used to ligate the DNA sequences coding for the present peptide, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., supra).

The host cell into which a DNA sequence or recombinant vector is introduced may be any cell which is capable of producing the present peptide and includes bacteria, yeast, fungi and higher eukaryotic cells. Examples of suitable host cells well known and used in the art are, without limitation, *E. coli*, *Saccharomyces cerevisiae*, or mammalian BHK or CHO cell lines. The peptide can also be produced by using in vitro transcription/translation systems commonly known in the art.

A variety of cellular proliferative disorders may be treated or prevented with the compounds described herein. "Proliferative disorder" refers to a disease or disorder characterised by aberrant cell proliferation, for example, where cells divide more than their counterpart normal cells. The aberrant proliferation may be caused by any mechanism of action or combination of mechanism of action. For example, the cell cycle of one or more cells may be affected such that cell(s) divide more frequently than their counterpart normal cells, or as another example, one or more cells may bypass inhibitory signals, which would normally limit their number of divisions. Proliferative disorders include, but are not limited to, carcinomas, sarcomas, leukaemias, neural cell tumours and non-invasive tumours. When

used to inhibit cellular proliferation, a compound may act for instance cytotoxically to kill the cell, or cytostatically to inhibit proliferation without killing the cell.

In one embodiment, the present invention provides a method of treatment or prophylaxis of a proliferative disorder, which comprises administration of the stabilised antagonist as hereinbefore defined. In one embodiment, there is provided a use of the stabilised antagonist as hereinbefore defined in the manufacture of a medicament for the treatment or prevention of a proliferative disorder. In one embodiment, there is provided a pharmaceutical composition comprising the stabilised antagonist as hereinbefore defined for use in the treatment of a proliferative disorder.

The term "treatment" and "treating" as used herein means the management and care of a patient for the purpose of combating a condition, such as a disease or a disorder. The term is intended to include the full spectrum of treatments for a given condition from which the patient is suffering, such as administration of the active compound to alleviate the symptoms or complications, to delay the progression of the disease, disorder or condition, to alleviate or relief the symptoms and complications, and/or to cure or eliminate the disease, disorder or condition as well as to prevent the condition, wherein prevention is to be understood as the management and care of a patient for the purpose of combating the disease, condition, or disorder and includes the administration of the active compounds to prevent the onset of the symptoms or complications. The patient to be treated is preferably a mammal, in particular a human being, but it may also include animals, such as dogs, cats, cows, sheep and pigs.

In one embodiment, the proliferative disorder is a cancer.

Cancers are traditionally classified based on the tissue and cell type from which cancer cells originate. Carcinomas are considered cancers arising from epithelial cells while sarcomas are considered cancers arising from connective tissues or muscle. Other cancer types include leukaemias, which arise from haematopoietic cells, and cancer of nervous system cells, which arise from neural tissue. For non-invasive tumours, adenomas are considered benign epithelial tumours with glandular organisation while chondomas are benign tumours arising from cartilage. According to the present invention, the stabilised antagonist may be used to treat proliferative disorders encompassed by carcinomas, sarcomas, leukaemias, lymphomas, neural cell tumours and non-invasive tumours.

In one embodiment, the stabilised antagonist is used to treat tumours arising from variant tissue types, including, but not limited to, cancers of the bone, breast, respiratory tract (e.g. lung), brain, reproductive organs (e.g. cervix), digestive tract (e.g. gastro-intestinal tract and colorectal tract), urinary tract, bladder, eye, liver, skin, head, neck, thyroid, parathyroid,

kidney, pancreas, blood, ovary, germ/prostate, neuronal tumors and metastatic forms thereof.

In one embodiment, the stabilised antagonist is used to treat estrogen dependent cancer.

5 In one embodiment, said proliferative disorder may include proliferative disorders of the breast, which include, but are not limited to, invasive ductal carcinoma, invasive lobular carcinoma, ductal carcinoma, lobular carcinoma *in situ*, medular carcinoma and metastatic breast cancer.

10 It is to be understood that the descriptions of proliferative disorders is not limited to the conditions described above, but encompasses other disorders characterised by uncontrolled growth and malignancy. It is further understood that proliferative disorders include various metastatic forms of the tumour and cancer types described herein. The compounds of the present invention may be tested for effectiveness against the disorders described herein, and therapeutically effective regimen established. Effectiveness includes
15 reduction or remission of the tumour, decreases in the rate of cell proliferation, induction of apoptosis, induction of cell senescence, or cytostatic or cytotoxic effect on cell growth.

The stabilised antagonist described herein may be used alone, in combination with one another, or as an adjunct to, or in conjunction with, other established anti-proliferative therapies. Thus, the compounds may be used with traditional cancer therapies, such as
20 ionisation radiation in the form of γ -rays and x-rays, delivered externally or internally by implantation of radioactive compounds, and as a follow-up to surgical removal of tumours. In another aspect, the compounds may be used with other chemotherapeutic agents.

The stabilised antagonist described herein may be used in combination with anti-estrogen therapies, inhibitors of growth factor receptors signalling, immunomodulators, anti-
25 angiogenic and anti-lymphogenic therapies.

The stabilised antagonist may also be administered in combination with agents useful to treat other disorders or maladies, such as steroids, membrane stabilisers and other modulators of intracellular signal transduction, protein kinase inhibitors, protein phosphatase inhibitors, cell cycle modulators and apoptosis inducing/modulating agents.

30 Examples of useful anti-cancer compounds are described in Merck Index, 13th Ed. (O'Neil M.J. et al., ed) Merck Publish Group (2001) and Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10th Edition, Hardman, J.G. and Limbird, L.E. eds., pg. 1381-1287, McGraw Hill (1996).

Examples of such combination therapies may include administration of a stabilised antagonist according to the present invention in combination with a medicament useful for treating cancer such as

conventional chemotherapeutic agents, such as anti-metabolites (such as

5 azathioprine, cytarabine, fludarabine phosphate, fludarabine, gemcitabine, cytarabine, cladribine, capecitabine 6-mercaptopurine, 6-thioguanine, methotrexate, 5-fluorouracil, and hydroxyurea) alkylating agents (such as melphalan, busulfan, cis-platin, carboplatin, cyclophosphamide, ifosfamide, dacarbazine, procarbazine, chlorambucil, thiotepa, lomustine, temozolamide) anti-mitotic agents (such as vinorelbine, vincristine, 10 vinblastine, docetaxel, paclitaxel) topoisomerase inhibitors (such as doxorubicin, amsacrine, irinotecan, daunorubicin, epirubicin, mitomycin, mitoxantrone, idarubicin, teniposide, etoposide, topotecan) antibiotics (such as actinomycin and bleomycin) asparaginase, or the anthracyclines or the taxanes;

certain monoclonal antibodies (mAbs), such as Rituximab, Alemtuzumab,

15 Trastuzumab, Gemtuzumab, Gemtuzumab-ozogamicin (Myelotarg ®, Wyeth) Cetuximab (Erbix™), Bevacizumab, HuMax-CD20, HuMax-EGFr, Zamy and Pertuzumab and/or such as an antibody against tissue factor, killer Ig-like receptors (KIR), laminin-5, EGF-R, VEGF-R, PDGF-R, HER-2/neu, or an antibody against a tumor antigen such as PSA, PSCA, CEA, CA125, KSA, etc.;

20 cell cycle control/apoptosis regulators, such as compounds, which target regulators such as (i) cdc-25, (ii) cyclin-dependent kinases that overstimulate the cell cycle (for instance flavopiridol (L868275, HMR1275; Aventis), 7-hydroxystaurosporine (UCN-01, KW-2401; Kyowa Hakko Kogyo) and roscovitine (R-roscovitine, CYC202; Cyclacel)), and (iii) telomerase (such as BIBR1532 and SOT-095, as well as drugs that interfere with apoptotic 25 pathways such as TNF-related apoptosis-inducing ligand (TRAIL)/apoptosis-2 ligand (Apo-2L), antibodies that activate TRAIL receptors, IFN α and anti-sense Bcl-2;

growth factor inhibitors, such as antibodies directed at the extracellular ligand binding domain of receptors of the epidermal growth factor receptor (EGF-R) family, and low molecular weight molecules that inhibit the tyrosine kinase domains of these receptors, for 30 instance Herceptin, cetuximab, Tarceva and Iressa;

inhibitors of tumor vascularisation (anti-angiogenesis drugs and anti-metastatic agents) such as endostatin, angiostatin, antibodies that block factors that initiate angiogenesis (for instance anti-VEGF – Avastin), and low molecular compounds that inhibit angiogenesis by inhibiting key elements in relevant signal transduction pathways;

anti-angiogenesis drugs, such as avastin, neovastat, thalidomide, PTK787, ZK222584, ZD-6474, SU6668, PD547,632, VEGF-Trap, CEP-7055, NM-3, SU11248

hormonal agents, such as estramustine phosphate, polyestradiol phosphate, estradiol, anastrozole, exemestane, letrozole, tamoxi-fen, megestrol acetate,

5 medroxyprogesterone acetate, octreotide, cyproterone acetate, bi-caltumide, flutamide, tritorelin, leuporelin, buserelin or goserelin;

agents that enhance the immune response against tumor cells or virus-infected cells, such as adjuvants, for instance vaccine adjuvants such as QS21, GM-CSF and CpG oligodeoxynucleotides, lipopolysaccharide, polyinosinic:polycytidylic acid, α -galactosylceramide
10 or analogues thereof, histamine dihydrochloride, or aluminum hydroxide;

cytokines, such as IFN- α , IFN- β , IFN- γ , IL-2, PEG-IL-2, IL-4, IL-6, IL-7, IL-12, IL-13, IL-15, IL-18, IL-23, IL-27, IL-28a, IL-28b, IL-29, GM-CSF, Flt3 ligand or stem cell factor or an analogue or derivative of any of these;

cisplatin, tamoxifen, DTIC, carmustine, carboplatin, vinblastine, vindesine, thymosin-
15 α , autologous LAK cells, gemcitabine;

agents that block inhibitory signalling in the immune system, such as mAbs specific for CTLA-4 (anti-CTLA-4), mAbs specific for KIR (anti-KIR), mAbs specific for LIR (anti-LIR), mAbs specific for CD94 (anti-CD94), or mAbs specific for NKG2A (anti-NKG2A);

anti-anergic agents, such as MDX-010 (Phan et al. Proc. Natl. Acad. Sci. USA 100,
20 8372 (2003));

antibodies against an inhibitory receptor expressed on an NK cell, a T cell or a NKT cell;

therapeutic vaccines;

agents that interfere with tumor growth, metastasis or spread of virus-infected cells;

25 and

immunosuppressive / immunomodulatory agents such as agents with influence on T-lymphocyte homing for instance FTY-720, calcineurin inhibitors such as valspodar, PSC 833, TOR-inhibitors, sirolimus, everolimus and rapamycin.

Such combination therapy may also include administration of a stabilised antagonist
30 according to the present invention together with radiotherapy, such as external beam radiation therapy (EBRT) or internal radiotherapy (brachytherapy (BT)), typical radioactive atoms that have been used include radium, Cesium-137, Iridium-192, Americium-241, Gold-198, Cobalt-57, Copper-67, Technetium-99, Iodide-123, Iodide-131 and Indium-111

Such combination therapy may also include administration of a stabilised antagonist
35 according to the present invention together with cellular immunotherapy, which may include

isolation of cells that can stimulate or exert an anti-cancer response from patients, expanding these into larger numbers, and reintroducing them into the same or another patient.

Such combination therapy may also include administration of a stabilised antagonist according to the present invention together with internal vaccination, which refers to drug- or radiation-induced cell death of tumor cells that leads to elicitation of an immune response directed towards (i) said tumor cells as a whole or (ii) parts of said tumor cells including (a) secreted proteins, glycoproteins or other products, (b) membrane-associated proteins or glycoproteins or other components associated with or inserted in membranes and (c) intracellular proteins or other intracellular components.

Such combination therapy may also include administration of a stabilised antagonist according to the present invention together with gene therapy, which includes transfer of genetic material into a cell to transiently or permanently alter the cellular phenotype.

The combination treatment may be carried out in any way as deemed necessary or convenient by the person skilled in the art and for the purpose of this specification, no limitations with regard to the order, amount, repetition or relative amount of the compounds to be used in combination is contemplated.

The stabilised antagonist of the present invention may be generally utilised as the free substance or as a pharmaceutically acceptable salt thereof.

The term "pharmaceutically acceptable salts" refers to salts which are not harmful to the patient. Such salts include pharmaceutically acceptable acid addition salts, pharmaceutically acceptable metal salts, ammonium and alkylated ammonium salts. Acid addition salts include salts of inorganic acids as well as organic acids. Representative examples of suitable inorganic acids include hydrochloric, hydrobromic, hydroiodic, phosphoric, sulfuric, nitric acids and the like. Representative examples of suitable organic acids include formic, acetic, trichloroacetic, trifluoroacetic, propionic, benzoic, cinnamic, citric, fumaric, glycolic, lactic, maleic, malic, malonic, mandelic, oxalic, picric, pyruvic, salicylic, succinic, methanesulfonic, ethanesulfonic, tartaric, ascorbic, pamoic, bismethylene salicylic, ethanedisulfonic, gluconic, citraconic, aspartic, stearic, palmitic, EDTA, glycolic, p-aminobenzoic, glutamic, benzenesulfonic, p-toluenesulfonic acids and the like. Further examples of pharmaceutically acceptable inorganic or organic acid addition salts include the pharmaceutically acceptable salts listed in J. Pharm. Sci. 1977, 66, 2, which is incorporated herein by reference. Examples of metal salts include lithium, sodium, potassium, magnesium salts and the like. Examples of ammonium and alkylated ammonium salts include ammonium, methylammonium, dimethylammonium, trimethylammonium, ethylammonium,

hydroxyethylammonium, diethylammonium, butylammonium, tetramethylammonium salts and the like.

Any salt of a stabilised antagonist according to the present invention, whether a pharmaceutical acceptable salt or not, is intended to be included with the mentioning of a "stabilised antagonist according to the present invention". The invention as presented in the claims thus encompasses the stabilised antagonist as well as any salt thereof, for instance a pharmaceutical salt.

The present invention also provides a pharmaceutical composition comprising the stabilised prolactin receptor antagonist of the invention. The composition may further comprise a buffer system, preservative(s), tonicity agent(s), chelating agent(s), stabilizers and surfactants. The composition may further comprise one or more therapeutic agents active against the same disease state.

In one embodiment of the invention the pharmaceutical composition is an aqueous composition, i.e. composition comprising water. Such composition is typically a solution or a suspension. In a further embodiment of the invention the pharmaceutical composition is an aqueous solution. The term "aqueous composition" is defined as a composition comprising at least 50 % w/w water. Likewise, the term "aqueous solution" is defined as a solution comprising at least 50 %w/w water, and the term "aqueous suspension" is defined as a suspension comprising at least 50 %w/w water.

In another embodiment the pharmaceutical composition is a freeze-dried composition, whereto the physician or the patient adds solvents and/or diluents prior to use.

In another embodiment the pharmaceutical composition is a dried composition (e.g. freeze-dried or spray-dried) ready for use without any prior dissolution.

In a further aspect the invention relates to a pharmaceutical composition comprising an aqueous solution of the stabilised antagonist as hereinbefore defined, and a buffer, wherein said composition has a pH from about 2.0 to about 10.0.

In another embodiment of the invention the pH of the composition is selected from the list consisting of 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, and 10.0.

In a further embodiment of the invention the buffer is selected from the group consisting of sodium acetate, sodium carbonate, citrate, glycylglycine, histidine, glycine, lysine, arginine, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium

phosphate, and tris(hydroxymethyl)-aminomethan, bicine, tricine, malic acid, succinate, maleic acid, fumaric acid, tartaric acid, aspartic acid or mixtures thereof. Each one of these specific buffers constitutes an alternative embodiment of the invention.

5 In a further embodiment of the invention the composition further comprises a pharmaceutically acceptable preservative. The use of a preservative in pharmaceutical compositions is well known to the skilled person. For convenience reference is made to Remington: The Science and Practice of Pharmacy, 20th edition, 2000.

10 In a further embodiment of the invention the composition further comprises an isotonic agent. The use of an isotonic agent in pharmaceutical compositions is well known to the skilled person. For convenience reference is made to Remington: The Science and Practice of Pharmacy, 20th edition, 2000.

15 In a further embodiment of the invention the composition further comprises a chelating agent. The use of a chelating agent in pharmaceutical compositions is well known to the skilled person. For convenience reference is made to Remington: The Science and Practice of Pharmacy, 20th edition, 2000.

In a further embodiment of the invention the composition further comprises a stabiliser. The use of a stabilizer in pharmaceutical compositions is well known to the skilled person. For convenience reference is made to Remington: The Science and Practice of Pharmacy, 20th edition, 2000.

20 In a further embodiment of the invention the composition further comprises a stabilizer selected from the group of high molecular weight polymers or low molecular compounds. In a further embodiment of the invention the stabilizer is selected from polyethylene glycol (e.g. PEG 3350), polyvinyl alcohol (PVA), polyvinylpyrrolidone, carboxy/hydroxycellulose or derivates thereof (e.g. HPC, HPC-SL, HPC-L and HPMC),
25 cyclodextrins, sulphur-containing substances as monothioglycerol, thioglycolic acid and 2-methylthioethanol, and different salts (e.g. sodium chloride). Each one of these specific stabilizers constitutes an alternative embodiment of the invention.

30 In a further embodiment of the invention the composition further comprises a surfactant. The use of a surfactant in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: The Science and Practice of Pharmacy, 20th edition, 2000.

It will be appreciated that following preparation, the composition may be packaged for storage, either in a liquid form, in a frozen state, or in a dried form for later reconstitution into a liquid form or other form suitable for administration to a subject.

By "dried form" is intended the liquid pharmaceutical composition or composition is dried either by freeze drying (i.e., lyophilization; see, for example, Williams and Polli (1984) J. Parenteral Sci. Technol. 38:48-59), spray drying (see Masters (1991) in Spray-Drying Handbook (5th ed; Longman Scientific and Technical, Essez, U.K.), pp. 491-676; Broadhead *et al.* (1992) Drug Devel. Ind. Pharm. 18:1169-1206; and Mumenthaler *et al.* (1994) Pharm. Res. 11:12-20), or air drying (Carpenter and Crowe (1988) Cryobiology 25:459-470; and Roser (1991) Biopharm. 4:47-53).

It is possible that other ingredients may be present in the pharmaceutical composition of the invention. Such additional ingredients may include wetting agents, emulsifiers, antioxidants, bulking agents, tonicity modifiers, chelating agents, metal ions, oleaginous vehicles, proteins (e.g., human serum albumin, gelatine or proteins) and a zwitterion (e.g., an amino acid such as betaine, taurine, arginine, glycine, lysine and histidine). Such additional ingredients, of course, should not adversely affect the overall stability of the pharmaceutical composition of the invention.

Pharmaceutical compositions containing a prolactin receptor antagonist according to the invention may be administered to a patient in need of such treatment at several sites, for example, at topical sites, for example, skin and mucosal sites, at sites which bypass absorption, for example, administration in an artery, in a vein, in the heart, and at sites which involve absorption, for example, administration in the skin, under the skin, in a muscle or in the abdomen.

Administration of pharmaceutical compositions according to the invention may be through several routes of administration, for example, oral, rectal, nasal, pulmonary, topical (including buccal and sublingual), transdermal, intracisternal, intraperitoneal, vaginal and parenteral (including subcutaneous, intramuscular, intrathecal, intravenous and intradermal) route. It will be appreciated that the preferred route will depend on the general condition and age of the subject to be treated, the nature of the condition to be treated and the active ingredient chosen.

Compositions of the current invention may be administered in several dosage forms, for example, as solutions, suspensions, emulsions, microemulsions, multiple emulsion, foams, salves, pastes, plasters, ointments, tablets, coated tablets, rinses, capsules, for example, hard gelatine capsules and soft gelatine capsules, suppositories, rectal capsules, drops, gels, sprays, powder, aerosols, inhalants, eye drops, ophthalmic ointments, ophthalmic rinses, vaginal pessaries, vaginal rings, vaginal ointments, injection solution, *in situ* transforming solutions, for example *in situ* gelling, *in situ* setting, *in situ* precipitating, *in situ* crystallization, infusion solution, and implants.

Compositions of the invention may further be compounded in, or attached to, for example through covalent, hydrophobic and electrostatic interactions, a drug carrier, drug delivery system and advanced drug delivery system in order to further enhance stability of the composition, increase bioavailability, increase solubility, decrease adverse effects, achieve chronotherapy well known to those skilled in the art, and increase patient compliance or any combination thereof. Examples of carriers, drug delivery systems and advanced drug delivery systems include, but are not limited to, polymers, for example cellulose and derivatives, polysaccharides, for example dextran and derivatives, starch and derivatives, poly(vinyl alcohol), acrylate and methacrylate polymers, polylactic and polyglycolic acid and block co-polymers thereof, polyethylene glycols, carrier proteins, for example albumin, gels, for example, thermogelling systems, for example block co-polymeric systems well known to those skilled in the art, micelles, liposomes, microspheres, nanoparticulates, liquid crystals and dispersions thereof, L2 phase and dispersions thereof, well known to those skilled in the art of phase behaviour in lipid-water systems, polymeric micelles, multiple emulsions, self-emulsifying, self-microemulsifying, cyclodextrins and derivatives thereof, and dendrimers.

Compositions of the current invention are useful in the composition of solids, semi-solids, powder and solutions for pulmonary administration of the prolactin receptor antagonist as hereinbefore defined, using, for example a metered dose inhaler, dry powder inhaler and a nebulizer, all being devices well known to those skilled in the art.

Compositions of the current invention are specifically useful in the composition of controlled, sustained, protracting, retarded, and slow release drug delivery systems. More specifically, but not limited to, compositions are useful in composition of parenteral controlled release and sustained release systems (both systems leading to a many-fold reduction in number of administrations), well known to those skilled in the art. Even more preferably, are controlled release and sustained release systems administered subcutaneous. Without limiting the scope of the invention, examples of useful controlled release system and compositions are hydrogels, oleaginous gels, liquid crystals, polymeric micelles, microspheres, nanoparticles.

Methods to produce controlled release systems useful for compositions of the current invention include, but are not limited to, crystallization, condensation, co-crystallization, precipitation, co-precipitation, emulsification, dispersion, high pressure homogenisation, en-capsulation, spray drying, microencapsulating, coacervation, phase separation, solvent evaporation to produce microspheres, extrusion and supercritical fluid processes. General reference is made to Handbook of Pharmaceutical Controlled Release

(Wise, D.L., ed. Marcel Dekker, New York, 2000) and Drug and the Pharmaceutical Sciences vol. 99: Protein Composition and Delivery (MacNally, E.J., ed. Marcel Dekker, New York, 2000).

Parenteral administration may be performed by subcutaneous, intramuscular, intraperitoneal or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a solution or suspension for the administration of the prolactin receptor antagonist in the form of a nasal or pulmonal spray. As a still further option, the pharmaceutical compositions containing the prolactin receptor antagonist of the invention can also be adapted to transdermal administration, e.g. by needle-free injection or from a patch, optionally an iontophoretic patch, or transmucosal, e.g. buccal, administration.

When the prolactin receptor antagonist as described herein or composition thereof is used in combination with a second therapeutic agent active against the same disease state, they may conveniently be administered alone or in combination, in either single or multiple doses, sequentially or simultaneously, by the same route of administration, or by a different route.

The prolactin receptor antagonist, or compositions thereof, will generally be used in an amount effective to achieve the intended result, for example in an amount effective to treat or prevent the particular disease being treated. The compound(s) may be administered therapeutically to achieve therapeutic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying disorder being treated and/or eradication or amelioration of one or more of the systems associated with the underlying disorder. Therapeutic benefit also includes halting or slowing the progression of the disease, regardless of whether improvement is realised.

The exact dosage will depend upon the frequency and mode of administration, the sex, age, weight and general condition of the subject treated, the nature and severity of the condition treated and any concomitant diseases to be treated and other factors evident to those skilled in the art. Determination of the effective dosage is well within the capabilities of those skilled in the art.

When the stabilised prolactin receptor antagonist, or compositions thereof, is used in combination with a second therapeutic agent active against the same disease state the dose of each compound may differ from that when the compound is used alone. Appropriate doses will be readily appreciated by those skilled in the art.

A composition comprising the prolactin receptor antagonist according to the invention can be used for any of the aforementioned purposes.

The following is a non-limiting list of embodiments of the present invention.

Embodiment 1: A stabilised prolactin receptor antagonist comprising one or more engineered linkers, wherein each linker is formed between two amino acid residues in the prolactin receptor antagonist.

5 Embodiment 2: An antagonist according to embodiment 1, wherein the linker comprises a disulfide bridge between two cysteine residues.

 Embodiment 3: An antagonist according embodiment 2, wherein one or more disulfide bridges may be formed by the substitution of cysteine residues in the positions selected from L1C/S135C, A22C/G129C, V23C/L186C, S26C/D183C, L32C/I119C,
10 S33C/L175C, S33C/R176C, S33C/S179C, M36C/K115C, F37C/L172C, T45C/I51C, S57C/N170C, H59C/P148C, L63C/S86C, P66C/Q71C, P66C/A72C, Q77C/V137C, K78C/K142C, K78C/H138C, L81C/V134C, S82C/E143C, S82C/N144C, V85C/N144C, S86C/I146C, L88C/L127C, R89C/Y147C, S90C/Y147C, E93C/W150C, L95C/E120C, V99C/A116C, V102C/L113C, M105C/A108C, H138C/T141C, M158C/R164C, and
15 D160C/S193C.

 Embodiment 4: An antagonist according embodiment 3, wherein one or more disulfide bridges may be formed by the substitution of cysteine residues in the positions selected from L32C/I119C, S90C/Y147C and L95C/E120C.

 Embodiment 5: An antagonist according to any of embodiments 1 to 4 for use in
20 therapy.

 Embodiment 6: An antagonist according to embodiment 5 for use in treating a proliferative disorder.

 Embodiment 7: An antagonist according to embodiment 6, wherein said proliferative disorder is a cancer.

25 Embodiment 8: An antagonist according to embodiment 7, wherein said cancer is selected from an estrogen dependent cancer, breast cancer, prostate cancer, lung cancer, colorectal cancer, head and neck cancer, ovarian cancer, cervical cancer, bladder cancer, pancreatic cancer, gastrointestinal cancer, leukaemia, skin cancer, and lymphoma.

 Embodiment 9: An antagonist according to embodiment 8, wherein said cancer is
30 breast cancer.

 Embodiment 10: A pharmaceutical composition comprising the antagonist according to any of embodiments 1 to 9.

 Embodiment 11: A pharmaceutical composition according to embodiment 10 for use in the treatment or prophylaxis of a proliferative disorder.

Embodiment 12: A pharmaceutical composition according to embodiment 11, wherein said proliferative disorder is a cancer.

Embodiment 13: A pharmaceutical composition according to embodiment 12, wherein said cancer is selected from an estrogen dependent cancer, breast cancer, prostate cancer, lung cancer, colorectal cancer, head and neck cancer, ovarian cancer, cervical cancer, bladder cancer, pancreatic cancer, gastrointestinal cancer, leukaemia, skin cancer, and lymphoma.

Embodiment 14: Use of an antagonist according to any of embodiments 1 to 4 for therapy.

Embodiment 15: Use of an antagonist according to any of embodiments 1 to 4 in the treatment or prophylaxis of a proliferative disorder.

Embodiment 16: Use of an antagonist according to any of embodiments 1 to 4 for the preparation of a pharmaceutical composition for the treatment or prophylaxis of a proliferative disorder.

Embodiment 17: Use according to embodiment 16, wherein said proliferative disorder is a cancer.

Embodiment 18: Use according to embodiment 17, wherein said cancer is selected from an estrogen dependent cancer, breast cancer, prostate cancer, lung cancer, colorectal cancer, head and neck cancer, ovarian cancer, cervical cancer, bladder cancer, pancreatic cancer, gastrointestinal cancer, leukaemia, skin cancer, and lymphoma.

Embodiment 19: A method of treatment or prophylaxis of a proliferative disorder, which comprises administration of the antagonist according to any of embodiments 1 to 4.

Embodiment 20: A method according to embodiment 19, wherein said proliferative disorder is a cancer.

Embodiment 21: A method according to embodiment 20, wherein said cancer is selected from an estrogen dependent cancer, breast cancer, prostate cancer, lung cancer, colorectal cancer, head and neck cancer, ovarian cancer, cervical cancer, bladder cancer, pancreatic cancer, gastrointestinal cancer, leukaemia, skin cancer, and lymphoma.

Embodiment 22: An antagonist according to any of embodiments 1 to 4 for use alone or in combination with anti-estrogen therapies.

Embodiment 23: An antagonist according to any of embodiments 1 to 4 for use alone or in combination with inhibitors of growth factor receptors signalling.

Embodiment 24: An antagonist according to any of embodiments 1 to 4 for use alone or in combination with anti-angiogenesis therapies.

Embodiment 25: An antagonist according to any of embodiments 1 to 4 for use alone or in combination with anti-lymphogenic therapies.

Embodiment 26: An antagonist according to any of embodiments 1 to 4 for use alone or in combination with immunomodulating therapies.

5 Embodiment 27: An antagonist according to any of embodiments 1 to 4 for use alone or in combination with chemotherapeutic agents.

Embodiment 28: An antagonist according to and used in any of embodiments 22 to 27 for treatment of estrogen dependent cancers.

10 Embodiment 29: An antagonist as defined and used in any of embodiments 22 to 27 for treatment of breast cancers.

Embodiment 30: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of prostate cancers.

Embodiment 31: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of lung cancers.

15 Embodiment 32: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of colorectal cancers.

Embodiment 33: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of head and neck cancers.

20 Embodiment 34: An antagonist as defined and used in any of embodiments 22 to 27 for treatment of ovarian cancers.

Embodiment 35: An antagonist as defined and used in any of embodiments 22 to 27 for treatment of cervical cancers.

Embodiment 36: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of bladder cancers.

25 Embodiment 37: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of pancreatic cancers.

Embodiment 38: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of gastro-intestinal cancers.

30 Embodiment 39: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of leukaemia.

Embodiment 40: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of skin cancers.

Embodiment 41: An antagonist as defined and used in any of embodiments 23 to 27 for treatment of lymphomas.

Embodiment 42: A method of stabilising a prolactin receptor antagonist which comprises the steps of:

- (a) substituting one or more pairs of existing amino acid residues within said antagonist for one or more pairs of cysteine residues; and
- 5 (b) forming one or more disulfide bridges between said one or more pairs of cysteine residues.

EXAMPLES

The invention will be further defined by reference to the following examples, which describe the preparation of the various compounds described herein and methods for
10 assaying their biological activity. It will be apparent to those skilled in the art that many modifications, both to the materials and methods may be practiced without departing from the scope of the invention.

Example 1

Protein expression and purification

15 The pET32-a(+) expression vector (Novagen, Madison WI) was used for expression of proteins. Recombinant Ser-hPRLR(1-210), PRL and mutated PRL monomers were produced as inclusion bodies in *Escherichia coli* BL21(DE3) cells co-transfected with pACYCDuet-MetAP plasmid, which express the *E. coli* MetAP protein. Solubilized in 8M urea, 0.1 M Tris, 2-20 mM DTT, pH 8.5 buffer and following refolding by dilution into a 20 mM
20 Tris, 0.05 % Tween 20, pH 8.0. Protein purification was performed using Source30Q ion exchange columns (Amersham Biosciences) followed by a macro-prep Caramic Hydroxyapatite column (BioRad) and a final size-exclusion chromatography on a Sephadex G25 column. PRL receptor was refolded in two dilution steps, first in 0.4M arginine pH 8.5 and then diluted further in 20 mM Tris, 0.05 % Tween 20, pH 8.0.

25 Example 2

Phospho-STAT3 ELISA

T47D cells grown to approximately 80% confluency were detached with trypsin; cell density was adjusted to 5x10⁵/ml in full growth medium (RPMI, 10% FCS, 2 mM L-glutamin, 0.2 U/ml bovine insulin). 200 µl of this suspension was plated per well of a 96-well plate. The
30 next day, growth medium was replaced with 150 µl starvation medium (growth medium omitting 10% FCS). The cells were starved for 24 hours prior to treatment with PRLR binding compounds. PRL and inhibitors were pre-mixed in starvation medium and 50 µl were added

per well to result in 10 nM PRL and varying concentrations of inhibitors indicated at Figure 1. The cells were incubated for 15 min at 37°C in a humidified CO₂ incubator. Medium was removed and the cells were washed with ice-cold PBS. Lysis of cells and ELISA were performed according to BioSource STAT-3 [pY705] phospho ELISA manual.

- 5 The results of the experiment are illustrated in Figure 1, which shows the effect of the various antagonists at 1:1 and 1:10 molecular ratio of hPRL to antagonist. It should be noted that 100% corresponds to STAT3 phosphorylation level in T47D cells 15 min after addition of 20nM hPRL.

Example 3

10 Prolactin receptor binding assessed by surface plasmon resonance measurements (BiaCore)

- The soluble form of the receptor (10 µg/ml in 10 mM sodium acetate, pH 4.0) was injected into a Biacore T100 instrument and coupled to a CM5 sensor chip by amine coupling chemistry. The immobilized level was about 500 RUs of coupled receptor. Prolactin antagonist and Wild Type prolactin (10, 5, 2.5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001 µg in
15 buffer; 20 mM Hepes, pH 7.4, containing 0.1 M NaCl, 2 mM CaCl₂ and 0.005% P20) were then injected over the immobilized receptor for 5 minutes at a flow rate of 30 µl/min, followed by a 10-min dissociation period during which buffer was injected, to assess receptor binding affinity. Regeneration was accomplished with 4.5 M MgCl₂ for 90 sec with a flow rate of 30 µl/min between runs. Data evaluation was performed in Biacore T100 Evaluation Software.

- 20 The results are shown in Table 2.

Table 2

	Prolactin Biacore 1:1 binding [KD (spr)] (nM)
hPRL	174 (4A)
PRL (12-199) Q12S S90C Y147C G129R	139.5 (1A)
PRL (12-199) Q12S L95C E120C G129R	63.61 (1A)
PRL (12-199) Q12S L32C I119C G129R	39.65 (1A)

CLAIMS

1. A stabilised prolactin receptor antagonist comprising one or more engineered linkers, wherein each linker is formed between two amino acid residues in the prolactin receptor antagonist.

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2. An antagonist according to claim 1, wherein the linker comprises a disulfide bridge between two cysteine residues.

3. An antagonist according claim 2, wherein one or more disulfide bridges may be formed by the substitution of cysteine residues in the positions selected from L1C/S135C,

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A22C/G129C, V23C/L186C, S26C/D183C, L32C/I119C, S33C/L175C, S33C/R176C, S33C/S179C, M36C/K115C, F37C/L172C, T45C/I51C, S57C/N170C, H59C/P148C, L63C/S86C, P66C/Q71C, P66C/A72C, Q77C/V137C, K78C/K142C, K78C/H138C, L81C/V134C, S82C/E143C, S82C/N144C, V85C/N144C, S86C/I146C, L88C/L127C, R89C/Y147C, S90C/Y147C, E93C/W150C, L95C/E120C, V99C/A116C, V102C/L113C, M105C/A108C, H138C/T141C, M158C/R164C, and D160C/S193C.

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4. An antagonist according claim 3, wherein one or more disulfide bridges may be formed by the substitution of cysteine residues in the positions selected from L32C/I119C, S90C/Y147C and L95C/E120C.

20

5. An antagonist according to any of claims 1 to 4 for use in therapy.

6. An antagonist according to claim 5 for use in treating a proliferative disorder.

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7. An antagonist according to claim 6, wherein said proliferative disorder is a cancer.

8. An antagonist according to claim 7, wherein said cancer is selected from an estrogen dependent cancer, breast cancer, prostate cancer, lung cancer, colorectal cancer, head and neck cancer, ovarian cancer, cervical cancer, bladder cancer, pancreatic cancer, gastrointestinal cancer, leukaemia, skin cancer, and lymphoma.

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9. An antagonist according to claim 8, wherein said cancer is breast cancer.

10. A pharmaceutical composition comprising the antagonist according to any of claims 1 to 9.
- 5 11. A pharmaceutical composition according to claim 10 for use in the treatment or prophylaxis of a proliferative disorder.
12. A pharmaceutical composition according to claim 11, wherein said proliferative disorder is a cancer.
- 10 13. A pharmaceutical composition according to claim 12, wherein said cancer is selected from an estrogen dependent cancer, breast cancer, prostate cancer, lung cancer, colorectal cancer, head and neck cancer, ovarian cancer, cervical cancer, bladder cancer, pancreatic cancer, gastrointestinal cancer, leukaemia, skin cancer, and lymphoma.
- 15 14. Use of an antagonist according to any of claims 1 to 4 for therapy.
15. Use of an antagonist according to any of claims 1 to 4 in the treatment or prophylaxis of a proliferative disorder.
- 20 16. Use of an antagonist according to any of claims 1 to 4 for the preparation of a pharmaceutical composition for the treatment or prophylaxis of a proliferative disorder.
17. Use according to claim 16, wherein said proliferative disorder is a cancer.
- 25 18. Use according to claim 17, wherein said cancer is selected from an estrogen dependent cancer, breast cancer, prostate cancer, lung cancer, colorectal cancer, head and neck cancer, ovarian cancer, cervical cancer, bladder cancer, pancreatic cancer, gastrointestinal cancer, leukaemia, skin cancer, and lymphoma.
- 30 19. A method of treatment or prophylaxis of a proliferative disorder, which comprises administration of the antagonist according to any of claims 1 to 4.
20. A method according to claim 19, wherein said proliferative disorder is a cancer.

21. A method according to claim 20, wherein said cancer is selected from an estrogen dependent cancer, breast cancer, prostate cancer, lung cancer, colorectal cancer, head and neck cancer, ovarian cancer, cervical cancer, bladder cancer, pancreatic cancer, gastrointestinal cancer, leukaemia, skin cancer, and lymphoma.

5

22. An antagonist according to any of claims 1 to 4 for use alone or in combination with anti-estrogen therapies.

23. An antagonist according to any of claims 1 to 4 for use alone or in combination with inhibitors of growth factor receptors signalling.

10

24. An antagonist according to any of claims 1 to 4 for use alone or in combination with anti-angiogenesis therapies.

25. An antagonist according to any of claims 1 to 4 for use alone or in combination with anti-lymphogenic therapies.

15

26. An antagonist according to any of claims 1 to 4 for use alone or in combination with immunomodulating therapies.

20

27. An antagonist according to any of claims 1 to 4 for use alone or in combination with chemotherapeutic agents.

28. An antagonist according to and used in any of claims 22 to 27 for treatment of estrogen dependent cancers.

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29. An antagonist as defined and used in any of claims 22 to 27 for treatment of breast cancers.

30. An antagonist as defined and used in any of claims 23 to 27 for treatment of prostate cancers.

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31. An antagonist as defined and used in any of claims 23 to 27 for treatment of lung cancers.

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32. An antagonist as defined and used in any of claims 23 to 27 for treatment of colorectal cancers.

33. An antagonist as defined and used in any of claims 23 to 27 for treatment of head and neck cancers.

34. An antagonist as defined and used in any of claims 22 to 27 for treatment of ovarian cancers.

35. An antagonist as defined and used in any of claims 22 to 27 for treatment of cervical cancers.

36. An antagonist as defined and used in any of claims 23 to 27 for treatment of bladder cancers.

37. An antagonist as defined and used in any of claims 23 to 27 for treatment of pancreatic cancers.

38. An antagonist as defined and used in any of claims 23 to 27 for treatment of gastrointestinal cancers.

39. An antagonist as defined and used in any of claims 23 to 27 for treatment of leukaemia.

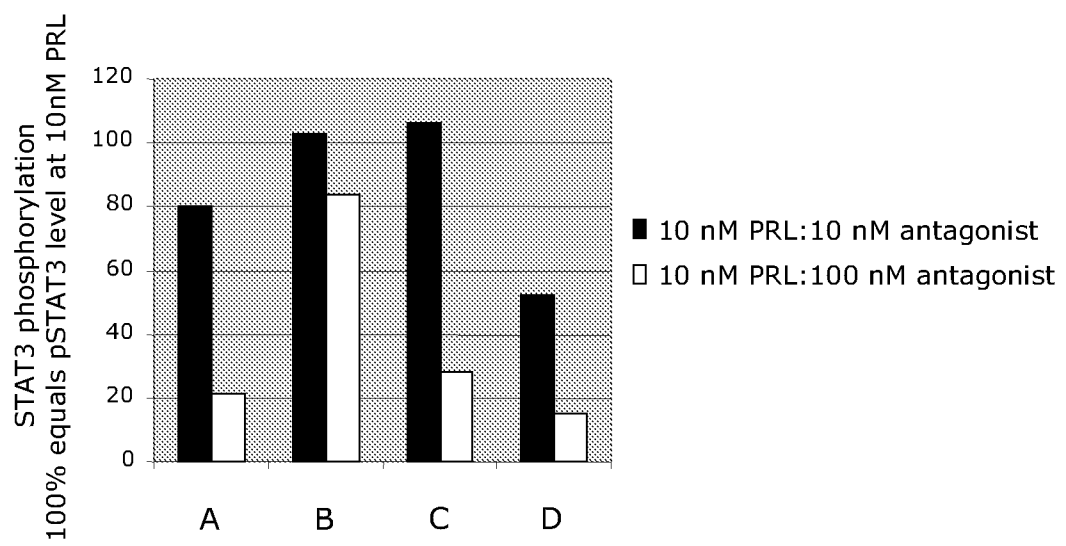
40. An antagonist as defined and used in any of claims 23 to 27 for treatment of skin cancers.

41. An antagonist as defined and used in any of claims 23 to 27 for treatment of lymphomas.

42. A method of stabilising a prolactin receptor antagonist which comprises the steps of:

- (a) substituting one or more pairs of existing amino acid residues within said antagonist for one or more pairs of cysteine residues; and
- (b) forming one or more disulfide bridges between said one or more pairs of cysteine residues.

FIGURE 1/1



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/058591

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/22 C07K14/575

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)
C07K

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, BIOSIS, WPI Data, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FUTAMI JUNICHIRO ET AL: "Stabilization of human RNase 1 by introduction of a disulfide bond between residues 4 and 118" JOURNAL OF BIOCHEMISTRY (TOKYO), vol. 128, no. 2, August 2000 (2000-08), pages 245-250, XP002478063 ISSN: 0021-924X	
A	GOFFIN V ET AL: "Development and potential clinical uses of human prolactin receptor antagonists" ENDOCRINE REVIEWS, BALTIMORE, MD, US, vol. 26, no. 3, 6 April 2005 (2005-04-06), pages 400-422, XP002408797	

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

☐ See patent family annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *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.
- *G* document member of the same patent family

Date of the actual completion of the international search

28 October 2008

Date of mailing of the international search report

07/11/2008

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2008/058591

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 19-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/058591

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DOMBKOWSKI ALAN A: "Disulfide by DesignTM: A computational method for the rational design of disulfide bonds in proteins." BIOINFORMATICS (OXFORD), vol. 19, no. 14, 22 September 2003 (2003-09-22), pages 1852-1853, XP002477809 ISSN: 1367-4803 -----	
A	PETERSEN MARIA TERESA NEVES ET AL: "Amino acid neighbours and detailed conformational analysis of cysteines in proteins" PROTEIN ENGINEERING, vol. 12, no. 7, July 1999 (1999-07), pages 535-548, XP002477810 ISSN: 0269-2139 -----	