

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
2 July 2009 (02.07.2009)

PCT

(10) International Publication Number
WO 2009/081136 A2

(51) International Patent Classification:

G01N 33/566 (2006.01) **C12N 15/10** (2006.01)
C07K 14/72 (2006.01)

(21) International Application Number:

PCT/GB2008/004223

(22) International Filing Date:

19 December 2008 (19.12.2008)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

0724860.2 20 December 2007 (20.12.2007) GB

(71) Applicant (for all designated States except US): **HEPTARES THERAPEUTICS LIMITED** [GB/GB]; 1-3 Burtonhole Lane, Mill Hill, London NW7 1AD (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **WEIR, Malcolm,**

Peter [GB/GB]; Heptares Therapeutic Limited, 1-3 Burtonhole Lane, Mill Hill, London NW7 1AD (GB). **MARSHALL, Fiona, Hamilton** [GB/GB]; Heptares Therapeutic Limited, 1-3 Burtonhole Lane, Mill Hill, London NW7 1AD (GB).

(74) Agent: **THOMAS, Philip**; Potter Clarkson LLP, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).

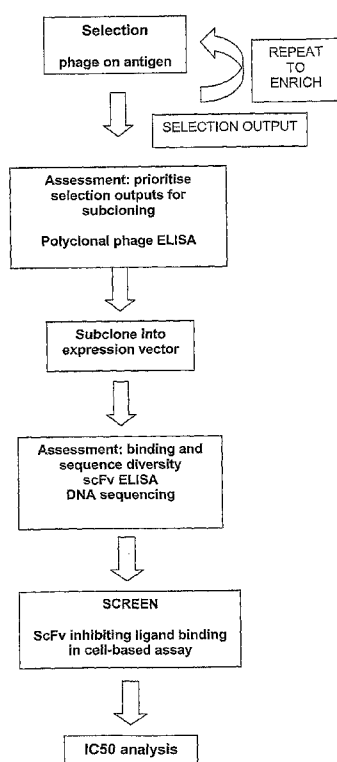
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

[Continued on next page]

(54) Title: SCREENING

Figure 29

Schematic representation of typical lead isolation process for the identification of inhibitory scFv binders



(57) Abstract: A method of producing a conformational specific binding partner of a GPCR, the method comprising: a) providing a mutant GPCR of a parent GPCR, wherein the mutant GPCR has increased stability in a particular conformation relative to the parent GPCR; b) providing a test compound; c) determining whether the test compound binds to the mutant GPCR when residing in a particular conformation; and d) isolating a test compound that binds to the mutant GPCR when residing in the particular conformation. Methods of producing GPCRs with increased stability relative to a parent GPCR are also disclosed.

WO 2009/081136 A2



(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL,

NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

SCREENING

The present invention relates to the screening of binding partners of G-protein coupled receptors (GPCRs) and particularly to conformation specific binding
5 partners of GPCRs.

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

10 GPCRs constitute a very large family of proteins that control many physiological processes and are the targets of many effective drugs. Thus, they are of considerable pharmacological importance. A list of GPCRs is given in Foord *et al* (2005) *Pharmacol Rev.* **57**, 279-288, which is incorporated herein by reference.

15 GPCRs are generally unstable when isolated, and despite considerable efforts, it has only been possible to crystallise bovine rhodopsin, which naturally is exceptionally stable and the beta 2 adrenergic receptor which was crystallised as a fusion protein or in complex with an antibody fragment.

20 GPCRs are druggable targets, and reference is made particularly to Overington *et al* (2006) *Nature Rev. Drug Discovery* **5**, 993-996 which indicates that over a quarter of present drugs have a GPCR as a target.

25 GPCRs are thought to exist in multiple distinct conformations which are associated with different pharmacological classes of ligand such as agonists and antagonists, and to cycle between these conformations in order to function (Kenakin T. (1997) *Ann N Y Acad Sci* **812**, 116-125). Switching between conformations contributes to the difficulty in obtaining crystal structures of receptors.

30 The generation of conformation specific binding partners to GPCRs is hindered by several factors. For example, GPCRs generally have poor stability when removed from their native membrane environment that severely restricts the range of conditions that can be explored without their immediate denaturation or
35 precipitation. The inability to produce purified GPCRs in their native conformation prevents their use in a wide range of screening paradigms which depend on the

use of purified receptors. Thus, GPCR screening has traditionally depended on assays in which the receptor is present in cell membranes or whole cells.

Many GPCRs represent important therapeutic targets which could be exploited by biotherapeutics such as antibodies. The generation of therapeutic antibodies for GPCRs has been extremely difficult due in part to the lack of a suitable immunogen. The usual route is to take small peptide fragments of the receptor for immunization however these do not retain their native conformation and often result in binding partners that can bind to and label the receptor but have no functional agonist or antagonist activity. Due to the unique physical conformation of GPCRs it is also known that biotherapeutics such as antibodies recognise combinations of polypeptide 'loops', features that are lost when peptide fragments are used in isolation. It is well known that the local membrane environment of GPCRs maintains the tertiary conformation of the protein, and governs which epitopes are present on the extracellular surface. These epitopes can in theory be recognised by biotherapeutics, however it is non-trivial to raise biotherapeutics, such as antibodies, to membranes or membrane fragments containing a target GPCR as these preparations inevitably contain other non-target GPCRs and membrane-associated proteins, and other membrane components such as lipoproteins, apolipoproteins, lipids, phosphoinositol lipids and liposacharides which can act as non-desired antigens in the biotherapeutic selection and production process.

We have developed a methodology for the stabilisation of a GPCR in a particular biologically relevant conformation. Such receptors have a number of advantages as immunogens and/or selection and screening reagents for the generation of binding partners. In particular, they are useful for the generation of conformation-specific binding partners, which will frequently have functional properties, and have previously proven very difficult to generate.

For example, the stability of native, correctly folded receptor throughout the expression, solubilisation and purification steps facilitates a high yield of purified GPCR (milligram quantities from lab-scale cell culture). Furthermore, the stability of folded protein in a range of detergents and solubilisation buffers and additives, without distortion of function, enables optimisation of conditions for immunization, for immobilization on solid surfaces without denaturation (e.g. plastic plates,

resins, beads or slides, directly or via affinity tags such as poly-Histidine tags), for the purpose of antibody production and screening or for library screening (such as affibody, antibody, phage or small molecule libraries). For library screening, reduction in non-specific binding by removal of membranous matter and cell-surface "sticky antigens" will give a major improvement in signal/noise. Use of short-chain detergents with highly polar or charged head groups (such as lauryldimethylamine-oxide, octyl D-glucoside or octyl D-maltoside) is also made possible, which will reveal a greater proportion of the antigenic surfaces of the GPCR which are masked by longer-chain detergents such as n-dodecyl beta-D-maltoside (Bamber *et al* PNAS 103 (2006) 16224-16229). The trapping of particular functional conformations of the receptor will also increase the likelihood of generating conformation-specific, functional binding partners.

Accordingly, a first aspect of the invention provides a method of producing a binding partner of a GPCR, the method comprising:

a) providing a mutant GPCR of a parent GPCR, wherein the mutant GPCR has increased stability in a particular conformation relative to the parent GPCR;

b) providing one or more test compounds;

c) determining whether the or each test compound binds to the mutant GPCR when residing in the particular conformation; and

d) isolating those test compounds that bind to the mutant GPCR when residing in the particular conformation.

By "binding partner" we mean a molecule that binds to a particular GPCR. Preferably, the molecule binds selectively to the GPCR. For example, it is preferred if the binding partner has a K_d value (dissociation constant) which is at least five or ten times lower (i.e. higher affinity) than for at least one other GPCR, and preferably more than 100 or 500 times lower. More preferably, the binding partner of a GPCR has a K_d value more than 1000 or 5000 times lower than for at least one other GPCR. However, it will be appreciated that the limits will vary dependent upon the nature of the binding partner. Thus, typically, for small molecule binding partners, the binding partner typically has a K_d value which is at least 50 times or 100 times lower than for at least one other GPCR. Typically, for antibody binding partners, the binding partner typically has a K_d value which is at least 500 or 1000 times lower than for at least one other GPCR.

The use of stabilised GPCRs trapped in particular conformations will increase the likelihood of producing conformation-specific binding partners. Accordingly, it is appreciated that the method may be used to produce a conformation-specific binding partner of a GPCR. Thus, the method may also be used to identify binding partners that have functional activity such as agonists or antagonists (or other pharmacological categories) which will be determined by the conformation in which the GPCR was stabilised.

By "conformation specific", we mean that the binding partner of the GPCR binds selectively to a particular conformation of the GPCR and thus has a K_d value for that conformation which is lower than for other conformations of the same GPCR. Thus, a conformation specific binding partner will bind to one conformation of a GPCR (and may cause the GPCR to adopt this conformation), but does not bind as strongly to another conformation that the GPCR may be able to adopt. It will be appreciated therefore that, while the difference in affinity between two conformations and the conformation specific binding partner may be small, typically it will be sufficient to alter the equilibrium between conformational states and encourage the GPCR to adopt a particular conformation. Thus, a conformational specific binding partner may be considered to be one which traps a GPCR in a conformation of biological relevance (e.g. ligand bound state). Typically, a conformation specific binding partner has a K_d value (dissociation constant) which is at least five or ten times lower (i.e. higher affinity) than for at least one other conformation of the GPCR conformation, and preferably between 100-10000 times lower.

Typically, the conformational specific binding partner binds to the GPCR with a K_d of from mM to pM or from mM to fM, such as in the range from μ M to nM or in the range from nM to pM.

Kd values can be determined readily using methods well known in the art and as described, for example, below.

At equilibrium $K_d = [R][L]/[RL]$

where the terms in brackets represent the concentration of

- Receptor-ligand complexes [RL],
- unbound receptor [R], and
- unbound ("free") ligand [L].

5 In order to determine the K_d the value of these terms must be known. Since the concentration of receptor is not usually known then the Hill-Langmuir equation is used where

$$\text{Fractional occupancy} = \frac{[L]}{[L] + K_d}.$$

10 In order to experimentally determine a K_d then, the concentration of free ligand and bound ligand at equilibrium must be known. Typically, this can be done by using a radio-labelled or fluorescently labelled ligand which is incubated with the receptor (present in whole cells or homogenised membranes) until equilibrium is reached. The amount of free ligand vs bound ligand must then be determined by separating the signal from bound vs free ligand. In the case of a radioligand this can be done by centrifugation or filtration to separate bound ligand present on
15 whole cells or membranes from free ligand in solution. Alternatively a scintillation proximity assay is used. In this assay the receptor (in membranes) is bound to a bead containing scintillant and a signal is only detected by the proximity of the radioligand bound to the receptor immobilised on the bead.

20 The affinity constant may also be determined in a functional assay (K_B). Here the receptor in a whole cell or membrane is activated by an agonist ligand and a response measured (e.g. mobilisation of intracellular calcium, G protein activation, increase or decrease in adenylate cyclase or cAMP, activation of a signal transduction pathway such as a MAP-kinase pathway or activation of gene transcription). The ability of an antagonist to inhibit agonist activity can be
25 measured and for a competitive antagonist is equal to the affinity constant.

The stability of the mutant GPCRs in a range of detergents, surfactants and solubilisation buffers enables their purification outside of their normal membrane environment. Therefore, the GPCR can be provided in an isolated form removed from non-desired antigens such as non-target GPCRs, membrane associated
30 proteins and other membrane components such as lipoproteins, apolipoproteins, lipids, phosphoinositol lipids and liposaccharides. Thus the method of the invention allows for the selection of a binding partner of a GPCR in the absence

of such non-desired antigens. Thus the invention produces binding partners that have enhanced selectivity for a GPCR over other membrane components.

Mutations of the parent GPCR that confer stability in a particular conformation are not expected to affect the binding of the parent GPCR residing in a particular conformation to a particular binding partner. However, it is appreciated that once a binding partner has been isolated by assessing binding to a mutant GPCR residing in a particular conformation, binding of that binding partner to the parent GPCR residing in the same particular conformation may also be assessed.

Thus, in one embodiment the method further comprises:

(e) determining whether the or each test compound binds to the parent GPCR when residing in the particular conformation; and

(f) isolating those test compounds that also bind the parent GPCR when residing in the particular conformation.

Typically, the selected binding partner binds to the mutant GPCR when residing in a particular conformation with a similar potency to its binding to the parent GPCR when residing in the same particular conformation. Typically, the K_d values for the particular binding partner binding the mutant GPCR and the parent GPCR are within 5-10 fold of each other, such as within 2-3 fold. Typically, the binding of the binding partner to the mutant GPCR compared to the parent GPCR would not be more than 5 times weaker and not more than 10 times stronger.

Typically, the selected binding partner binds to the mutant GPCR with approximately equal affinity (that is to say typically within 2-3 fold) or greater affinity than does the parent receptor, when residing in the same conformation. For agonist-conformation mutants, the mutants typically bind the agonist-conformation specific binding partners with the same or higher affinity than the parent GPCR and typically bind antagonist-conformation specific binding partners with the same or lower affinity than the parent GPCR. Similarly for antagonist-conformation mutants, the mutants typically bind the antagonist-conformation specific binding partners with the same or higher affinity than the parent GPCR and typically bind agonist-conformation specific binding partners with the same or lower affinity than the parent GPCR.

Selectivity of binding partners for particular conformations of GPCR or for particular GPCRs and calculation of K_d s can be determined using binding assays well known in the art and as described, for example, below. Typically, K_d values are calculated using conventional GPCR assays in membranes wherein the binding affinity at different compound concentrations is measured. Examples of suitable assays include surface plasmon resonance assays and competition assays which are well known in the art and are described below.

Typically, the mutant GPCR used in the present aspect is selected and prepared using any of the methods as described below.

Providing a mutant GPCR of a parent GPCR

A mutant GPCR with increased stability relative to its parent GPCR can be provided by the methods described below, and by any of the methods disclosed in PCT applications WO 2008/114020 and PCT/GB2008/004032.

Method 1

A method for selecting a mutant G-protein coupled receptor (GPCR) with increased stability, comprises

(a) providing one or more mutants of a parent GPCR,

(b) selecting a ligand, the ligand being one which binds to the parent GPCR when the GPCR is residing in a particular conformation,

(c) determining whether the or each mutant GPCR has increased stability with respect to binding the selected ligand compared to the stability of the parent GPCR with respect to binding that ligand, and

(d) selecting those mutants that have an increased stability compared to the parent GPCR with respect to binding of the selected ligand.

The inventors have appreciated that, in order to improve the likelihood of crystallisation of a GPCR in a biologically relevant form (which is therefore pharmacologically useful), it is desirable not only to increase the stability of the protein, but also for the protein to have this increased stability when in a particular conformation. The conformation is determined by a selected ligand, and is a biologically relevant conformation in particular a pharmacologically relevant conformation. Thus, the method of selection may be considered to be a method for selecting mutants of a GPCR which have increased stability of a particular conformation, for example they may have increased conformational thermostability. The method may be used to create stable, conformationally locked GPCRs by mutagenesis. The selected mutant GPCRs are effectively purer forms of the parent molecules in that a much higher proportion of them occupies a particular conformational state. The deliberate selection of a chosen receptor conformation resolved from other conformations by use of a ligand (or ligands) that bind preferentially to this conformation is therefore an important feature of the selection method. The method may also be considered to be a method for selecting mutant GPCRs which are more tractable to crystallisation. This is because it is well known that decreased homogeneity or increased pleiotropy within a population of molecules does not favour crystallisation, and further, that an increased number of conformations of a particular molecule does not favour crystallisation.

Thus a further method for selecting a mutant G-protein coupled receptor (GPCR) with increased stability, comprises

- (a) providing one or more mutants of a parent GPCR,
- (b) selecting a ligand, the ligand being one which binds to the parent GPCR when the GPCR is residing in a particular conformation,
- (c) determining whether the or each mutant GPCR when residing in the particular conformation has increased stability with respect to binding the selected ligand compared to the stability of the parent GPCR when residing in the same particular conformation with respect to binding that ligand, and

- (d) selecting those mutants that have an increased stability compared to the parent GPCR with respect to binding of the selected ligand.

In a review of the druggable genome by Hopkins & Groom (2002) *Nature Rev. Drug Discovery* 1, 727-730, Table 1 contains a list of protein families many of which are GPCRs. Overington *et al* (2006) *Nature Rev. Drug Discovery* 5, 993-996 provides more details of drug targets, and Figure 1 indicates that more than a quarter of current drugs target GPCRs. There are 52 GPCR targets for orally available drugs out of a total of 186 total targets in this category.

Suitable GPCRs for use in the practice of the invention include, but are not limited to β -adrenergic receptor, adenosine receptor, in particular adenosine A_{2a} receptor, and neurotensin receptor (NTR). Other suitable GPCRs are well known in the art and include those listed in Hopkins & Groom *supra*. In addition, the International Union of Pharmacology produce a list of GPCRs (Foord *et al* (2005) *Pharmacol. Rev.* 57, 279-288, incorporated herein by reference and this list is periodically updated at <http://www.iuphar-db.org/GPCR/ReceptorFamiliesForward>). It will be noted that GPCRs are divided into different classes, principally based on their amino acid sequence similarities. They are also divided into families by reference to the natural ligands to which they bind. All GPCRs are included in the scope of the invention.

The amino acid sequences (and the nucleotide sequences of the cDNAs which encode them) of many GPCRs are readily available, for example by reference to GenBank. In particular, Foord *et al supra* gives the human gene symbols and human, mouse and rat gene IDs from Entrez Gene (<http://www.ncbi.nlm.nih.gov/entrez>). It should be noted, also, that because the sequence of the human genome is substantially complete, the amino acid sequences of human GPCRs can be deduced therefrom.

Although the GPCR may be derived from any source, it is particularly preferred if it is from a eukaryotic source. It is particularly preferred if it is derived from a vertebrate source such as a mammal or a bird. It is particularly preferred if the GPCR is derived from rat, mouse, rabbit or dog or non-human primate or man, or from chicken or turkey. For the avoidance of doubt, we include within the meaning of "derived from" that a cDNA or gene was originally obtained using

genetic material from the source, but that the protein may be expressed in any host cell subsequently. Thus, it will be plain that a eukaryotic GPCR (such as an avian or mammalian GPCR) may be expressed in a prokaryotic host cell, such as *E. coli*, but be considered to be avian- or mammalian-derived, as the case may be.

In some instances, the GPCR may be composed of more than one different subunit. For example, the calcitonin gene-related peptide receptor requires the binding of a single transmembrane helix protein (RAMP1) to acquire its physiological ligand binding characteristics. Effector, accessory, auxiliary or GPCR-interacting proteins which combine with the GPCR to form or modulate a functional complex are well known in the art and include, for example, receptor kinases, G-proteins and arrestins (*Bockaert et al (2004) Curr Opinion Drug Discov and Dev* **7**, 649-657).

The mutants of the parent GPCR may be produced in any suitable way and provided in any suitable form. Thus, for example, a series of specific mutants of the parent protein may be made in which each amino acid residue in all or a part of the parent protein is independently changed to another amino acid residue. For example, it may be convenient to make mutations in those parts of the protein which are predicted to be membrane spanning. The three-dimensional structure of rhodopsin is known (*Li et al (2004) J Mol Biol* **343**, 1409-1438; *Palczewski et al (2000) Science* **289**, 739-745), and it is possible to model certain GPCRs using this structure. Thus, conveniently, parts of the GPCR to mutate may be based on modelling. Similarly, computer programs are available which model transmembrane regions of GPCRs based on hydrophobicity (Kyle & Dolittle (1982) *J. Mol. Biol.* **157**, 105-132), and use can be made of such models when selecting parts of the protein to mutate. Conventional site-directed mutagenesis may be employed, or polymerase chain reaction-based procedures well known in the art may be used. It is possible, but less desirable, to use ribosome display methods in the selection of the mutant protein.

Typically, each selected amino acid is replaced by Ala (ie Ala-scanning mutagenesis), although it may be replaced by any other amino acid. If the selected amino acid is Ala, it may conveniently be replaced by Leu. Alternatively, the amino acid may be replaced by Gly (ie Gly-scanning mutagenesis), which

may allow a closer packing of neighbouring helices that may lock the protein in a particular conformation. If the selected amino acid is Gly, it may conveniently be replaced by Ala.

5 Although the amino acid used to replace the given amino acid at a particular position is typically a naturally occurring amino acid, typically an "encodeable" amino acid, it may be a non-natural amino acid (in which case the protein is typically made by chemical synthesis or by use of non-natural amino-acyl tRNAs). An "encodeable" amino acid is one which is incorporated into a
10 polypeptide by translation of mRNA. It is also possible to create non-natural amino acids or introduce non-peptide linkages at a given position by covalent chemical modification, for example by post-translational treatment of the protein or semisynthesis. These post-translational modifications may be natural, such as phosphorylation, glycosylation or palmitoylation, or synthetic or biosynthetic.

15

Alternatively, the mutants may be produced by a random mutagenesis procedure, which may be of the whole protein or of a selected portion thereof. Random mutagenesis procedures are well known in the art.

20 Conveniently, the mutant GPCR has one replaced amino acid compared to the parent protein (ie it is mutated at one amino acid position). In this way, the contribution to stability of a single amino acid replacement may be assessed. However, the mutant GPCR assayed for stability may have more than one replaced amino acid compared to the parent protein, such as 2 or 3 or 4 or 5 or 6
25 replacements.

As is discussed in more detail below, combinations of mutations may be made based on the results of the selection method. It has been found that in some specific cases combining mutations in a single mutant protein leads to a further
30 increase in stability. Thus, it will be appreciated that the method of selection can be used in an iterative way by, for example, carrying it out to identify single mutations which increase stability, combining those mutations in a single mutant GPCRs which is the GPCR then provided in part (a) of the method. Thus, multiply-mutated mutant proteins can be selected using the method.

35

The parent GPCR need not be the naturally occurring protein. Conveniently, it may be an engineered version which is capable of expression in a suitable host organism, such as *Escherichia coli*. For example, as described in Example 1, a convenient engineered version of the turkey β -adrenergic receptor is one which is truncated and lacks residues 1-33 of the amino acid sequence (ie βAR_{34-424}). The parent GPCR may be a truncated form of the naturally occurring protein (truncated at either or both ends), or it may be a fusion, either to the naturally occurring protein or to a fragment thereof. Alternatively or additionally, the parent GPCR, compared to a naturally-occurring GPCR, may be modified in order to improve, for example, solubility, proteolytic stability (eg by truncation, deletion of loops, mutation of glycosylation sites or mutation of reactive amino acid side chains such as cysteine). In any event, the parent GPCR is a protein that is able to bind to the selected ligand which ligand is one which is known to bind the naturally occurring GPCR. Conveniently, the parent GPCR is one which, on addition of an appropriate ligand, can affect any one or more of the downstream activities which are commonly known to be affected by G-protein activation.

However, it will be appreciated that the stability of the mutant is to be compared to a parent in order to be able to assess an increase in stability.

A ligand is selected, the ligand being one which binds to the parent GPCR when residing in a particular conformation. Typically, the ligand will bind to one conformation of the parent GPCR (and may cause the GPCR to adopt this conformation), but does not bind as strongly to another conformation that the GPCR may be able to adopt. Thus, the presence of the ligand may be considered to encourage the GPCR to adopt the particular conformation. Thus, the method may be considered to be a way of selecting mutant GPCRs which are trapped in a conformation of biological relevance (eg ligand bound state), and which are more stable with respect to that conformation.

Preferably the particular conformation in which the GPCR resides in step (c) corresponds to the class of ligand selected in step (b).

Preferably the selected ligand is from the agonist class of ligands and the particular conformation is an agonist conformation, or the selected ligand is from

the antagonist class of ligands and the particular conformation is an antagonist conformation.

5 Preferably the selected ligand is from the agonist class of ligands and the particular conformation in which the GPCR resides in step (c) is the agonist conformation.

10 Preferably, the selected ligand binding affinity for the mutant receptor should be equal to or greater than that for the wild type receptor; mutants that exhibit significantly reduced binding to the selected ligand are typically rejected.

15 By "ligand" we include any molecule which binds to the GPCR and which causes the GPCR to reside in a particular conformation. The ligand preferably is one which causes more than half of the GPCR molecules overall to be in a particular conformation.

Many suitable ligands are known.

20 Typically, the ligand is a full agonist and is able to bind to the GPCR and is capable of eliciting a full (100%) biological response, measured for example by G-protein coupling, downstream signalling events or a physiological output such as vasodilation. Thus, typically, the biological response is GDP/GTP exchange in a G-protein, followed by stimulation of the linked effector pathway. The measurement, typically, is GDP/GTP exchange or a change in the level of the
25 end product of the pathway (eg cAMP, cGMP or inositol phosphates). The ligand may also be a partial agonist and is able to bind to the GPCR and is capable of eliciting a partial (<100%) biological response.

30 The ligand may also be an inverse agonist, which is a molecule which binds to a receptor and reduces its basal (ie unstimulated by agonist) activity sometimes even to zero.

The ligand may also be an antagonist, which is a molecule which binds to a receptor and blocks binding of an agonist, so preventing a biological response.
35 Inverse agonists and partial agonists may under certain assay conditions be antagonists.

The above ligands may be orthosteric, by which we include the meaning that they combine with the same site as the endogenous agonist; or they may be allosteric or allotropic, by which we include the meaning that they combine with a site distinct from the orthosteric site. The above ligands may be syntopic, by which we include the meaning that they interact with other ligand(s) at the same or an overlapping site. They may be reversible or irreversible.

In relation to antagonists, they may be surmountable, by which we include the meaning that the maximum effect of agonist is not reduced by either pre-treatment or simultaneous treatment with antagonist; or they may be insurmountable, by which we include the meaning that the maximum effect of agonist is reduced by either pre-treatment or simultaneous treatment with antagonist; or they may be neutral, by which we include the meaning the antagonist is one without inverse agonist or partial agonist activity. Antagonists typically are also inverse agonists.

Ligands for use in the selection method may also be allosteric modulators such as positive allosteric modulators, potentiators, negative allosteric modulators and inhibitors. They may have activity as agonists or inverse agonists in their own right or they may only have activity in the presence of an agonist or inverse agonist in which case they are used in combination with such molecules in order to bind to the GPCR.

Neubig *et al* (2003) *Pharmacol. Rev.* **55**, 597-606, incorporated herein by reference, describes various classes of ligands.

Preferably, the above-mentioned ligands are small organic or inorganic moieties, but they may be peptides or polypeptides. Typically, when the ligand is a small organic or organic moiety, it has a M_r of from 50 to 2000, such as from 100 to 1000, for example from 100 to 500.

Typically, the ligand binds to the GPCR with a K_d of from mM to pM, such as in the range of from μ M (micromolar) to nM. Generally, the ligands with the lowest K_d are preferred.

Small organic molecule ligands are well known in the art, for example see the Examples below. Other small molecule ligands include 5HT which is a full agonist at the 5HT_{1A} receptor; eltoprazine which is a partial agonist at the 5HT_{1A} receptor (see Newman-Tancredi *et al* (1997) *Neuropharmacology* **36**, 451-459); (+)-butaclamol and spiperone are dopamine D₂ receptor inverse agonists (see Roberts & Strange (2005) *Br. J. Pharmacol.* **145**, 34-42); and WIN55212-3 is a neutral antagonist of CB₂ (Savinainen *et al* (2005) *Br. J. Pharmacol.* **145**, 636-645).

10 The ligand may be a peptidomimetic, a nucleic acid, a peptide nucleic acid (PNA) or an aptamer. It may be an ion such as Na⁺ or Zn²⁺, a lipid such as oleamide, or a carbohydrate such as heparin.

The ligand may be a polypeptide which binds to the GPCR. Such polypeptides (by which we include oligopeptides) are typically from M_r 500 to M_r 50,000, but may be larger. The polypeptide may be a naturally occurring GPCR-interacting protein or other protein which interacts with the GPCR, or a derivative or fragment thereof, provided that it binds selectively to the GPCR in a particular conformation. GPCR-interacting proteins include those associated with signalling and those associated with trafficking, which often act via PDZ domains in the C terminal portion of the GPCR.

Polypeptides which are known to bind certain GPCRs include any of a G protein, an arrestin, a RGS protein, G protein receptor kinase, a RAMP, a 14-3-3 protein, a NSF, a periplakin, a spinophilin, a GPCR kinase, a receptor tyrosine kinase, an ion channel or subunit thereof, an ankyrin and a Shanks or Homer protein. Other polypeptides include NMDA receptor subunits NR1 or NR2a, calcyon, or a fibronectin domain framework. The polypeptide may be one which binds to an extracellular domain of a GPCR, such as fibulin-1. The polypeptide may be another GPCR, which binds to the selected GPCR in a hetero-oligomer. A review of protein-protein interactions at GPCRs is found in Milligan & White (2001) *Trends Pharmacol. Sci.* **22**, 513-518, or in Bockaert *et al* (2004) *Curr. Opinion Drug Discov. Dev.* **7**, 649-657 incorporated herein by reference.

35 The polypeptide ligand may conveniently be an antibody which binds to the GPCR. By the term "antibody" we include an immunoglobulin whether natural or

partly or wholly synthetically produced. Examples include immunoglobulin isotypes and their isotypic subclasses, and monoclonal antibodies and fragments thereof comprising an antigen binding domains such as Fab, F(ab')₂, single chain Fv (scFv), Fv, domain antibodies (dAbs) and diabodies. Mention is also
5 made of camelid antibodies and engineered camelid antibodies. Such molecules which bind GPCRs are known in the art and in any event can be made using well known technology. Suitable antibodies include ones presently used in radioimmunoassay (RIAs) for GPCRs since they tend to recognise conformational epitopes.

10 The polypeptide may also be a binding protein based on a modular framework, such as ankyrin repeat proteins, armadillo repeat proteins, leucine rich proteins, tetratriopeptide repeat proteins or Designed Ankyrin Repeat Proteins (DARPs) or proteins based on lipocalin or fibronectin domains or Affilin scaffolds based on
15 either human gamma crystalline or human ubiquitin.

In one embodiment of the selection method, the ligand is covalently joined to the GPCR, such as a G-protein or arrestin fusion protein. Some GPCRs (for example thrombin receptor) are cleaved N-terminally by a protease and the new
20 N-terminus binds to the agonist site. Thus, such GPCRs are natural GPCR-ligand fusions.

It will be appreciated that the use of antibodies, or other "universal" binding polypeptides (such as G-proteins which are known to couple with many different
25 GPCRs) may be particularly advantageous in the use of the method on "orphan" GPCRs for which the natural ligand, and small molecule ligands, are not known.

Once the ligand has been selected, it is then determined whether the or each mutant GPCR has increased stability with respect to binding the selected ligand
30 compared to the parent GPCR with respect to binding that ligand. It will be appreciated that this step (c) is one in which it is determined whether the or each mutant GPCR has an increased stability (compared to its parent) for the particular conformation which is determined by the selected ligand. Thus, the mutant GPCR has increased stability with respect to binding the selected ligand
35 as measured by ligand binding or whilst binding the selected ligand. As is

discussed below, it is particularly preferred if the increased stability is assessed whilst binding the selected ligand.

The increased stability is conveniently measured by an extended lifetime of the mutant under the imposed conditions which may lead to instability (such as heat, harsh detergent conditions, chaotropic agents and so on). Destabilisation under the imposed condition is typically determined by measuring denaturation or loss of structure. As is discussed below, this may manifest itself by loss of ligand binding ability or loss of secondary or tertiary structure indicators.

As is described with respect to Figure 12 below (which depicts a particular, preferred embodiment), there are different assay formats which may be used to determine stability of the mutant GPCR.

In one embodiment the mutant GPCR may be brought into contact with a ligand before being subjected to a procedure in which the stability of the mutant is determined (the mutant GPCR and ligand remaining in contact during the test period). Thus, for example, when the method is being used to select for mutant GPCRs which in one conformation bind to a ligand and which have improved thermostability, the receptor is contacted with the ligand before being heated, and then the amount of ligand bound to the receptor following heating may be used to express thermostability compared to the parent receptor. This provides a measure of the amount of the GPCR which retains ligand binding capacity following exposure to the denaturing conditions (eg heat), which in turn is an indicator of stability.

In an alternative (but less preferred) embodiment, the mutant GPCR is subjected to a procedure in which the stability of the mutant is determined before being contacted with the ligand. Thus, for example, when the method is being used to select for mutant membrane receptors which in one conformation bind to a ligand and which have improved thermostability, the receptor is heated first, before being contacted with the ligand, and then the amount of ligand bound to the receptor may be used to express thermostability. Again, this provides a measure of the amount of the GPCR which retains ligand binding capacity following exposure to the denaturing conditions.

In both embodiments, it will be appreciated that the comparison of stability of the mutant is made by reference to the parent molecule under the same conditions.

5 It will be appreciated that in both of these embodiments, the mutants that are selected are ones which have increased stability when residing in the particular conformation compared to the parent protein.

10 The preferred route may be dependent upon the specific GPCR, and will be dependent upon the number of conformations accessible to the protein in the absence of ligand. In the embodiment described in Figure 12, it is preferred if the ligand is present during the heating step because this increases the probability that the desired conformation is selected.

15 From the above, it will be appreciated that the selection method includes a method for selecting a mutant GPCR with increased thermostability, the method comprising (a) providing one or more mutants of a parent GPCR, (b) selecting an antagonist or an agonist which binds the parent GPCR, (c) determining whether the or each mutant has increased thermostability when in the presence of the said antagonist or agonist by measuring the ability of the mutant GPCR to bind
20 the selected said antagonist or agonist at a particular temperature and after a particular time compared to the parent GPCR and (d) selecting those mutant GPCRs that bind more of the selected said antagonist or agonist at the particular temperature and after the particular time than the parent GPCR under the same conditions. In step (c), a fixed period of time at the particular temperature is
25 typically used in measuring the ability of the mutant GPCR to bind the selected said antagonist or agonist. In step (c), typically a temperature and a time is chosen at which binding of the selected said antagonist or agonist by the parent GPCR is reduced by 50% during the fixed period of time at that temperature (which is indicative that 50% of the receptor is inactivated; "quasi" T_m).

30

Conveniently, when the ligand is used to assay the GPCR (ie used to determine if it is in a non-denatured state), the ligand is detectably labelled, eg radiolabelled or fluorescently labelled. In another embodiment, ligand binding can be assessed by measuring the amount of unbound ligand using a secondary
35 detection system, for example an antibody or other high affinity binding partner covalently linked to a detectable moiety, for example an enzyme which may be

used in a colorimetric assay (such as alkaline phosphatase or horseradish peroxidase). FRET methodology may also be used. It will be appreciated that the ligand used to assay the mutant GPCR in determining its stability need not be the same ligand as selected in step (b) of the method.

5

Although it is convenient to measure the stability of the parent and mutant GPCR by using the ability to bind a ligand as an indicator of the presence of a non-denatured protein, other methods are known in the art. For example, changes in fluorescence spectra can be a sensitive indicator of unfolding, either by use of intrinsic tryptophan fluorescence or the use of extrinsic fluorescent probes such as 1-anilino-8-naphthaleneulfonate (ANS), for example as implemented in the Thermofluor™ method (Mezzasalma et al, J Biomol Screening, 2007, Apr;12(3):418-428). Proteolytic stability, deuterium/hydrogen exchange measured by mass spectrometry, blue native gels, capillary zone electrophoresis, circular dichroism (CD) spectra and light scattering may also be used to measure unfolding by loss of signals associated with secondary or tertiary structure. However, all these methods require the protein to be purified in reasonable quantities before they can be used (eg high pmol/nmol quantities), whereas the method described in the Examples makes use of pmol amounts of essentially unpurified GPCR.

20

In a preferred embodiment, in step (b) two or more ligands of the same class are selected, the presence of each causing the GPCR to reside in the same particular conformation. Thus, in this embodiment, one or more ligands (whether natural or non-natural) of the same class (eg full agonist or partial agonist or antagonist or inverse agonist) may be used. Including multiple ligands of the same class in this process, whether in series or in parallel, minimises the theoretical risk of inadvertently engineering and selecting multiply mutated receptor conformations substantially different to the parent, for example in their binding site, but still able, due to compensatory changes, to bind ligand. The following steps may be used to mitigate this risk:

25

30

1. Select a chemically distinct set (eg $n=2-5$) of ligands, in a common pharmacological class as evidenced by for example a binding or functional or spectroscopic assay. These ligands should be thought to bind to a common spatial region of the receptor, as evidenced for example by competitive binding

35

studies using wild type and/or mutated receptors, and/or by molecular modelling, although they will not necessarily express a common pharmacophore.

2. Make single or multiple receptor mutants intended to increase stability, and assay for tight binding using the full set of ligands. The assays can be parallelised, multiplexed or run in series.

3. Confirm authenticity of stabilised receptor mutant by measurement for example of the binding isotherm for each ligand, and by measurement of the stability shift with ligand (the window should typically be narrowed compared to wild type).

In order to guard against changes in apparent affinity caused by perturbations to the binding site upon mutation, preferably ligands of the same pharmacological class, but different chemical class, should be used to profile the receptor. These should typically show similar shifts in affinity (mutant versus parent, e.g. wild type) in spite of having different molecular recognition properties. Binding experiments should preferably be done using labelled ligand within the same pharmacological class.

Nonetheless it should be recognised that conformational substrates may exist that are specific to chemical classes of ligand within the same pharmacological class, and these may be specifically stabilised in the procedure depending on the chemical class of the selected ligand.

Typically the selected ligand binds to the mutant GPCR with a similar potency to its binding to the parent GPCR. Typically, the K_d values for the particular ligand binding the mutant GPCR and the parent GPCR are within 5-10 fold of each other, such as within 2-3 fold. Typically, the binding of the ligand to the mutant GPCR compared to the parent GPCR would be not more than 5 times weaker and not more than 10 times stronger.

Typically, mutant receptors which have been stabilised in the selected conformation should bind the selected ligand with approximately equal affinity (that is to say typically within 2-3 fold) or greater affinity than does the parent receptor. For agonist-conformation mutants, the mutants typically bind the

agonists with the same or higher affinity than the parent GPCR and typically bind antagonists with the same or lower affinity than the parent GPCR. Similarly for antagonist-conformation mutants, the mutants typically bind the antagonists with the same or higher affinity than the parent GPCR and typically bind agonists with
5 the same or lower affinity than the parent GPCR.

Mutants that exhibit a significant reduction (typically greater than 2-3 fold) in affinity for the selecting ligand are typically rejected.

10 Typically, the rank order of binding of a set of ligands of the same class are comparable, although there may be one or two reversals in the order, or there may be an out-lier from the set.

In a further embodiment, two or more ligands that bind simultaneously to the
15 receptor in the same conformation may be used, for example an allosteric modulator and orthosteric agonist.

For the avoidance of doubt, and as is evident from the Examples, it is not necessary to use multiple ligands for the method to be effective.

20

In a further embodiment, it may be advantageous to select those mutant GPCRs which, while still being able to bind the selected ligand, are not able to bind, or bind less strongly than the parent GPCR, a second selected ligand which is in a different class to the first ligand. Thus, for example, the mutant GPCR may be
25 one that is selected on the basis that it has increased stability with respect to binding a selected antagonist, but the mutant GPCR so selected is further tested to determine whether it binds to a full agonist (or binds less strongly to a full agonist than its parent GPCR). Mutants are selected which do not bind (or have reduced binding of) the full agonist. In this way, further selection is made of a
30 GPCR which is locked into one particular conformation.

It will be appreciated that the selected ligand (with respect to part (b) of the method) and the further (second) ligand as discussed above, may be any pair of ligand classes, for example: antagonist and full agonist; full agonist and
35 antagonist; antagonist and inverse agonist; inverse agonist and antagonist; inverse agonist and full agonist; full agonist and inverse agonist; and so on.

It is preferred that the mutant receptor binds the further (second) ligand with an affinity which is less than 50% of the affinity the parent receptor has for the same further (second) ligand, more preferably less than 10% and still more preferably less than 1% or 0.1% or 0.01% of affinity for the parent receptor. Thus, the K_d for the interaction of the second ligand with mutant receptor is higher than for the parent receptor. As is shown in Example 1, the mutant β -adrenergic receptor β AR-m23 (which was selected by the selection method using an antagonist) binds an agonist 3 orders of magnitude more weakly than its parent (ie K_d is 1000 x higher). Similarly, in Example 2, the mutant adenosine A2a receptor Rant21 binds agonist 2-4 orders of magnitude more weakly than its parent.

This type of counter selection is useful because it can be used to direct the mutagenesis procedure more specifically (and therefore more rapidly and more efficiently) along a pathway towards a pure conformation as defined by the ligand.

Preferably, the mutant GPCR is provided in a suitable solubilised form in which it maintains structural integrity and is in a functional form (eg is able to bind ligand). An appropriate solubilising system, such as a suitable detergent (or other amphipathic agent) and buffer system is used, which may be chosen by the person skilled in the art to be effective for the particular protein. Typical detergents which may be used include, for example, dodecylmaltoside (DDM) or CHAPS or octylglucoside (OG) or many others. It may be convenient to include other compounds such as cholesterol hemisuccinate or cholesterol itself or heptane-1,2,3-triol. The presence of glycerol or proline or betaine may be useful. It is important that the GPCR, once solubilised from the membrane in which it resides, must be sufficiently stable to be assayed. For some GPCRs, DDM will be sufficient, but glycerol or other polyols may be added to increase stability for assay purposes, if desired. Further stability for assay purposes may be achieved, for example, by solubilising in a mixture of DDM, CHAPS and cholesterol hemisuccinate, optionally in the presence of glycerol. For particularly unstable GPCRs, it may be desirable to solubilise them using digitonin or amphipols or other polymers which can solubilise GPCRs directly from the membrane, in the absence of traditional detergents and maintain stability typically by allowing a significant number of lipids to remain associated with the

GPCR. Nanodiscs may also be used for solubilising extremely unstable membrane proteins in a functional form.

Typically, the mutant GPCR is provided in a crude extract (eg of the membrane fraction from the host cell in which it has been expressed, such as *E. coli*). It may be provided in a form in which the mutant protein typically comprises at least 75%, more typically at least 80% or 85% or 90% or 95% or 98% or 99% of the protein present in the sample. Of course, it is typically solubilised as discussed above, and so the mutant GPCR is usually associated with detergent molecules and/or lipid molecules.

A mutant GPCR may be selected which has increased stability to any denaturant or denaturing condition such as to any one or more of heat, a detergent, a chaotropic agent or an extreme of pH.

In relation to an increased stability to heat (ie thermostability), this can readily be determined by measuring ligand binding or by using spectroscopic methods such as fluorescence, CD or light scattering at a particular temperature. Typically, when the GPCR binds to a ligand, the ability of the GPCR to bind that ligand at a particular temperature may be used to determine thermostability of the mutant. It may be convenient to determine a "quasi T_m " ie the temperature at which 50% of the receptor is inactivated under stated conditions after incubation for a given period of time (eg 30 minutes). Mutant GPCRs of higher thermostability have an increased quasi T_m compared to their parents.

In relation to an increased stability to a detergent or to a chaotrope, typically the GPCR is incubated for a defined time in the presence of a test detergent or a test chaotropic agent and the stability is determined using, for example, ligand binding or a spectroscopic method as discussed above.

In relation to an extreme of pH, a typical test pH would be chosen (eg in the range 4.5 to 5.5 (low pH) or in the range 8.5 to 9.5 (high pH)).

Because relatively harsh detergents are used during crystallisation procedures, it is preferred that the mutant GPCR is stable in the presence of such detergents. The order of "harshness" of certain detergents is DDM, $C_{11} \rightarrow C_{10} \rightarrow C_9 \rightarrow C_8$

maltoside or glucoside, lauryldimethylamine oxide (LDAO) and SDS. It is particularly preferred if the mutant GPCR is more stable to any of C₉ maltoside or glucoside, C₈ maltoside or glucoside, LDAO and SDS, and so it is preferred that these detergents are used for stability testing.

5

Because of its ease of determination, it is preferred that thermostability is determined, and those mutants which have an increased thermostability compared to the parent protein with respect to the selected condition are chosen. It will be appreciated that heat is acting as the denaturant, and this can readily be removed by cooling the sample, for example by placing on ice. It is believed that thermostability may also be a guide to the stability to other denaturants or denaturing conditions. Thus, increased thermostability is likely to translate into stability in denaturing detergents, especially those that are more denaturing than DDM, eg those detergents with a smaller head group and a shorter alkyl chain and/or with a charged head group. We have found that a thermostable GPCR is also more stable towards harsh detergents.

10

15

When an extreme of pH is used as the denaturing condition, it will be appreciated that this can be removed quickly by adding a neutralising agent. Similarly, when a chaotrope is used as a denaturant, the denaturing effect can be removed by diluting the sample below the concentration in which the chaotrope exerts its chaotropic effect.

20

In a particular embodiment of the selection method, the GPCR is β -adrenergic receptor (for example from turkey) and the ligand is dihydroalprenolol (DHA), an antagonist.

25

In a further preferred embodiment of the selection method, the GPCR is the adenosine A_{2a} receptor (A_{2a}R) (for example, from man) and the ligand is ZM 241385 (4-[2-[[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3- α][1,3,5]triazin-5-yl]amino]ethyl]phenol), an antagonist or NECA (5'-N-ethylcarboxamido adenosine), an agonist.

30

In a still further preferred embodiment of the selection method, the GPCR is the neurotensin receptor (NTR) (for example, from rat) and the ligand is neurotensin, an agonist.

35

Method 2

A method for preparing a mutant GPCR selected as above comprises:

5

(a) carrying out a method to select a mutant GPCR as described above,

10

(b) identifying the position or positions of the mutated amino acid residue or residues in the mutant GPCR or GPCRs which has been selected for increased stability, and

(c) synthesising a mutant GPCR which contains a mutation at one or more of the positions identified.

15

As can be seen in the Examples, surprisingly, changes to a single amino acid within the GPCR may increase the stability of the protein compared to the parent protein with respect to a particular condition in which the protein resides in a particular conformation. Thus, in one embodiment of the method of the second aspect of the invention, a single amino acid residue of the parent protein is changed in the mutant protein. Typically, the amino acid residue is changed to the amino acid residue found in the mutant tested in the method of the first aspect of the invention. However, it may be replaced by any other amino acid residue, such as any naturally-occurring amino acid residue (in particular, a
20 “codeable” amino acid residue) or a non-natural amino acid. Generally, for convenience, the amino acid residue is replaced with one of the 19 other codeable amino acids. Preferably, it is the replaced amino acid residue which is present in the mutant selected in the selection method described above.

25

Also as can be seen in the Examples, a further increase in stability may be obtained by replacing more than one of the amino acids of the parent protein. Typically, each of the amino acids replaced is one which has been identified using the selection method described above. Typically, each amino acid identified is replaced by the amino acid present in the mutant protein although, as
30 noted above, it may be replaced with any other amino acid.

35

Typically, the mutant GPCR contains, compared to the parent protein, from 1 to 10 replaced amino acids, preferably from 1 to 8, typically from 2 to 6 such as 2, 3, 4, 5 or 6 replaced amino acids.

- 5 It will be appreciated that the multiple mutants may be subject to the selection method. In other words, multiple mutants may be provided in step (a) of the selection. It will be appreciated that multiply mutagenised GPCRs may be made, whose conformation has been selected to create a very stable multiple point mutant protein.

10

- The mutant GPCRs may be prepared by any suitable method. Conveniently, the mutant protein is encoded by a suitable nucleic acid molecule and expressed in a suitable host cell. Suitable nucleic acid molecules encoding the mutant GPCR may be made using standard cloning techniques, site-directed mutagenesis and
- 15 PCR as is well known in the art. Suitable expression systems include constitutive or inducible expression systems in bacteria or yeasts, virus expression systems such as baculovirus, semliki forest virus and lentiviruses, or transient transfection in insect or mammalian cells. Suitable host cells include *E. coli*, *Lactococcus lactis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces*
- 20 *pombe*, *Pichia pastoris*, *Spodoptera frugiperda* and *Trichoplusiani* cells. Suitable animal host cells include HEK 293, COS, S2, CHO, NSO, DT40 and so on. It is known that some GPCRs require specific lipids (eg cholesterol) to function. In that case, it is desirable to select a host cell which contains the lipid. Additionally or alternatively the lipid may be added during isolation and purification of the
- 25 mutant protein. It will be appreciated that these expression systems and host cells may also be used in the provision of the mutant GPCR in part (a) of the selection method.

- Molecular biological methods for cloning and engineering genes and cDNAs, for
- 30 mutating DNA, and for expressing polypeptides from polynucleotides in host cells are well known in the art, as exemplified in "Molecular cloning, a laboratory manual", third edition, Sambrook, J. & Russell, D.W. (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, incorporated herein by reference.

- 35 It is appreciated that it may be determined whether the selected or prepared mutant GPCR is able to couple to a G protein. It is also preferred if it is

determined whether the selected or prepared mutant GPCR is able to bind a plurality of ligands of the same class as the selecting ligand with a comparable spread and/or rank order of affinity as the parent GPCR.

5 Method 3

As shown in Examples 1-3 and described above, thermostabilising mutations are scattered widely throughout the sequences of the turkey beta1 adrenergic receptor, human adenosine receptor, rat neurotensin receptor and human
10 muscarinic receptor. Figure 17 provides an alignment of these sequences with the sequence of the human beta-2AR such that when the thermostabilising mutations are positioned onto the sequences then, in 11 instances out of a total of 70, two sequences contain mutations at the same position (denoted in Figure 17 with a star). Thus it will be appreciated that once one or more stabilising
15 mutations have been identified in one GPCR, a further GPCR with increased stability can be generated by aligning the amino acid sequences of the GPCRs and making the same one or more mutations at the corresponding position or positions. This concept is clearly exemplified in Figure 26 wherein the six thermostabilising mutations in turkey β 1-m23 were transferred directly to the
20 human β 2 receptor. The resultant mutant, β 2-m23, had a T_m 12°C higher than that of the human β 2 receptor.

Accordingly, a further method for producing a mutant GPCR with increased stability relative to its parent GPCR comprises

25

- (i) identifying in the amino acid sequence of one or more mutants of a first parent GPCR with increased stability relative to the first parent GPCR, the position or positions at which the one or more mutants have at least one different amino acid residue compared to the
30 first parent GPCR, and

30

- (ii) making one or more mutations in the amino acid sequence that defines a second GPCR at the corresponding position or positions, to provide one or more mutants of a second parent GPCR with
35 increased stability relative to the second parent GPCR.

35

The one or more mutants of a first parent GPCR may be selected or prepared according to the selection or preparation methods described above. Hence, this method may also be used to create stable, conformationally locked GPCRs by mutagenesis. For example, following the selection of mutant GPCRs which have
5 increased stability in a particular conformation, the stabilising mutation can be identified and the amino acid at a corresponding position in a second GPCR replaced to produce a mutant GPCR with increased stability in a particular conformation relative to a second parent GPCR.

10 For the avoidance of doubt the first parent GPCR may be a GPCR which has a naturally-occurring sequence, or it may be a truncated form or it may be a fusion, either to the naturally-occurring protein or to a fragment thereof, or it may contain mutations compared to the naturally-occurring sequence, providing that it retains ligand-binding ability.

15 Typically, identifying the position or positions at which the one or more mutants have at least one different amino acid residue compared to the first parent GPCR involves aligning their amino acid sequences with that of the parent GPCR, for example using the Clustal W program (Thompson *et al.*, 1994).

20 By "corresponding position or positions", we include the meaning of the position in the amino acid sequence of a second GPCR which aligns to the position in the amino acid sequence of the first GPCR, when the first and second GPCRs are compared by alignment, for example by using MacVector and Clustal W. For
25 example, as shown in the alignment in Figure 17, the six stabilising mutations in turkey $\beta 1$ -m23, R68S, M90V, Y227A, A282L, F327A and F338M, are at positions which correspond to residues K60, M82, Y219, C265, L310 and F321 respectively in the human $\beta 2$ receptor.

30 Having identified the corresponding position or positions in the amino acid sequence of a second GPCR, the amino acids at those positions are replaced with another amino acid. Typically, the amino acids are replaced with the same amino acids which replaced the amino acids at the corresponding positions in the mutant of the first parent GPCR (unless they are already that residue). For
35 example, at position 68 in turkey $\beta 1$ -m23 (R68S), an arginine residue was replaced with a serine residue. Therefore, at the corresponding position in the

human $\beta 2$ receptor, position 60 (K60), the lysine residue is preferably replaced with a serine residue.

Mutations can be made in an amino acid sequence, for example, as described above and using techniques well-established in the art.

It will be appreciated that the second GPCR may be any other GPCR. For example, stabilising mutations in a GPCR from one species may be transferred to a second GPCR from another species. Similarly, stabilising mutations in one particular GPCR isoform may be transferred to a second GPCR which is a different isoform. Preferably, the second parent GPCR is of the same GPCR class or family as the first parent GPCR. Phylogenetic analyses have divided GPCRs into three main classes based on protein sequence similarity, i.e., classes 1, 2, and 3 whose prototypes are rhodopsin, the secretin receptor, and the metabotropic glutamate receptors, respectively (Foord *et al* (2005) *Pharmacol. Rev.* **57**, 279-288). Thus, the second GPCR may be a GPCR which is of the same GPCR class as the first parent GPCR. Similarly, GPCRs have been divided into families by reference to natural ligands such as glutamate and GABA. Thus, the second GPCR may be of the same GPCR family as the first parent GPCR. A list of GPCR classes and families has been produced by the International Union of Pharmacology (Foord *et al* (2005) *Pharmacol. Rev.* **57**, 279-288) and this list is periodically updated at <http://www.iuphar-db.org/GPCR/ReceptorFamiliesForward>.

It will be appreciated that the second parent GPCR must be able to be aligned with the first parent GPCR such that the corresponding positions of the mutations in the first GPCR can be determined in the second GPCR. Thus typically, the second parent GPCR has at least 20% sequence identity to the first parent GPCR and more preferably at least 30%, 40%, 50%, 60%, 70%, 80% or 90% sequence identity to the first parent GPCR. However, some GPCRs have low sequence identity (e.g. family B and C GPCRs) and at the same time are very similar in structure. Thus the 20% sequence identity threshold is not absolute.

Method 4

The inventors have reasoned that the identification of structural motifs in which the one or more mutations in a mutant GPCR with increased stability reside, will be useful in producing further mutant GPCRs with increased stability.

- 5 Accordingly, a further method for producing a mutant G-protein coupled receptor (GPCR) with increased stability relative to its parent GPCR comprises:
- a. providing one or more mutants of a first parent GPCR with increased stability relative to the first parent GPCR
 - 10 b. identifying in a structural membrane protein model the structural motif or motifs in which the one or more mutants have at least one different amino acid residue compared to the first parent GPCR, and
 - 15 c. making one or more mutations in the amino acid sequence that defines a corresponding structural motif or motifs in a second parent GPCR, to provide one or more mutants of a second parent GPCR with increased stability relative to the second
 - 20 parent GPCR.

Mapping stabilising mutations onto one or more known structural models can be used to identify particular structural motifs in which such stabilising mutations reside. We have mapped stabilising mutations of the β 1-adrenergic receptor onto structural models of the β 2-adrenergic receptor (Rasmussen *et al* (2007) *Nature* 25 450, 383-387; Cherezov *et al* (2007) *Science* 318:1258-65; Rosenbaum *et al* (2007) *Science* 318:1266-1273) in order to identify such motifs. For example, Table (vi) lists the turkey β 1-adrenergic receptor mutations which we have mapped onto the human β 2-adrenergic receptor and describes the corresponding structural motifs in which they reside. As discussed in Example 4, mapping of the Y227A mutation (equivalent to Y219 in the human β 2 receptor) onto the human β 2-adrenergic receptor reveals its position at the interface between helices such that the mutation may improve packing at the helical interface (see Figures 15, 16 and 23). Similarly, mapping of the M90V mutation (equivalent to M82 in the human β 2 receptor) onto the human β 2-adrenergic receptor reveals it to be in helix 35 2 at a point where the helix is kinked (see Figures 15, 16 and 20). Other

mutations were found to reside in further structural motifs including transmembrane helix surfaces pointing into the lipid bilayer, hydrophobic-hydrophilic boundary regions, protein binding pockets and loop regions (see Table (vi) and Figures 18-19, 21-22 and 24-25).

5

Such structural motifs, by virtue of them containing stabilising mutations, are important in determining protein stability. Therefore, targeting mutations to these motifs will facilitate the generation of stabilised mutant GPCRs. Indeed, there were several instances where more than one mutation mapped to the same structural motif. For example, the Y227A, V230A and A234L mutations in the turkey β 1 adrenergic receptor mapped to the same helical interface, the V89L and M90V mutations mapped to the same helical kink and the F327A and A334L mutations mapped to the same helical surface pointing towards the lipid bilayer (Table (vi)). Thus, when one stabilising mutation has been identified, the determination of the structural motif in which that mutation is located will enable the identification of further stabilising mutations.

10

15

20

25

The one or more mutants of a first parent GPCR may be selected or prepared according to any of the methods described above. Hence, this method may also be used to create stable, conformationally locked GPCRs by mutagenesis. For example, following the selection of mutant GPCRs which have increased stability in a particular conformation, the structural motifs in which such stabilising mutations reside can be identified. Making one or more mutations in the amino acid sequence that defines the corresponding structural motif in another GPCR can then be used to produce a mutant GPCR with increased stability in a particular conformation relative to its parent GPCR.

30

35

We have performed a multiple sequence alignment of the human beta-2AR, rat NTR1, turkey beta-1 AR, human Adenosine A2aR and human muscarinic M1 receptor amino acid sequences (Figure 17) which shows that, when the thermostabilising mutations identified (see Examples 1-3) are positioned on the sequences then, in 11 instances out of a total of 70, two sequences contain mutations at the same position (denoted in Figure 17 with a star). Without wishing to be bound by any theory, the inventors believe that thermostabilising mutations at these positions should be of enhanced transferability for mapping onto a structural membrane protein model. Thus in one embodiment, the mutant

of the first parent GPCR is a mutant human beta-2AR, rat NTR1, turkey beta-1 AR, human Adenosine A2aR or human muscarinic M1 receptor which, when compared to its corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of the following positions according to the numbering of the human beta2 AR as set out in Figure 17: Ala 59, Val 81, Ser 143, Lys 147, Val 152, Glu 180, Val 222, Ala 226, Ala 271, Leu 275 and Val 317.

In order to identify the structural motif or motifs, the stabilising mutations are mapped onto a known structure of a membrane protein.

By "membrane protein" we mean a protein that is attached to or associated with a membrane of a cell or organelle. Preferably, the membrane protein is an integral membrane protein that is permanently integrated into the membrane and can only be removed using detergents, non-polar solvents or denaturing agents that physically disrupt the lipid bilayer.

The structural model of a membrane protein may be any suitable structural model. For example, the model may be a known crystal structure. Examples of GPCR crystal structures include bovine rhodopsin (Palczewski, K. *et al.*, *Science* 289, 739-745. (2000)) and human β_2 adrenergic receptor (Rasmussen *et al*, *Nature* 450, 383-7 (2007); Cherezov *et al* (2007) *Science* 318:1258-65; Rosenbaum *et al* (2007) *Science* 318:1266-1273). The coordinates for the human β_2 adrenergic receptor structure can be found in the RCSB Protein Data Bank under accession codes: 2rh1, 2r4r and 2r4s. Alternatively, the structural model may be a computer generated model based upon homology or using *de novo* structure prediction methods (Qian *et al* *Nature* (2007) 450: 259-64).

It will be appreciated that stabilising mutations of a given mutant GPCR can be mapped onto a structural model of any membrane protein which has sufficient structural similarity to the GPCR. In particular, the domain of the membrane protein must have sufficient structural similarity to the GPCR domain in which the stabilising mutation resides, for a given mutation to be transferable.

A protein domain is typically defined as a discretely folded assembly of secondary structure elements which may stand alone as a single protein or be

part of a larger protein in combination with other domains. It is commonly a functional evolutionary unit.

GPCRs are essentially single domain proteins excluding those with large N-terminal domains. Therefore, typically, the structural model is of a membrane protein which comprises at least one domain that has sufficient structural similarity to the GPCR.

Structural similarity can be determined indirectly by the analysis of sequence identity, or directly by comparison of structures.

With regard to sequence identity, the amino acid sequence encoding the GPCR domain in which the mutant has at least one different amino acid residue compared to the first parent GPCR, is aligned with an amino acid sequence encoding a domain of a membrane protein for which a structural model is available. It will be appreciated that one or more of these sequences may contain an inserted sequence or N-terminal or C-terminal extensions which are additional to the core conserved domain. For optimal alignment, such sequences are removed so as not to skew the analysis. Membrane proteins with sufficient sequence identity across the domain in question may then be used as the structural model for mapping mutations. It has been shown for soluble protein domains that their 3D structure is broadly conserved above 20% sequence identity and well conserved above 30% identity, with the level of structural conservation increasing as sequence identity increases up to 100% (Ginalski, K. Curr Op Struc Biol (2006) 16, 172-177). Thus, it is preferred if the structural membrane protein model is a model of a membrane protein which contains a domain that shares at least 20% sequence identity with the mutant GPCR domain containing the at least one different amino acid residue compared to the first parent GPCR, and more preferably at least 30%, 40%, 50%, 60%, 70%, 80% or 90% sequence identity, and yet more preferably at least 95% or 99% sequence identity.

Sequence identity may be measured by the use of algorithms such as BLAST or PSI-BLAST (Altschul et al, NAR (1997), 25, 3389-3402) or methods based on Hidden Markov Models (Eddy S et al, J Comput Biol (1995) Spring 2 (1) 9-23). Typically, the percent sequence identity between two polypeptides may be

determined using any suitable computer program, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequence has been aligned optimally. The alignment may alternatively be carried out using the
5 Clustal W program (Thompson *et al.*, 1994). The parameters used may be as follows: Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent. Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05. Scoring matrix: BLOSUM.

10 In addition to sequence identity, structural similarity can be determined directly by comparison of structural models. Structural models may be used to detect regions of structural similarity not evident from sequence analysis alone, and which may or may not be contiguous in the sequence. For example, family B and
15 C GPCRs are thought to share similar structures; however, their sequence identity is very low. Similarly, the water transporting aquaporins spinach SoPip2, *E. coli* AqpZ, Methanococcus AqpM, rat Aqp4, human Aqp1 and sheep Aqp0 share low sequence identity but all have similar structures.

20 Structural models of high fidelity may be constructed for proteins of unknown structure using standard software packages such as MODELLER (Sali A and Blundell T, J Mol Biol (1993) 234(3) 779-815), wherein the structure is modelled on a known structure of a homologous protein. Such modelling improves with increasing sequence identity. Typically, the sequence identity between the
25 sequence of unknown structure and a sequence of known 3D structure is more than 30% (Ginalski, K. Curr Op Struc Biol (2006) 16, 172-177). In addition, *de novo* structure prediction methods based on sequence alone may be used to model proteins of unknown structure (Qian *et al.*, (2007) Nature 450:259-64). Once structures have been experimentally determined or derived by modelling,
30 regions of structural similarity may be detected by direct comparison of two or more 3D structures. They may, for example, comprise secondary structure elements of a particular architecture and topology which can be detected by the use of software such as DALI (Holm, L and Sander, C (1996) Science 273, 595-603). They may comprise local arrangements of amino acid side chains and the
35 polypeptide backbone, or specific sets of atoms or groups of atoms in a particular spatial arrangement, which may for example also be detected by the use of graph

theoretical representations (Artymiuk, P et al, (2005) *J Amer Soc Info Sci Tech* 56 (5) 518-528). In this approach, the atoms or groups of atoms within the proteins or regions of proteins to be compared are typically represented as the nodes of a graph, with the edges of the graph describing the angles and distances between the nodes. Common patterns in these graphs indicate common structural motifs. This approach may be extended to include any descriptor of atoms or groups of atoms, such as hydrogen bond donor or acceptor, hydrophobicity, shape, charge or aromaticity; for example proteins may be spatially mapped according to such descriptors using GRID and this representation used as a basis for similarity searching (Baroni et al (2007) *J Chem Inf Mod* 47, 279-294). Descriptions of the methods, availability of software, and guidelines for user-defined selection of parameters, thresholds and tolerances are described in the references given above.

In a preferred embodiment, the structural membrane protein model is a structural GPCR model. It will be appreciated that the structural model of a GPCR may be a model of the first parent GPCR. For example, stabilising mutations within a mutant GPCR having increased stability can be directly mapped onto the first parent GPCR structure and the structural motifs in which such mutations are located, identified. Where the structure of the first parent GPCR is unknown, structural models of other GPCRs may be used. For example, stabilising mutations in a GPCR from one species may be mapped onto a known structural model of the same GPCR from another species. Similarly, stabilising mutations in one particular GPCR isoform may be mapped onto a known structural model of another GPCR isoform. Moreover, stabilising mutations from one GPCR may be mapped onto a GPCR of the same class or family. A list of GPCR classes and families has been produced by the International Union of Pharmacology (Foord *et al* (2005) *Pharmacol. Rev.* 57, 279-288) and this list is periodically updated at <http://www.iuphar-db.org/GPCR/ReceptorFamiliesForward>.

As described above, it will be appreciated that the structural model may be of any GPCR provided it has sufficient structural similarity across the domain in which the mutant GPCR has at least one different amino acid compared to the first parent GPCR. Thus, it is preferred if the GPCR shares at least 20% sequence identity with the mutant of the first parent GPCR across the protein domain containing the at least one different amino acid residue compared to the first

parent GPCR, and more preferably at least 30%, 40%, 50%, 60%, 70%, 80% or 90% sequence identity, and yet more preferably at least 95% or 99% sequence identity. However, the inventors recognise that the 20% sequence identity threshold is not absolute. GPCRs with less than 20% sequence identity to the first parent GPCR may also serve as a structural model to which stabilising mutations are transferred, wherein the low sequence identity is counterbalanced by other similarities, including, for example, the presence of the same sequence motifs, binding to the same G-protein or having the same function, or having substantially the same hydropathy plots compared to the first parent GPCR.

Mapping of stabilising mutations onto the structural model can be done using any suitable method known in the art. For example, typically, the amino acid sequence of the GPCR for which the structural model is available is aligned with the amino acid sequence of the mutant of the first parent GPCR. The position or positions of the at least one different amino acid residue in the mutant GPCR relative to the first parent GPCR can then be located in the amino acid sequence of the GPCR for which a structural model is available.

By 'structural motif' we include the meaning of a three dimensional description of the location in a GPCR structural model of a thermostabilising mutation. For example, the structural motif may be any secondary or tertiary structural motif within the GPCR. By 'tertiary structural motif' we include any descriptor of atoms or groups of atoms, such as hydrogen bond donor or acceptor, hydrophobicity, shape, charge or aromaticity. For example, proteins may be spatially mapped according to such descriptors using GRID and this representation used as a basis for defining a structural motif (Baroni et al (2007) J Chem Inf Mod 47, 279-294).

Table (vi) lists the structural motifs in which the turkey $\beta 1$ adrenergic receptor stabilising mutations were found to reside. As seen from the table, the mutations are positioned in a number of distinct localities. Three mutations are in loop regions that are predicted to be accessible to aqueous solvent. Eight mutations are in the transmembrane α -helices and point into the lipid bilayer; three of these mutations are near the end of the helices and may be considered to be at the hydrophobic-hydrophilic boundary layer. Eight mutations are found at the interfaces between transmembrane α -helices, three of which are either within a

kinked or distorted region of the helix and another two mutations occur in one helix but are adjacent to one or more other helices which contain a kink adjacent in space to the mutated residue. These latter mutations could affect the packing of the amino acids within the kinked region, which could result in
5 thermostabilisation. Another mutation is in a substrate binding pocket.

Accordingly, in one embodiment, the structural motif is any of a helical interface, a helix kink, a helix opposite a helix kink, a helix surface pointing into the lipid bilayer, a helix surface pointing into the lipid bilayer at the hydrophobic-
10 hydrophilic boundary layer, a loop region or a protein binding pocket.

Identifying a structural motif in which a stabilising mutation resides suggests the importance of that motif in protein stability. Therefore, making one or more mutations in the amino acid sequence that defines a corresponding structural
15 motif or motifs in a second parent GPCR, should provide one or more mutants of a second parent GPCR with increased stability relative to the second parent GPCR.

The amino acid sequence which defines a structural motif is the primary amino
20 acid sequence of the amino acid residues which combine in the secondary or tertiary structure of the protein to form the structural motif. It will be appreciated that such a primary amino acid sequence may comprise contiguous or non-contiguous amino acid residues. Thus, identifying the amino acid sequence which defines the structural motif will involve determining the residues involved
25 and subsequently defining the sequence. Mutations can be made in an amino acid sequence, for example as described above and using techniques well-established in the art.

By "corresponding structural motif or motifs", we mean the analogous structural
30 motif or motifs identified in the structural model which are present in the second parent GPCR. For example, if a helical interface was identified, the corresponding helical interface in the second parent GPCR would be the interface between the helices which are analogous to the helices present in the structural model. If a helical kink was identified, the corresponding helical kink would be the
35 kink in the helix which is analogous to the kinked helix present in the structural model. An analogous structural motif or motifs in the second parent GPCR can

be identified by searching for similar amino acid sequences in the sequence of the second parent GPCR which define the motif or motifs in the structural model, for example, by sequence alignment. Moreover, computer based algorithms are widely available in the art that can be used to predict the presence of protein motifs based on an amino acid sequence. Thus, based upon the relative position of a particular motif within the amino acid sequence and its position relative to other motifs, an analogous structural motif can readily be identified. It will be appreciated that if a structural model of the second parent GPCR is available, the analogous structural motif or motifs can be directly mapped onto the structure of the protein. Typically, the amino acid sequence defining the analogous structural motif has at least 20% sequence identity with the sequence defining the motif in the structural model, more preferably at least 30%, 40%, 50%, 60%, 70%, 80% and 90% sequence identity and yet more preferably 95% and 99% sequence identity.

In one embodiment, the second parent GPCR is the first parent GPCR. For the avoidance of doubt, the second parent GPCR may have the naturally-occurring sequence of the first parent GPCR, or it may be a truncated form or it may be a fusion, either to the naturally occurring protein or to a fragment thereof, or it may contain mutations compared to the naturally-occurring sequence, providing that it retains ligand-binding.

In an alternative embodiment, the second parent GPCR is not the first parent GPCR. For example, a mutant of a first parent GPCR may have been identified that has increased stability but it is desired to generate a mutant of a different GPCR with increased stability. Preferably, the second parent GPCR is of the same GPCR class or family as the first parent GPCR as described above. However, it will be appreciated that the second parent GPCR may be any known GPCR provided that it shares sufficient structural similarity with the first parent GPCR, such that it contains a corresponding structural motif in which the stabilising mutation of the mutant of the first parent GPCR resides. Thus typically, the second parent GPCR has at least 20% sequence identity to the first parent GPCR and more preferably at least 30%, 40%, 50%, 60%, 70%, 80% or 90% sequence identity. However, as mentioned above, some GPCRs have low sequence identity (e.g. family B and C GPCRs) but are similar in structure. Thus the 20% sequence identity threshold is not absolute.

Since there are potentially thousands of mutations that can be screened in a GPCR for increased stability, it is advantageous to target particular mutations which are known to be important in conferring stability. Therefore, it will be appreciated that the methods 3 and 4 may be used in a method of selecting mutant GPCRs with increased stability. In particular, carrying out methods 3 and 4 can be used to target mutations to particular amino acid residues or to amino acid sequences which define structural motifs important in determining stability.

Accordingly, in one embodiment, methods 3 and 4 further comprise:

(I) selecting a ligand, the ligand being one which binds to the second parent GPCR when the GPCR is residing in a particular conformation

(II) determining whether the or each mutant of the second parent GPCR when residing in a particular conformation has increased stability with respect to binding the selected ligand compared to the stability of the second parent GPCR when residing in the same particular conformation with respect to binding that ligand, and

(III) selecting those mutants that have an increased stability compared to the second parent GPCR with respect to binding the selected ligand.

It will be noted that steps (I), (II) and (III) correspond to steps (b), (c) and (d) of method 1 described above. Accordingly, preferences for the ligand and methods of assessing stability are as defined above with respect to method 1.

Any mutant GPCR with increased stability relative to its parent GPCR, for example those provided by any of methods 1-4 described above, may be used in the present invention. For example, mutant GPCRs with increased stability compared to their parent GPCRs, particularly those with increased thermostability may be used. Particular examples of mutant GPCRs suitable for use in the present invention are provided below.

In one embodiment, the mutant GPCR is a mutant GPCR which has, compared to its parent receptor, at least one different amino acid at a position which corresponds to any one or more of the following positions: (i) according to the numbering of the turkey β -adrenergic receptor as set out in Figure 9: Ile 55, Gly 67, Arg 68, Val 89, Met 90, Gly 67, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313, Lys 315, (iii) according to the numbering of the rat neurotensin receptor as set out in Figure 11: Ala 69, Leu 72, Ala 73, Ala 86, Ala 90, Ser 100, His 103, Ser 108, Leu 109, Leu 111, Asp 113, Ile 116, Ala 120, Asp 139, Phe 147, Ala 155, Val 165, Glu 166, Lys 176, Ala 177, Thr 179, Met 181, Ser 182, Arg 183, Phe 189, Leu 205, Thr 207, Gly 209, Gly 215, Val 229, Met 250, Ile 253, Leu 256, Ile 260, Asn 262, Val 268, Asn 270, Thr 279, Met 293, Thr 294, Gly 306, Leu 308, Val 309, Leu 310, Val 313, Phe 342, Asp 345, Tyr 349, Tyr 351, Ala 356, Phe 358, Val 360, Ser 362, Asn 370, Ser 373, Phe 380, Ala 385, Cys 386, Pro 389, Gly 390, Trp 391, Arg 392, His 393, Arg 395, Lys 397, Pro 399, and (iv) according to the numbering of the muscarinic receptor as set out in Figure 17: Leu 65, Met 145, Leu 399, Ile 383 and Met 384.

Alignment of the turkey β_1 AR, human adenosine receptor, rat neurotensin receptor and human muscarinic receptor amino acid sequences in Figure 17, shows that in 11 instances out of 70, two sequences contain mutations at the same position, namely at the following positions according to the numbering of the human beta2 AR as set out in Figure 17: Ala 59, Val 81, Ser 143, Lys 147, Val 152, Glu 180, Val 222, Ala 226, Ala 271, Leu 275 and Val 317. Therefore, in a further embodiment, the mutant GPCR is one which has, compared to its parent receptor, a different amino acid at any one of these positions.

Mutant β -adrenergic receptor

β -adrenergic receptors are well known in the art. They share sequence homology to each other and bind to adrenalin.

In one embodiment, the mutant GPCR is a mutant β -adrenergic receptor which, when compared to the corresponding wild-type β -adrenergic receptor, has a different amino acid at a position which corresponds to any one or more of the

following positions according to the numbering of the turkey β -adrenergic receptor as set out in Figure 9: Ile 55, Gly 67, Arg 68, Val 89, Met 90, Gly 98, Ile 129, Ser 151, Val 160, Gln 194, Gly 197, Leu 221, Tyr 227, Arg 229, Val 230, Ala 234, Ala 282, Asp 322, Phe 327, Ala 334, Phe 338.

5

The mutant β -adrenergic receptor may be a mutant of any β -adrenergic receptor provided that it is mutated at one or more of the amino acid positions as stated by reference to the given turkey β -adrenergic receptor amino acid sequence.

10 It is particularly preferred if the mutant GPCR is one which has at least 20% amino acid sequence identity when compared to the given turkey β -adrenergic receptor sequence, as determined using MacVector and CLUSTALW (Thompson *et al* (1994) *Nucl. Acids Res.* **22**, 4673-4680). More preferably, the mutant receptor has at least 30% or at least 40% or at least 50% amino acid
15 sequence identity. There is generally a higher degree of amino acid sequence identity which is conserved around the orthosteric ("active") site to which the natural ligand binds.

As is described in Example 1 and Figure 1 below, individual replacement of the
20 following amino acid residues in the parent turkey β -adrenergic sequence (as shown in Figure 9) lead to an increase in thermostability: Ile 55, Gly 67, Arg 68, Val 89, Met 90, Gly 98, Ile 129, Ser 151, Val 160, Gln 194, Gly 197, Leu 221, Tyr 227, Arg 229, Val 230, Ala 234, Ala 282, Asp 322, Phe 327, Ala 334, Phe 338.

25 Thus, a mutant turkey β -adrenergic receptor in which, compared to its parent, one or more of these amino acid residues have been replaced by another amino acid residue may be used. Mutant β -adrenergic receptors from other sources in which one or more corresponding amino acids in the parent receptor are replaced by another amino acid residue may also be used.

30

In one embodiment the mutant GPCR is a mutant β -adrenergic receptor which has at least one different amino acid residue in a structural motif in which the mutant receptor compared to its parent receptor has a different amino acid at a position which corresponds to any of the following positions according to the
35 numbering of the turkey β -adrenergic receptor as set out in Figure 9: Ile 55, Gly

67, Arg 68, Val 89, Met 90, Gly 98, Ile 129, Ser 151, Val 160, Gln 194, Gly 197, Leu 221, Tyr 227, Arg 229, Val 230, Ala 234, Ala 282, Asp 322, Phe 327, Ala 334, Phe 338.

- 5 For the avoidance of doubt, the parent may be a β -adrenergic receptor which has a naturally-occurring sequence, or it may be a truncated form or it may be a fusion, either to the naturally occurring protein or to a fragment thereof, or it may contain mutations compared to the naturally-occurring sequenced provided that it retains ligand-binding ability.

10

By "corresponding amino acid residue" we include the meaning of the amino acid residue in another β -adrenergic receptor which aligns to the given amino acid residue in turkey β -adrenergic receptor when the turkey β -adrenergic receptor and the other β -adrenergic receptor are compared using MacVector and CLUSTALW.

15

Figure 9 shows an alignment between turkey β -adrenergic receptor and human β 1, β 2 and β 3 β -adrenergic receptors.

- 20 It can be seen that Ile 72 of human β 1 corresponds to Ile 55 of turkey β -adrenergic receptor; Ile 47 of human β 2 corresponds to Ile 55 of turkey β -adrenergic receptor; and Thr51 of human β 3 corresponds to Ile 55 of turkey β -adrenergic receptor. Other corresponding amino acid residues in human β 1, β 2 and β 3 can readily be identified by reference to Figure 9.

25

It is preferred that the particular amino acid is replaced with an Ala. However, when the particular amino acid residue is an Ala, it is preferred that it is replaced with a Leu (for example, see turkey β -adrenergic Ala 234, Ala 282 and Ala 334 in Figure 1).

30

It is preferred if the mutant β -adrenergic receptor has a different amino acid compared to its parent at more than one amino acid position since this is likely to give greater stability. Particularly preferred human β 1 receptor mutants are those in which one or more of the following amino acid residues are replaced with another amino acid residue: K85, M107, Y244, A316, F361 and F372. Typically,

35

the given amino acid residue is replaced with Ala or Val or Met or Leu or Ile (unless they are already that residue).

Mutant human $\beta 1$ receptors which have combinations of 3 or 4 or 5 or 6 mutations as described above are preferred.

Particularly preferred human $\beta 2$ receptor mutants are those in which one or more of the following amino acids are replaced with another amino acid residue: K60, M82, Y219, C265, L310 and F321. Typically, the given amino acid residue is replaced with Ala or Val or Met or Leu or Ile (unless they are already that residue).

Mutant human $\beta 2$ receptors which have combinations of 3 or 4 or 5 or 6 mutations as described above are preferred.

Figure 26 shows the effect on thermostability when six thermostabilising mutations in $\beta 1$ -m23 (R68S, M90V, Y227A, A282L, F327A, F338M) were transferred directly to the human $\beta 2$ receptor (equivalent mutations K60S, M82V, Y219A, C265L, L310A, F321M), making human $\beta 2$ -m23. The Tms for human $\beta 2$ and $\beta 2$ -m23 were 29°C and 41°C respectively, thus exemplifying the transferability of thermostabilising mutations from one receptor to another receptor. Accordingly, a particularly preferred human $\beta 2$ receptor mutant is one which comprises the mutations K60S, M82V, Y219A, C265L, L310A, F321M.

Particularly preferred human $\beta 3$ receptor mutants are those in which one or more of the following amino acids are replaced with another amino acid residue: W64, M86, Y224, P284, A330 and F341. Typically, the given amino acid residue is replaced with Ala or Val or Met or Leu or Ile (unless they are already that residue).

Mutant human $\beta 3$ receptors which have combinations of 3 or 4 or 5 or 6 mutations as described above are preferred.

Particularly preferred combinations of mutations are described in detail in Tables 1 and 2 in Example 1, and suitable mutants include the mutant turkey β -

adrenergic receptors, and also include mutant β -adrenergic receptors where amino acids in corresponding position have been replaced by another amino acid, typically the same amino acid as indicated in Tables 1 and 2 in Example 1.

- 5 Particularly preferred mutants are those which contain mutations in the amino acids which correspond to the given amino acid residue by reference to turkey β -adrenergic receptor: (R68S, Y227A, A282L, A334L) (see m6-10 in Table 2 below); (M90V, Y227A, F338M) (see m7-7 in Table 2 below); (R68S, M90V, V230A, F327A, A334L) (see m10-8 in Table 2 below); and (R68S, M90V, Y227A, 10 A282L, F327A, F338M) (see m23 in Table 2 below).

Mutant adenosine receptor

- Adenosine receptors are well known in the art. They share sequence homology 15 to each other and bind to adenosine.

- In one embodiment, the mutant GPCR is a mutant adenosine receptor which, when compared to the corresponding wild-type adenosine, has a different amino acid at a position which corresponds to any one or more of the following positions 20 according to the numbering of the human adenosine A_{2a} receptor as set out in Figure 10: Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313, Lys 315, Ala 54, Val 57, His 75, Thr 88, Gly 114, Gly 118, Thr 119, Lys 122, 25 Gly 123, Pro 149, Glu 151, Gly 152, Ala 203, Ala 204, Ala 231, Leu 235, Val 239.

- The mutant adenosine receptor may be a mutant of any adenosine receptor provided that it is mutated at one or more of the amino acid positions as stated by reference to the given human adenosine A_{2a} receptor amino acid sequence. 30

- It is particularly preferred if the mutant GPCR is one which has at least 20% amino acid sequence identity when compared to the given human adenosine A_{2a} receptor sequence, as determined using MacVector and CLUSTALW. Preferably, the mutant GPCR has at least 30% or at least 40% or at least 50% or 35 at least 60% sequence identity. Typically, there is a higher degree of sequence conservation at the adenosine binding site.

As is described in Example 2 below, individual replacement of the following amino acid residues in the human adenosine A_{2a} receptor sequence (as shown in Figure 10) lead to an increase in thermostability when measured with the agonist

5 5'-N-ethylcarboxamidoadenosine (NECA):

Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313, Lys
10 315.

Replacement of the following amino acid residues in the human A_{2a} receptor sequence (as shown in Figure 10) lead to an increase in thermostability when measured with the antagonist ZM 241385 (4-[2-[[7-amino-2-(2-furyl) [1,2,4]-
15 triazolo[2,3- α][1,3,5]triazin-5-yl]amino]ethyl]phenol):

Ala 54, Val 57, His 75, Thr 88, Gly 114, Gly 118, Thr 119, Lys 122, Gly 123, Pro 149, Glu 151, Gly 152, Ala 203, Ala 204, Ala 231, Leu 235, Val 239.

20 Thus, a mutant human adenosine A_{2a} receptor in which, compared to its parent, one or more of these amino acid residues have been replaced by another amino acid residue may be used. Mutant adenosine receptors from other sources in which one or more corresponding amino acids in the parent receptor are replaced by another amino acid residue may also be used.

25

In one embodiment, the mutant GPCR is a mutant adenosine receptor which has at least one different amino acid residue in a structural motif in which the mutant receptor compared to its parent receptor has a different amino acid at a position which corresponds to any of the following positions according to the numbering of
30 the human adenosine A_{2a} receptor as set out in Figure 10: Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313, Lys 315.

35 For the avoidance of doubt, the parent may be an adenosine receptor which has a naturally-occurring sequence, or it may be a truncated form or it may be a

fusion, either to the naturally-occurring protein or to a fragment thereof, or it may contain mutations compared to the naturally-occurring sequence, provided that it retains ligand-binding ability.

- 5 By "corresponding amino acid residue" we include the meaning of the amino acid residue in another adenosine receptor which aligns to the given amino acid residue in human adenosine A_{2a} receptor when the human adenosine A_{2a} receptor and the other adenosine receptor are compared using MacVector and CLUSTALW.

10

Figure 10 shows an alignment between human adenosine A_{2a} receptor and three other human adenosine receptors (A_{2b}, A₃ and A₁).

15

It can be seen that, for example, Ser 115 in the A_{2b} receptor (indicated as AA2BR) corresponds to Gly 114 in the A_{2a} receptor. Similarly, it can be seen that Ala 60 in the A₃ receptor (indicated as AA3R) corresponds to Ala 54 in the A_{2a} receptor, and so on. Other corresponding amino acid residues in human adenosine receptors A_{2b}, A₃ and A₁ can readily be identified by reference to Figure 10.

20

It is preferred that the particular amino acid in the parent is replaced with an Ala. However, when the particular amino acid residue in the parent is an Ala, it is preferred that it is replaced with a Leu.

25

It is preferred that the mutant adenosine receptor has a different amino acid compared to its parent at more than one amino acid position. Particularly preferred human adenosine A_{2b} receptors are those in which one or more of the following amino acid residues are replaced with another amino acid residue: A55, T89, R123, L236 and V240. Typically, the given amino acid residue is replaced with Ala or Val or Met or Leu or Ile (unless they are already that residue).

30

Mutant human adenosine A_{2b} receptors which have combinations of 3 or 4 or 5 mutations as described above are preferred.

35

Particularly preferred human adenosine A3 receptors are those in which one or more of the following amino acid residues are replaced with another amino acid residue: A60, T94, W128, L232 and L236. Typically, the given amino acid residue is replaced with Ala or Val or Met or Leu or Ile (unless they are already that residue).

Mutant human adenosine A3 receptors which have combinations of 3 or 4 or 5 mutations as described above are preferred.

Particular preferred human adenosine A1 receptors are those in which one or more of the following residues are replaced: A57, T91, A125, L236, and L240. Typically, the given amino acid residue is replaced with Ala or Val or Met or Leu or Ile (unless they are already that residue).

Particularly preferred combinations of mutations are described in detail in Example 2. Suitable mutants include these mutant human adenosine A_{2a} receptors, and also include other mutant adenosine receptors where amino acids in corresponding positions have been replaced by another amino acid, typically the same amino acid as indicated in Example 2.

Particularly preferred adenosine receptor mutants are those which contain mutations in the amino acids which correspond to the given amino residue by reference to human adenosine A_{2a} receptor: (A54L, K122A, L235A) (Rant 17); (A54L, T88A, V239A, A204L) (Rant 19); and (A54L, T88A, V239A, K122A) (Rant 21).

Mutant neurotensin receptor

Neurotensin receptors are known in the art. They share sequence homology and bind neurotensin.

In one embodiment, the mutant GPCR is a mutant neurotensin receptor which, when compared to the corresponding wild-type neurotensin receptor, has a different amino acid at a position which corresponds to any one or more of the following positions according to the numbering of the rat neurotensin receptor as set out in Figure 11: Ala 69, Leu 72, Ala 73, Ala 86, Ala 90, Ser 100, His 103, Ser

108, Leu 109, Leu 111, Asp 113, Ile 116, Ala 120, Asp 139, Phe 147, Ala 155,
 Val 165, Glu 166, Lys 176, Ala 177, Thr 179, Met 181, Ser 182, Arg 183, Phe
 189, Leu 205, Thr 207, Gly 209, Gly 215, Val 229, Met 250, Ile 253, Leu 256, Ile
 260, Asn 262, Val 268, Asn 270, Thr 279, Met 293, Thr 294, Gly 306, Leu 308,
 5 Val 309, Leu 310, Val 313, Phe 342, Asp 345, Tyr 349, Tyr 351, Ala 356, Phe
 358, Val 360, Ser 362, Asn 370, Ser 373, Phe 380, Ala 385, Cys 386, Pro 389,
 Gly 390, Trp 391, Arg 392, His 393, Arg 395, Lys 397, Pro 399.

10 It is particularly preferred if the mutant GPCR is one which has at least 20%
 amino acid sequence identity when compared to the given rat neurotensin
 receptor sequence, as determined using MacVector and CLUSTALW.
 Preferably, the mutant GPCR has at least 30% or at least 40% or at least 50%
 amino acid sequence identity.

15 The mutant neurotensin receptor may be a mutant of any neurotensin receptor
 provided that it is mutated at one or more of the amino acid positions as stated
 by reference to the given rat neurotensin receptor amino acid sequence.

20 As is described in Example 3 below, individual replacement of the following
 amino acid residues in the rat neurotensin receptor sequence (as shown in
 Figures 11 and 28) lead to an increase in thermostability when considered with
 respect to the absence of neurotensin. Leu 72, Ala 86, Ala 90, Ser 100, His 103,
 Ser 108, Leu 109, Leu 111, Asp 113, Ile 116, Ala 120, Asp 139, Phe 147, Ala
 155, Lys 176, Thr 179, Met 181, Ser 182, Phe 189, Leu 205, Thr 207, Gly 209,
 25 Gly 215, Leu 256, Asn 262, Val 268, Met 293, Asp 345, Tyr 349, Tyr 351, Ala
 356, Phe 358, Ser 362, Ala 385, Cys 386, Trp 391, Arg 392, His 393, Lys 397,
 Pro 399.

30 As is described in Example 3 below, individual replacement of the following
 amino acid residues in the rat neurotensin receptor sequence (as shown in
 Figures 11 and 28) lead to an increase in thermostability when considered with
 respect to the presence of neurotensin. Ala 69, Ala 73, Ala 86, Ala 90, His 103,
 Val 165, Glu 166, Ala 177, Arg 183, Gly 215, Val 229, Met 250, Ile 253, Ile 260,
 Thr 279, Thr 294, Gly 306, Leu 308, Val 309, Leu 310, Val 313, Phe 342, Phe
 35 358, Val 360, Ser 362, Asn 370, Ser 373, Phe 380, Ala 385, Pro 389, Gly 390,
 Arg 395.

Thus, a mutant rat neurotensin receptor in which, compared to its parent, one or more of these amino acid residues have been replaced by another amino acid residue may be used. Mutant neurotensin receptors from other sources in which one or more corresponding amino acids in the parent receptor are replaced by another amino acid residue may also be used.

In one embodiment the mutant GPCR is a mutant neurotensin receptor which has at least one different amino acid residue in a structural motif in which the mutant receptor compared to its parent receptor has a different amino acid at a position which corresponds to any of the following positions according to the numbering of the rat neurotensin receptor as set out in Figure 11: Ala 69, Leu 72, Ala 73, Ala 86, Ala 90, Ser 100, His 103, Ser 108, Leu 109, Leu 111, Asp 113, Ile 116, Ala 120, Asp 139, Phe 147, Ala 155, Val 165, Glu 166, Lys 176, Ala 177, Thr 179, Met 181, Ser 182, Arg 183, Phe 189, Leu 205, Thr 207, Gly 209, Gly 215, Val 229, Met 250, Ile 253, Leu 256, Ile 260, Asn 262, Val 268, Asn 270, Thr 279, Met 293, Thr 294, Gly 306, Leu 308, Val 309, Leu 310, Val 313, Phe 342, Asp 345, Tyr 349, Tyr 351, Ala 356, Phe 358, Val 360, Ser 362, Asn 370, Ser 373, Phe 380, Ala 385, Cys 386, Pro 389, Gly 390, Trp 391, Arg 392, His 393, Arg 395, Lys 397, Pro 399.

For the avoidance of doubt the parent may be a neurotensin receptor which has a naturally-occurring sequence, or it may be a truncated form or it may be a fusion, either to the naturally-occurring protein or to a fragment thereof, or it may contain mutations compared to the naturally-occurring sequence, providing that it retains ligand-binding ability.

By "corresponding amino acid residue" we include the meaning of the amino acid residue in another neurotensin receptor which aligns to the given amino acid residue in rat neurotensin receptor when the rat neurotensin receptor and the other neurotensin receptor are compared using MacVector and CLUSTALW.

Figure 11 shows an alignment between rat neurotensin receptor and two human neurotensin receptors 1 and 2. It can be seen, for example, that Ala 85 of the human neurotensin receptor 1 corresponds to Ala 86 of the rat neurotensin receptor, that Phe 353 of the human neurotensin receptor 1 corresponds to Phe

358 of the rat neurotensin receptor, and so on. Other corresponding amino acid residue in the human neurotensin receptors 1 and 2 can readily be identified by reference to Figure 11.

- 5 It is preferred that the particular amino acid in the parent is replaced with an Ala. However, when the particular amino acid residue in the parent is an Ala, it is preferred that it is replaced with a Leu.

10 It is preferred that the mutant neurotensin receptor has a different amino acid compared to its parent at more than one amino acid position. Particularly preferred human neurotensin receptors (NTR1) are those in which one or more of the following amino acid residues are replaced with another amino acid residue: Ala 85, His 102, Ile 259, Phe 337 and Phe 353. Typically, the given amino acid residues is replaced with Ala or Val or Met or Leu or Ile (unless they
15 are already that residue).

Mutant human neurotensin receptors (NTR1) which have combinations of 3 or 4 or 5 mutations as described above are preferred.

20 Particularly preferred human neurotensin receptors (NTR2) are those in which one or more of the following amino acid residues are replaced with another amino acid residue: V54, R69, T229, P331 and F347. Typically, the given amino acid residue is replaced with Ala or Val or Met or Leu or Ile (unless they are already that residue). Mutant human neurotensin receptors (NTR2) which have
25 combinations of 3 or 4 or 5 mutations as described above are preferred.

Particularly preferred combinations of mutations are described in detail in Example 3. Suitable mutants include these mutant rat neurotensin receptors, and also include other mutant neurotensin receptors where amino acids in
30 corresponding positions have been replaced by another amino acid, typically the same amino acid as indicated in Example 3.

Particularly preferred neurotensin receptor mutants are those which contain mutations in the amino acid residues which correspond to the given amino acid
35 residue by reference to the rat neurotensin receptor: (F358A, A86L, I260A, F342A) (Nag7m); (F358A, H103A, I260A, F342A) (Nag7n).

Mutant muscarinic receptor

Muscarinic receptors are known in the art. They share sequence homology and
5 bind muscarine.

In one embodiment, the mutant GPCR is a mutant muscarinic receptor which,
when compared to the corresponding wild-type muscarinic receptor, has a
different amino acid at a position which corresponds to any one or more of the
10 following positions according to the numbering of the human muscarinic receptor
M1 as set out in Figure 17: Leu 65, Met 145, Leu 399, Ile 383 and Met 384.

It is particularly preferred if the mutant GPCR is one which has at least 20%
amino acid sequence identity when compared to the given human muscarinic
15 receptor sequence, as determined using MacVector and CLUSTALW.
Preferably, the mutant GPCR has at least 30% or at least 40% or at least 50%
amino acid sequence identity.

The mutant muscarinic receptor may be a mutant of any muscarinic receptor
20 provided that it is mutated at one or more of the amino acid positions as stated
by reference to the given muscarinic receptor amino acid sequence.

Thus, a mutant human muscarinic receptor in which, compared to its parent, one
or more of these amino acid residues have been replaced by another amino acid
25 residue may be used. Mutant muscarinic receptors from other sources in which
one or more corresponding amino acids in the parent receptor are replaced by
another amino acid residue may also be used.

For the avoidance of doubt the parent may be a muscarinic receptor which has a
30 naturally-occurring sequence, or it may be a truncated form or it may be a fusion,
either to the naturally-occurring protein or to a fragment thereof, or it may contain
mutations compared to the naturally-occurring sequence, providing that it retains
ligand-binding ability.

35 In one embodiment, the mutant GPCR is a mutant muscarinic receptor. For
example, the mutant muscarinic receptor may have at least one different amino

acid residue in a structural motif in which the mutant receptor compared to its parent receptor has a different amino acid at a position which corresponds to any of the following positions according to the numbering of the human muscarinic receptor as set out in Figure 17: Leu 65, Met 145, Leu 399, Ile 383 and Met 384.

5

By "corresponding amino acid residue" we include the meaning of the amino acid residue in another muscarinic receptor which aligns to the given amino acid residue in human muscarinic receptor when the human muscarinic receptor and the other muscarinic receptor are compared using MacVector and CLUSTALW.

10

It is preferred that the particular amino acid is replaced with an Ala. However, when the particular amino acid residue is an Ala, it is preferred that it is replaced with a Leu.

15 It is preferred that the mutant GPCRs used in the invention have increased stability to any one of heat, a detergent, a chaotropic agent and an extreme of pH.

It is preferred if the mutant GPCRs used in the invention have increased
20 thermostability.

It is preferred that the mutant GPCRs used in the invention, including the mutant β -adrenergic, adenosine and neurotensin receptors, have an increased thermostability compared to its parent when in the presence or absence of a
25 ligand thereto. Typically, the ligand is an antagonist, a full agonist, a partial agonist or an inverse agonist, whether orthosteric or allosteric. As discussed above, the ligand may be a polypeptide, such as an antibody.

It is preferred that the mutant GPCRs used in the invention, for example a mutant
30 β -adrenergic receptor or a mutant adenosine receptor or a mutant neurotensin receptor or a mutant muscarinic receptor is at least 2°C more stable than its parent preferably at least 5°C more stable, more preferably at least 8°C more stable and even more preferably at least 10°C or 15°C or 20°C more stable than its parent. Typically, thermostability of the parent and mutant receptors are
35 measured under the same conditions. Typically, thermostability is assayed under a condition in which the GPCR resides in a particular conformation.

Typically, this selected condition is the presence of a ligand which binds the GPCR.

It is preferred that the mutant GPCRs used in the invention, when solubilised and purified in a suitable detergent has a similar thermostability to bovine rhodopsin purified in dodecyl maltoside. It is particularly preferred that the mutant GPCR retains at least 50% of its ligand binding activity after heating at 40°C for 30 minutes. It is further preferred that the mutant GPCR retains at least 50% of its ligand binding activity after heating at 55°C for 30 minutes.

For the avoidance of doubt, the mutant GPCR provided in step (a) of the method of the first aspect of the invention may be extended or truncated, contain internal deletions or insertions or otherwise altered beyond introduction of stabilising mutations; for example by introduction or deletion of sites for posttranslational modification e.g. glycosylation or phosphorylation or fatty acylation. It may also be chemically modified synthetically, for example by peptide semisynthesis or crosslinking or alkylation. In any event, the mutant GPCR provided has increased stability in a particular conformation relative to its parent GPCR.

Selection of binding partners

Selection for binding partners against membrane proteins has previously proven to be a difficult task. The preparation of the required pure antigen is problematic. Membrane proteins are often detergent-solubilised and they are purified as protein-detergent complexes. The type and concentration of detergent is crucial to keep the protein in its native conformation. Some detergents may prevent proteins from binding to plastic and polystyrene surfaces used as common ELISA supports. In addition, adsorption to the solid phase can cause partial denaturation of the protein.

In contrast, the increased stability of mutant GPCRs confers several advantages when the GPCRs are used as immunogens or selection reagents for screening, enabling them to be used as such in a variety of contexts.

Accordingly, in one embodiment, the mutant GPCR may be in a whole cell preparation, a cell membrane fragment, solubilised in detergent or it may be

incorporated into a lipid monolayer, a lipid bilayer, a bead-linked lipid particle, another solid-supported lipid layer or a proteoliposome. It is appreciated that when the GPCR is immobilised, the lipid layers may be supported directly as a layer on the surface of the solid support or may be tethered as a layer or vesicle as described in Cooper M. A. J Mol Recognit. 2004 Jul-Aug;17(4):286-315.

The inventors recognise that high throughput membrane-receptor screening is facilitated by immobilising membranes on beads or on surfaces that can be arrayed or otherwise multiplexed. Typically, membrane proteins are deposited on a surface together with lipid in the form of proteoliposomes. The most commonly employed methods for the formation of proteoliposomes start with either prokaryote or eukaryote cells. Membrane proteins can be isolated either in a mixed micelle with detergent, dissolved in an organic solvent or aggregated as membrane fragments by sonication in buffer. Once isolated and purified, the membrane proteins can be reconstituted into vesicles by: a) organic solvent-mediated reconstitutions (for example reverse-phase evaporation, rehydration of lipid-protein films), b) mechanical means (for example sonication, French press, freeze-thaw), or c) detergent-mediated means (for example dialysis, dilution, or direct incorporation into pre-formed vesicles or into bicelles)

The detergent solubilised form of the mutant GPCR may be a partly pure or highly pure preparation. Purification, enabled by the improved stability and optimisation of solubilisation conditions, confers the advantage of removal of extraneous "sticky" antigens and lipids and other cell surface material such as carbohydrate to which, for example, phage may stick to. This is particularly beneficial when the 'extraneous' material is charged or hydrophobic since phage particles, for example, are known to stick to hydrophobic or charged surfaces non-specifically. Moreover, certain phage antibodies may themselves bind promiscuously to antigens. A normal level of purity, as assessed by SDS-PAGE, is 80-95%. Therefore, preferably the detergent solubilised form of the mutant GPCR is at least 80% pure, or at least 85% pure, and still more preferably at least 90%, or at least 93%, or at least 95%, pure of other proteins. As is known in the art, higher levels of purity, e.g. at least 99%, can be achieved using additional purification techniques.

Solubilised receptor preparations are typically made in a buffer of 20mM Tris (pH 7.0), 0.1M (NH₄)₂SO₄, 10% glycerol, 0.07% CHS, 0.33% DOM, 0.33% Chaps, 0.33mM DOPC/DOPS (7:3), and 1 protease inhibitor tablet per 50 ml buffer. For solubilization trials, 0.5ml of this solubilization buffer is added to approximately 2x10⁶ cells, and these cell suspensions are sonicated using a probe sonicator (6x1-s pulses) and placed on a rocker at 4 °C. After 2 h, the solutions are centrifuged at 4 °C for 20min at 14,000rpm using a tabletop centrifuge. The supernatants containing solubilised receptor are then transferred to new tubes and kept frozen at -80 °C until analysis.

Preparations of mutant GPCRs formed from membrane fragments or membrane-detergent extracts are reviewed in detail in Cooper M. A. *J Mol Recognit.* 2004 Jul-Aug;17(4):286-315, incorporated herein by reference. Of particular interest are methods adapted from Graham, J. M.; Higgins, J. A. *Membrane Analysis*; Springer-Verlag: New York, 1997 and Dignam, J. D. *Methods in Enzymology* **1990**, 182, 194-203. For example, one method is as follows; i) Spin 500 ml of a suitable cell culture (e.g. CHO, Sf9) at 1000 g for 10 min, re-suspend pellet in ca. twice the pellet volume of a suitable ice-cold 20 mM tris-HCl pH 8, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 2 µg/ml aprotinin, and 10 µg/ml leupeptin, ii) Homogenize with a Dounce homogenizer (type A) for 10 strokes, then centrifuge at 30,000 g for 20 min, iii) Re-suspend pellet with 50 ml of 20 mM tris HCl pH 8, 3 mM MgCl, 10 µg/ml DNAase I plus PMSF, 2 µg/ml aprotinin and 10 µg/ml leupeptin (solution B) and re-homogenize, iv) Centrifuge at 30,000 g for 20 min. and resuspend pellet in 20 ml of solution B and homogenize again, v) Store at 4°C for immediate use after snap freezing.

The mutant GPCR may be engineered to include a molecular tag at the C terminus or N-terminus as is well known in the art. The tag may be any of a FLAG tag, a His tag, a c-Myc tag, a DDDDK tag, an HSV tag, a Halo tag or a biotin tag. Such tags can be used to facilitate phage-based selection protocols in solution and may also be used to confer binding to a solid support. Moreover, such tags will facilitate selection and enrichment of mutant GPCRs from impure preparations using affinity columns, affinity filters, magnetic beads and other examples of selective solid supported reagents, filtration, centrifugation, size-exclusion chromatography and dialysis amongst other methods

The increased stability of mutant GPCRs in a range of detergents and solubilisation buffers and additives lends them particularly well to being immobilised onto solid surfaces. Thus, in one embodiment the mutant GPCR is immobilised onto a solid support. Various supports are known in the art and include, for example, beads, columns, slides, chips or plates. Immobilisation may be via covalent or non-covalent interaction.

Where immobilisation is via a non-covalent interaction, the support may be coated with any of avidin, streptavidin, a metal ion, an antibody to the parent GPCR or an antibody to a molecular tag attached to the mutant GPCR. For example, the tag may be one recognised by an antibody such as a FLAG tag, or may be a poly-histidine tag enabling binding to a metal ion such as nickel or cobalt, as described for example in Venturi *et al*, *Biochemica et Biophysica Acta* 1610 (2003) 46-50. Alternatively, the mutant GPCR may be chemically modified for example with a biotin tag which can be bound to a surface coated with avidin or streptavidin. Moreover, a mutant GPCR may be immobilised via an antibody raised to the native receptor sequence.

Where immobilisation is via a covalent interaction, the support may be coated with a polymeric support such as carboxylated dextran. For example, the mutant GPCR may be covalently immobilised onto a surface coated with a carboxylated polymer via amine coupling. For example, water-soluble carbodiimide mediated activation of a carboxymethylated support such as dextran or hyaluronic acid allows for direct covalent capture of a mutant GPCR via available amino moieties of the protein to form a stable amide linkage. Alternatively, GPCRs can be engineered or further derivatised with sulfhydryl-reactive reagents (e.g. pyridinyldithioethanamine (PDEA) or 3-(2-pyridinyldithio)propionic acid N-hydroxysuccinimide ester (SPDP)) which allows reaction with free surface thiols (e.g. native free Cys, Met residues or with an engineered C-terminal Cys residue) to form a reversible disulfide linkage. In a similar manner, stable thioether bonds may be formed using maleimide coupling reagents such as sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexanecarboxylate (Sulfo-SMCC) and *N*-(γ -maleimidobutyroloxy)sulfosuccinimide ester (GMBS). A solid support may also be derivatised with cystamine to effect coupling with disulfide-activated GPCRs. Furthermore, treatment with hydrazine followed by a reductive amination enables coupling with aldehydes. The aldehyde groups may be native to the receptor or

formed by mild oxidation of any *cis*-diols present in the solid support (present in sugar residues of carbohydrates such as dextran, agarose, sepharose, hyaluronic acid and polyaginate). In addition to the above, amino-presenting surfaces can be treated with commercially available bifunctional linking reagents to effect coupling with free amino or sulfhydryl groups on the receptor as described by (Ernst, O. P. et al., *Meth. Enzymol.* **2000**, 315, 471-489 and Nunomura, W.e et al. *J. Biol. Chem.* **2000**, 275, 24540-24546).

The orientation of the mutant GPCR will depend on the desired outcome of the binding partner identification. For example, for the identification of therapeutic small molecules or antibodies, the mutant GPCR will typically be immobilised via the C-terminus or other intracellular domain to enable the extracellular domains to be outward facing in the assay. To identify native intracellular GPCR binding partners or agents which interfere with GPCR binding, the mutant GPCR is typically immobilised by the N-terminus or extracellular domain. Immobilisation by the N-terminus or extracellular domain may also identify therapeutic molecules that bind to the intracellular domain. However, it will be appreciated that for such molecules to be active *in vivo*, they must be able to pass through the cell membrane.

In an alternative embodiment, the mutant GPCR is not immobilised and is, for example, solubilised in detergent or present in a whole cell preparation. In this case, the test compound (for example, a library of test compounds) may be immobilised on a solid support, such as a chip surface. A variety of techniques are known in the art for immobilising compounds onto a chip surface, and any may be utilised. For example, suitable techniques include photolithography (Affymetrix, Santa Clara, Calif.), mechanical microspotting (Schena et al., *Science* (1995) vol. 270, p. 467-470; Synteni, Fremont, Calif.) and ink jetting (Incyte Pharmaceuticals, Palo Alto, Calif.; and Protogene, Palo Alto, Calif.). The address of the test compound/GPCR mutant pair on the chip is used to reveal the identity of the test compound. Other suitable methods are described, for example, in Fang, Y. et al. *Drug Discovery Today* **2003**, 8, 755-761, and references Alves, I. D. et al. *Curr. Prot. & Peptide Sci.* 2005, 6, 293-312.; Barry, R. et al. *Proteomics* 2004, 4, 3717-3726.; Besenicar, M. et al. *Chem. Phys. Lipids* 2006, 141, 169-178.; Cocklin, S. et al. *Prot. Sci.* 2004, 13, 194-194.; Cooper, M. A. *J. of Mol. Recognit.* 2004, 17, 286-315.; Fang, Y. et al. *Drug Discov. Today* 2003, 8, 755-

- 761.; Ferracci, G.*et al.* Anal. Biochem. 2004, 334, 367-375.; Graneli, A.*et al.* Anal. Biochem. 2007, 367, 87-94.; Graneli, A.*et al.* Biosens. Bioelectron. 2004, 20, 498-504.; Groves, J. T. Curr. Op. Drug Discov. Develop. 2002, 5, 606-612.; Groves, J. T.*et al.* J. Immunol. Meth. 2003, 278, 19-32.; Harding, P. J.*et al.* Eur. Biophys. J. Biophys. Lett. 2006, 35, 709-712.; Komolov, K. E.*et al.* Anal. Chem. 2006, 78, 1228-1234.; Kuroda, K.*et al.* App. Psychophys. Biofeedback 2006, 31, 127-136.; Lang, M. J.*et al.* Curr. Prot. Peptide Sci. 2006, 7, 335-353.; Leifert, W. R.*et al.* J. Biomol. Screening 2005, 10, 765-779.; Martin-Garcia, J.*et al.* J. Virology 2005, 79, 6703-6713.; Minic, J.*et al.* Biochim. Biophys. Acta-General Subjects 2005, 1724, 324-332.; Mozsolits, H.*et al.* J. Peptide Sci. 2003; 9, 77-89.; Navratilova, I.*et al.* Anal. Biochem. 2006, 355, 132-139.; Ott, D.*et al.* Prot. Eng. Design & Selection 2005, 18, 153-160.; Park, P. S. H.*et al.* Febs Lett. 2004, 567, 344-348.; Sobek, J.*et al.* Combinat. Chem. & High Throughput Screening 2006, 9, 365-380.; Stenlund, P.*et al.* Analytical Biochemistry 2003, 316, 243-250.; Winter, E. M.*et al.* Anal. Chem. 2006, 78, 174-180.; Yokogawa, M.*et al.* J. Am. Chem. So. 2005, 127, 12021-12027.; Zurawski, J. A.*et al.* Biopolymers 2003, 71, 388-389, all of which are incorporated herein by reference.

In one embodiment, neither the GPCR nor the test compound are immobilised, for example in phage-display selection protocols in solution.

The ability to produce milligram quantities of purified GPCRs, stabilised in a particular conformation, enables screening approaches which would not be available to native GPCRs present in biological membranes. Thus, the method of the invention maybe used to identify ligands of 'orphan' GPCRs where the natural ligand is unknown. Ligands of orphan GPCRs may be identified from biological samples such as blood or tissue extracts or from libraries of ligands. Similarly, ligands of mutant GPCRs may be identified where the ligands were interacting proteins such as accessory proteins. It is also appreciated that peptide or protein therapeutics may be identified by the methods of the invention as could conformation-specific antibodies. For example, antibodies prepared as described below could be assessed for conformational-specific GPCR binding. In particular, antibodies may be identified from supernatants obtained from B-cells taken from immunised animals, from hybridomas obtained following immortalisation of B-cells from the immunised animal or from recombinant antibody libraries which may be expressed on phage particles or through an *in*

vitro expression system such as ribosome display. The methods of the invention may also be used to determine the mechanism of action of compounds where the mechanism of action has not previously been known. For example, a mixture of GPCRs representing the 'GPCRome' or a subset thereof could be screened
5 against compounds to identify if their mechanism of action was via binding to a GPCR. In addition, the invention may be used as a biochemical affinity purification system wherein particular compounds are purified from a mixture of compounds.

10 The test compound may be provided as a biological sample. In particular, the sample could be any suitable sample taken from an individual. For example, the sample may be a fluid sample such as blood, serum, plasma or spinal fluid. Alternatively, the sample could be a tissue or cell extract.

15 In one embodiment, the one or more test compounds is a polypeptide. For example, the test compound may be a particular type of polypeptide which is known to bind to certain GPCRs but where the identification of a conformation-specific polypeptide is desired. Alternatively, the polypeptide may be a candidate therapeutic molecule, for example an anticancer (Skerra *J Biotechnol* (2001)
20 74(4):257-75).

In one embodiment, the one or more test compounds is a peptide.

In one embodiment, the one or more test compounds is an affibody, a
25 peptidomimetic, a nucleic acid, a peptide nucleic acid (PNA) or an aptamer, or a lipid or a carbohydrate.

In one embodiment, the one or more test compounds is a binding protein based on a modular framework, such as ankyrin repeat proteins, armadillo repeat
30 proteins, leucine rich proteins, tetrariopeptide repeat proteins or Designed Ankyrin Repeat Proteins (DARPs) or proteins based on lipocalin or fibronectin domains or Affilin scaffolds based on either human gamma crystalline or human ubiquitin.

In one embodiment, the one or more test compounds is a small molecule, for example a molecule less than 5000 daltons, or the one or more test compounds is a natural product.

5 In one embodiment, the one or more test compounds is an antibody. For example, the test compound may be an antibody that has been raised against a mutant GPCR of a parent GPCR, wherein the mutant GPCR has increased stability in a particular conformation relative to the parent GPCR. Preferably, the mutant GPCR is the same mutant GPCR as was provided in step (a).

10

As used herein, the term "antibody" includes but is not limited to polyclonal, monoclonal, chimaeric, single chain, Fab fragments and fragments produced by a Fab expression library. Such fragments include fragments of whole antibodies which retain their binding activity for a target substance, Fv, F(ab') and F(ab')₂ fragments, as well as genetically engineering derivatives of antibodies such as single chain antibodies (scFv), fusion proteins, domain antibodies (dAbs) and diabodies. For example, it will be appreciated that recombinant DNA technology may be used to produce further antibodies or chimeric molecules which retain the binding specificity of an original antibody. Such technology may involve fusing the DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions of a different immunoglobulin, as described, for example, in EP-A-184187, GB 2188638A or EP-A-239400. Moreover, a hybridoma or other cell producing an antibody may be subject to genetic mutation or other changes which may or may not alter the binding specificity of antibodies produced. Thus, since antibodies can be modified in a number of ways, the term "antibody" is to be construed as covering any specific binding member or substance having a binding domain with the required specificity. The term therefore includes antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent fused to another polypeptide are therefore included. Furthermore, antibodies and fragments thereof may be human or humanised antibodies, as is well known in the art.

15
20
25
30
35

Various procedures known within the art may be used to raise antibodies against a mutant GPCR, or against fragments or fusions thereof.

For example, an antibody to a mutant GPCR having increased stability relative to
5 a parent GPCR may be produced by immunising a lymphocyte with an immunogen of the mutant GPCR, screening the antibodies so produced for an antibody which binds to the GPCR, and isolating the antibody. By 'immunising a lymphocyte' we include both *in vivo* immunisation, where a whole animal is immunised and *in vitro* immunisation, where lymphocytes are immunised *in vitro*,
10 for example as described in US 5,290,681.

Preferably, the immunogen of a mutant GPCR is generally all of the mutant GPCR but may also be a part of the mutant GPCR, for example a fragment of the mutant GPCR. For example, the production of a stabilised GPCR may facilitate
15 identification of a part of the GPCR not previously obvious from the native conformation that would benefit from a targeted immunisation approach. The fragment of the mutant GPCR may be any part of the GPCR which is able to elicit an immune response such as an antibody response. It is known that peptides having as few as 5 amino acids may elicit an antibody response, although
20 typically larger peptides are used. Thus, the fragment of the immunogen may have at least 5 amino acids, typically from 5 to 1000 amino acids, such as 5 to 500, 5 to 200, 5 to 100, 5 to 50, 5 to 40, 5 to 30, 5 to 20, for example 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids.

25 Furthermore, the immunogen may be a fusion of the mutant GPCR, wherein the GPCR is fused to a second protein known to be immunogenic to lymphocytes. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. The immunogen may also further include an adjuvant to enhance the
30 immunological response to a mutant GPCR in a mammal *in vivo*. Various adjuvants used to increase the immunological response include, but are not limited to, Freund's (complete and incomplete), mineral gels (e.g., aluminum hydroxide), surface-active substances (e.g., lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, etc.), adjuvants usable in
35 humans such as Bacille Calmette-Guerin and *Corynebacterium parvum*, or similar immunostimulatory agents. An additional example of an adjuvant that can be

employed includes MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The choice of adjuvant can be important in maintaining the structure of the GPCR and, for this reason, Titremax and oil in water emulsions such as Ribi's adjuvant emulsion are particularly preferred.

5 Stabilised mutant GPCRs may also be linked together on a backbone to produce polyvalent molecules to further increase antigenicity.

It is appreciated that the immunogen of the mutant GPCR may be a variant of the mutant GPCR, provided that it is able to elicit an immune response to the mutant
10 GPCR and does not significantly reduce the stability of the mutant GPCR. Such variants include polypeptides which have one or more amino acid substitutions compared to the mutant GPCR, and as many as 5% substitutions. Typically, the substitutions are conservative substitutions where, for example, a "variant" refers to a protein wherein at one or more positions there have been amino acid
15 insertions, deletions, or substitutions, either conservative or non-conservative, provided that such changes result in a protein that is still able to elicit an immune response against the mutant GPCR and does not significantly reduce the stability of the mutant GPCR. By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe,
20 Tyr. Such variants of a mutant GPCR can be made using standard methods of protein engineering and site-directed mutagenesis.

It is appreciated that the immunogen may also be a polynucleotide encoding the stabilised GPCR or fragment thereof. For example, the polynucleotide may be
25 taken up into cells *in vivo* and expressed on the cell surface where it will stimulate an immune response.

The immunogen of a mutant GPCR may be provided as a whole cell preparation, in a cell membrane fragment, solubilised in detergent, in a lipid monolayer, in a
30 lipid bilayer, in a bead-linked lipid particle, in a solid-supported lipid layer or in a proteoliposome, as described above.

For the production of polyclonal antibodies, various suitable host animals (e.g., rabbit, goat, chicken, mouse or other mammal) may be immunized by one or
35 more injections with the immunogen. The polyclonal antibody molecules directed against the immunogenic protein can be isolated from the mammal (e.g., from the

serum or egg yolk) and further purified by well known techniques, such as affinity chromatography using protein A or protein G, which provide primarily the IgG fraction of immune serum

- 5 Monoclonal antibodies can be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent.
- 10

Generally, either peripheral blood lymphocytes are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, (1986) pp. 59-103). Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells can be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

15

20

25

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, Calif. and the American Type Culture Collection, Manassas, Va. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. *Immunol.*, 133:3001 (1984); Brodeur *et al.*,

30

35

Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63).

Alternatively, monoclonal antibodies can be generated using the 'Selected
5 Lymphocyte Antibody Method' (SLAM) as is well known in the art and described,
for example, in Babcook *et al.* (1996) *Proc. Natl. Acad. Sci.* 93:7843-7848.
Briefly, a single lymphocyte that is producing an antibody with a desired
specificity or function within a large population of lymphoid cells is identified.
Usually, lymphoid cells derived from an *in vivo* immunization are screened for
10 those that produce antibodies which bind to selected antigens using an adapted
haemolytic plaque assay (Jerne & Nordin, 1963, *Science*, 140:405) and the
genetic information that encodes the specificity of the antibody subsequently
retrieved from that lymphocyte.

15 Monoclonal antibodies can also be made by recombinant DNA methods, such as
those described in U.S. Pat. No. 4,816,567. DNA encoding monoclonal
antibodies raised against mutant GPCRs with increased stability can be readily
isolated and sequenced using conventional procedures (e.g., by using
oligonucleotide probes that are capable of binding specifically to genes encoding
20 the heavy and light chains of murine antibodies). The hybridoma cells described
above serve as a preferred source of such DNA. Once isolated, the DNA can be
placed into expression vectors, which are then transfected into host cells such as
simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do
not otherwise produce immunoglobulin protein, to obtain the synthesis of
25 monoclonal antibodies in the recombinant host cells. The DNA also can be
modified, for example, by substituting the coding sequence for human heavy and
light chain constant domains in place of the homologous murine sequences (U.S.
Pat. No. 4,816,567; Morrison, *Nature* 368, 812-13 (1994)) or by covalently joining
to the immunoglobulin coding sequence all or part of the coding sequence for a
30 non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can
be substituted for the constant domains of an antibody of the invention, or can be
substituted for the variable domains of one antigen-combining site of an antibody
of the invention to create a chimeric bivalent antibody.

35 It will be appreciated that single-chain antibodies specific to GPCRs can also be
produced (see e.g., U.S. Pat. No. 4,946,778). Antibody fragments that contain

the idiotypes to the mutant GPCR may also be produced by techniques known in the art including, but not limited to: (i) an $F_{(ab')_2}$ fragment produced by pepsin digestion of an antibody molecule; (ii) an F_{ab} fragment generated by reducing the disulfide bridges of an $F_{(ab')_2}$ fragment; (iii) an F_{ab} fragment generated by the treatment of the antibody molecule with papain and a reducing agent and (iv) F_v fragments. A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is found in Winter & Milstein (1991) *Nature* **349**, 293-299.

Bispecific antibodies may also be produced. Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for a mutant GPCR. The second binding target is any other antigen, and advantageously is a cell-surface protein or receptor, including another GPCR or receptor subunit. For example, bispecific antibodies would be useful for pairs of GPCRs which can form heterodimers (Milligan. *Biochim Biophys Acta*. 2007 Apr;1768(4):825-35.). In this case, the bispecific antibody may selectively target specific heterodimeric receptors. Bispecific antibodies may also be useful for viral entry co-receptors where one of the co-receptors is a GPCR, e.g CD4 and the GPCRs CCR5 or CXCR4 (Alkhatib G, Berger EA. *Eur J Med Res*. 2007 Oct 15;12(9):375-84). Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, *Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published May 13, 1993, and in Traunecker *et al.*, 1991 *EMBO J.*, 10:3655-3659.

It is appreciated that in some instances high throughput screening of test compounds is preferred and that the method may be used as a "library screening" method, a term well known to those skilled in the art. Thus, the test compound may be a library of test compounds. For example, the library may be a peptide or

protein library produced, for example, by ribosome display or an antibody library prepared either *in vivo*, *ex vivo* or *in vitro*. Methodologies for preparing and screening such libraries are known in the art.

5 Thus, rather than the test compound being an antibody raised against a mutant GPCR with increased stability in a particular conformation relative to its parent GPCR and then testing its binding to that GPCR, the test compound may be an antibody library. Thus another method for generating antibodies specific to a GPCR involves screening expression libraries encoding immunoglobulin genes,
10 or portions thereof, expressed in bacteria, yeast, filamentous phages, ribosomes or ribosomal subunits or other display systems. In this method, large libraries of antibody sequences or antibody fragment sequences are obtained from diverse sources such as healthy donors, patients or animals (healthy or not). These sequences are cloned and expressed in an appropriate system and antibodies
15 typically selected by binding to a GPCR with increased stability immobilised on a solid surface.

A particular example of an antibody library is a recombinant combinatorial antibody library, for example a scFv or Fab phage display library, prepared using
20 human VL and VH cDNAs prepared from mRNA derived from human lymphocytes (McCafferty et al., Nature 348:552-553 (1990)). According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M 13 or fd, and displayed as functional antibody fragments on the surface of the phage particle.
25 Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the ability of the antibody to bind to the GPCR with increased stability also result in selection of the gene encoding the antibody exhibiting those properties. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S. and Chiswell, David J.,
30 Current Opinion in Structural Biology 3:564-571 (1993). Moreover, examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, Ladner et al. U.S. Pat. No. 5,223,409; Kang et al. PCT Publication No. WO 92/18619; Dower et al. PCT Publication No. WO 91/17271; Winter et al. PCT Publication No. WO 92/20791;
35 Markland et al PCT Publication No. WO 92/15679; Breitling et al. PCT Publication No. WO 93/01288; McCafferty et al. PCT Publication No. WO 92/01047; Garrard

et al. PCT Publication No. WO 92/09690; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; McCafferty et al., *Nature* (1990) 348:552-554; Griffiths et al. (1993) *EMBO J.* 12:725-734; Hawkins et al. (1992) *J. Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

The methods may also be used to identify a polynucleotide capable of expressing a polypeptide binding partner of a GPCR, for example as described above in relation to phage display methods. Aliquots of an expression library in a suitable vector may be tested for the ability to give the required result. It will be appreciated that several cycles of identifying pools of polynucleotides comprising a polynucleotide having the required property and then rescreening those polynucleotides may be required in order to identify a single species of polynucleotide with the required property.

The invention includes screening methods to identify drugs or lead compounds of use in treating a disease or condition. It is appreciated that screening assays which are capable of high throughput operation are particularly preferred.

It is appreciated that in the methods described herein, which may be drug screening methods, a term well known to those skilled in the art, the test compound may be a drug-like compound or lead compound for the development of a drug-like compound.

The term "drug-like compound" is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons and which may be water-soluble. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to

penetrate target cellular membranes or the blood:brain barrier, but it will be appreciated that these features are not essential.

The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, poorly soluble, difficult to synthesise or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics.

Thus in one embodiment, the method further comprises modifying a test compound which has been shown to bind to the mutant GPCR when residing in a particular conformation, and determining whether the modified test compound binds to the mutant GPCR when residing in the particular conformation. It will be appreciated that it may be further determined whether the modified test compound also binds to the parent GPCR when residing in the particular conformation.

Various methods may be used to determine binding between a GPCR and a test compound including, for example, enzyme linked immunosorbent assays (ELISA), surface plasmon resonance assays, chip-based assays, immunocytofluorescence, yeast two-hybrid technology and phage display which are common practice in the art and are described, for example, in Plant *et al* (1995) *Analyt Biochem*, 226(2), 342-348. and Sambrook *et al* (2001) *Molecular Cloning A Laboratory Manual*. Third Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. Other methods of detecting binding between a test compound and the GPCR include ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Fluorescence Energy Resonance Transfer (FRET) methods, for example, well known to those skilled in the art, may be used, in which binding of two fluorescent labelled entities may be measured by measuring the interaction of the fluorescent labels when in close proximity to each other.

Where the mutant GPCR is provided as a whole cell preparation, a membrane protoplast or a proteoliposome, it will be appreciated that biophysical techniques such as patch clamping, magic angle spinning NMR, fluorescence correlation

spectroscopy, fluorescence resonance energy transfer and analytical ultracentrifugation may be used to analyse binding of the mutant GPCR to the test compound (as described in New, R. C., *Liposomes: a practical approach*. 1st ed.; Oxford University Press: Oxford, 1990, and Graham, J. M.; Higgins, J. A., *Membrane Analysis*. Springer-Verlag: New York, 1997.) Methods which allow quantitative, non-invasive determination of both the affinity and kinetics of such interactions include direct assays that allow continuous monitoring of membrane-protein binding, or in some cases by ultra-rapid separation of bound and free interactants followed by quantification of the membrane-bound and membrane-free analyte.

It will be appreciated that a bound test compound can be detected using a unique label or tag associated with the compound such as a peptide label, a nucleic acid label (Kerr et al., *JACS* (1993) vol. 115, p. 2529-2531; and Brenner & Lerner, *Proc. Natl. Acad. Sci. USA* (1992) vol. 89, p. 5381-5383), a chemical label (Ohlmeyer et al., *Proc. Natl. Acad. Sci. USA* (1993) vol. 90, p. 10922-10926; and Maclean et al., *Proc. Natl. Acad. Sci. USA* (1997) vol. 94, p. 2805-2810); a fluorescent label (Yamashita & Weinstock (SmithKline Beecham Corporation), WO95/32425 (1995); and Sebestyen et al., *Pept. Proc. Eur. Pept. Symp.* 22nd 1992 (1993), p. 63-64), or a radio frequency tag (Nicolau et al., *Angew. Chem. Int. Ed. Engl.* (1995) vol. 34, p. 2289-2291; and Moran et al., *JACS* (1995) vol. 117, p. 10787-10788).

Where the test compound is an antibody against a mutant GPCR with increased stability, produced using any of the methods described above, binding is preferably assayed using immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. For example, in an ELISA, typically, the stabilised GPCR is immobilised on a microtitre plate or other solid surface. The plates are first incubated with BSA or other similar protein to block non-specific binding sites. Samples containing the antibody (such as serum or egg yolk or hybridoma cell culture supernatant) are then added to the plate and the antibodies allowed to bind to the immobilised GPCR. Bound antibodies are detected by the addition of a second detection antibody which binds to the first antibody and allows detection via its conjugation to an enzyme which catalyzes a reaction which can be subsequently detected. In addition, the

binding affinity of antibodies can also be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem, 107:220 (1980). Preferably, antibodies having a high degree of specificity and a high binding affinity for the target GPCR are isolated.

5

It will be appreciated that where the antibody is a monoclonal antibody derived from a hybridoma, the hybridoma clones identified as expressing specific antibody can subsequently be subcloned by limiting dilution procedures and grown using standard methods. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells can be grown *in vivo* as ascites in a mammal. The monoclonal antibodies secreted by the subclones can then be isolated or further purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

Where the test compound is a phage display antibody library, binding to a stabilised GPCR may be assessed as follows. Typically, the mutant GPCR with increased stability is coated onto wells of a microtiter plate overnight at 4 °C. The wells are washed in PBS and blocked for 1 hour at 37 °C in MPBS (3% milk powder in PBS). Purified phage from a phagemid library e.g. expressing a repertoire of human scFv (10 transducing units (tu)) are then blocked for 1 hour in a final volume of 100µl of 3% MPBS. The blocked phage are added to the blocked GPCR wells and incubated for 1 hour. Wells are washed 5 times with PBST (PBS containing 0.1 % v/v Tween 20) before being wash 5 times with PBS. The bound phage particles are then eluted and used to infect 10 ml exponentially growing E. coli TG1. The infected cells are grown in 2TY broth for 1 hour at 37 °C, then spread onto 2TYAG plates and incubated overnight at 30 °C. Cultures from this first round of panning selection are superinfected with helper phage and rescued to give, for example, scFv antibody-expressing phage particles for the second round of panning.

An alternative binding assay for phage display antibodies includes the use of soluble selections using biotinylated mutant GPCR protein at a final concentration typically of 100 nM. In this case, purified scFv phage (10¹² tu) from a scFv

phagemid library (as described above) is suspended in 1 ml 3% MPBS and blocked for 30 minutes. The biotinylated GPCR is then added and incubated at room temperature for 1 hour. The phage/antigen complexes are subsequently added to 250 μ l of Dynal M280 Streptavidin magnetic beads that have been
5 blocked for 1 hour at 37 °C in 1 ml of 3% MPBS, and incubated with the beads for a further 15 minutes at room temperature. The beads are captured using a magnetic rack and washed 4 times in 1 ml of 3% MPBS/0.1% (v/v) Tween 20 followed by 3 washes in PBS. After the last PBS wash, beads are resuspended in 100 μ l PBS and used to infect 5 ml exponentially growing E.coli. Again, cultures
10 from this first round of soluble selection would be superinfected with helper phage and rescued to give scFv antibody-expressing phage particles for a second round of soluble selection.

It is appreciated that screening assays which are capable of high throughput
15 operation are particularly preferred to determine binding to a mutant GPCR, for example chip-based assays. Stabilised mutant GPCRs are particularly suited to such assays unlike their parent GPCRs which are not stable enough when purified to be used in these formats. In particular, technology called VLSIPS™ has enabled the production of extremely small chips that contain hundreds of
20 thousands or more of different molecular probes, i.e. the test compounds. These biological chips have probes arranged in arrays, each probe assigned a specific location. Biological chips have been produced in which each location has a scale of, for example, ten microns. The chips can be used to determine whether target molecules interact with any of the probes on the chip. After exposing the array to
25 target molecules under selected test conditions, scanning devices can examine each location in the array and determine whether a target molecule has interacted with the probe at that location.

A test compound to a mutant GPCR on a chip surface may be detected by
30 scanning the chip surface for radioactivity or fluorescence. The address of the interacting pair on the chip reveals the identity of the test compound or where there is an array of mutant GPCRs on the chip, the identity of the receptor (see, for example, Kuimelis et al., Addressable Protein Arrays, U.S. Ser. No. 60/080,686, Apr. 3, 1998, now abandoned, and U.S. Ser. No. 09/282,734, Mar.
35 31, 1999). In the latter case the array of mutant GPCRs may be used as a

method for obtaining selectivity data on compounds either for various conformations of the same GPCR or for various GPCRs.

Alternative methods of detecting binding of a mutant GPCR to a test compound, for example DNA, RNA, proteins and phospholipids, small molecules and natural products include surface plasmon resonance assays (SPA), for example as described in Plant *et al* (2005) *Analyt Biochem* **226(2)**, 342-348. The mutant GPCR, immobilised on a SPA bead, may be incubated with a single ligand which is labelled for example with a fluorescent group or the ligand may be a radioligand. The ability of a test compound to bind to the mutant GPCR may then be determined via its ability to displace the fluorescent ligand or the radioligand. In another example, the mutant GPCR is immobilised on a chip surface and binding of test compounds is detected by surface plasmon resonance and related techniques employing evanescent waves. Changes in refractive index can be used to determine the amount of bound compound, the affinity of interaction and the association and dissociation kinetics. An example of this approach has been described for rhodopsin which was immobilised on carboxylated dextran surfaces modified with long alkyl groups. Following amine coupling of the detergent-solubilised receptor, lipid/detergent-mixed micelles were adhered over the immobilized surface. The detergent was eluted in the subsequent buffer flow and the remaining lipid formed a bilayer on the chip surface. (Karlsson OP, Lofas S.. *Anal Biochem*. 2002 Jan 15;300(2):132-8.

Where the test compound is a peptide or protein, for example, a ligand for an orphan receptor or an interacting protein, the bound ligand may be eluted and then identified by mass spectrometry such as matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) or electrospray ionization mass spectrometry (ESI-MS) (Williams C. Addona TA. *Trends Biotechnol*. 2000 Feb;18(2):45, Williams C *Curr Opin Biotechnol*. 2000 Feb;11(1):42-6). The mutant GPCR may be immobilised on a column or bead, or expressed in tagged form in a cell and co-purified with the ligand from such complex mixtures using reagents directed to the tag or directly to the GPCR (Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Séraphin B. *Nat Biotechnol*. 1999 Oct;17(10):1030-2).

The ability to generate high affinity conformation specific binding partners to GPCRs will facilitate the production of therapeutic GPCR binding partners. Thus, it will be appreciated that in addition to establishing binding to a GPCR, it will also be desirable to determine the functional effect of a binding partner on the GPCR.

5

Accordingly, in an embodiment of the invention, the method further comprises determining if the binding partner affects the function of the GPCR to which it binds and isolating a test compound that affects the function of the GPCR.

10 For example, in one embodiment, it is determined whether the binding partner alters the binding of the GPCR to its ligand. By ligand, we include any molecule which binds to the GPCR and which causes the GPCR to reside in a particular conformation as described above. Preferably, the ligand is the natural ligand of that GPCR or an analogue thereof. Binding of a GPCR to its ligand can be
15 assayed using standard ligand binding methods known in the art and, for example, as described above. For example, the ligand may be radiolabelled or fluorescently labelled. The binding assay can be performed using the stabilised mutant GPCR or the parent GPCR. Typically, the stabilised GPCR is purified or expressed in a cell such as a mammalian, bacterial or insect cell. Typically, the
20 parent receptor is expressed in a cell such as a mammalian, bacterial or insect cell. The assay may be carried out on whole cells or on membranes obtained from the cells. The binding partner will be characterised by its ability to alter the binding of the labelled ligand.

25 In one embodiment, the binding partner decreases binding between the GPCR and its ligand. For example, the binding partner may decrease binding by a factor of at least 2 fold, 3 fold, 4 fold, 5 fold, 10 fold, 15 fold, 20, fold, 50 fold, 100 fold, 250 fold, 500 fold or 1000 fold. Preferably, the binding partner decreases binding by a factor of between 100-1000 fold, such as between 10-100 fold.

30

In one embodiment, the binding partner increases binding between the GPCR and its ligand. For example, the binding partner may increase binding by a factor of at least 2 fold, 3 fold, 4 fold, 5 fold, 10 fold, 15 fold, 20, fold, 50 fold, 100 fold, 250 fold, 500 fold or 1000 fold. Preferably, the binding partner increases binding
35 by a factor of between 100-1000 fold, such as between 10-100 fold.

In a further embodiment, it is determined whether the binding partner modulates activation of a GPCR. For example, if a mutant GPCR stabilised in an agonist conformation was provided in step (a) of the method of the invention, the binding partner selected may be an agonist binding partner and thus increase activation of the GPCR. If a mutant GPCR stabilised in an antagonist conformation was provided in step (a) of the method of the invention, the binding partner selected may be an antagonist binding partner and thus decrease activation of the GPCR.

In this assay the parent GPCR or stabilised mutant GPCR is expressed *in vivo*, for example, in mammalian or insect cells where the GPCR is allowed to couple to well known GPCR signal transduction pathways (Eglén R.M. Functional G protein-coupled receptor assays for primary and secondary screening. Comb Chem High Throughput Screen. 2005 Jun;8(4):311-8). Such assays include: calcium mobilisation (Gonzalez JE, Maher MP. Cellular fluorescent indicators and voltage/ion probe reader (VIPR) tools for ion channel and receptor drug discovery. Receptors Channels. 2002;8(5-6):283-95, Dupriez VJ, Maes K, Le Poul E, Burgeon E, Detheux M. Aequorin-based functional assays for G-protein-coupled receptors, ion channels, and tyrosine kinase receptors. Receptors Channels. 2002;8(5-6):319-30), changes in cAMP levels (Weber M, Ferrer M, Zheng W, Inglese J, Strulovici B, Kunapuli P.A 1536-well cAMP assay for Gs- and Gi-coupled receptors using enzyme fragmentation complementation. Assay Drug Dev Technol. 2004 Feb;2(1):39-49.), activation of kinase pathways (Leroy D, Missotten M, Waltzinger C, Martin T, Scheer A.G protein-coupled receptor-mediated ERK1/2 phosphorylation: towards a generic sensor of GPCR activation. J Recept Signal Transduct Res. 2007;27(1):83-97), regulation of gene transcription for example via the use of a reporter gene (Liu B, Wu D. Analysis of the coupling of G12/13 to G protein-coupled receptors using a luciferase reporter assay. Methods Mol Biol. 2004;237:145-9, Kent TC, Thompson KS, Naylor LH. Development of a generic dual-reporter gene assay for screening G-protein-coupled receptors J Biomol Screen. 2005 Aug;10(5):437-46), recruitment of β -arrestin (Hudson CC, Oakley RH, Sjaastad MD, Loomis CR. High-content screening of known G protein-coupled receptors by arrestin translocation Methods Enzymol. 2006;414:63-78), activation of G proteins such as measuring GTPase activity (Jameson EE, Roof RA, Whorton MR, Mosberg HI, Sunahara RK, Neubig RR, Kennedy RT. Real-time detection of basal and stimulated G protein GTPase activity using fluorescent GTP analogues. J Biol Chem. 2005 Mar

4;280(9):7712-9) or measuring [³⁵S]GTPgammaS binding (Rodgers G, Hubert C, McKinzie J, Suter T, Statnick M, Emmerson P, Stancato L. Development of displacement binding and GTPgammaS scintillation proximity assays for the identification of antagonists of the micro-opioid receptor. Assay
5 Drug Dev Technol. 2003 Oct;1(5):627-36).

Binding partners are typically selected which modulate the activation of the receptor.

10 For agonist binding partners the binding partner will typically mimic the activity of the natural ligand of the receptor and produce an increase in receptor activation, G protein activation or signal transduction. This will occur in the absence of an additional agonist. An agonist binding partner may increase receptor activation by a factor of at least 2 fold, 3 fold, 4 fold, 5 fold, 10 fold, 15 fold, 20, fold, 50 fold,
15 100 fold, 250 fold, 500 fold, 1000 fold, or 10000 fold.

It will be appreciated that there are two ways in which a binding partner may increase receptor activation. For example, the binding partner may act as a direct agonist, in which case receptor activation is typically increased by between
20 2-1000 fold. In another method, the binding partner may act to amplify the activity of an agonist. For example, the binding partner may increase the potency of the agonist, in which case receptor activation is typically increased by between 2-1000 fold, such as between 10-100 fold, or the binding partner may increase the maximal response produced by the agonist, in which case receptor activation
25 is typically increased by between 2-10 fold. It will be appreciated that activating/amplifying the activity of receptors that are already switched on by endogenous ligand may be preferable to turning on all available receptors, since it is more physiologically specific and may mitigate issues such as desensitisation and undesirable side-effects (Christopoulos A (2002) Nat Rev Drug Discov
30 1:198-210).

In the case of antagonist binding partners the binding partner will typically act to block the activity of the receptor or its activation by an agonist. The antagonist binding partner may do this by blocking the binding of the agonist or by locking
35 the receptor in an inactive form such that it is unable to couple to G proteins. An antagonist binding partner may decrease receptor activation by a factor of at least

2 fold, 3 fold, 4 fold, 5 fold, 10 fold, 15 fold, 20, fold, 50 fold, 100 fold, 250 fold, 500 fold, 1000 fold or 10000 fold. Typically, an antagonist binding partner would reduce receptor activation to a non-detectable level.

5 It will be appreciated that it may be desirable to produce a test compound or combination of test compounds that bind to more than one GPCR. For example, the test compound may bind to more than one GPCR of the same class of family. A list of GPCR classes and families has been produced by the International Union of Pharmacology (Foord *et al* (2005) *Pharmacol. Rev.* **57**, 279-288) and
10 this list is periodically updated at <http://www.iuphar-db.org/GPCR/ReceptorFamiliesForward>. The test compound may bind to one target GPCR and at least one further GPCR. The at least one further GPCR may be a GPCR that has been implicated in a disease pathway, or a GPCR that is involved in the same signalling pathway as the target GPCR, for example a
15 signalling pathway that regulates or modulates a disease pathway. In addition, the at least one further GPCR may be one which enhances or suppresses the action of a test compound on the target GPCR. Such enhancement or suppression of action can be determined using methods well known in the art including binding assays and functional assays as described, for example,
20 above.

Accordingly, in one embodiment more than one mutant GPCR is provided in step (a). For example, at least 2, 3, 4 or 5 mutant GPCRs of a different parent GPCR may be provided in step (a). Thus, in this embodiment, test compounds are
25 selected for which bind to more than one GPCR. The compound may be a cross-reactive compound including, for example, a small molecule, affibody, antibody or diabody. It will be appreciated that such an approach may lead to improvements in a binding partner's efficacy or potency.

30 Thus, a test compound may be selected which binds to a first and second GPCR, where the first and second GPCRs may be any pair of GPCRs. For example, the method can be used to select for a bivalent partner that binds to GPCRs which form a heterodimer, in which case the bivalent partner could bind to both receptors at the same time. Examples of appropriate GPCR heterodimers in this
35 context include dopamine D1 and adenosine A1, opioid receptor heterodimers, cannabinoid CB1 and orexin receptors (Marshall FH. Heterodimerization of G-

protein-coupled receptors in the CNS. *Curr Opin Pharmacol.* 2001 Feb;1(1):40-4; Kent T, McAlpine C, Sabetnia S, Presland J. G-protein-coupled receptor heterodimerization: assay technologies to clinical significance. *Curr Opin Drug Discov Devel.* 2007 Sep;10(5):580-9). Moreover, a binding partner could be selected for which binds to and modulates the activity of two independent receptors for the purpose of enhanced therapeutic activity. For example, CCK1/opioid receptor peptides bind to both CCK1 and the opioid receptor (García-López MT, González-Muñiz R, Martín-Martínez M, Herranz R. Strategies for design of non peptide CCK1R agonist/antagonist ligands. *Curr Top Med Chem.* 2007;7(12):1180-94). Other appropriate examples include a combined beta 2 agonist and muscarinic antagonist; a dopamine D2 antagonist and 5HT2 antagonist; a D2 antagonist and 5HT6 antagonist; and a M1 agonist and 5HT6 antagonist.

It will be appreciated that the more than one GPCR provided in step (a) may or may not reside in the same conformation. For example, a diabody or similar bivalent binding partner may agonise at one end of the molecule and antagonise at another end, in which case the GPCRs would not have to reside in the same conformation.

Typically, where a test compound is selected that binds to more than one GPCR, the test compound binds to each GPCR with a similar potency. Typically, the K_d values for the particular binding partner binding to each of the respective GPCRs are within 5-10 fold of each other, such as within 2-3 fold.

It will be appreciated that the methods of the invention allow for combinations of test compounds that bind to one or more GPCRs to be isolated, either by repeating the method with single test compounds, providing multiple test compounds in one cycle of the method or by using a library of test compounds in the method.

In a further embodiment, it may be advantageous to select those test compounds which, while still able to bind to a first GPCR, are not able to bind, or bind less strongly than to the first GPCR, to at least one other GPCR, for example a second GPCR. It will be appreciated that the first and second GPCRs may be any pair of GPCRs. Thus, for example, the test compound may be one that is

selected on the basis that it binds to a first GPCR, but the test compound so selected is further tested to determine whether it binds a second GPCR (or binds less strongly to a second GPCR than the first GPCR). Test compounds are selected which do not bind (or have reduced binding to) the second GPCR.

- 5 Where the binding partner is therapeutic molecule, such an approach may help to reduce the binding partner's toxicity.

It is preferred that the test compound binds the further (second) GPCR with an affinity which is less than 50% of the affinity the compound has for first GPCR,
10 more preferably less than 10% and still more preferably less than 1% or 0.1% or 0.01% of the affinity the compound has for the first GPCR. Thus, the K_d for the interaction of the test compound with the first GPCR is higher than for the second GPCR.

- 15 A second aspect of the invention provides a method for producing a binding partner of a GPCR, the method comprising synthesising a binding partner identifiable by carrying out the method according to the first aspect of the invention.

20 The binding partners can be synthesised by any suitable method known in the art including the techniques of organic chemistry, molecular biology or biochemistry. For example, if the binding partner is a polypeptide, the binding partner may be made by expressing the nucleic acid molecule encoding the binding partner in a suitable host cell as is known in the art. Antibodies may be synthesised using
25 any of the methods described above including, for example, recombinant DNA technology.

A third aspect of the invention provides a binding partner obtained by any of the methods of the first aspect of the invention.

30

In an embodiment, the binding partner is a conformation-specific binding partner, as described above.

A fourth aspect of the invention provides a binding partner, for example a
35 conformation-specific binding partner, obtainable by any of the methods of the first aspect of the invention.

The binding partner may be any of a polypeptide; an anticalin; a peptide; an antibody; a chimeric antibody; a single chain antibody; an aptamer; a darpin; a Fab, F(ab')₂, Fv, ScFv or dAb antibody fragment; a small molecule; a natural product; an affibody; a peptidomimetic; a nucleic acid; a peptide nucleic acid molecule; a lipid; a carbohydrate; a protein based on a modular framework including ankyrin repeat proteins, armadillo repeat proteins, leucine rich proteins, tetrariopeptide repeat proteins or Designed Ankyrin Repeat Proteins (DARPs); or proteins based on lipocalin or fibronectin domains or Affilin scaffolds based on either human gamma crystalline or human ubiquitin.

In a preferred embodiment, the binding partner is an antibody. For example, the antibody may be specific to a non-contiguous epitope in the GPCR or may be specific to a contiguous epitope in the GPCR. Preferably, the relevant epitopes in the parent and mutant GPCR recognised by the antibody are similar, whether they be contiguous or non-contiguous. Specifically, extracellular epitopes such as C- or N-termini or polypeptide loops of the parent and mutant GPCR are preferably similar.

Typically the binding partner to the mutant GPCR with a similar potency to its binding to the parent GPCR. Typically, the K_d values for the particular binding partner binding the mutant GPCR and the parent GPCR are within 5-10 fold of each other, such as within 2-3 fold. Typically, the binding of the binding partner to the mutant GPCR compared to the parent GPCR would be not more than 5 times weaker and not more than 10 times stronger.

Typically, mutant receptors which have been stabilised in the selected conformation should bind the binding partner with approximately equal affinity (that is to say typically within 2-3 fold) or greater affinity than does the parent receptor. For agonist-conformation binding partners, the mutants typically bind the agonists with the same or higher affinity than the parent GPCR and typically bind antagonists with the same or lower affinity than the parent GPCR. Similarly for antagonist-conformation binding partners, the mutants typically bind the antagonists with the same or higher affinity than the parent GPCR and typically bind agonists with the same or lower affinity than the parent GPCR.

It is appreciated that the methods of the invention may be used as a biosensor to detect target substances such as molecules, especially biomolecules. For example, the biosensor may be used to detect biomarkers of disease or drug treatment which may be used as a diagnostic or prognostic. The mutant GPCR
5 may be immobilised on a sensor surface and binding of compounds detected, for example, by surface plasmon resonance as described above. In a further example of a biosensor, compound binding to the receptor can be detected by changes in intrinsic tryptophan fluorescence or using fluorescence resonance energy transfer between an intrinsic tryptophan resident donor and a fluorescent
10 acceptor (Lakowicz JR 1999. Principles of fluorescence spectroscopy, Plenum New York, Martin DD, Budamagunta MS, Ryan RO, Voss JC, Oda MN. J Biol Chem. 2006 Jul 21;281(29):20418-26). Alternatively, mutant GPCRs may be used in acoustic biosensors, wherein the mutant GPCRs are immobilised on quartz crystal resonator sensors (QCRS) and the acoustic sensor response used
15 to detect compound – mutant GPCR binding interactions (Cooper MA, Drug Discov Today. 2006 Dec;11(23-24):1068-74. Epub 2006 Oct 20).

Accordingly, a fifth aspect of the invention provides a biosensor comprising a mutant GPCR of a parent GPCR wherein the mutant GPCR has increased
20 stability in a particular conformation relative to the parent GPCR, and wherein when a target substance binds to said mutant GPCR, a detectable signal is produced.

Preferences for the mutant GPCRs and their methods of production are as
25 defined above with respect to the first aspect of the invention.

Preferably, the biosensor is in a chip form or a bead supported form, where the mutant GPCRs are immobilised on a chip or bead and used to detect target substances. However, it will be appreciated that the mutant GPCRs may be
30 provided in soluble form, in which case the biosensor would comprise a solution.

Immobilisation of mutant GPCRs onto a solid support for incorporation into a biosensor can be performed using methods well known in the art and as described above with respect to the first aspect of the invention. Typically,
35 mutant GPCRs are reconstituted onto chip surfaces suitable for direct biosensor analysis via flow-mediated surface reconstitution (Karlsson et al., Analytical

Biochemistry 300, 132–138 (2002)). For example, rapid immobilization and reconstitution of GPCRs on carboxylated dextran surfaces modified with long alkyl groups can be achieved following amine coupling of a detergent-solubilized receptor; lipid/detergent-mixed micelles are adhered as they are injected over the immobilized surface, taking advantage of integrated flow cells present in many biosensor systems. The detergent can then be eluted in the subsequent buffer flow leaving functional, intact mutant GPCRs for subsequent screening and analysis. Such mutant GPCR preparations containing detergent are ideally suited for use in a flow-based biosensor, such as quartz crystal microbalance biosensor, an evanescent wave biosensor, a planar wave guide biosensor, a surface Raman sensor, or a surface plasmon resonance biosensor. In the latter case, solubilized receptors can be captured on a GE Healthcare (Biacore) CM4 or CM5 dextran sensor chip. The dextran matrix of the sensor chip is activated by 35 μ L of 50 mM *N*-hydroxysuccinimide and 200 mM *N*-ethyl-*N*-[(dimethylamino)propyl]carbodiimide at a flow rate of 5 μ L/min, followed by a 7-min injection of 0.1 mg/mL detergent-solubilised GPCR receptor (for example with 25 mM CHAPS in 10 mM MOPS, pH 7.5). Any remaining reactive carboxy groups are deactivated using a 7-min pulse of 1 M ethanolamine hydrochloride, pH 8.5. After the injection, the biosensor chip is washed at high flow rate with the surface plasmon resonance running solution until a stable baseline is restored (ca. 30 min). This washing step works like a flow dialysis procedure and ensures the removal of the detergent from the sensor chip surface; however, it will be appreciated that hydrophobic parts of the GPCR may still be attached to some lipid or detergent molecules in order to maintain functional integrity.

The target substance may be any of a molecule, a biomolecule, a peptide, a protein, a carbohydrate, a lipid, a GPCR ligand, a synthetic molecule, a drug, a drug metabolite or a disease biomarker.

In an embodiment, the detectable signal is any of a change in colour; fluorescence; evanescence; surface plasmon resonance; electrical conductance or charge separation; ultraviolet, visible or infrared absorption; luminescence; chemiluminescence; electrochemiluminescence; fluorescence anisotropy; fluorescence intensity; fluorescence lifetime; fluorescence polarisation; fluorescence energy transfer; molecular mass; electron spin resonance; nuclear magnetic resonance; hydrodynamic volume or radius; specific gravity;

scintillation; field effect resistance; electrical impedance; acoustic impedance; quantum evanescence; resonant scattering; fluorescent quenching; fluorescence correlation spectroscopy; acoustic load; acoustic shear wave velocity; binding force; or interfacial stress.

5

The invention will now be described in more detail with respect to the following Figures and Examples wherein:

Figure 1 Amino acid changes in β AR that lead to thermostability. Stability quotient indicates the % remaining binding activity of the mutants after heating the sample for 30 min at 32°C. All values are normalized to β AR₃₄₋₄₂₄ (50%, showed as a discontinuous line) to remove any experimental variability between assays. Bars show the stability for each mutant. The letters on the x-axis indicate the amino acid present in the mutant. The original amino acid and its position in β AR₃₄₋₄₂₄ is indicated below. Bars corresponding to the same amino acid in β AR₃₄₋₄₂₄ are in the same colour with arrows indicating the best mutations. Errors were calculated from duplicate measurements; the best mutants were subsequently re-assayed to determine the T_m for each individual mutation and to give an accurate rank order of stability for each mutant (see Example 1).

20

Figure 2 Side chains in rhodopsin that are at equivalent positions to the thermostable mutations in β AR₃₄₋₄₂₄. The equivalent amino acid residues in rhodopsin to the amino acid residues mutated in β AR₃₄₋₄₂₄ were located in the rhodopsin structure, based upon an alignment among rhodopsin, β 1 adrenergic receptor, neurotensin receptor, and adenosine A_{2a} receptor (data not shown). Side chains in the same transmembrane helix are shown as space filling models in the same colour. The name and position of the amino acid residues are those in rhodopsin.

Figure 3 Evolution of thermostability in β AR. Starting from β AR-m10-8, combinations of mutations were rearranged systematically to find the optimum combination of mutations (see also Table 2).

Figure 4 Stability of β AR-m23 and β AR₃₄₋₄₂₄ in the apo-state or containing the bound antagonist [³H]-DHA. To determine T_m in the absence of ligand (apo-state, discontinuous lines), detergent-solubilised receptors were incubated

for 30 minutes at the temperatures indicated before carrying out the binding assay. For the T_m determination of the antagonist-bound form (continuous lines), detergent-solubilised receptors were pre-incubated with [3 H]-DHA, followed by incubation at the temperatures indicated. β AR-m23 (circles), and β AR₃₄₋₄₂₄ (squares). Data points are from duplicates measurements in a representative experiment.

Figure 5 Competition binding of agonists to β AR-m23 and β AR₃₄₋₄₂₄. Binding assays were performed on receptors partially purified in DDM; β AR-m23 (triangles) and β AR₃₄₋₄₂₄ (squares). [3 H]-DHA was used at a concentration three times greater than the K_D of partially purified receptor (see Methods). [3 H]-DHA binding was competed with increasing concentrations of the agonists, norepinephrine (a) and isoprenaline (b), or with an antagonist, alprenolol (c). LogEC₅₀ and corresponding EC₅₀ values for the different ligands were calculated by nonlinear regression using GraphPad Prism software and the error for logEC₅₀s were lower than 10%. The EC₅₀s for ligand binding to β AR₃₄₋₄₂₄ and β AR-m23 are: norepinephrine, β AR₃₄₋₄₂₄ 1.5 μ M, β AR-m23 3.7 mM; isoprenaline, β AR₃₄₋₄₂₄ 330 nM, β AR-m23 20 μ M; alprenolol, β AR 78 nM, β AR-m23 112 nM.

Figure 6 Stability of β AR-m23 and β AR₃₄₋₄₂₄ in five different detergents. Samples of β AR₃₄₋₄₂₄ (a), and β AR-m23 (b) solubilized in DDM were partially purified on Ni-NTA agarose columns allowing the exchange into various different detergents: DDM (squares), DM (triangles), OG (inverted triangles), LDAO (diamonds) and NG (circles). β AR is so unstable in OG, NG and LDAO that it was not possible to measure any activity after purification at 6°C. Assays were carried out as described in the Methods and the T_m is shown at the intersection between the curves and the discontinuous line. Results are from duplicate measurements in a representative experiment performed in parallel. (c) Photomicrograph of a crystal of β AR-m23 mutant, which showed good order by X-ray diffraction.

Figure 7 Curve of thermostability of β AR₃₄₋₄₂₄ (T_m). Binding assays were performed using [3 H]-dihydroalprenolol (DHA) as radioligand as described under "Methods". Samples were heated for 30 minutes at different temperatures before the assay. T_m represents the temperature at which the binding decreased to the 50%, value showed as a discontinuous line. Data points are from duplicates of

one single experiment. This experiment has been repeated several times with similar results.

Figure 8 Saturation binding assays of membranes of βAR_{34-424} and $\beta\text{AR-m23}$.

5 Binding assays were performed as described in "Methods" using [^3H]-dihydroalprenolol (DHA) as radioligand; βAR_{34-424} (a) and $\beta\text{AR-m23}$ (b). Scatchard plots are shown as insets along with the corresponding values for B_{max} and K_D . Data points are from duplicates of two independent experiments for each protein. Data were analyzed by nonlinear regression using Prism software
10 (GraphPad).

Figure 9 Alignment of the turkey β -adrenergic receptor with human $\beta 1$, $\beta 2$ and $\beta 3$ receptors.

15 **Figure 10** Alignment of human adenosine receptors.

Figure 11 Alignment of neurotensin receptors.

Figure 12 Flow chart showing the two different assay formats of ligand (+) and
20 ligand (-) used to determine receptor thermostability.

Figure 13 Pharmacological profile of thermostable mutant adenosine A2a receptor, Rant21. Saturation binding of (A) antagonist and (B) agonist to solubilised receptors. (C-F) Inhibition of [^3H]ZM241385 binding by increasing
25 concentrations of antagonists (C) XAC and (D) Theophylline, and agonists (E) NECA and (F) R-PIA; binding of [^3H]ZM241385 (10 nM) in the absence of unlabelled ligand was set to 100%. Each solubilised receptor was incubated with ligands for one hour on ice in binding buffer (50mM Tris pH7.5 and 0.025% DDM) containing 400 mM NaCl (A, C-F). Data shown are from two independent
30 experiments with each data point measured in triplicate. K_D and K_i values are given in Table (iii).

Figure 14 Thermostable mutants show a decreased dependence on lipids (A) and an increased survival at higher concentration of DDM (B) upon heating
35 compared to the wild-type receptor. Receptors were solubilised in 1% DDM (diluted in 50 mM Tris pH7.5 and 400 mM NaCl) and immobilised on Ni-NTA

agarose for the IMAC step. Exchange of buffer containing the appropriate concentration of DDM and/or lipids was performed during washes and elution from the Ni-NTA beads.

- 5 **Figure 15** Mapping of the M90V, Y227A, A282L and F338M m23 mutations in turkey beta1 adrenergic receptor onto homologous residues (M82, Y219, C265 and A321 respectively) in the human beta2 adrenergic receptor structure (Rasmussen *et al* (2007) Nature 15:383-387; pdb accession codes 2R4R and 2R4S) reveals their position at a helical interface and helical kink respectively.
- 10 Amino acid residues in equivalent positions to the thermostabilising mutations in the turkey β 1 adrenergic receptor are shown as labelled space filling models.

- Figure 16** Mapping of m23 mutations in turkey beta1 adrenergic receptor onto homologous residues in the human beta2 adrenergic receptor structure
- 15 (Cherezov *et al* (2007) Science, 318:1258-65; pdb accession code 2RH1). The α trace of the β 2AR is shown with the fusion moiety (T4 lysozyme) removed. The six mutations in β AR-m23 (R68S, M90V, Y227A, A282L, F327A, F338M) are equivalent to amino acid residues K60, M82, Y219, C265, L310, F321 in the human β 2AR. Lys60 is on the intracellular end of Helix 1 and points into the lipid-
- 20 water interface. Met82 is near the middle of Helix 2 and points into the ligand binding pocket; the nearest distance between the substrate carazolol and the Met side chain is 5.7 Å. Tyr219 is towards the intracellular end of helix 5 and is at the helix5-helix 6 interface. Cys265 is at the end of the loop region between helices 5 and 6 and points away from the transmembrane regions. Leu310 and Phe321
- 25 are both in helix 7 and both point out into the lipid bilayer.

- Figure 17** Multiple sequence alignment of human beta-2AR, rat NTR1, turkey beta-1 AR, human Adenosine A2aR and human muscarinic M1 receptors. In each sequence, thermostabilising mutations are marked with a box. Mutations
- 30 occurring in two or more sequences are denoted with a star.

- Figure 18** Mapping of turkey beta1AR mutation I55A (human beta2AR I47) onto human beta2AR structure (pdb accession code 2RH1). Mutation is at the interface between 3 helices (H1, H2 kink, H7 kink). Left: side view; right: top
- 35 view.

Figure 19 Mapping of turkey beta1AR V89L mutation (human beta2AR V81) onto human beta2AR structure (pdb accession code 2RH1). Mutation is in the kink in helix 2. The helices are numbered and the bound antagonist is shown as a space filling model. Amino acid residues in equivalent positions to the thermostabilising mutations in the turkey β 1 adrenergic receptor are shown as space filling models and are arrowed for clarity. Left: side view; right: top view.

Figure 20 Mapping of turkey beta1AR M90V mutation (human beta2AR M82) onto human beta2AR structure (pdb accession code 2RH1). Mutation is in kink in helix 2 oriented towards the binding pocket. The helices are numbered and the bound antagonist is shown as a space filling model. Amino acid residues in equivalent positions to the thermostabilising mutations in the turkey β 1 adrenergic receptor are shown as space filling models and are arrowed for clarity. Left: side view; right: top view.

Figure 21 Mapping of turkey beta1AR I129V mutation (human beta2AR I121) onto human beta2AR structure (pdb accession code 2RH1). Mutation is opposite a kink in helix 5. The helices are numbered and the bound antagonist is shown as a space filling model. Amino acid residues in equivalent positions to the thermostabilising mutations in the turkey β 1 adrenergic receptor are shown as space filling models and are arrowed for clarity. Left: side view; right: bottom view.

Figure 22 Mapping of turkey beta1AR F338M mutation (human beta2AR F321) onto human beta2AR structure (pdb accession code 2RH1). Mutation is in kink in helix 7. The helices are numbered and the bound antagonist is shown as a space filling model. Amino acid residues in equivalent positions to the thermostabilising mutations in the turkey β 1 adrenergic receptor are shown as space filling models and are arrowed for clarity. Left: side view; right: top view.

Figure 23 Mapping of turkey beta1AR Y227A mutation (human beta2AR Y219) onto human beta2AR structure (pdb accession code 2RH1). Mutation is at helix-helix interface. The helices are numbered and the bound antagonist is shown as a space filling model. Amino acid residues in equivalent positions to the thermostabilising mutations in the turkey β 1 adrenergic receptor are shown as

space filling models and are arrowed for clarity. Left: side view; right: bottom view.

Figure 24 Mapping of turkey beta1AR A282L mutation (human beta2AR C265) onto human beta2AR structure (pdb accession code 2RH1). Mutation is in loop region. The helices are numbered and the bound antagonist is shown as a space filling model. Amino acid residues in equivalent positions to the thermostabilising mutations in the turkey β 1 adrenergic receptor are shown as space filling models and are arrowed for clarity. Left: side view; right: top view.

Figure 25 Mapping of turkey beta1AR R68S mutation (human beta2AR K60) onto human beta2AR structure (pdb accession code 2RH1). Mutation is at the lipid-water boundary, pointing into the solvent. The helices are numbered and the bound antagonist is shown as a space filling model. Amino acid residues in equivalent positions to the thermostabilising mutations in the turkey β 1 adrenergic receptor are shown as space filling models and are arrowed for clarity. Left: side view; right: angled top view.

Figure 26 Comparison of the thermostabilities of three β adrenergic receptors (turkey β 1 (■), human β 1 (▼) and human β 2 (●)) and two thermostabilised receptors (turkey β 1-m23 (▲) and human β 2-m23 (◆)). The six thermostabilising mutations in β 1-m23 (R68S, M90V, Y227A, A282L, F327A, F338M) were all transferred directly to the human β 2 receptor (K60S, M82V, Y219A, C265L, L310A, F321M) making β 2-m23, based upon the alignment in Figure 9. The resulting mutants were transiently expressed in mammalian cells, solubilised in 0.1% dodecylmaltoside and assayed for thermostability in the minus-ligand format (heating the apo-state, quenching on ice, adding 3H-DHA). The apparent Tms for turkey β 1 and β 2-m23 were 23°C and 45°C respectively, giving a ΔT_m of 22°C as seen previously in *E.coli* expressed receptor. The Tms for human β 2 and β 2-m23 were 29°C and 41°C respectively, showing that the apo receptor was stabilised by 12°C. This exemplifies the principle of the transferability of thermostabilising mutations from one receptor to another receptor, which in this case are 59% identical. The human β 1 receptor ($T_m \sim 12^\circ\text{C}$) is much less stable than the turkey β 1 receptor.

Figure 27 Percentage identity of the turkey $\beta 1$ adrenergic receptor, human adenosine receptor and rat neurotensin receptor to human β adrenergic receptors, human adenosine receptors and human neurotensin receptors, respectively.

5

Figure 28 Alignment of neurotensin receptors

Figure 29 Schematic representation of typical lead isolation process for the identification of inhibitory scFv binders.

10

Figure 30 Results of (A) polyclonal and (B) monoclonal phage ELISAs. (B) Columns 1-9 rows A-H of a 96 well ELISA plate were used to screen 69 anti β -AR phage clones

15 **Figure 31** Specificity phage ELISA using β -adrenergic receptor and 3 unrelated control proteins (CD86-CD4, Notch1-Fc and the NRR region of Notch1). Proteins coated on two ELISA plates, amino-plate (A) and His-plate (B) are shown. Beta-AR phage clones (hashed bars) from left to right (C1, E2, A3, G3, C4, D4, F4, H4, D5, F5, G5, C6, D6, C7, F7, B8 and C8) names originates from the screen shown
20 in Figure 30. In the graph, sticky anti β -AR clones are indicated by their clone names over the corresponding bar. Also shown are binding of control phage populations specific to the control proteins, anti-CD86 (grey bars), anti-N1 EGF (white bars) and anti-N1 NRR (dotted bars).

25 **Figure 32** Anti- β -AR antibody clones (white bars) and positive control antibody (black bar) and a no antibody negative control (black bar) are shown.

Figure 33 Two capture stages of the biotinylated $\beta 1$ AR onto a streptavidin-coated flow cell. A ~1200 RU captured; B ~4000 RU captured.

30

Figure 34 Biacore responses for alprenolol. The highest concentration is 666 nM and each concentration was tested three times in a three-fold dilution series. The responses are concentration dependent and fairly reproducible. The lines depict the fit of a simple 1:1 interaction model and the parameters determined

from this fit are listed in the inset (the number in parentheses is the error in the last digit).

Figure 35 Normalised responses to alprenolol. The responses are normalized with respect to the Rmax determined (from the fitting) for each curve.

Figure 36 Biacore responses for propranolol. The highest concentration is 111 nM and each concentration was tested two or three times in a three-fold dilution series.

Figure 37 Normalised responses to propranolol. The responses are normalized with respect to the Rmax determined (from the fitting) for each curve.

Figure 38 Biacore responses to alprenolol on β 1AR surface that was almost three days old.

Figure 39 Capture of β 1AR to a density of 8000 RU.

Figure 40 Biacore response to alprenolol binding to 8000 RU β 1AR surface.

Figure 41 Biacore responses to salmeterol using 2 different surfaces. Salmeterol was tested using a highest concentration of 1.67 μ M, with each concentration tested two or three times. A. 4200 RU surface; B. 8000 RU surface. Larger responses are observed with the 8000 RU surface.

Figure 42 Biacore responses for salmeterol, shown as normalised responses. A. 4200 RU surface; B. 8000 RU surface.

Figure 43 Biacore responses to isoproterenol. Isoproterenol was tested using a highest concentration of 2 μ M. A. 4200 RU surface. B. 8000 RU surface.

Figure 44 Biacore responses for carvedilol. A. 5200 RU surface; B. 8000 RU surface.

Figure 45 Competition binding curves to a range of compounds tested for activity at the stabilised adenosine A2a receptor (Rant22) using [3 H]ZM241385.

Example 1: Conformational stabilisation of the β -adrenergic receptor in detergent-resistant form

5 *Summary*

There are over 500 non-odorant G protein-coupled receptors (GPCRs) encoded by the human genome, many of which are predicted to be potential therapeutic targets, but there is only one structure available, that of bovine rhodopsin, to represent the whole of the family. There are many reasons for the lack of progress in GPCR structure determination, but we hypothesise that improving the detergent-stability of these receptors and simultaneously locking them into one preferred conformation will greatly improve the chances of crystallisation. A generic strategy for the isolation of detergent-solubilised thermostable mutants of a GPCR, the β -adrenergic receptor, was developed based upon alanine scanning mutagenesis followed by an assay for receptor stability. Out of 318 mutants tested, 15 showed a measurable increase in stability. After optimisation of the amino acid residue at the site of each initial mutation, an optimally stable receptor was constructed by combining specific mutations. The most stable mutant receptor, β AR-m23, contained 6 point mutations that led to a T_m 21°C higher than the native protein and, in the presence of bound antagonist, β ARm23 was as stable as bovine rhodopsin. In addition, β AR-m23 was significantly more stable in a wide range of detergents ideal for crystallisation and was preferentially in an antagonist conformation in the absence of ligand.

25

Results

Selection of single mutations that increase the thermostability of the β 1 adrenergic receptor

30

β AR from turkey erythrocytes is an ideal target for structural studies because it is well characterised and is expressed at high-levels in insect cells using the baculovirus expression system[10,11]. The best overexpression of β AR is obtained using a truncated version of the receptor containing residues 34-424 (β AR₃₄₋₄₂₄) [9] and this was used as the starting point for this work. Alanine scanning mutagenesis was used to define amino residues in β AR₃₄₋₄₂₄ that, when

35

mutated, altered the thermostability of the receptor; if an alanine was present in the sequence it was mutated to a leucine residue. A total of 318 mutations were made to amino acid residues 37-369, a region that encompasses all seven transmembrane domains and 23 amino acid residues at the C terminus; mutations at 15 amino residues were not obtained due to strong secondary structure in the DNA template. After sequencing each mutant to ensure the presence of only the desired mutation, the receptors were functionally expressed in *E. coli* and assayed for stability.

The assay for thermostability was performed on unpurified detergent-solubilised receptors by heating the receptors at 32°C for 30 minutes, quenching the reaction on ice and then performing a radioligand binding assay, using the antagonist [³H]-dihydroalprenolol, to determine the number of remaining functional βAR_{34-424} molecules compared to the unheated control. Heating the unmutated βAR_{34-424} at 32°C for 30 min before the assay reduced binding to approximately 50% of the unheated control (Fig. 7); all the data for the mutants were normalised by including the unmutated βAR_{34-424} as a control in every assay performed. In the first round of screening, eighteen mutants showed an apparent increase in stability, maintaining more than 75% of antagonist binding after heating and being expressed in *E. coli* to at least 50% of the native βAR_{34-424} levels. In view of the possibility of increasing further the stability of these mutants, each of the 18 residues was mutated to 2-5 alternative amino acid residues of varying size or charge (Fig. 1). Out of these 18 mutants, 12 were not improved by further changes, 5 had better thermostability if another amino acid was present and one mutation from the first screen turned out to be a false positive. In addition, three residues that were not stabilised upon mutation to alanine (V89, S151, L221) were mutated to a range of other amino acid residues; the two positions that when mutated to alanine did not affect thermostability, were also unaffected by other changes. In contrast, V89 showed less thermostability when mutated to alanine, but thermostability increased when it was mutated to Leu. Thus the initial alanine scanning successfully gave two-thirds of the best amino acid residues of those tested for any given position.

The position and environment predicted for each of the 16 amino residues that gave the best increases in thermostability when mutated were determined by aligning the βAR sequence with that of rhodopsin whose structure is known

(Fig. 2). Fourteen of these residues were predicted to be present in transmembrane α -helices, with five of the residues predicted to be lipid-facing, 4 being deeply buried and the remainder were predicted to be at the interfaces between the helices. Some of these residues would be expected to interact with each other in the β AR structure, such as the consecutive amino acids G67 and R68 (V63 and Q64 in rhodopsin), or the amino acids within the cluster Y227, R229, V230 and A234 in helix 5 (Y223, Q225, L226 and V230 in rhodopsin). Other amino acid residues that could interact in β AR were Q194A in external loop 2 and D322A in external loop 3 (G182 and P285 in rhodopsin, respectively).

The increase in stability that each individual mutation gave to β AR₃₄₋₄₂₄ was determined by measuring the T_m for each mutant (results not shown); T_m in this context is the temperature that gave a 50% decrease in functional binding after heating the receptor for 30 minutes. Each mutation increased the T_m of β AR₃₄₋₄₂₄ by 1-3°C, with the exception of M90A and Y227A that increased the T_m by 8°C.

Combining mutations to make an optimally stable receptor

Initially, mutations that improved thermostability that were adjacent to one another in the primary amino sequence of β AR were combined. Constructions containing the mutations G67A and R68S, or different combinations of the mutations at the end of helix 5 (Y227A, R229Q, V230A and A234L) were expressed and assayed; the T_m values (results not shown) were only 1-3°C higher than the T_m for β AR₃₄₋₄₂₄ and one mutant was actually slightly less stable, suggesting that combining mutations that are adjacent to one another in the primary amino acid sequence does not greatly improve thermostability. Subsequently, mutations predicted to be distant from one another in the structure were combined. PCR reactions were performed using various mixes of primers to combine up to 5 different mutations in a random manner and then tested for thermostability (Table 1). The best of these combinations increased the T_m more than 10°C compared to the T_m of β AR₃₄₋₄₂₄. In some cases, there was a clear additive effect upon the T_m with the sequential incorporation of individual mutations. This is seen in a series of 3 mutants, m4-1, m4-7 and m4-2, with the addition of V230A to m4-1 increasing the T_m by 2°C and the additional mutation D332A in m4-7 increasing the T_m a further 3°C. Mutants that contained Y227A and M90A all showed an increase in T_m of 10°C or more. Just these two

mutations together increased the T_m of βAR_{34-424} by 13°C (m7-5), however, the total antagonist binding was less than 50% of βAR_{34-424} suggesting impaired expression of this mutant. The addition of F338M to m7-5 did not increase the thermostability, but it increased levels of functional expression in *E. coli*.

5

Table 1 Combinations of mutations by PCR. 10 PCR reactions were performed combining different pairs of primers that contained the selected mutations. Successful PCR reactions are shown in the table. The stability of these new mutants was assayed as described in Figure 7 and the T_m calculated. The results are shown as the mean \pm S.E. from duplicates.

10

PCR	Receptor	Mutations	T_m (°C)
	βAR_{34-424}		31.7 \pm 0.1
4	m4-1	G67A, G98A	35.5 \pm 0.9
	m4-2	G67A, G98A, V230A, D322A	40.9 \pm 0.9
	m4-6	G98A, D322A	35.0 \pm 0.2
	m4-7	G67A, G98A, V230A	38.0 \pm 1.2
6	m6-1	Y227A, A234L, A282L, A334L	41.6 \pm 0.9
	m6-4	R68S, Y227A, A234L, A282L	41.6 \pm 0.1
	m6-5	R68S, A234L, A282L, A334L	41.9 \pm 0.5
	m6-9	R68S, Y227A, A234L, A282L, A334L	43.7 \pm 0.4
	m6-10	R68S, Y227A, A282L, A334L	47.4 \pm 1.1
	m6-11	R68S, A282L, A334L	39.1 \pm 0.5
7	m7-1	M90V, A282L, F338M	43.0 \pm 0.8
	m7-2	M90V, A282L	38.9 \pm 0.6
	m7-5	M90V, Y227A	45.2 \pm 1.0
	m7-6	M90V, I129V	40.0 \pm 0.6
	m7-7	M90V, Y227A, F338M	45.2 \pm 2.0
10	m10-4	R68S, M90V, V230A, A334L	46.9 \pm 1.0
	m10-8	R68S, M90V, V230A, F327A, A334L	47.3 \pm 1.4

The most thermostable mutants obtained, which were still expressed at high levels in *E. coli*, were m6-10, m7-7 and m10-8. These mutants contained collectively a total of 10 different mutations, with 8 mutations occurring in at least two of the mutants. A second round of mutagenesis was performed using m10-8 as the template and adding or replacing mutations present in m6-10 and m7-7 (Fig. 3); some of these mutations were very close in the primary amino acid sequence of βAR and therefore were not additive as noted above, but many mutations improved the T_m further (Table 2). For example, exchanging two mutations in m10-8, to create m18, raised the T_m to 49.6°C and adding A282L to make m23 increased the T_m a further 3°C to 52.8°C. This produced the most thermostable βAR_{34-424} mutant so far and will be referred to as βAR -m23.

15

20

Table 2 Improvement of best combination of mutations. These new mutants were obtained mixing the changes present in mutants m6-10, m7-7 and m10-8 by PCR. The stability of these new mutants was assayed as described in Figure 7 and the T_m calculated. The results are shown as the mean \pm S.E. from duplicates.

	Mutations								T_m (°C)
m17	R68S	M90V	Y227A	V230A	-	F327A	A334L	-	48.2 \pm 1.4
m18	R68S	M90V	Y227A	V230A	A282L	F327A	-	F338M	49.6 \pm 0.9
m19	R68S	M90V	Y227A	-	A282L	F327A	-	F338M	49.0 \pm 0.8
m20	R68S	M90V	-	-	-	F327A	A334L	-	48.4 \pm 0.7
m21	R68S	M90V	Y227A	-	-	F327A	A334L	-	47.0 \pm 1.3
m22	R68S	M90V	Y227A			F327A	A334L	-	47.4 \pm 0.5
m23	R68S	M90V	Y227A	-	A282L	F327A	-	F338M	52.8 \pm 1.4

The thermostability assays used to develop βAR_{34-424} mutants were performed by heating the receptor in the absence of the antagonist, but it is well known that bound ligand stabilises receptors. Therefore, stability assays for βAR_{34-424} and βAR -m23 were repeated with antagonist bound to the receptors during the heating step (Fig. 4). As expected, the T_m of the receptor that contained bound antagonist during the incubation was higher than that for the receptor without antagonist. For βAR_{34-424} the T_m was 6°C higher with bound antagonist and for βAR -m23 the T_m increased 2°C to 55°C; the smaller increase in thermostability observed for βAR -m23 when antagonist binds suggests that the receptor is already in a more stable conformation similar to the antagonist bound state than βAR_{34-424} (see also below). The T_m of βAR -m23 with antagonist bound is very similar to the T_m of dark-state rhodopsin in dodecylmaltoside (DDM)[12], whose structure has been solved by two independent laboratories[13,14]. This suggested that βAR -m23 is sufficiently stable for crystallisation.

Characterization of βAR -m23

The three characteristic activities measured for βAR -m23 and βAR_{34-424} to identify the effect of the six mutations were the affinity of antagonist binding, the relative efficacies of agonist binding and the ability of βAR -m23 to couple to G proteins.

Saturation binding experiments to membranes using the antagonist [³H]-dihydroalprenolol (Figure 8) showed that the affinity of binding to β AR-m23 (K_D 6.5 ± 0.2 nM, $n=2$) was slightly lower than for β AR₃₄₋₄₂₄ (K_D 2.8 ± 0.1 nM, $n=2$), suggesting that there are no large perturbations in the structure of β ARm23 in the antagonist-bound conformation. This is consistent with the observation that none of the mutations in β AR-m23 correspond with amino acids believed to be implicated in ligand binding. In contrast to antagonist binding, the efficacy of agonist binding by β AR-m23 is 3 orders of magnitude weaker than for β AR₃₄₋₄₂₄ (Fig. 5). The potency of the agonist isoprenaline is consistently lower in β AR-m23 and β AR₃₄₋₄₂₄ than for the native agonist norepinephrine, indicating that the agonist-bound conformation for the two receptors is likely to be similar. However, the large decrease in agonist efficacy in β AR-m23 compared to β AR₃₄₋₄₂₄ indicates that the 6 mutations in β AR-m23 have locked the receptor preferentially in an antagonist-bound conformation. From a crystallisation perspective, this is an added bonus to thermostabilisation, because it is essential to have a conformationally homogeneous protein population for the production of diffraction-quality crystals.

All of the thermostability assays used to derive β AR-m23 were performed on receptors solubilised in DDM. The aim of the thermostabilisation process was to produce a receptor that is ideal for crystallography, which means being stable in a variety of different detergents and not just DDM. We therefore tested the stability of β AR-m23 and β AR in a variety of different detergents, concentrating on small detergents that are preferentially used in crystallising integral membrane proteins. Membranes prepared from *E. coli* expressing β AR-m23 or β AR₃₄₋₄₂₄ were solubilised in DDM, bound to Ni-NTA agarose then washed with either DDM, decylmaltoside (DM), octylglucoside (OG), lauryldimethylamine oxide (LDAO) or nonylglucoside (NG). Stability assays were performed on the receptors in each of the different detergents (Fig 6). β AR₃₄₋₄₂₄ was only stable in DDM and DM, with no active receptors eluting from the resin washed with OG, NG or LDAO. In contrast, functional β AR-m23 was still present in all detergents and the T_m could be determined. As expected, the smaller detergents were considerably more denaturing than either DDM (T_m 52°C) or DM (T_m 48°C), with T_m s of 25°C (NG), 23°C (LDAO) and 17°C (OG). The difference in T_m between β AR-m23 and β AR₃₄₋₄₂₄ is about 20°C, irrespective of whether the receptors were solubilised in either DDM or DM; it is therefore not surprising that no active β AR₃₄₋₄₂₄ could be

found in even NG, because the predicted T_m would be about 5°C, thus resulting in rapid inactivation of the receptor under the conditions used for purification. The selection strategy used for the generation of βAR -m23 was chosen deliberately to be based upon thermostability, because it is far simpler to apply than selecting for stability in detergents of increasing harshness. However, it is clear that increasing the thermostability of βAR_{34-424} also resulted in increasing tolerance to small detergents ideal for crystallising integral membrane proteins.

Crystallisation of mutant GPCR

Earlier attempts to crystallise several different constructs of turkey beta-adrenergic receptor failed. Despite experimenting with a variety of conditions, using both the native sequence and several truncated and loop-deleted constructs, over many years, no crystals were obtained.

However, once the stabilising mutations from βAR -m23 were transferred into the constructs, several different crystals were obtained in different detergents and different conditions.

The crystals that have been most studied so far were obtained using the purified beta-36 construct (amino acid residues 34-367 of the turkey beta receptor containing the following changes: point mutations C116L and C358A; the 6 thermostabilising point mutations in m23; replacement of amino acid residues 244-278 with the sequence ASKRK; a C terminal His6 tag) expressed in insect cells using the baculovirus expression system, after transferring the receptor into the detergent octyl-thioglucoside. The precipitant used was PEG600 or PEG1000 and the crystals obtained are elongated plates.

Experiments have also been carried out to see whether, once the crystallisation conditions had been defined using the stabilised receptor, it was possible to get crystals using the original non-stabilised construct. It was possible that similar or perhaps very small crystals could have been obtained, but, in fact, the "wild type" (i.e. the starting structure from which the mutagenesis began) never gave any crystals.

The crystals are plate-shaped with space group C2 and diffract well, though the cell dimensions do vary depending on the freezing conditions used.

In general, once a GPCR has been stabilised it may be subjected to a variety of well-known techniques for structure determination. The most common technique for crystallising membrane proteins is by vapour diffusion (20, 21), usually using initially a few thousand crystallisation conditions set up using commercial robotic devices (22). However, sometimes the crystals formed by vapour diffusion are small and disordered, so additional techniques may then be employed. One technique involves the co-crystallisation (by vapour diffusion) of the membrane protein with antibodies that bind specifically to conformational epitopes on the proteins' surface (23, 24); this increases the hydrophilic surface of the protein and can form strong crystal contacts. A second alternative is to use a different crystallisation matrix that is commonly called either lipidic cubic phase or lipidic mesophase (25, 26), which has also been developed into a robotic platform (27). This has proven very successful for producing high quality crystals of proteins with only small hydrophilic surfaces e.g. bacteriorhodopsin (28). Membrane protein structures can also be determined to high-resolution by electron crystallography (29).

The evolution of β AR-m23 from β AR₃₄₋₄₂₄ by a combination of alanine scanning mutagenesis and the selection of thermostable mutants has resulted in a GPCR that is ideal for crystallography. The T_m for β AR-m23 is 21°C higher than for β AR₃₄₋₄₂₄ and, in the presence of antagonist, β AR-m23 has a similar stability to rhodopsin. The increased T_m of β AR-m23 has resulted in an increased stability in a variety of small detergents that inactivate β AR₃₄₋₄₂₄. In addition, the selection strategy employed resulted in a receptor that is preferentially in the antagonist-bound conformation, which will also improve the chances of obtaining crystals, because the population of receptor conformations will be more homogeneous than for wild type β AR₃₄₋₄₂₄. Thus we have achieved a process of conformational stabilisation in a single selection procedure.

It is not at all clear why the particular mutations we have introduced lead to the thermostabilisation of the receptor. Equivalent positions in rhodopsin suggest that the amino acid residues mutated could be pointing into the lipid bilayer, into the centre of the receptor or at the interfaces between these two environments.

Given the difficulties in trying to understand the complexities of the thermostabilisation of soluble proteins[15], it seems unlikely that membrane proteins will be any easier to comprehend; we found that there was no particular pattern in the amino acid residues in β AR that, when mutated, led to thermostability. However, since nearly 5% of the mutants produced were more stable than the native receptor, alanine scanning mutagenesis represents an efficient strategy to rapidly identify thermostable mutants.

The procedure we have used to generate β AR-m23 is equally applicable to any membrane protein that has a convenient assay for detecting activity in the detergent solubilized form. While we have selected for stability as a function of temperature as the most convenient primary parameter, the procedure can easily be extended to test primarily for stability, for example, in a harsh detergent, an extreme of pH or in the presence of chaotropic salts. Conformational stabilisation of a variety of human receptors, channels and transporters will make them far more amenable to crystallography and will also allow the improvement in resolution of membrane proteins that have already been crystallised. It is to be hoped that conformational stabilisation will allow membrane protein crystallisation to become a far more tractable problem with a greater probability of rapid success than is currently the case. This should allow routine crystallisation of human membrane proteins in the pharmaceutical industry, resulting in valuable structural insights into drug development.

METHODS

Materials. The truncated β 1 adrenergic receptor from turkey (β AR₃₄₋₄₂₄)[9] was kindly provided by Dr Tony Warne (MRC Laboratory of Molecular Biology, Cambridge, UK). This β AR construct encoding residues 34-424 contains the mutation C116L to improve expression[11], and a C-terminal tag of 10 histidines for purification. 1-[4,6-propyl-³H]-dihydroalprenolol ([³H]-DHA) was supplied by Amersham Bioscience, (+) L-norepinephrine bitartrate salt, (-) isoprenaline hydrochloride, (-) alprenolol tartrate salt and s-propranolol hydrochloride were from Sigma.

Mutagenesis of β AR. The β AR cDNA was ligated into pRGIII to allow the functional expression of β AR in *E. coli* as a MalE fusion protein[16]. Mutants

were generated by PCR using the expression plasmid as template using the QuikChange II methodology (Stratagene). PCR reactions were transformed into XL10-Gold ultracompetent cells (Stratagene) and individual clones were fully sequenced to check that only the desired mutation was present. Different mutations were combined randomly by PCR by including all the pairs of primers that introduced the following mutations: Mut4, G67A, G068A, V230A, D322A and F327A; Mut6, R068S, Y227A, A234L, A282L and A334L; Mut7, M90V, I129V, Y227A, A282L and F338M; Mut10, R68S, M90V, V230A, F327A and A334L. The PCR mixes were transformed and the clones sequenced to determine exactly which mutations were introduced.

Protein expression and membrane preparations. Expression of β AR and the mutants was performed in XL10 cells (Stratagene). Cultures of 50 ml of 2xTY medium containing ampicillin (100 μ g/ml) were grown at 37°C with shaking until $OD_{600}=3$ and then induced with 0.4 mM IPTG. Induced cultures were incubated at 25°C for 4h and then cells were harvested by centrifugation at 13,000 xg for 1 min (aliquots of 2 ml) and stored at -20°C. For the assays, cells were broken by freeze-thaw (five cycles), resuspended in 500 μ l of buffer [20mM Tris pH 8, 0.4 M NaCl, 1mM EDTA and protease inhibitors (Complete™, Roche)]. After an incubation for 1h at 4°C with 100 μ g/ml lysozyme and DNase I (Sigma), samples were solubilized with 2% DDM on ice for 30 minutes. Insoluble material was removed by centrifugation (15,000xg, 2 min, 4°C) and the supernatant was used directly in radioligand binding assays.

For large-scale membrane preparations, 2L and 6L of *E. coli* culture of β AR and Mut23, respectively, were grown as described above. Cells were harvested by centrifugation at 5,000 xg for 20 min, frozen in liquid nitrogen and stored at -80°C. Pellets were resuspended in 10 ml of 20 mM Tris pH 7.5 containing 1x protease inhibitor cocktail (Complete™ EDTA-free, Roche); 1 mg DNase I (Sigma) was added and the final volume was made to 100 ml. Cells were broken by a French press (2 passages, 20,000 psi), and centrifuged at 12,000 xg for 45 min at 4°C to remove cell debris. The supernatant (membranes) was centrifuged at 200,000 xg for 30 min at 4°C; the membrane pellet was resuspended in 15 ml of 20 mM Tris pH 7.5 and stored in 1 ml aliquots at -80°C after flash-freezing in liquid nitrogen. The protein concentration was determined by the amido black

method[17]. These samples were used in radioligand binding assays after thawing and being solubilized in 2% DDM as above.

For competition assays, as well as testing different detergents, DDM-solubilized β AR was partially purified with Ni-NTA agarose (Qiagen). 200 μ l of Ni-NTA agarose was added to 2 ml of solubilized samples (10 mg/ml of membrane protein) in 20mM Tris pH 8, 0.4 M NaCl, 20 mM imidazole pH 8 and incubated for 1 h at 4°C. After incubation, samples were centrifuged at 13,000 x g for 30 sec and washed twice with 250 μ l of buffer (20mM Tris pH 8, 0.4 M NaCl, 20 mM imidazole) containing detergent (either 0.1% DDM, 0.1% DM, 0.1% LDAO, 0.3% NG or 0.7% OG).

Receptors were eluted in 2 x 100 μ l of buffer (0.4 M NaCl, 1mM EDTA, 250 mM imidazole pH 8, plus the relevant detergent). The K_D for [3 H]-DHA binding to semipurified β AR₃₄₋₄₂₄ and β AR-m23 was, respectively 3.7 nM and 12.5 nM and the final concentration of [3 H]-DHA used in the competition assays was 3 times the K_D ie 12 nM for β AR₃₄₋₄₂₄ and 40 nM for β AR-m23.

Radioligand binding and thermostability assays. Single point binding assays contained 20mM Tris pH 8, 0.4 M NaCl, 1mM EDTA, 0.1% DDM (or corresponding detergent) with 50 nM [3 H]-DHA and 20-100 μ g membrane protein in a final volume of 120 μ l; equilibration was for 1 h at 4°C. Thermostability was assessed by incubating the binding assay mix, with or without [3 H]-DHA at the specified temperature for 30 minutes; reactions were placed on ice and [3 H]-DHA added as necessary and equilibrated for a further hour. Receptor-bound and free radioligand were separated by gel filtration as described previously[18]. Non-specific binding was determined in the presence of 1 μ M of s-propranolol. Saturation curves were obtained using a range of [3 H]-DHA concentration from 0.4 nM to 100 nM. Competition assays were performed using a concentration of [3 H]-DHA of 12 nM for β AR₃₄₋₄₂₄ and 40 nM for β AR-m23 (ie three times the K_D) and various concentrations of unlabeled ligands (0-100 mM). Radioactivity was counted on a Beckman LS6000 liquid scintillation counter and data were analyzed by nonlinear regression using Prism software (GraphPad).

Location of β AR-m23 thermostable mutations in rhodopsin structure. The pdb file for the rhodopsin structure, accession code 1GZM[14], was downloaded

from the Protein Data Bank website (www.pdb.org) and displayed in the program PyMOLX11Hybrid (DeLano Scientific). The equivalent amino acid residues in rhodopsin for the thermostable mutations in β AR were located in the rhodopsin structure based upon an alignment among the four GPCRs with which we are most familiar, namely rhodopsin, β 1 adrenergic receptor, neurotensin receptor and adenosine A_{2a} receptor[19].

Example 2: Mutants of the adenosine A_{2a} receptor ($A_{2a}R$) with increased thermostability

1. 315 site-directed mutants made between residues 2-316 of $A_{2a}R$.
2. All of these mutants have been assayed for thermostability using an assay measuring agonist and antagonist binding after the heating step (Ligand(-) format as described in Figure 12).
 - a. 26 mutants showed improved thermostability when measured with 3H -NECA (agonist): G114 A, G118A, L167A, A184L, R199A, A203L, L208A, Q210A, S213A, E219A, R220A, S223A, T224A, Q226A, K227A, H230A, L241A, P260A, S263A, L267A, L272A, T279A, N284A, Q311A, P313A, K315A.
 - b. 18 mutants showed improved thermostability when assayed with 3H -ZM241385 (antagonist): A54L, V57A, H75A, T88A, G114A, G118A, T119A, K122A, G123A, P149A, E151A, G152A, A203L, A204L, A231L, L235A, V239A.
3. Mutations have been combined to generate mutants in a putative antagonist conformation. Wildtype $A_{2a}R$ has a T_m of 31°C with ZM241385 bound.

a. Rant17 A54L+K122A+L235A	T_m 48°C (ZM241385 bound)
b. Rant19 A54L,T88A,V239A+A204L	T_m 47°C (ZM241385 bound)
c. Rant21 A54L,T88A,V239A+K122A	T_m 49°C (ZM241385 bound)
4. Mutations from the agonist screen have been combined, but have led to only a very low level of improvement in T_m of +2°C.

Table (i). List of A2aR stabilising mutations. Mutants were expressed in *E. coli*, solubilised in 2% DDM + 10% glycerol and tested for ligand-binding, using the agonist [³H]-NECA (on the right) and the antagonist [³H]-ZM241385 (left). Concentrations of radioligands were 6-10-fold above their K_D measured for the wild-type receptor. Expression of active receptor was evaluated by ligand binding at 4°C. Stability was assayed by heating the solubilised receptor in its apo-state at 30°C for 30 minutes and then measuring residual binding activity. Under these conditions, wild-type activity decays to 50% (S.D.=15%). Data obtained for expression and stability were normalised to wild-type values. Mutations included in subsequent rounds of mutagenesis were those whose expression was ≥ 30-40% and stability ≥ 130-140% compared to the wild-type. Bold lines indicate cluster of mutations.

Agonist			Antagonist		
Mutation	Expression (%)	Stability (%)	Mutation	Expression (%)	Stability (%)
wt	100	100	wt	100	100
S090A	151	151	A054L	90	140
G114A	62	143	V057A	44	144
G118A	71	151	H075A	82	152
L167A	41	174	T088A	67	230
A184L	140	150	G114A	73	153
R199A	73	202	G118A	84	148
A203L	42	172	T119A	90	148
L208A	276	215	K122A	52	153
Q210A	46	155	G123A	90	158
S213A	40	140	P149A	54	215
E219A	96	221	E151A	63	173
R220A	84	250	G152A	70	156
S223A	57	146	A203L	111	132
T224A	142	276	A204L	40	181
Q226A	119	217	A231L	90	148
K227A	87	222	L235A	85	140
H230A	57	154	V239A	91	134
L241A	139	156			
P260A	70	169			
S263A	60	158			
L267A	40	187			
L272A	34	157			
T279A	125	158			
N284A	64	151			
Q311A	49	164			
P313A	44	148			
K315A	64	186			

Table (ii). Stability of best combinations. Receptors were solubilised in 1% DDM (no glycerol). A melting profile was obtained by heating the solubilised receptor at different temperatures in absence (apo-state) or presence of ligand (ligand-occupied state). Data shown are representative of at least three independent experiments. S.D. is < 1°C.

	T _m (°C)			T _m (°C)	
	-	+		-	+
	agonist	agonist		antagonist	antagonist
Wt	21	29	wt	31	32
Rag 1 (A184L/R199A/L272A)	26	34	Rant 5 (A54L/T88A/V239A)	42	46
Rag 23 (Rag 1+F79A /L208A)	22	38	Rant 21 (Rant 5+K122A)	41	49

Table (iii). Summary of results for competition assays of detergent-solubilised wild-type A2aR and thermo-stable mutant Rant 21. Values are

representative of two independent experiments. Each data point was assayed in triplicate and plotted as mean \pm SD. Each solubilised receptor was incubated with ligands for one hour on ice in binding buffer (50 mM Tris pH 7.5 and 0.025% DDM) containing 400 mM NaCl. Binding of [³H]ZM241385 (10 nM) in the absence of unlabeled ligand was set to 100%. Data shown are from two independent experiments with each data point measured in triplicate. Incubation of samples with ligands was for 1 hour on ice with [³H]ZM241385 at a concentration of 10 nM. K_i values were calculated according to the Cheng and Prusoff equation using the non-linear regression equation of the software Prism, applying a K_D for [³H]ZM241385 of 12 nM for the wild-type and 15 nM for Rant 21. Rant 21 did not bind NECA sufficiently for an accurate K_i determination (hence indicated as $>1 \times 10^{-1}$). The affinity of Rant21 for agonist binding is weakened 232 fold for R-PIA and at least by 1900 fold for NECA.

Competitor	K _i (M)	
	wt	Rant 21
XAC	2.3×10^{-6}	2.3×10^{-6}
Theophylline	1.5×10^{-3}	0.9×10^{-3}
NECA	7.0×10^{-6}	$>1 \times 10^{-1}$
R-PIA	1.6×10^{-5}	3.6×10^{-3}

Table (iv). Summary of results for saturation assays of detergent-solubilised wild-type A2aR and thermo-stable mutants. Values are

representative of three independent experiments. Each data point was assayed in triplicate and plotted as mean \pm SD. Data were fitted to the Michaelis-Menten equation using the non-linear regression equation of the software Prism.

Receptor	K_D (nM)	
	$[^3H]$ NECA (agonist)	$[^3H]$ ZM241385 (antagonist)
wt	32 ± 1	12 ± 3
Rag 1	26 ± 0.4	26 ± 0.5
Rag 23	21 ± 1	62 ± 1
Rant 21	>450	15 ± 3

5 **Table (v). Summary of stability of wild-type and mutant receptors in different detergents.** Solubilisation of receptors and detergent exchange was performed during the IMAC step. S.D. is $< 1^\circ\text{C}$. It was not possible to determine the T_m for some receptor-detergent combinations, because the receptor was too unstable (\dagger).

	T_m ($^\circ\text{C}$)			
	Agonist-binding		Antagonist-binding	
	wt	Rag 23	wt	Rant 21
0.01% DDM	27	34	25	39
0.1% DM	23	29	10	28
0.3% NM	22	28	< 4	25
0.3% NG	\dagger	\dagger	\dagger	22
0.6% OG	<9	16	\dagger	23
0.003% LDAO	28	38	32	42
0.006% FC12	37	39	43	49

10

Example 3: Mutants of the neurotensin receptor (NTR) with increased thermostability

15 1. 340 site-directed mutants have been made between residues 61-400 of NTR.

2. Initially, all of these mutants were assayed for thermostability using an assay measuring ^3H -neurotensin (agonist) binding after the heating step. 24 mutations led to a small but significant increase in thermostability: A356L, H103A, D345A, A86L, A385L, Y349A, C386A, K397A, H393A, I116A, F358A, S108A, M181A, R392A, D113A, G209A, L205A, L72A, A120L, P399A, Y351A, V268A, T207A, A155L, S362A, F189A, N262A, L109A, W391A, T179A, S182A, M293A, L256A, F147A, D139A, S100A, K176A, L111A, A90L, N270A.

3. Mutants tested for thermostability by heating in the absence of the agonist were re-tested using a slightly different assay where the mutants were heated in the presence of ^3H -neurotensin (Ligand(+)) format in Figure 12). Mutants with improved thermostability are: A69L, A73L, A86L, A90L, H103A, V165A, E166A, G215A, V229A, M250A, I253A, A177L, R183A, I260A, T279A, T294A, G306A, L308A, V309A, L310A, V313A, F342A, F358A, V360A, S362A, N370A, S373A, F380A, A385L, P389A, G390A, R395A.

4. There are also mutants that have a significantly enhanced expression level compared to the wildtype receptor and could be used to boost preceptor production levels for crystallisation: A86L, H103A, F358A, S362A, N370A, A385L, G390A. All of these also have increased thermostability.

5. Preferred combinations are

a. Nag7m F358A+A86L+I260A+F342A Tm 51°C (neurotensin bound)

b. Nag7n F358A+H103A+I260A+F342A Tm 51°C (neurotensin bound)

Wildtype NTR has a Tm of 35°C with neurotensin bound.

Example 4: Identification of structural motifs in which stabilising GPCR mutations reside.

The structure of the β_2 adrenergic receptor has been determined (20, 21), which is 59% identical to the turkey β_1 receptor, but with a distinctly different pharmacological profile (22, 23). In order to determine the structural motifs in which the stabilising mutations of the turkey β_1 receptor reside, we mapped the mutations onto the human β_2 structure (21).

The beta adrenergic receptors were first aligned using ClustalW in the MacVector package; thermostabilising mutations in turkey β_1 were highlighted along with the corresponding residue in the human β_2 sequence. The human β_2 model (pdb accession code 2RH1) was visualised in Pymol and the desired amino acids were shown as space filling models by standard procedures known in the art. The structural motifs in which the stabilising mutations were located, were determined by visual inspection.

Table (vi) lists the equivalent positions in the β_2 receptor corresponding to the thermostabilising mutations in βAR -m23 and the structural motifs in which they reside.

As seen from Table (vi), the mutations are positioned in a number of distinct localities. Three mutations are in loop regions that are predicted to be accessible to aqueous solvent (loop). Eight mutations are in the transmembrane α -helices and point into the lipid bilayer (lipid); three of these mutations are near the end of the helices and may be considered to be at the hydrophilic boundary layer (lipid boundary). Eight mutations are found at the interfaces between transmembrane α -helices (helix-helix interface), three of which are either within a kinked or distorted region of the helix (kink) and another two mutations occur in one helix but are adjacent to one or more other helices which contain a kink adjacent in space to the mutated residue (opposite kink). These latter mutations could affect the packing of the amino acids within the kinked region, which could result in thermostabilisation. Another mutation is in a substrate binding pocket (pocket).

	Turkey β 1	Human β 2	Description	
Helix 1	I55A	I47	3-helix kink interface	Fig 18
Helix 1	G67A	A59	lipid boundary	
Helix 1	R68S	K60	lipid boundary	Fig 25
Helix 2	V89L	V81	kink	Fig 19
Helix 2	M90V	M82	kink	Fig 20
Helix 2	G98A	G90	pocket	
Helix 3	I129V S151E	I121 S143	opposite kink loop	Fig 21
Helix 4	V160A Q194A	V152 A186	lipid loop	
Helix 5	L221V	V213	lipid	
Helix 5	Y227A	Y219	helix-helix interface	Fig 23
Helix 5	R229Q	R221	lipid	
Helix 5	V230A	V222	helix-helix interface	
Helix 5	A234L	A226	helix-helix interface	
Helix 6	A282L D322A	C265 K305	loop lipid boundary	Fig 24
Helix 7	F327A	L310	lipid	
Helix 7	A334L	V317	lipid	
Helix 7	F338M	F321	kink	Fig 22

Table (vi) Position in the human β 2 structure of the amino acid residues equivalent to the thermostabilising mutations found in the turkey β 1 receptor and the structural motifs in which they reside.

Such structural motifs, by virtue of them containing stabilising mutations, are important in determining protein stability. Therefore, targeting mutations to these motifs will facilitate the generation of stabilised mutant GPCRs. Indeed, there were several instances where more than one mutation mapped to the same structural motif. For example, the Y227A, V230A and A234L mutations in the turkey β 1 adrenergic receptor all mapped to the same helical interface, the V89L and M90V mutations mapped to the same helical kink and the F327A and A334L

mutations mapped to the same helical surface pointing towards the lipid bilayer (Table (vi)). Thus, when one stabilising mutation has been identified, the determination of the structural motif in which that mutation is located will enable the identification of further stabilising mutations.

5

Example 5: Generation of conformation specific binding partners of GPCRs

The generation of mutant GPCRs having increased stability in a particular conformation relative to a parent GPCR provides a number of advantages for screening of binding partners. For example, the present methods reduce the amount of material required for a screen. In standard screens, GPCRs are present in whole cells or in membranes from whole cells which are usually screened by incubation with individual compounds in isolated chambers rather than libraries of compounds. Therefore the present invention provides advantages in terms of time required to carry out a compound screen. The ability to lock a GPCR in a particular conformation provides advantages in that it increases the likelihood of identifying a ligand with the required pharmacological properties. In standard binding screens GPCRs are able to assume a number of different conformations and binding compounds will be identified across different pharmacological types. Reagent costs can be reduced due to the ability to miniaturize the assay formats and this is facilitated by the present method.

METHODS

Compound screening

A mutant GPCR having increased stability in a particular conformation is immobilised on a solid surface and incubated with buffer containing an encoded library of compounds. After a suitable period of time, to allow binding between the mutant GPCR and compounds from the library which selectively bind to the mutant GPCR, the buffer is removed. Next there follows a number of wash steps to remove compounds which have not bound specifically to the mutant GPCR. The reading code, tag or address (such as DNA or RNA) is then used to identify the small molecule bound either whilst still bound to the mutant GPCR or following elution from the GPCR. The conformation specific binding partner is subsequently isolated.

Selection of antibodies using immobilised GPCRS

Immobilised stabilised GPCRs can be used to select antibodies to the receptor from mixtures of antibodies such as would be present in plasma from an animal immunised with the stabilised GPCR, the native receptor or a peptide from the receptor. Antibodies could be identified from supernatants obtained from B-cells taken from immunised animals or from hybridomas obtained following immortalization of B-cells from the immunised animal or from recombinant antibody libraries which may be expressed on phage particles or through an in vitro expression system such as ribosome display. The method has the advantage of selecting antibodies to particular conformations of a receptor. A stabilised GPCR locked in the antagonist or ground state of the receptor would increase the probability of selecting an antagonistic antibody whereas a stabilised GPCR locked in the activated or R* state would increase the probability of selecting an activating antibody. In standard screens antibodies are often selected to GPCRs that bind to peptide epitopes of the receptor but do not have antagonist or agonist properties and therefore are not useful as therapeutic agents.

Example 6: Antibody phage selection on β -adrenergic receptor

Summary

We have used stabilised β -adrenergic receptor (β -AR) to generate antibodies using phage display. Positive phage clones showed specificity for β -AR and sub-cloning of selected antibody genes led to successful production of anti- β -AR specific scFv antibodies.

Introduction

The therapeutic application of antibodies in the area of receptor-ligand systems has great potential. However, the primary issue in generating antibodies (either by in vitro or in vivo methods) to G protein coupled receptors (GPCRs) concerns the immunogenic recognition of a specific conformation in a homogeneous antigen preparation, i.e. either an agonistic conformation or antagonistic conformation, rather than creating a pool of antibody binders that recognises

purified antigen in multiple conformations in a heterogeneous preparation. In addition, recombinant receptor antigen is usually available only in the extracellular domain form, which precludes any tertiary structure involving other parts of the receptor. The proposed solution to this issue is the application of stabilized GPCRs as the target antigen.

We have demonstrated the utility of stabilised GPCRs (StaRs™) in the generation of recombinant antibodies by the in vitro method of phage display such that antibodies which bind the β adrenergic receptor stabilised in the antagonist conformation may be isolated. Such antibodies can then be subject to functional assays e.g. in ligand binding assays.

Overview of process

A typical phage display strategy comprises several stages in the process to identify inhibitory scFv clones (Figure 29). The first part is the selection of phage libraries on antigen to isolate a population of phage antibody binders using various methodologies (for example, panning selection, soluble selection, etc.). This resulting population of phage antibody binders is referred to as a selection output. This process is repeated 2-4 times to enrich for specific antigen binders.

A pool of clones representing a selection output (polyclonal phage ELISA) is assessed for recognition of antigen by phage ELISA. Individual clones can also be assessed by monoclonal phage ELISA and for diversity by sequence analysis, however the preferred method is to subclone the selected population into a recombinant antibody expression vector (pSANG 10-3F) and then perform the assessment by monoclonal scFv ELISA and DNA sequencing. This circumvents the problem of identifying phage antibody binders that subsequently exhibit poor expression as scFv fragments. Selection outputs yielding diverse ELISA positives can then be prioritised for functional screening of larger panels of phage antibodies.

Selection outputs are subjected to a screening campaign, employing a functional high-throughput assay, of periplasmic extracts in order to identify a population of scFv inhibitors. Lastly, the hits from the screening campaign are profiled as scFv

by further functional assays, as well as IC50 analysis which assess the efficiency of their inhibitory action.

Methods and Results

5

2-3 rounds of antibody selections were carried out using stabilised β -AR as antigen and using the antibody phage display library described in Schofield *et al*, 2007 (24). Selections were carried out in PBS in the presence (A) or absence (B) of 20nM ligand (-)-alprenolol during binding and washing steps. Further, for β -AR protein handling, all coating, washing and blocking buffers were supplemented with 0.1% detergent decylmaltoside (Anatrace, Anagrade). The relative success of the selectives was determined using polyclonal phage ELISA and monoclonal phage ELISA.

10

15 **Optimisation to immobilise antigen**

Immobilisation strategies were based on taking advantage of the His tagged antigen. In the first instance, immobilisation was done using control proteins rather than β AR-m23. A variety of surfaces, including Ni-NTA plates, were employed in the initial assessment. All were compared against standard passive absorption onto Nunc plates. In all cases, the outcome was evaluated using polyclonal phage ELISA after 2 rounds of selection.

20

Antibody selection

25

Selection, elution and rescue of the library was as described in Schofield *et al* (2007). 150 μ l of β -AR at concentration of 20 μ g/ml was coated over night at +4°C in two (24) wells of a Nickel chelate-plate (Nunc). The receptor was diluted in coating buffer which is 20mM Tris pH8, 100mM NaCl, 0.1 % decylmaltoside (dec-M) and also 20nM ligand for selection A. Next day, the wells were rinsed with PBS and blocked for 1 h at room temperature with 3% Marvel milk protein in PBS supplemented with 0.1% dec-M (PBS-M). After coating, the well was rinsed in PBS and 100 μ l of phage library pre-blocked in 2% Marvel/PBS was added and incubated for 1h at room temperature. Following binding, samples were washed 6 times in PBS/0.1% Tween supplemented with 0.1% dec-M and six times in PBS supplemented with 0.1% dec-M. Bound phage was then eluted with Trypsin (24).

30

35

Eluted phage were added to exponentially growing TG1 cells (at $OD_{600} = 0.5$) and grown at 37°C for 1h. Infected cells were plated onto TY plates supplemented with 100µg/ml ampicillin, 2% glucose and grown overnight at 30°C. Next day, plates were scraped in TY medium supplemented with 100µg/ml ampicillin, 15% glycerol for storage. The population from this first round of selection was rescued with helper phage and PEG precipitated and 100 µl of these were used in a second round of selections using the same conditions and procedures as described for round 1.

10 Polyclonal phage ELISA

For ELISA, β AR was covalently immobilised on Amino plates (Nunc Catalog No: 436008). Coating and washing buffers were supplemented with 20nM ligand and 0.1% dec-M. The ELISA plate was coated overnight with β -AR at 24 µg/ml and 2 control proteins (CD86 and Notch1) at 5 µg/ml. Next day the wells were washed and blocked with PBS-M. 50 µl/well of polyclonal phage from 2 rounds of selection were added (in PBS-M, at a concentration of 0.1x relative to the initial culture volume) and incubated for 1 hour at room temperature. Wells were washed and incubated with an α -M13 antibody (GE healthcare product No: 27-9421-01), for 1 hour at room temperature. Plates were washed and incubated with Europium labelled anti-mouse antibody (Perkin Elmer, product No: AD 0207), for 1 hour at room temperature. Wells were washed and 50 µl/well of enhancement solution (Perkin Elmer, product No: 4001-0010) added and incubated for 10 min. The results are shown in Figure 30A, which suggests specific enrichment of β -AR binding phage in experiments A and B following 2 rounds of selection. Signals were higher from phage selection A (with ligand present in buffers).

Monoclonal phage ELISA

For this assay, individual clones of round 2 phage from selection A were picked, rescued and PEG precipitated. Again, coating, washing, blocking and antibody detection buffers were supplemented with 20nM ligand and 0.1% detergent. Wells of a Nunc Amino plate were coated with β -AR for 1 h 30 minutes at room temperature. Coated wells were washed 3 times with PBST and 3 times with PBS and blocked with PBS-M and 50 ul/well of 0.1x phage was added and incubated

for 1h at room temperature. Wells were washed 6 times with PBS and incubated with α -M13 antibody for 1h at room temperature. After washing 6 times with PBS the wells were incubated with anti-mouse-Eu antibody, 1h at room temperature. Next, wells were washed and incubated with enhancement solution for 10 min.

5 Out of 69 screened clones, 20 clones were detected as positive (Figure 30B).

Specificity phage ELISA

17 of the positive phage clones from the monoclonal ELISA were tested for
10 binding specificity to β -AR and 3 unrelated proteins. This includes N1-EGF, (EGF domains 1-12 of murine Notch1 fused to a human Fc domain from R&D Systems, catalogue number 1057-TK). The other control proteins include the extracellular domain of murine CD86 and the negative regulatory region of murine Notch1 expressed as a CD4 fusion (CD86 and N1(NRR) respectively) in a transient
15 expression system as described in Chapple et al 2006 (25). In this ELISA both nickel chelate and amino-plate were used for comparison. Again, for wells containing β -AR protein, coating, washing, blocking and antibody binding buffers were supplemented with 20nM ligand and 0.1% detergent. Wells were washed and incubated with antibodies as described for the monoclonal ELISA in previous
20 section. The assay showed that both His and Amino-plates can be used and that the majority of the anti- β -AR clones do not cross react with the unrelated proteins (Figure 31).

Expression and screening of monoclonal scFv

25 Sub-cloning, antibody single chain Fv (scFv) expression and purification were as described in Schofield *et al* (2007). Selected antibody genes within the round 2 phage population (selection A) were sub-cloned into the pSANG10-3F vector (26) and transformed into BL21(DE3) cells. 96 colonies were picked and periplasmic
30 expression of the scFv antibody was induced in a 96 well format using standard methods in the art. scFv were recovered from the periplasm and used for ELISA on an Amino plate (Nunc). Washed and blocked wells were incubated for 1h with 50 μ l/well of scFv. Plates were washed and incubated with Europium labelled anti-FLAG antibody for 1h at room temperature. 12 clones gave a signal above
35 1000 units with background levels of less than 50 for negative clones (Figure 32).

Positive clones are selected with binding and specifically confirmed in a secondary ELISA, and clones of interest sequenced. Antibody sequences are analysed to identify the number of unique binding clones that have been isolated.

- 5 To test for blocking of ligand binding by isolated antibodies, unique positive antibody clones are selected for larger scale preparation (50-500ml) using periplasmic extraction and immobilised metal affinity chromatography. This material is assessed for the ability to interfere in ligand binding to receptor using robust reporter assays (e.g. inhibition of cAMP generation or inhibition of ligand
10 binding to transfected cells).

Example 7: Assessment of compound interaction with β 1-AR

Methods and Results:

15

Binding studies were performed at 10°C using a Biacore S51 optical biosensor equipped with a streptavidin-coated CM5 chip and equilibrated with running buffer (20 mM trisHCl, 150 mM NaCl, 1 mM EDTA, 1% DMSO, 0.1% decyl maltoside, pH 7.8).

20

β 1AR immobilization.

25

β 1AR36-M23 was minimally biotinylated using EZ-link sulfo-NHS-LC-LC biotin (Pierce #21338): The biotin was added to the receptor preparation, (spiked with
100 μ M alprenolol) and allowed to react for three hours at 4°C, after which free biotin was removed via column chromatography.

30

Figure 33 shows the two capture stages of the biotinylated β 1AR onto a streptavidin-coated flow cell. In the first stage, we captured ~1200 RU; in the second, ~4000 RU.

35

The biacore sensor chips coated in the stabilised beta receptor could be used to characterise the binding of drugs with activity at the beta receptor. Flow through of the compound allowed the on rate to be determined. Subsequent washing enabled determination of the off rate. These parameters could then be used to determine a kinetic affinity measurement (K_d).

Figure 34 shows the responses for alprenolol which was tested in replicate in a three-fold dilution series for binding to the receptor surface. The highest concentration is 666 nM and each concentration was tested three times. The responses are concentration dependent and are reproducible. The grey lines depict the fit of a simple 1:1 interaction model and the parameters determined from this fit are listed in the inset (the number in parentheses is the error in the last digit).

Figure 35 is an alternative depiction of the data set in Figure 34. The responses are normalized with respect to the R_{max} determined (from the fitting) for each curve.

The alprenolol data demonstrates that the biotinylated β_1AR is active and this biosensor approach can be used to characterize the compound/receptor interactions.

Figure 36 shows the responses for propranolol binding to β_1AR . 111 nM is the highest concentration and each concentration was tested two or three times. The propranolol data are plotted as normalized responses in Figure 37.

When the β_1AR surface was almost three days old, we retested alprenolol binding to determine how much activity the receptor had lost over time. Figure 38 shows the alprenolol binding responses with 333 nM as the highest concentration. The receptor appeared to be nearly as active as when it was first captured.

β_1AR capture on another spot.

The S51 biosensor has the ability to monitor two reaction spots at one time so we captured the receptor to a density of 8000 RU on another streptavidin-coated spot (Figure 39).

The data from a test run of alprenolol binding to the 8000-RU β_1AR spot are provided in Figure 40.

The beta receptor agonist salmeterol was tested using a highest concentration of 1.67 μ M, with each concentration tested two or three times (Figure 41A and B). The responses are concentration dependent and mostly reproducible. Also, the responses from the 8000 RU surface (Figure 41B) were larger than from the 4200
5 RU surface (Figure 41A), as expected.

Salmeterol dissociated much faster and bound the receptor much more weakly than alprenolol or propranolol did. This is to be expected since salmeterol has a low affinity for the β 1-AR and in particular has a low affinity for the antagonist
10 stabilised form of the receptor which is used here. In addition, we detected some complexity in the interaction, as indicated by the poor fit of the model to the responses during the dissociation phase ($t > 60$ sec). Figure 42A and B shows the data plotted as normalized responses.

15 Isoproterenol, a non-selective β -adrenergic agonist, was tested using a highest concentration of 2 μ M (Figure 43A and B). In this analysis, isoproterenol displayed a much slower association rate than the other compounds. The compound was injected for 90 sec.

20 Figure 44A and B show responses for 111 μ M and 333 nM carvedilol binding to the two b1AR surfaces. Carvedilol displays a much slower dissociation rate than the other compounds examined so far.

Conclusion

- 25
1. Minimal biotinylation and capture by streptavidin produced active b1AR surfaces that can be used to measure compound binding.
 2. At 10°C, the b1AR surfaces remained active over several days.
 - 30 3. For the compounds tested including agonists and antagonists, we observed differences in both the association and dissociation rates, as well as the affinities. This demonstrates the biosensor assay is a viable approach to characterising panels of compounds binding to this b1AR
35 preparation.

4. Binding parameters were determined for several compounds as shown in Table A below, all of which were measured at 10°C.

Table A

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
Alprenolol	$(1.453 \pm 0.007) \times 10^5$	$(6.3 \pm 0.1) \times 10^{-3}$	44 ± 1
Propranolol	$(5.94 \pm 0.02) \times 10^5$	$(2.17 \pm 0.04) \times 10^{-3}$	3.64 ± 0.07
Salmeterol ¹	$(3.8 \pm 0.3) \times 10^4$	$(9.5 \pm 0.4) \times 10^{-2}$	2500 ± 300
Isoproterenol ^{1,2}	$(6 \pm 2) \times 10^3$	$(4.4 \pm 0.2) \times 10^{-3}$	800 ± 300
Carvedilol ^{1,2}	$(2.3 \pm 0.9) \times 10^5$	$(1.1 \pm 0.1) \times 10^{-3}$	5 ± 3

¹ averaged from two b1AR surfaces

² preliminary results

Example 8: Use of the adenosine A2a StaR for compound screening in drug discovery

Methods

A thermostabilised adenosine A2a receptor (A2a StaR) conformationally selected in the antagonist form (referred to as Rant22) was used to screen compounds from a library in order to identify compounds with activity at the A2a receptor. The StaR was generated as previously described (Magnani et al, Co-evolving stability and conformational homogeneity of the human adenosine A2a receptor. Proc Natl Acad Sci U S A. 2008 Aug 5;105 (31):10744-9). HEK293T cells transfected with Rant22 A2a receptors were grown in a monolayer in T-175 flasks at 37°C and 5% CO₂ in Dulbeccos Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells were harvested by scraping the cells from the T-175 surface and collected by centrifugation.

Membrane Preparation.

Cell pellets were resuspended in 10ml 20mM HEPES, pH 7.4 plus protease inhibitor cocktail tablets (Roche) and were homogenised for 30s at 20,500 rpm using a tissuemizer. Homogenates were centrifuged at 200xg for 15 min at 4°C.

The supernatant was removed and reserved on ice. This procedure was repeated a further two times and the pooled supernatants were then centrifuged at 40,000xg for 45 min at 4°C. Membranes were resuspended in 1ml aliquots of 20mM HEPES, pH 7.4 plus protease inhibitor tablets. Protein concentration was
5 determined by a BCA protein assay (Pierce).

Screening assay.

10µg aliquots of prepared membrane were incubated with [³H]ZM241385 3.7nM
10 and an appropriate amount of unlabelled ligand for 60 min at room temperature. A serial dilution ranging from 10mM – 1µM was screened in a 96 well format. The filter GFC plates were pre soaked in 0.1% PEI for 60 min. Radioactivity was determined by liquid scintillation counting using a Microbeta counter at 3 min/well.

Results

Data was analysed using GraphPad prism to fit concentration response curves. The IC₅₀ of compounds was calculated as the concentration resulting in 50% inhibition of the specific binding of [³H]ZM241385. The data presented in Figure
20 45 and in Table B below demonstrates that the compounds tested were able to inhibit binding to the A2a receptor StaR and had a range of activities in this assay. This data demonstrates the utility of StaRs for compound screening.

Table B

	HTL0245	HTL0246	HTL0247	HTL0248	HTL0249	HTL0250	HTL0251	HTL0252	CGS15943
Best-fit values									
Bottom	105.2	151.2	47.23	44.21	25.74	13.98	-5.831	176.4	22.40
Top	1428	1414	1406	1519	1527	1382	1446	1257	1530
LogIC50	-6.795	-6.442	-7.552	-8.098	-8.431	-4.112	-2.874	-4.658	-8.661
IC50	1.605e-007	3.614e-007	2.804e-008	7.985e-009	3.709e-009	7.735e-005	0.001337	2.199e-005	2.183e-009
Std.Error									
Bottom	29.07	70.00	40.67	18.05	7.115	104.4	79.15	154.9	29.97
Top	44.68	83.73	96.91	50.84	20.46	38.13	26.10	74.89	40.93
LogIC50	0.07888	0.1886	0.1338	0.05205	0.02427	0.1202	0.2821	0.2878	0.08677

References

1. S. H. White (2004) *Protein Sci* **13**, 1948-1949.
- 5 2. C. G. Tate (2001) *FEBS Lett* **504**, 94-98.
3. R. Grisshammer, C. G. Tate (1995) *Q Rev Biophys* **28**, 315-422.
4. J. U. Bowie (2001) *Curr Opin Struct Biol* **11**, 397-402.
5. F. W. Lau, S. Nauli, Y. Zhou, J. U. Bowie (1999) *J Mol Biol* **290**, 559-564.
6. Y. Zhou, J. U. Bowie (2000) *J Biol Chem* **275**, 6975-6979.
- 10 7. S. Faham, D. Yang, E. Bare, S. Yohannan, J. P. Whitelegge, J. U. Bowie (2004) *J Mol Biol* **335**, 297-305.
8. Y. Yarden, H. Rodriguez, S. K. Wong, D. R. Brandt, D. C. May, J. Burnier, R. N. Harkins, E. Y. Chen, J. Ramachandran, A. Ullrich, *et al* (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6795-6799.
- 15 9. T. Warne, J. Chirside, G. F. Schertler (2003) *Biochim Biophys Acta* **1610**, 133-140.
10. E. M. Parker, E. M. Ross (1991) *J Biol Chem* **266**, 9987-9996.
11. E. M. Parker, K. Kameyama, T. Higashijima, E. M. Ross (1991) *J Biol Chem* **266**, 519-527.
- 20 12. W. J. Degrip (1982) *Methods in Enzymology* **81**, 256-265.
13. K. Palczewski, T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. Le Trong, D. C. Teller, T. Okada, R. E. Stenkamp, *et al* (2000) *Science* **289**, 739-745.
14. J. Li, P. C. Edwards, M. Burghammer, C. Villa, G. F. Schertler (2004) *J Mol Biol* **343**, 1409-1438.
- 25 15. R. Jaenicke, G. Bohm (1998) *Current Opinion in Structural Biology* **8**, 738-748.
16. J. Tucker, R. Grisshammer (1996) *Biochem J* **317** (Pt 3), 891-899.
17. W. Schaffner, C. Weissmann (1973) *Anal. Biochem.* **56**, 502-514.
- 30 18. C. G. Tate (1998) *Methods Enzymol* **296**, 443-455.
19. H. M. Weiss, R. Grisshammer (2002) *Eur J Biochem* **269**, 82-92.
20. Rasmussen, S. G., Choi, H. J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., Burghammer, M., Ratnala, V. R., Sanishvili, R., Fischetti, R. F., Schertler, G. F., Weis, W. I. and Kobilka, B. K. (2007) *Nature* **447**, 383-387.
- 35

21. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G.,
Thian, F. S., Kobilka, T. S., Choi, H. J., Kuhn, P., Weis, W. I., Kobilka, B.
K. and Stevens, R. C. (2007) *Science* 318:1258-1265.
22. Minneman, K. P., Weiland, G. A. and Molinoff, P. B. (1980) *Mol*
5 *Pharmacol* 17:1-7.
23. Parker, E. M., Swigart, P., Nunnally, M. H., Perkins, J. P. and Ross, E. M.
(1995) *J Biol Chem* 270:6482-6487.
24. Schofield DJ, Pope A, Clementel V, Buckell J, Chapple SDJ,, Clarke KF,
Conquer JS, Crofts AM, Crowther SRE, Dyson MR, Flack G, Griffin GJ,
10 Hooks Y, Howat WJ, Kolb-Kokocinski A,, Kunze S, Martin CD, Maslen
GL,, Mitchell JM, O'Sullivan M, Perera RL, Roake W, Shadbolt SP,
Vincent KJ, Warford A, Wilson WE, Xie J, Young JL, McCafferty J (2007)
Application of phage display to high throughput antibody generation and
characterisation. *Genome Biology*. 8 (11) R254
- 15 25. Chapple SD, Crofts AM, Shadbolt SP, McCafferty J, Dyson MR. (2006)
Multiplexed expression and screening for recombinant protein production
in mammalian cells. *BMC Biotechnol*. 2006 Dec 22;6:49.
<http://www.biomedcentral.com/1472-6750/6/49>
26. Martin C, Rojas G, Mitchell JN, Vincent KJ, Wu J, McCafferty J, Schofield
20 DJ (2006) A simple vector system to improve performance and utilisation
of recombinant antibodies. *BMC Biotechnology* 6:46 doi:10.1186/1472-
6750-6-46 <http://www.biomedcentral.com/1472-6750/6/46/abstract>
27. Warne *et al* (2008) Structure of a β adrenergic G protein coupled receptor
Nature 454: 486-491

25

CLAIMS

1. A method of producing a binding partner of a GPCR, the method comprising:
 - 5 a) providing a mutant GPCR of a parent GPCR, wherein the mutant GPCR has increased stability in a particular conformation relative to the parent GPCR;
 - b) providing one or more test compounds;
 - c) determining whether the or each test compound binds to the
10 mutant GPCR when residing in a particular conformation; and
 - d) isolating one or more test compounds that bind to the mutant GPCR when residing in the particular conformation.
2. A method according to Claim 2, the method further comprising:
 - 15 e) determining whether the or each test compound binds to the parent GPCR when residing in the particular conformation and
 - f) isolating the or each test compound that also binds the parent GPCR when residing in the particular conformation.
- 20 3. A method according to Claim 1 or 2, wherein the mutant GPCR is immobilised onto a solid support.
4. A method according to Claim 3, wherein the support is any of a bead, a column, a slide, a chip or a plate.
- 25 5. A method according to Claim 3 or 4, wherein the mutant GPCR is immobilised onto the support via a covalent interaction.
6. A method according to Claim 5, wherein the support is coated with a
30 polymeric support
7. A method according to Claim 6, wherein the polymeric support is carboxylated dextran.
- 35 8. A method according to Claim 3 or 4, wherein the mutant GPCR is immobilised on the support via a non-covalent interaction.

9. A method according to Claim 8, wherein the support is coated with any of avidin, streptavidin, a metal ion, an antibody to the parent GPCR or an antibody to a molecular tag attached to the mutant GPCR.
- 5
10. A method according to any of Claims 3-9, wherein the mutant GPCR is immobilised via the C-terminus or an intracellular domain such that the extracellular domains are outward facing.
- 10 11. A method according to any of Claims 3-9, wherein the mutant GPCR is immobilised via the N-terminus or an extracellular domain such that the intracellular domains are outward facing.
12. A method according to any of Claims 1-11, wherein the mutant GPCR
15 comprises a molecular tag at the C-terminus or N-terminus.
13. A method according to Claim 10, wherein the tag is any of a FLAG tag, a His tag, a c-Myc tag, a DDDDK tag, an HSV tag, a Halo tag or a biotin tag.
- 20 14. A method according to any of Claims 1-13, wherein the mutant GPCR is in a whole cell preparation, in a cell membrane fragment, solubilised in detergent, in a lipid monolayer, in a lipid bilayer, in a bead-linked lipid particle, in a solid-supported lipid layer or in a proteoliposome.
- 25 15. A method according to any of Claims 1, 2, 12-14, wherein the test compound is immobilised on a solid support.
16. A method according to Claim 15, wherein the solid support is any of a bead, a column, a slide, a chip or a plate.
- 30
17. A method according to any of Claims 1, 2, 12-14, wherein the mutant GPCR and the test compound are not immobilised on a solid support.
- 35 18. The method according to any of Claims 1-17 wherein the test compound is any of a polypeptide; an anticalin; a peptide; an antibody; a chimeric antibody; a single chain antibody; an aptamer; a darpin; a Fab, F(ab')₂, Fv,

ScFv or dAb antibody fragment; a small molecule; a natural product; an affibody; a peptidomimetic; a nucleic acid; a peptide nucleic acid molecule; a lipid; a carbohydrate; a protein based on a modular framework including ankyrin repeat proteins, armadillo repeat proteins, leucine rich proteins, tetrariopeptide repeat proteins or Designed Ankyrin Repeat Proteins (DARPs); or proteins based on lipocalin or fibronectin domains or Affilin scaffolds based on either human gamma crystalline or human ubiquitin.

19. A method according to any of Claims 1-18 wherein the test compound is provided as a biological sample.

20. A method according to Claim 19, wherein the sample is any of blood, serum, plasma, spinal fluid, a tissue extract or a cell extract.

21. A method according to any of Claims 1-18 wherein the test compound is a library of test compounds.

22. A method according to Claim 20, wherein the library is any of a peptide library, a protein library, an antibody library, a recombinant combinatorial antibody library or a scFV or Fab phage display library.

23. A method according to any of Claims 1-22, wherein the test compound is labelled with any of a peptide tag, a nucleic acid tag, a chemical tag, a fluorescent tag or a radio frequency tag.

24. A method according to Claim 18, wherein the antibody is an antibody to a mutant GPCR of a parent GPCR, wherein the mutant GPCR has increased stability in a particular conformation relative to the parent GPCR.

25. A method according to Claim 24, wherein the antibody is produced by immunising a lymphocyte with an immunogen of the mutant GPCR.

26. A method according to Claim 25, wherein the lymphocyte is immunised *in vivo*.

27. A method according to Claim 25, wherein the lymphocyte is immunised *in vitro*.

28. A method according to any of Claims 25-27, wherein the immunogen of the mutant GPCR is all of the mutant GPCR, a fragment thereof or a fusion protein thereof.

29. A method according to any of Claims 24-28, wherein the immunogen of a mutant GPCR is provided in a whole cell preparation, in a cell membrane fragment, solubilised in detergent, in a lipid monolayer, in a lipid bilayer, in a bead-linked lipid particle, in a solid-supported lipid layer or in a proteoliposome.

30. A method according to Claims 24-29, wherein the immunogen is provided with an adjuvant.

31. A method according to Claim 30, wherein the adjuvant is Titremax or Ribi's adjuvant emulsion.

32. A method according to any of Claims 1-31, the method further comprising modifying the isolated test compound that binds to a mutant GPCR when residing in a particular conformation and determining whether the modified test compound binds to the mutant GPCR when residing in a particular conformation.

33. A method according to Claim 32, further comprising determining whether the modified test compound binds to the parent GPCR when residing in the particular conformation.

34. A method according to any of Claims 1-33, wherein more than one mutant GPCR is provided in step (a), it is determined whether the test compound binds to each mutant GPCR when residing in a particular conformation; and the test compound which binds to each mutant GPCR when residing in the particular conformation is isolated.

35. A method according to Claim 34, wherein a mutant GPCR of a first parent GPCR and a mutant GPCR of a second parent GPCR are provided in step (a), it is determined whether the test compound binds to each mutant GPCR when residing in a particular conformation; and the test compound which binds to each mutant GPCR when residing in the particular conformation is isolated.
36. A method according to any of Claims 1-33, wherein more than one mutant GPCR is provided in step (a) and a test compound is selected that binds to a first mutant GPCR but which does not bind, or binds less strongly than to the first mutant GPCR, to at least one other mutant GPCR.
37. A method according to any of Claims 1-36, further comprising (i) determining if the isolated test compound affects the function of the GPCR to which it binds and (ii) isolating a test compound that affects the function of the GPCR to which it binds.
38. A method according to Claim 37, wherein in step (i) it is determined if the isolated test compound affects the binding of the GPCR to its natural ligand or analog thereof.
39. A method according to Claim 38, wherein in step (ii) a test compound that decreases binding between the GPCR and its natural ligand or analog thereof is isolated.
40. A method according to Claim 38, wherein in step (ii) a test compound that increases binding between the GPCR and its natural ligand or analog thereof is isolated.
41. A method according to any of Claims 37-40, wherein in step (i) it is determined if the isolated test compound modulates activation of the GPCR to which it binds.
42. A method according to Claim 41, wherein in step (ii) a test compound which modulates any of calcium mobilisation, cAMP levels, kinase pathway activity, gene transcription from a reporter gene under control of the GPCR

to which the test compound binds, β -arrestin recruitment, activation of G proteins, GTPase activity or [35S]GTP γ S binding is selected.

5 43. A method according to Claim 41 or 42, wherein in step (ii) an agonist test compound that increases activation of the GPCR to which it binds, is isolated.

10 44. A method according to Claim 41 or 42, wherein in step (ii) an antagonist test compound that decreases activation of the GPCR to which it binds, is isolated.

45. A method according to any of Claims 1-44, wherein the mutant GPCR is provided by:

15 (a) providing one or more mutants of a parent GPCR,

(b) selecting a ligand, the ligand being one which binds to the parent GPCR when the GPCR is residing in a particular conformation,

20 (c) determining whether the or each mutant GPCR has increased stability with respect to binding the selected ligand compared to the stability of the parent GPCR with respect to binding that ligand, and

25 (d) selecting those mutants that have an increased stability compared to the parent GPCR with respect to binding the selected ligand.

46. A method according to Claim 45, wherein the one or more mutants are brought into contact with the selected ligand prior to step (c).

30 47. A method according to Claim 45 or 46, wherein the one or more mutants are provided in a solubilised form.

35 48. A method according to any of Claims 45-47 wherein the particular conformation in which the GPCR resides in step (c) corresponds to the class of ligand selected in step (b).

49. A method according to Claim 48, wherein the selected ligand is from the agonist class of ligands and the particular conformation is an agonist conformation, or the selected ligand is from the antagonist class of ligands and the particular conformation is an antagonist conformation.

5

50. A method according to Claim 49 wherein the selected ligand is from the agonist class of ligands and the particular conformation in which the GPCR resides in step (c) is the agonist conformation.

10 51. A method according to any of Claims 45 to 50 wherein the binding affinity of the mutant for the selected ligand is substantially the same or greater than the binding affinity of the parent for the selected ligand.

15 52. A method as claimed in any of Claims 45 to 51 wherein the method is repeated for one or more rounds, with the selected mutants having increased stability in step (a) representing the parent GPCR in a subsequent round of the method.

20 53. A method according to any of Claims 45 to 52 wherein a mutant GPCR is selected which has increased stability to any one or more of heat, a detergent, a chaotropic agent and an extreme of pH.

25 54. A method according to Claim 53 wherein a mutant GPCR with increased thermostability is selected.

55. A method according to any of Claims 45 to 54 wherein the ligand is any one of a full agonist, a partial agonist, an inverse agonist, an antagonist.

30 56. A method according to any of Claims 45 to 55 wherein the ligand is a polypeptide which binds to the GPCR.

35 57. A method according to Claim 56 wherein the polypeptide is any of an antibody, an ankyrin, a G protein, an RGS protein, an arrestin, a GPCR kinase, a receptor tyrosine kinase, a RAMP, a NSF, a GPCR, an NMDA receptor subunit NR1 or NR2a, or calcyon, a fibronectin domain framework, or a fragment or derivative thereof that binds to the GPCR.

58. A method according to any of Claims 45 to 57 wherein in step (b) two or more ligands are selected, the presence of each causes the GPCR to reside in the same particular conformation.

5

59. A method according to any one of Claims 45-58 wherein a mutant GPCR is selected which has reduced ability to bind a ligand of a different class to the ligand selected in step (b) compared to its parent.

10 60. A method according to any one of Claims 45-59 wherein the GPCR is any one of a β -adrenergic receptor, an adenosine receptor and a neurotensin receptor.

15 61. A method according to any one of Claims 1-44, wherein the mutant GPCR is provided by:

(a) carrying out the method of any one of Claims 45-60,

20 (b) identifying the position or positions of the mutated amino acid residue or residues in the mutant GPCR or GPCRs which has been selected for increased stability, and

(c) synthesising a mutant GPCR which contains a replacement amino acid at one or more of the positions identified.

25

62. A method according to Claim 61 wherein the mutant GPCR contains a plurality of mutations compared to the parent GPCR.

30 63. A method according to any one of Claims 1-44, wherein the mutant GPCR is provided by:

35 (i) identifying in the amino acid sequence of one or more mutants of a first parent GPCR with increased stability relative to the first parent GPCR, the position or positions at which the one or more mutants have at least one different amino acid residue compared to the first parent GPCR, and

- (ii) making one or more mutations in the amino acid sequence that defines a second GPCR at the corresponding position or positions, to provide one or more mutants of a second parent GPCR with increased stability relative to the second parent GPCR.

64. A method according to Claim 63 wherein the one or more mutants of a first parent GPCR are provided according to the methods of any of Claims 45-62.

65. A method according to Claim 63 or 64, wherein the second GPCR is of the same GPCR class or family as the first parent GPCR.

66. A method according to any of Claims 63-65, wherein the second GPCR is a GPCR which has at least 20% sequence identity with the first parent GPCR.

67. A method according to any of Claims 1-44, wherein of producing a mutant GPCR with increased stability relative to its parent GPCR, the method comprising:

- (i) providing one or more mutants of a first parent GPCR with increased stability relative to the first parent GPCR

- (ii) identifying in a structural membrane protein model the structural motif or motifs in which the one or more mutants have at least one different amino acid residue compared to the first parent GPCR, and

- (iii) making one or more mutations in the amino acid sequence that defines a corresponding structural motif or motifs in a second parent GPCR, to provide one or more mutants of a second parent GPCR with increased stability relative to the second parent GPCR.

68. A method according to Claim 67, wherein the structural membrane protein model is a model of an integral membrane protein.
- 5 69. A method according to Claim 67 or 68, wherein the integral membrane protein has at least 20% sequence identity with the mutant of the first parent GPCR in step (i) across the protein domain in which the mutant has at least one different amino acid relative to the first parent GPCR.
- 10 70. A method according to Claim 68 or 69, wherein the integral membrane protein is a GPCR.
71. A method according to Claim 70, wherein the GPCR is of the same GPCR class or family as the first parent GPCR.
- 15 72. A method according to any of Claims 67-71, wherein the structural membrane protein model is a model of human β_2 adrenergic receptor or bovine rhodopsin.
- 20 73. A method according to any of Claims 67-72, wherein the structural motif is any of a helical interface, a helix kink, a helix opposite a helix kink, a helix surface pointing into the lipid bilayer, a helix surface pointing into the lipid bilayer at the hydrophobic-hydrophilic boundary layer, a loop region or a protein binding pocket.
- 25 74. A method according to any of Claims 67-73, wherein the second parent GPCR is the first parent GPCR.
75. A method according to any of Claims 67-73, wherein the second parent GPCR is not the first parent GPCR.
- 30 76. A method according to Claim 74 or 75, wherein the second parent GPCR is a GPCR which has at least 20% sequence identity with the first parent GPCR.
- 35 77. A method according to any of Claims 74-76, wherein the second GPCR is of the same GPCR class or family as the first parent GPCR.

78. A method according to any of Claims 63-77, further comprising:

5 (I) selecting a ligand, the ligand being one which binds to the second parent GPCR when the GPCR is residing in a particular conformation

10 (II) determining whether the or each mutant of the second parent GPCR when residing in a particular conformation has increased stability with respect to binding the selected ligand compared to the stability of the second parent GPCR when residing in the same particular conformation with respect to binding that ligand, and

15 (III) selecting those mutants that have an increased stability compared to the second parent GPCR with respect to binding the selected ligand.

79. A method according to Claim 78, wherein the particular conformation in which the GPCR resides in step (II) corresponds to the class of ligand selected in step (I).

20 80. A method according to Claim 79, wherein the selected ligand is from the agonist class of ligands and the particular conformation is an agonist conformation, or the selected ligand is from the antagonist class of ligands and the particular conformation is an antagonist conformation.

25 81. A method according to any one of Claims 78-80, wherein the ligand is as defined in any of Claims 55-57.

30 82. A method according to any one of Claims 78-81, wherein the binding affinity of the one or more mutants of the second GPCR is substantially the same or greater than the binding affinity of the second parent GPCR for the selected ligand.

35 83. A method according to any of Claims 1-82 wherein the mutant GPCR provided in step (a) is any one of a β -adrenergic receptor, an adenosine receptor, a neurotensin receptor or a muscarinic receptor.

84. A method according to any of Claims 1-82 wherein the mutant GPCR provided in step (a) has, compared to its parent receptor, at least one different amino acid at a position which corresponds to any one or more of the following positions: (i) according to the numbering of the turkey β -adrenergic receptor as set out in Figure 9: Ile 55, Gly 67, Arg 68, Val 89, Met 90, Gly 98, Ile 129, Ser 151, Val 160, Gln 194, Gly 197, Leu 221, Tyr 227, Arg 229, Val 230, Ala 234, Ala 282, Asp 322, Phe 327, Ala 334, Phe 338, (ii) according to the numbering of the human adenosine A_{2a} receptor as set out in Figure 10: Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313, Lys 315, (iii) according to the numbering of the rat neurotensin receptor as set out in Figure 11: Ala 69, Leu 72, Ala 73, Ala 86, Ala 90, Ser 100, His 103, Ser 108, Leu 109, Leu 111, Asp 113, Ile 116, Ala 120, Asp 139, Phe 147, Ala 155, Val 165, Glu 166, Lys 176, Ala 177, Thr 179, Met 181, Ser 182, Arg 183, Phe 189, Leu 205, Thr 207, Gly 209, Gly 215, Val 229, Met 250, Ile 253, Leu 256, Ile 260, Asn 262, Val 268, Asn 270, Thr 279, Met 293, Thr 294, Gly 306, Leu 308, Val 309, Leu 310, Val 313, Phe 342, Asp 345, Tyr 349, Tyr 351, Ala 356, Phe 358, Val 360, Ser 362, Asn 370, Ser 373, Phe 380, Ala 385, Cys 386, Pro 389, Gly 390, Trp 391, Arg 392, His 393, Arg 395, Lys 397, Pro 399, and (iv) according to the numbering of the muscarinic receptor as set out in Figure 17: Leu 65, Met 145, Leu 399, Ile 383 and Met 384.

85. A method according to any of Claims 1-82 wherein the mutant GPCR provided in step (a) is a mutant β -adrenergic receptor which, when compared to its corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of the following positions according to the numbering of the turkey β -adrenergic receptor as set out in Figure 9: Ile 55, Gly 67, Arg 68, Val 89, Met 90, Gly 98, Ile 129, Ser 151, Val 160, Gln 194, Gly 197, Leu 221, Tyr 227, Arg 229, Val 230, Ala 234, Ala 282, Asp 322, Phe 327, Ala 334, Phe 338.

86. A method according to Claim 86, wherein the mutant β -adrenergic receptor has an amino acid sequence which is at least 20% identical to

that of the turkey β -adrenergic receptor whose sequence is set out in Figure 9.

87. A method according to any of Claims 1-82 wherein the mutant GPCR provided in step (a) is a mutant β -adrenergic receptor which has at least one different amino acid residue in a structural motif in which the mutant receptor compared to its parent receptor has a different amino acid at a position which corresponds to any of the following positions according to the numbering of the turkey β -adrenergic receptor as set out in Figure 9: Ile 55, Gly 67, Arg 68, Val 89, Met 90, Gly 98, Ile 129, Ser 151, Val 160, Gln 194, Gly 197, Leu 221, Tyr 227, Arg 229, Val 230, Ala 234, Ala 282, Asp 322, Phe 327, Ala 334, Phe 338.

88. A method according to any of Claims 1-82 wherein the mutant GPCR provided in step (a) is a mutant adenosine receptor which, when compared to its corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of the following positions according to the numbering of the human adenosine A_{2a} receptor as set out in Figure 10: Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His 230, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313, Lys 315.

89. A method according to Claim 88 wherein the mutant adenosine receptor has an amino acid sequence which is at least 20% identical to that of the human adenosine A_{2a} receptor whose sequence is set out in Figure 10.

90. A method according to any of Claims 1-82 wherein the mutant GPCR provided in step (a) is a mutant adenosine receptor which has at least one different amino acid residue in a structural motif in which the mutant receptor compared to its parent receptor has a different amino acid at a position which corresponds to any of the following positions according to the numbering of the human adenosine A_{2a} receptor as set out in Figure 10: Gly 114, Gly 118, Leu 167, Ala 184, Arg 199, Ala 203, Leu 208, Gln 210, Ser 213, Glu 219, Arg 220, Ser 223, Thr 224, Gln 226, Lys 227, His

230, Leu 241, Pro 260, Ser 263, Leu 267, Leu 272, Thr 279, Asn 284, Gln 311, Pro 313, Lys 315.

- 5 91. A method according to any of Claims 1-82 wherein the mutant GPCR provided in step (a), is a mutant neurotensin receptor which, when compared to its corresponding parent receptor, has a different amino acid at a position which corresponds to any one or more of the following positions according to the numbering of the rat neurotensin receptor as set out in Figure 11: Ala 69, Leu 72, Ala 73, Ala 86, Ala 90, Ser 100, His 103, Ser 108, Leu 109, Leu 111, Asp 113, Ile 116, Ala 120, Asp 139, Phe 147, Ala 155, Val 165, Glu 166, Lys 176, Ala 177, Thr 179, Met 181, Ser 182, Arg 183, Phe 189, Leu 205, Thr 207, Gly 209, Gly 215, Val 229, Met 250, Ile 253, Leu 256, Ile 260, Asn 262, Val 268, Asn 270, Thr 279, Met 293, Thr 294, Gly 306, Leu 308, Val 309, Leu 310, Val 313, Phe 342, Asp 15 345, Tyr 349, Tyr 351, Ala 356, Phe 358, Val 360, Ser 362, Asn 370, Ser 373, Phe 380, Ala 385, Cys 386, Pro 389, Gly 390, Trp 391, Arg 392, His 393, Arg 395, Lys 397, Pro 399.
- 20 92. A method according to Claim 91 wherein the mutant neurotensin receptor has an amino acid sequence which is at least 20% identical to that of the rat neurotensin receptor whose sequence is set out in Figure 11.
- 25 93. A mutant GPCR according to any of Claims 1-82, wherein the mutant GPCR provided in step (a) is a mutant neurotensin receptor which has at least one different amino acid residue in a structural motif in which the mutant receptor compared to its parent receptor has a different amino acid at a position which corresponds to any of the following positions according to the numbering of the rat neurotensin receptor as set out in Figure 11: Ala 69, Leu 72, Ala 73, Ala 86, Ala 90, Ser 100, His 103, Ser 108, Leu 109, Leu 111, Asp 113, Ile 116, Ala 120, Asp 139, Phe 147, Ala 155, Val 30 165, Glu 166, Lys 176, Ala 177, Thr 179, Met 181, Ser 182, Arg 183, Phe 189, Leu 205, Thr 207, Gly 209, Gly 215, Val 229, Met 250, Ile 253, Leu 256, Ile 260, Asn 262, Val 268, Asn 270, Thr 279, Met 293, Thr 294, Gly 306, Leu 308, Val 309, Leu 310, Val 313, Phe 342, Asp 345, Tyr 349, Tyr 35 351, Ala 356, Phe 358, Val 360, Ser 362, Asn 370, Ser 373, Phe 380, Ala

385, Cys 386, Pro 389, Gly 390, Trp 391, Arg 392, His 393, Arg 395, Lys 397, Pro 399.

5 94. A method according to any of Claims 1-82, wherein the mutant GPCR provided in step (a) is a mutant muscarinic receptor which, when compared to the corresponding wild-type muscarinic receptor, has a different amino acid at a position which corresponds to any one or more of the following positions according to the numbering of the human muscarinic receptor as set out in Figure 17: Leu 65, Met 145, Leu 399, Ile 10 383 and Met 384.

95. A method according to Claim 94 wherein the mutant muscarinic receptor has an amino acid sequence which is at least 20% identical to that of the rat neurotensin receptor whose sequence is set out in Figure 17

15 96. A method according to any of Claims 1-82 wherein the mutant GPCR provided in step (a) is a mutant muscarinic receptor which has at least one different amino acid residue in a structural motif in which the mutant receptor compared to its parent receptor has a different amino acid at a position which corresponds to any of the following positions according to 20 the numbering of the human muscarinic receptor as set out in Figure 17: Leu 65, Met 145, Leu 399, Ile 383 and Met 384.

25 97. A method of producing a binding partner of a GPCR, the method comprising synthesising a binding partner identifiable by carrying out the method of any one of Claims 1-96.

98. A binding partner obtained by any of the methods according to Claims 1-97.

30 99. A binding partner according to Claim 98, wherein the binding partner is conformation-specific.

35 100. A binding partner according to Claim 98 or 99, wherein the binding partner is any of a polypeptide; an anticalin; a peptide; an antibody; a chimeric antibody; a single chain antibody; an aptamer; a darpin; a Fab, F(ab')₂, Fv,

ScFv or dAb antibody fragment; a small molecule; a natural product; an affibody; a peptidomimetic; a nucleic acid; a peptide nucleic acid molecule; a lipid; a carbohydrate; a protein based on a modular framework including ankyrin repeat proteins, armadillo repeat proteins, leucine rich proteins, tetrariopeptide repeat proteins or Designed Ankyrin Repeat Proteins (DARPs); or proteins based on lipocalin or fibronectin domains or Affilin scaffolds based on either human gamma crystalline or human ubiquitin.

- 10 101. A binding partner according to Claim 100, wherein the binding partner is an antibody.
- 15 102. A binding partner according to any of Claims 98-101, wherein the binding affinity of the mutant GPCR for the binding partner is substantially the same or greater than the binding affinity of the parent GPCR for the binding partner.
- 20 103. A biosensor comprising a mutant GPCR of a parent GPCR, wherein the mutant GPCR has increased stability in a particular conformation relative to the parent GPCR and wherein when a target substance binds to said mutant GPCR, a detectable signal is produced.
- 25 104. A biosensor according to Claim 103, wherein the mutant GPCR is provided as defined in any of Claims 45-82.
105. A biosensor according to Claim 103 or 104, wherein the mutant GPCR is as defined in any of Claims 83-96.
- 30 106. A biosensor according to any of Claims 103-105, wherein the detectable signal is any of a change in colour; fluorescence; evanescence; surface plasmon resonance; electrical conductance or charge separation; ultraviolet, visible or infrared absorption; luminescence; chemiluminescence; electrochemiluminescence; fluorescence anisotropy; fluorescence intensity; fluorescence lifetime; fluorescence polarisation; fluorescence energy transfer; molecular mass; electron spin resonance; nuclear magnetic resonance; hydrodynamic volume or radius; specific
- 35

gravity; scintillation; field effect resistance; electrical impedance; acoustic impedance; quantum evanescence; resonant scattering; fluorescent quenching; fluorescence correlation spectroscopy; acoustic load; acoustic shear wave velocity; binding force; or interfacial stress.

5

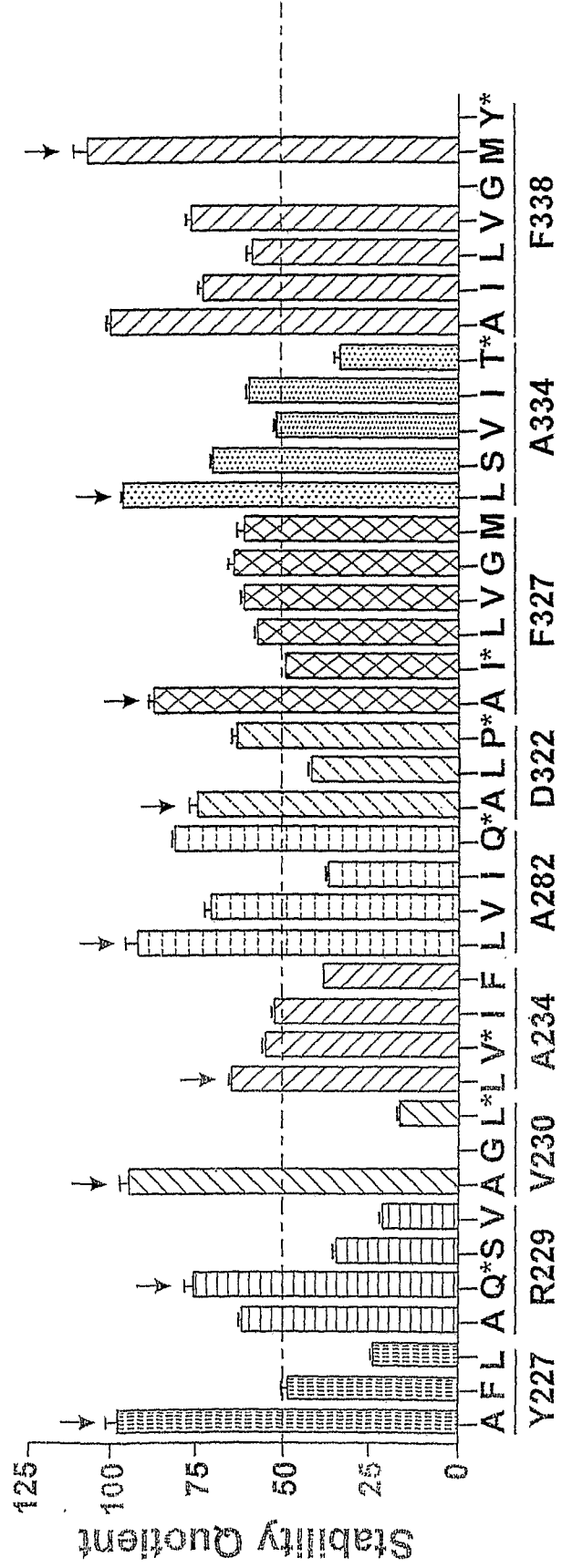
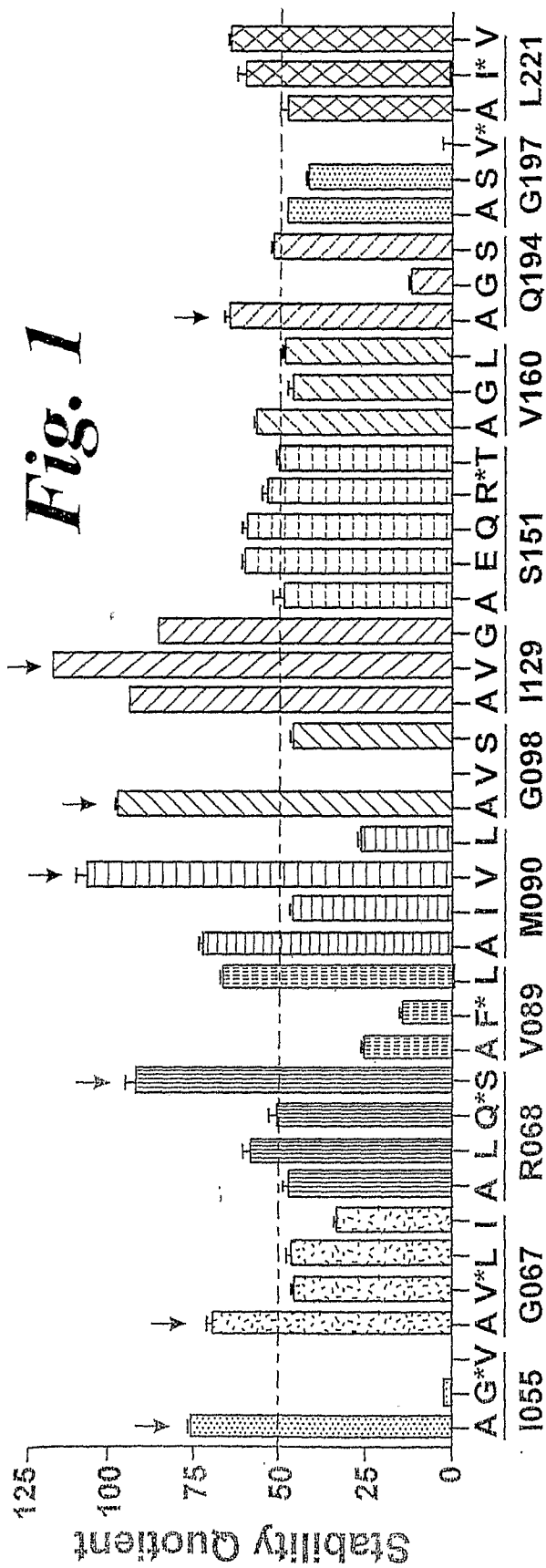
107. A biosensor according to any of Claims 103-106 wherein the biosensor is a flow-based biosensor, such as a quartz crystal microbalance biosensor, an evanescent wave biosensor, a planar wave guide biosensor, a surface Raman sensor, or a surface plasmon resonance biosensor.

10

108. A biosensor according to any of Claims 103-107, wherein the target substance is any of a molecule, a biomolecule, a peptide, a protein, a carbohydrate, a lipid, a GPCR ligand, a synthetic molecule, a drug, a drug metabolite or a disease biomarker.

15

Fig. 1



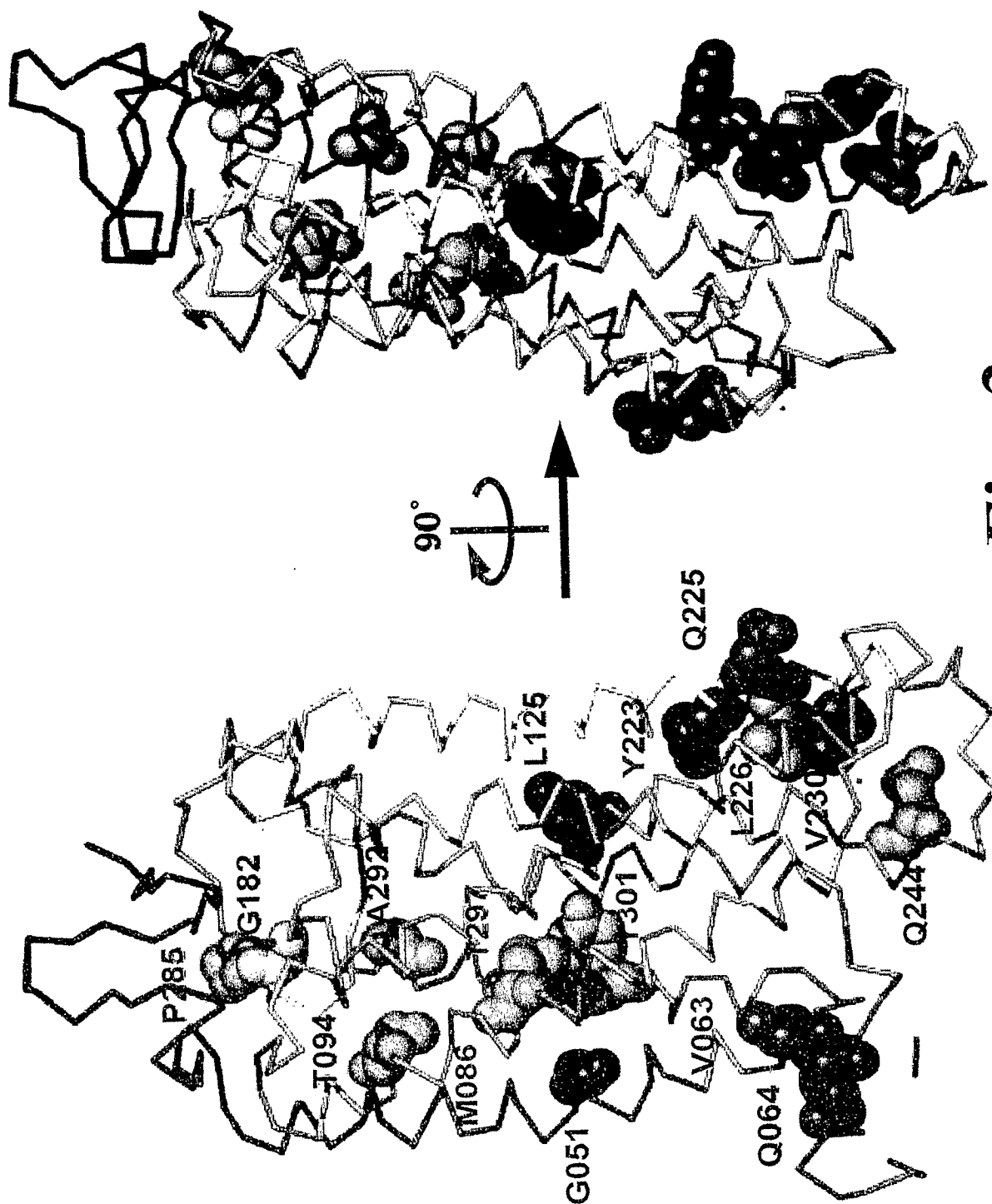


Fig. 2

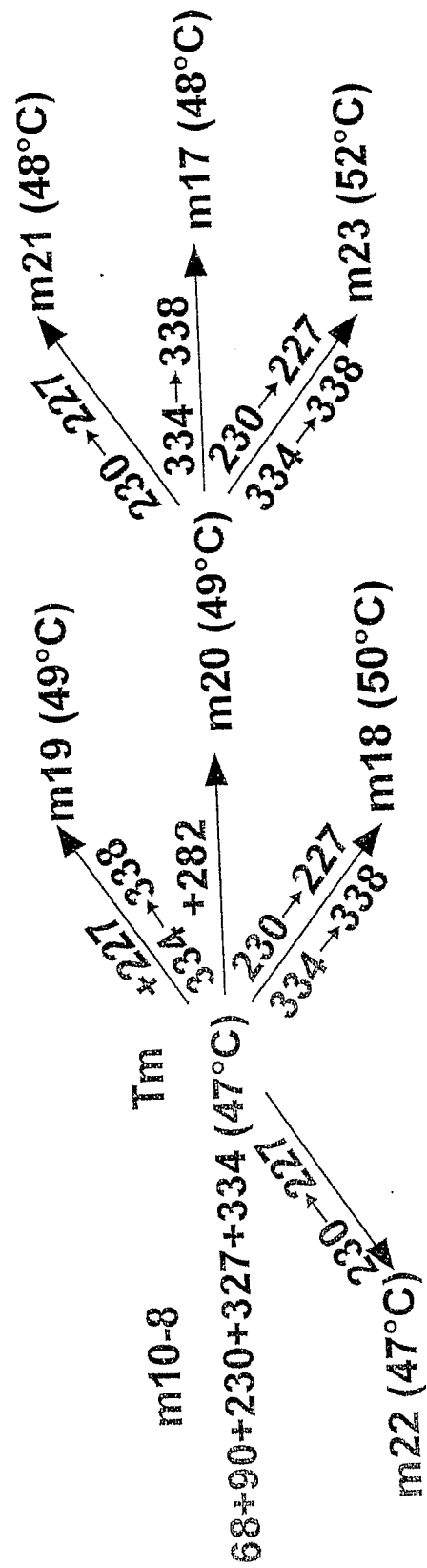


Fig. 3

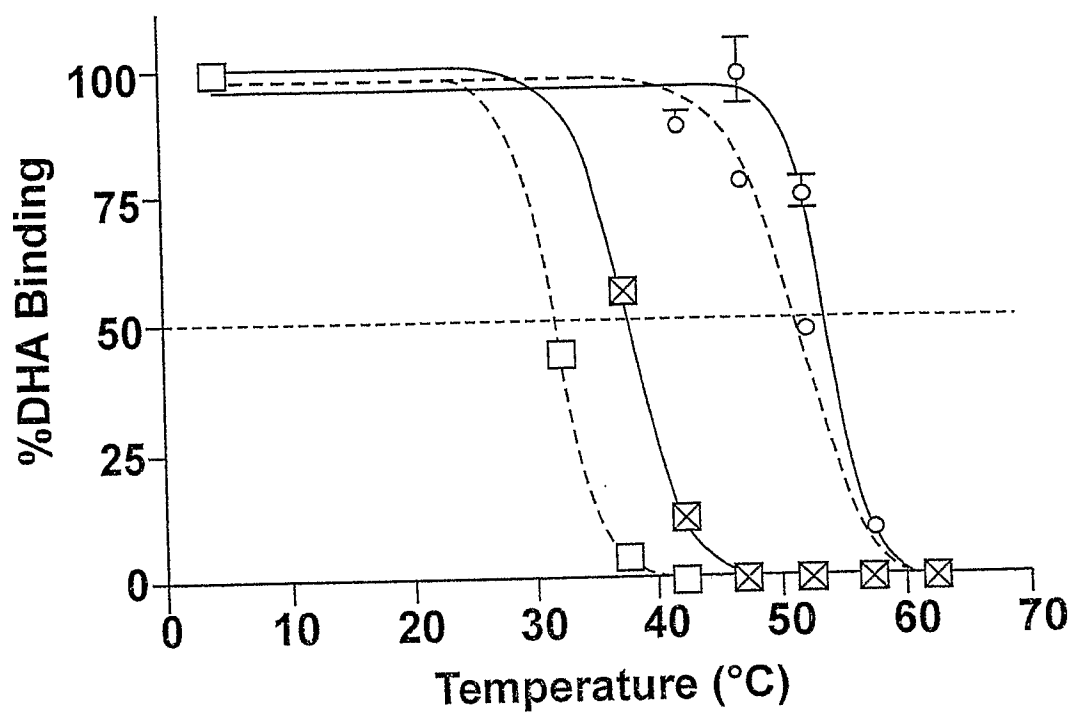
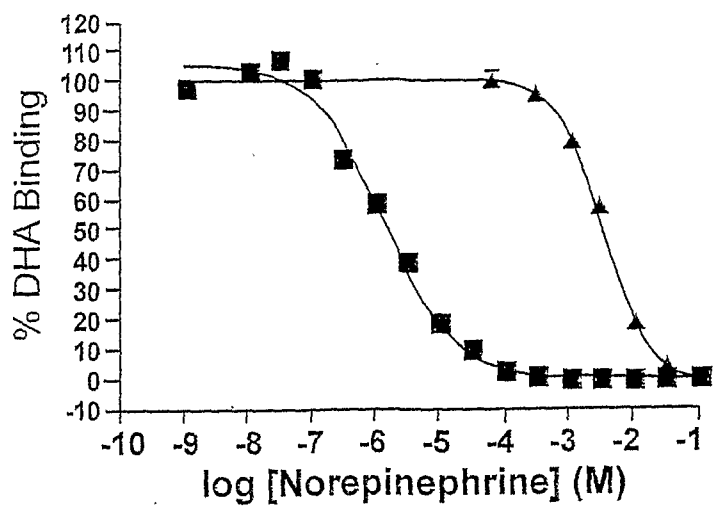
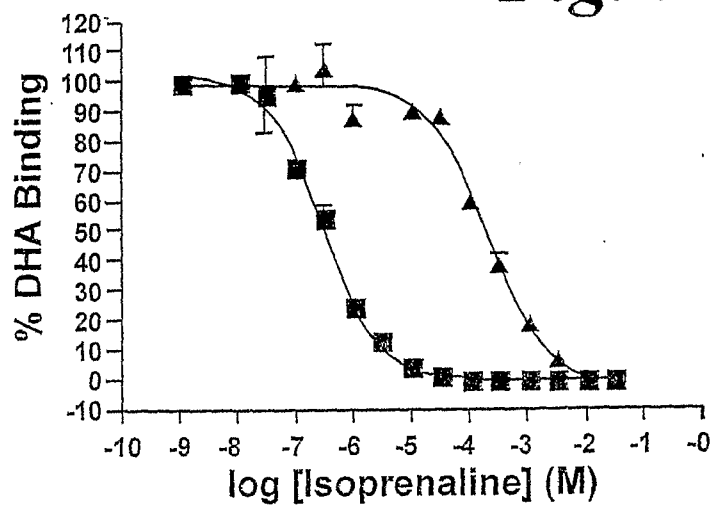
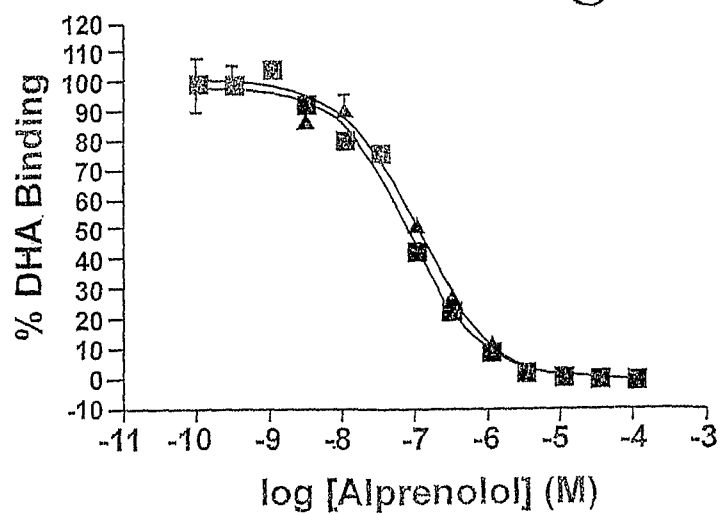
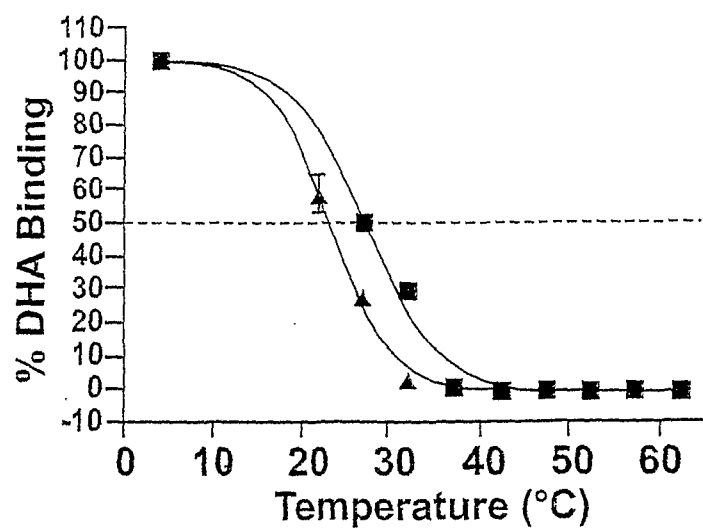
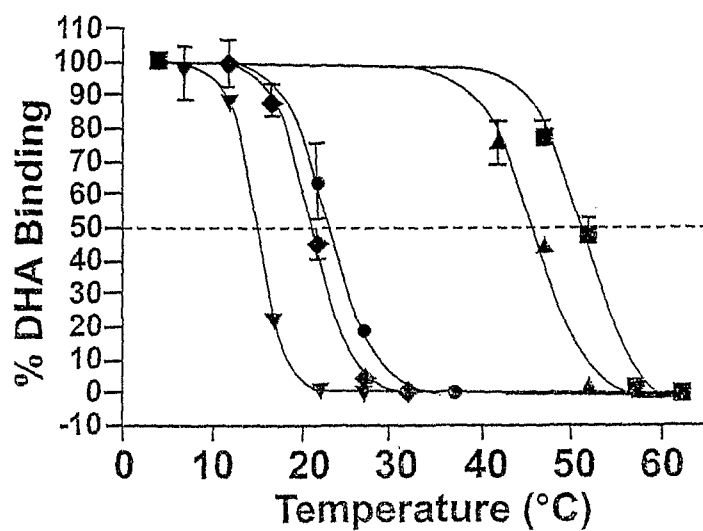
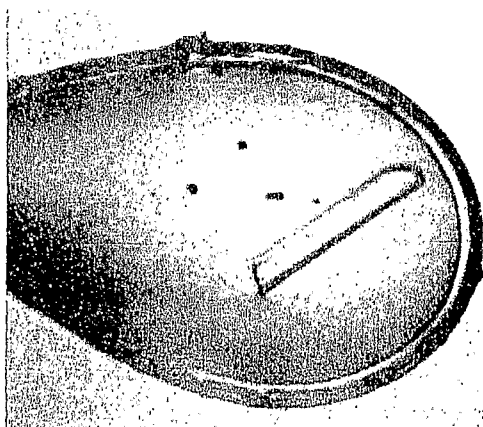


Fig. 4

*Fig. 5a**Fig. 5b**Fig. 5c*

*Fig. 6a**Fig. 6b**Fig. 6c*

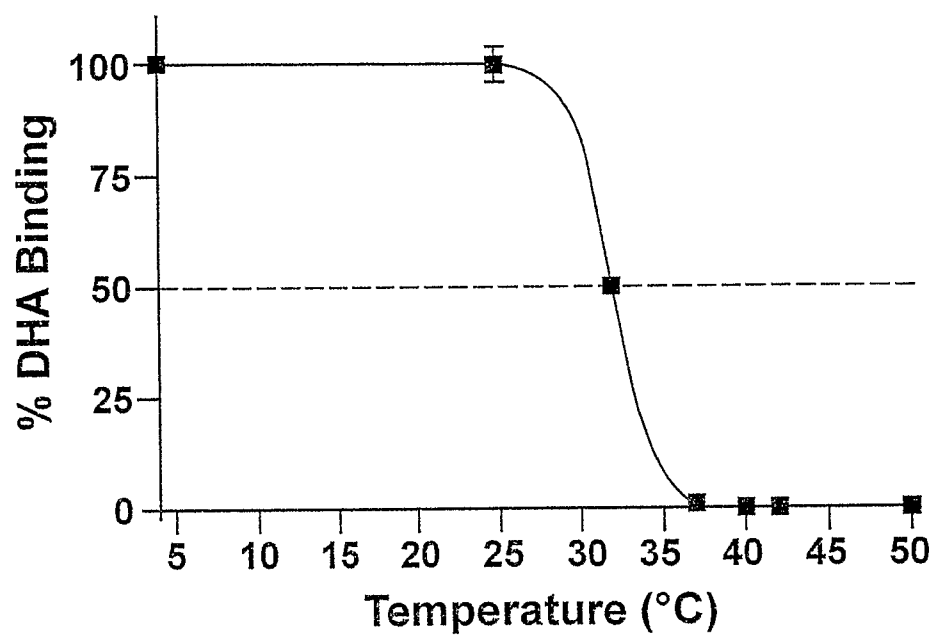
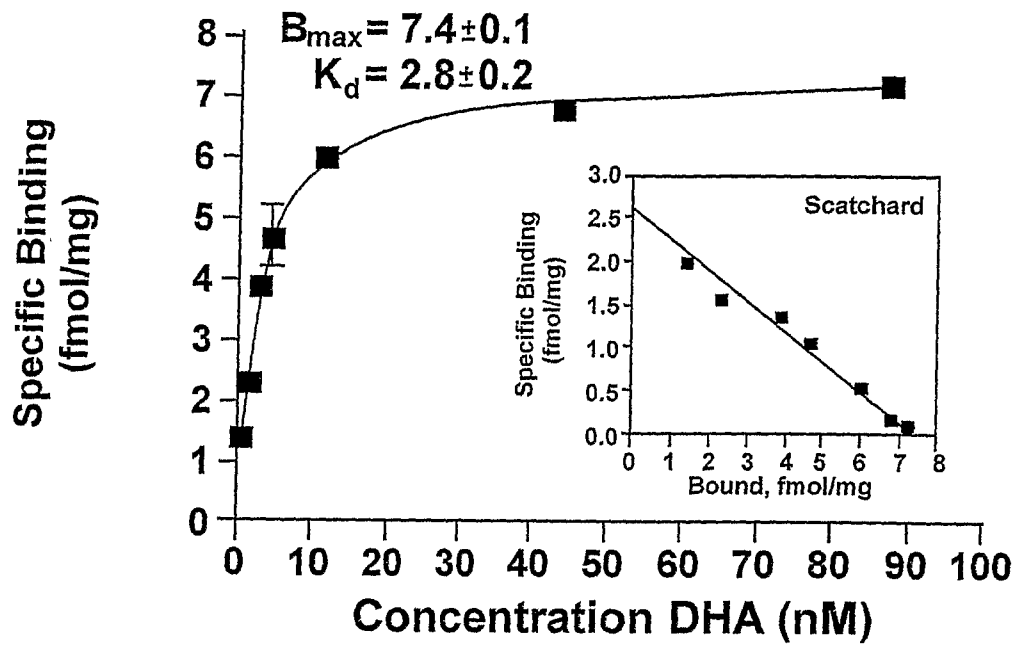
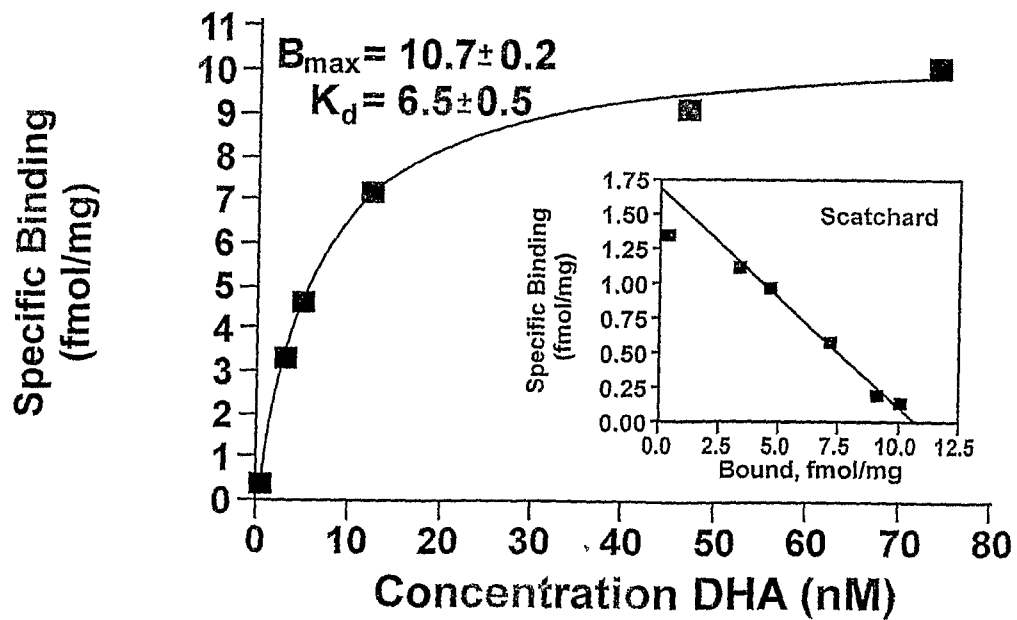


Fig. 7

*Fig. 8a**Fig. 8b*

Alignment of the turkey β -adrenergic receptor with human $\beta 1$, $\beta 2$ and $\beta 3$

adrb1_melga	1	MGDGWLPPDCGPHNRSGGGGATAAPTGSR-----	29
adrb1_human	1	MGAGVLVLGASE-----PGNLSSAAPLPDGAATAARLLVPASPPASLLP	44
adrb2_human	1	MGQ-----PGNGSAFLLAPNRSHAPD-----	21
adrb3_human	1	MAPW-----PHENSSLAPWPDLPPLAP-----N	23
		* *	
adrb1_melga	30	-QVSAEL-LSQQWEAGMSLLMALVLLI ^{bc} VAGNVLVIAAI ^a ETQRLQTLTN	77
adrb1_human	45	PASESPEPLSQQWTAGMGLLMALIVLLI ^{bc} VAGNVLVIVIAIAKTPRLQTLTN	94
adrb2_human	22	-HDVTQQ-RDEVWVVGMI ^{bc} MSLIVLAIVFGNVLVITAIKFERLQTVTN	69
adrb3_human	24	TANTSGLPGVPWEAALAGALLA ^{bc} VLATVGGNLLVIVIAIAWT ^a PRLQTM ^{bc} TN	73
		bc	
adrb1_melga	78	LFITSLACADLV ^{bc} GLLVVPF ^{bc} EATLVVRGTWLGWSFLCECWTS ^{bc} LDVLCVTA	127
adrb1_human	95	LFIMSLASADLV ^{bc} MGLLVVPF ^{bc} GATIVVWGRWEYGSFFCELWTS ^{bc} VDVLCVTA	144
adrb2_human	70	YFITSLACADLV ^{bc} MGLAVVPF ^{bc} GAHILMKMWTFGNFWCEFWTS ^{bc} SIDVLCVTA	119
adrb3_human	74	VFVTS ^{bc} LAAADLV ^{bc} MGLLVPPA ^{bc} ATLALT ^{bc} GHWPLGATGCELWTS ^{bc} VDVLCVTA	123
		d e	
adrb1_melga	128	SIETLCVIAIDRYLAITS ^d PF ^e RYQSLMTRARAK ^{bc} VIICTVW ^{bc} AI ^{bc} SALVSFLPI	177
adrb1_human	145	SIETLCVIALDRYLAITS ^d PF ^e RYQSL ^{bc} LTRARARGLVCTVW ^{bc} AI ^{bc} SALVSFLPI	194
adrb2_human	120	SIETLCVIAVDRYFAITS ^d PF ^e KYQSL ^{bc} LTKNKARVIILMVWIVSGLTSFLPI	169
adrb3_human	124	SIETLCALAVDRYLA ^d VTNPLRYGALVTKRCARTAVVLVWV ^{bc} SAAVSFAP ^{bc} I	173
		f	
adrb1_melga	178	MMHWWRDEDP-QALKCY ^{bc} QDPGCCDFVTNRAYAIASSII ^{bc} SFYIPL ^{bc} IIMIFV	226
adrb1_human	195	LMHWWRAESD-EARRCYNDPKCCDFVTNRAYAIASSVVSFYVPLCIMA ^{bc} FV	243
adrb2_human	170	QMHWYRATHQ-EAINCYANETCCDFFTNQAYAIASSIVSFYVPLVIMV ^{bc} FV	218
adrb3_human	174	MSQWWRVGADAEAR ^{bc} CHSNPRCCAFASNMPYVLLSSSVSFYLP ^{bc} LLV ^{bc} MLFV	223
		g	
adrb1_melga	227	YLRVYRE ^{bc} AK ^{bc} EQIRKIDRCEGRFYGSQ ^{bc} E-----QPQ--PPPLPQHQPILG-	268
adrb1_human	244	YLRVFREAQKQVKKIDSCERRFLGGPARPPSPSPSPVPAPAPPPGPP ^{bc} PPA	293
adrb2_human	219	YSRVFQ ^{bc} EAKRQLQKIDKSEGRFHVQ ^{bc} N-----LSQVEQDGR-	253
adrb3_human	224	YARVFVATRQLRLRLRGELGRFPPEES-PPAPSRSLAPAPVGT ^{bc} CAPPE--	270
		h	
adrb1_melga	269	-----NGRASKRKTSRV ^{bc} MREHKALKKTLGIIMGVFTLCWL ^{bc} PFFLV	309
adrb1_human	294	AAAATAPLANGRAGKRRPSRLVALREQKALKKTLGIIMGVFTLCWL ^{bc} PFFLA	343
adrb2_human	254	-----TGHLRR--SSKFCLKEHKALKKTLGIIMGTFTLCWL ^{bc} PFFIV	292
adrb3_human	271	-----GVPACGRRPARLLPLREHRALCTGLIMGTFTLCWL ^{bc} PFFLA	311
		i j k l	
adrb1_melga	310	NIVNVFN ⁱ NR-DLV ^j PDWLFV ^k FNWLGY ^{bc} ANSA ^{bc} NP ^{bc} IIYCRSPDFRKA ^{bc} FKRLLC	358
adrb1_human	344	NVVKAFHR-ELVPDR ⁱ LFVFF ^j NWLGY ^{bc} ANSA ^{bc} NP ^{bc} IIYCRSPDFRKA ^{bc} FQRLLC	392
adrb2_human	293	NIVHVIQD-NLIRKEVYILLNWIGYVNSGFNPLIYCRSPDFRIAFQ ^{bc} ELLC	341
adrb3_human	312	NVLRALGGPSLVPGPAFLANWLGY ^{bc} ANSA ^{bc} NP ^{bc} IIYCRSPDFRS ^{bc} AFRLLC	361

Fig. 9 (Page 1 of 2)

```

adrb1_melga 359 FPRKADRRRLHAGGQPAPLPGGFISTLGSPHEHSPGGTWSDCNGGTRGGSES 408
adrb1_human 393 CARRAARRRHATHGDRPR-----ASGCLARPGPPPS 423
adrb2_human 342 LRRSSLKAYGNG-----YS-----SNGNTGEQSG--- 365
adrb3_human 362 RCGRRLP-----PEP-----CAAARPALFPS 382

adrb1_melga 409 SLEERHSKTSRSESKMEREKNILATTRFYCTFLGNGDKAVFCTVLRIVKL 458
adrb1_human 424 PGAASDDDD-----DDVVGATPPARLLEPWAGCNGGAAADSDSSLDE 465
adrb2_human 366 ----YHVEQ-----EKENK-----LLCEDLPGTEDFVGHQGTVPSPDN 398
adrb3_human 383 GVPAARS-----SPAQPRLCQRLDGASWGV 408

adrb1_melga 459 FEDATCTCPHTHKLKMKWRFKQHQ 483
adrb1_human 466 PCRPGFAESKV 477
adrb2_human 399 IDSQGRNCSTNDSLL 413
adrb3_human 409 408

```

SEE BELOW FOR KEY



Position of mutations in m23
 Position of other thermostabilising mutations
 Position of transmembrane domains
 Position of helix 8

Where other amino acid substitutions gave significant thermostability, the position is labelled with a lower case letter and the mutations are listed below in order of decreasing thermostability.

- a. R68S
- b. V89L
- c. M90V, A
- d. I129V, A, G
- e. S151E, Q
- f. L221V, I
- g. R229Q, A
- h. A282L, V, Q
- i. D322A, P
- j. F327A, G, M, V
- k. A334L, S, I
- l. F338M, A, V, I

Fig. 9 (Page 2 of 2)

Alignment of human adenosine receptors

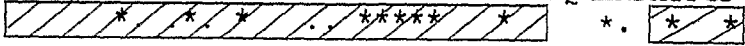
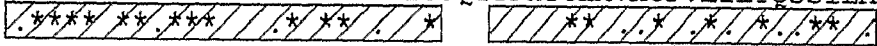
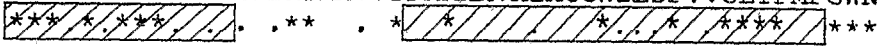
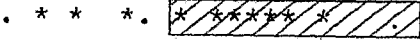
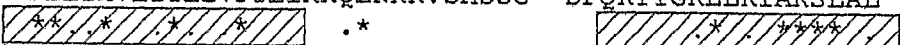
AA2AR_human	1	MPIMGSSVYITVELAIAVLAILGNVLVCWAVWLNSNLQNVVTNYF	44
AA2BR_human	1	MLLETQDALYVALELVIAALSVAAGNVLCVCAAVGTANTLQTPNTNYF	45
AA3R_human	1	MPNNSTALSLANVTYITMEIFIGLCAIVGNVLVICVVKLNPSLQTTTFYF	50
AA1R_human	1	MP---PSISAFQAAYIGIEVLIALVSVPGNVLVWAVKVNQALRDATFCF	47
<div style="text-align: center;">  </div>			
AA2AR_human	45	VVSLAAADIAVGVLAIPFAITISTGFCAACHGCLFIACFVLVLQSSIFS	94
AA2BR_human	46	LVSLAAADVAVGLFAIPFAITISLGFCTDFYGCFLACFVLVLQSSIFS	95
AA3R_human	51	IVSLALADIAVGVLVMP LAIVVSLGITIHFYSCLFMTCLLLIFTHASIMS	100
AA1R_human	48	IVSLAVADVAVGALVIPLAILINIGPQTYFHTCLMVACPVILITQSSILA	97
<div style="text-align: center;">  </div>			
AA2AR_human	95	LLAIAIDRYIAIRIPLRYNGLVTGTRAKGIIAICWVLSFAIGLTPMLGWN	144
AA2BR_human	96	LLAVAVDRYLAICVPLRYKSLVTGTRARGVIAVLWVLAFGIGLTPFLGWN	145
AA3R_human	101	LLAIAVDRYLVRVCLTVRYKRVTTTHRIWLALGLCWLVSFLVGLTPMFGWN	150
AA1R_human	98	LLAIAVDRYLVRKIPLRYKMVVTPRRAAVAIAGCWILSFVVGLTPMFGWN	147
<div style="text-align: center;">  </div>			
AA2AR_human	145	-----NCGQPKEGKNHSQGC GEGQVACLFEDVVP MNM VYFNFFACVL	187
AA2BR_human	146	SKDSATNNCTEPWDGTTNESCC---LVKCLFENVVPMSYMVYFNFFGCVL	192
AA3R_human	151	-----MKLTSEYHRNVT-----FLSCQFVSVMRMDYMVYFSFLTWIF	187
AA1R_human	148	-----NLSAVERAWAANGSMGEPVIKCEFEKVISMEYMVYFNFFVWVL	190
<div style="text-align: center;">  </div>			
AA2AR_human	188	VPLLLMLGVYLRIFLAARRQLKQMESQPLPGERARSTLQKEVHAAKSLAI	237
AA2BR_human	193	PPLLIMLVIIYIKIFLVACRQLQRTELMDHS----RTTLQREIHAAKSLAM	238
AA3R_human	188	IPLVVMCAIYLDIFYIIRNKLNLNSNSK---ETGAFYGREFKTAKSLFL	234
AA1R_human	191	PPLLLMVLIIYLEVFYLIRKQLNKKVSASSG--DPQKYYGKELKIAKSLAL	238
<div style="text-align: center;">  </div>			

Fig. 10 (Page 1 of 2)

A Mutations determined by agonist binding
 Mutations determined by antagonist binding
 Position of transmembrane domains
 Position of helix 8

12/54

Alignment of neurotensin receptors

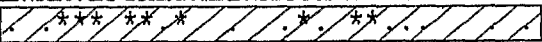
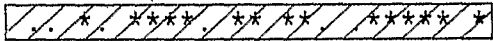
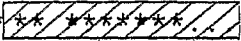
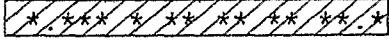
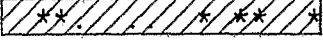
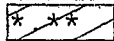
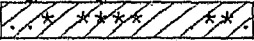
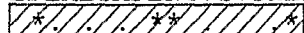
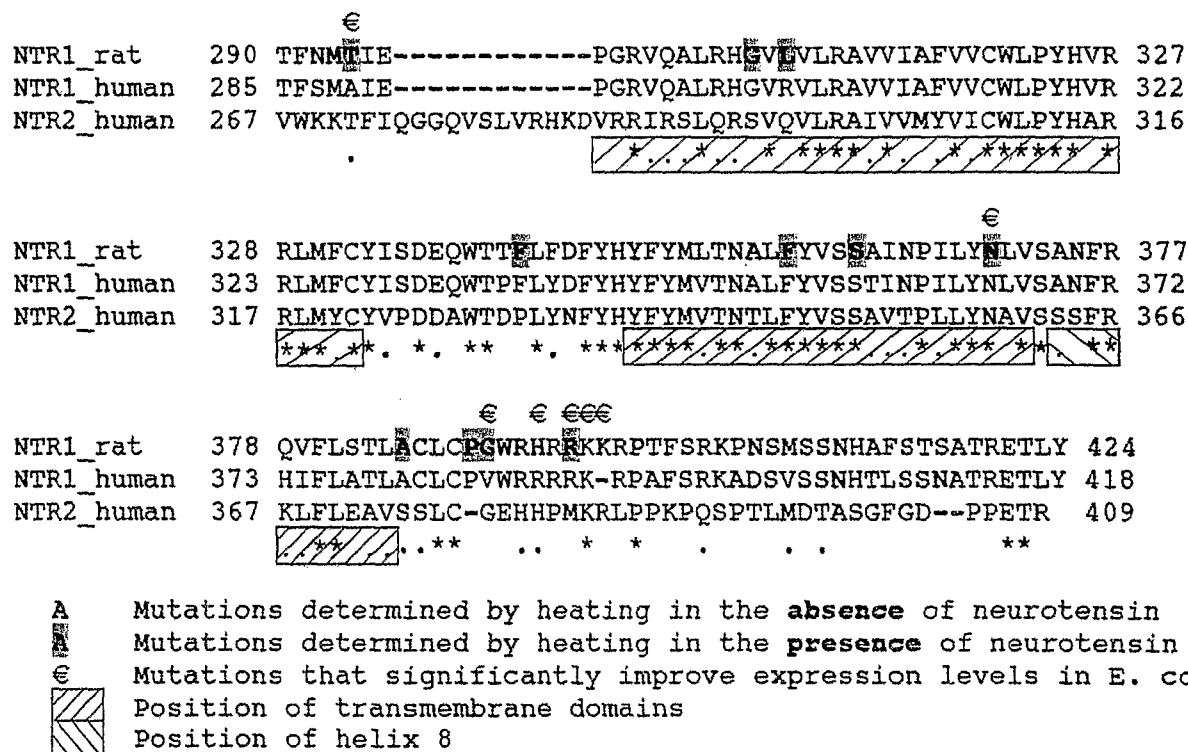
NTR1_rat	1	MHLNSSVPQGTTPGEPDAQPFSGPQSEMEATFLALSLNNGSGNTSES	50
NTR1_human	1	MRLNSSAP-GTPGTPAADPFQRAQAGLEEALLAPGFGNASGNASERV	49
NTR2_human	1	METSSP--RPPRPSSNPG-----LS	18
		. . * * * . . *	
			€
NTR1_rat	51	PNSDLDVNTDIYSKVLVT ¹⁰⁰ AIYL ¹⁰⁰ ALFVVGVTVGNSVT ¹⁰⁰ FTL ¹⁰⁰ ARKKSLSQSLQS	100
NTR1_human	50	PSSELDVNTDIYSKVLVTAVYLALFVVGVTVGNTVTFTLARKKSLSQSLQS	99
NTR2_human	19	LDARLGVDTRLWAKVLTALYALI ¹⁰⁰ WALGAAGNALS ¹⁰⁰ VHVVLKAR--AGRAG	66
		. * * * *  .	
		a	
NTR1_rat	101	TV ¹⁰⁰ HYHLGSLALSDDLILLAMPVELYNFIWVHH ¹⁰⁰ PWAFGDAGCRGYYFLRD	150
NTR1_human	100	TVHYHLGSLALSDDLTLAMPVELYNFIWVHH ¹⁰⁰ PWAFGDAGCRGYYFLRD	149
NTR2_human	67	RLRHHVLSLALAGLLLLLVGV ¹⁰⁰ PVELYSFVWFHYPVWFGDLGCRGYYFVHE	116
		 . * * * * 	
NTR1_rat	151	ACTYATALNVASLS ¹⁰⁰ VERYLAICH ¹⁰⁰ PFKA ¹⁰⁰ KTLM ¹⁰⁰ SR ¹⁰⁰ RTKKFISAIWLASALL	200
NTR1_human	150	ACTYATALNVASLSVERYLAICH ¹⁰⁰ PFKA ¹⁰⁰ KTLM ¹⁰⁰ SR ¹⁰⁰ RTKKFISAIWLASALL	199
NTR2_human	117	LCAYATVLSVAGLSAERCLAVCOPLRARSLLTPR ¹⁰⁰ TRWLVALSWAASLGL	166
		 . * 	
NTR1_rat	201	AIPMLFTMGLQNR--SGDG-THPGGLVCTPIVD ¹⁰⁰ TATVKVVIQVNTFMSFL	247
NTR1_human	200	AVPMLFTMGEQNR--SADG-QHAGGLVCTPTIHTATVKVVIQVNTFMSFI	246
NTR2_human	167	ALPMAVIMGQKHELETADGEPEPASRVCTVLVSR ¹⁰⁰ TALOVFIQVNVLVSEV	216
		 ** .. . ** *** . . 	
			€
NTR1_rat	248	FPMLVISILNTV ¹⁰⁰ LANKLTVMVHQA ¹⁰⁰ AEQ---G-----RV ¹⁰⁰ CTV ¹⁰⁰ GTHNGLEHS	289
NTR1_human	247	FPMVVISVLNTIIANKLTVMVRQA ¹⁰⁰ AEQ---G-----QVCTVGG---EHS	284
NTR2_human	217	LPLALTAFLNGVT ¹⁰⁰ VS ¹⁰⁰ HLLALCSQVPSTSTPGSSTPSRLELLSEEGLLSFI	266
		 . * * * * . * . . .	

Fig. 11 (Page 1 of 2)



(a) H103: Thermostability obtained with A, N, S, V, L, M
 Only H103N and H103S gave wt levels of expression

Fig. 11 (Page 2 of 2)

Flow chart showing the two different assay formats of ligand(+) and ligand(-) used to determine receptor thermostability

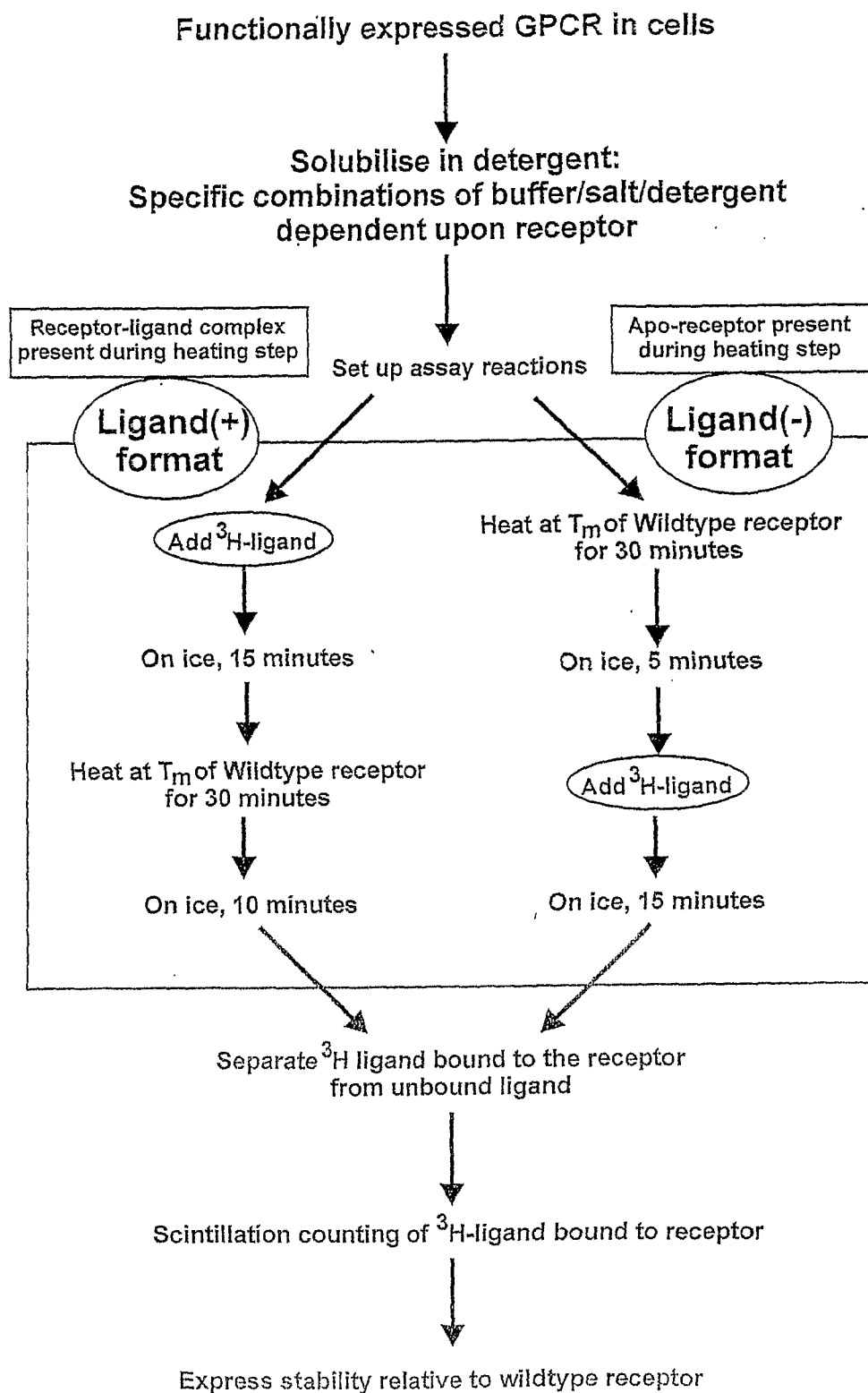
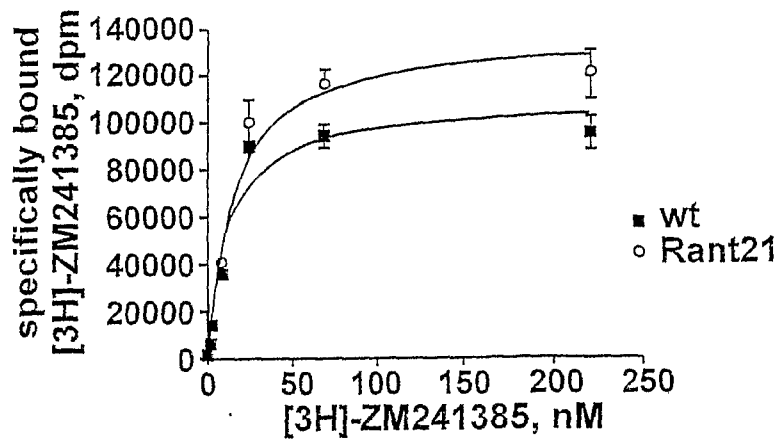
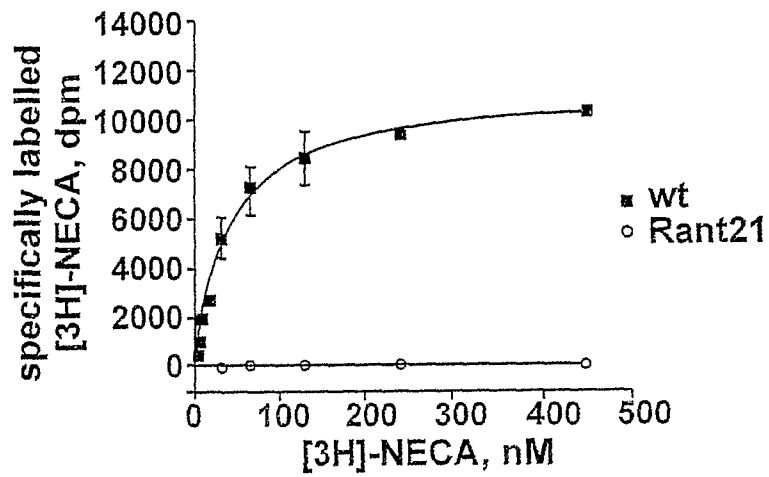
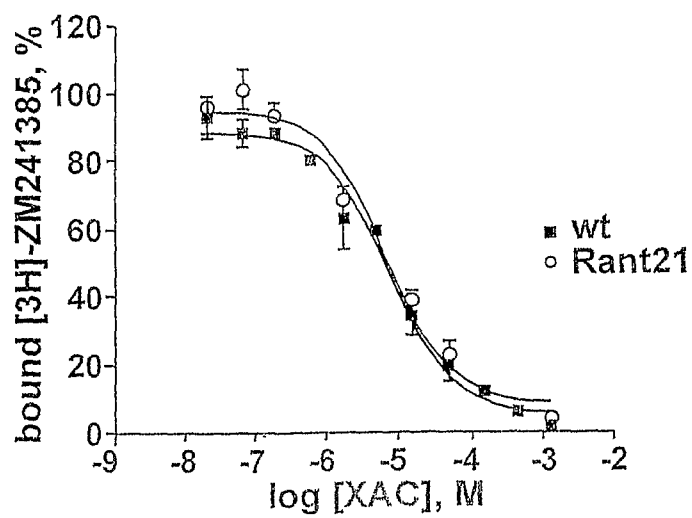
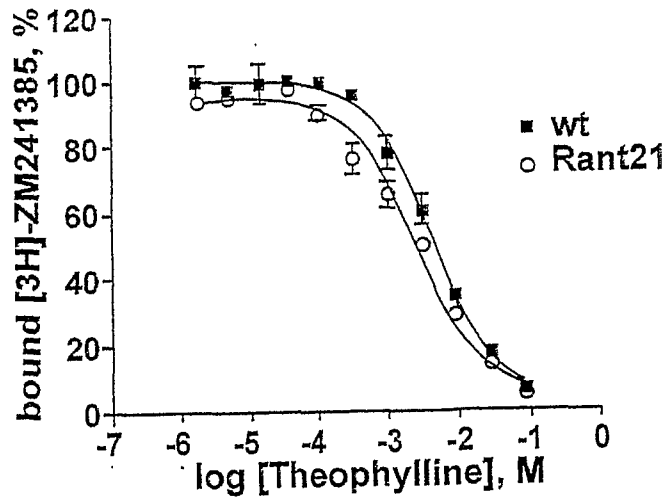
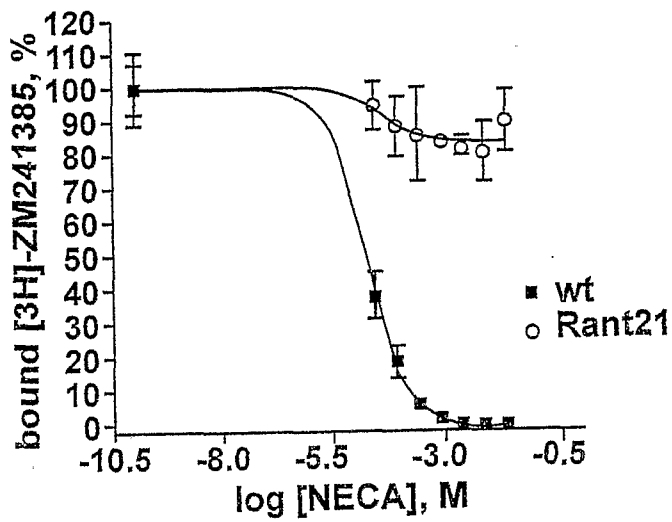
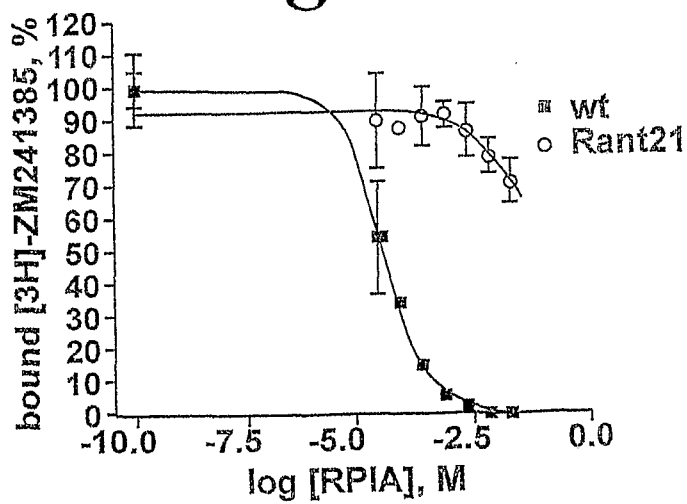
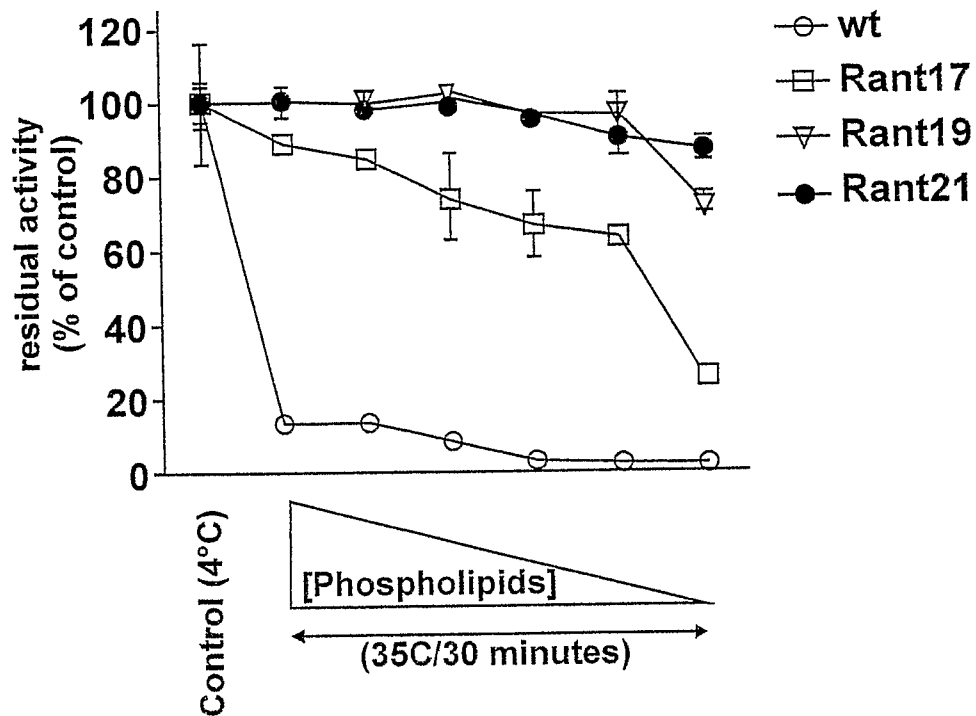
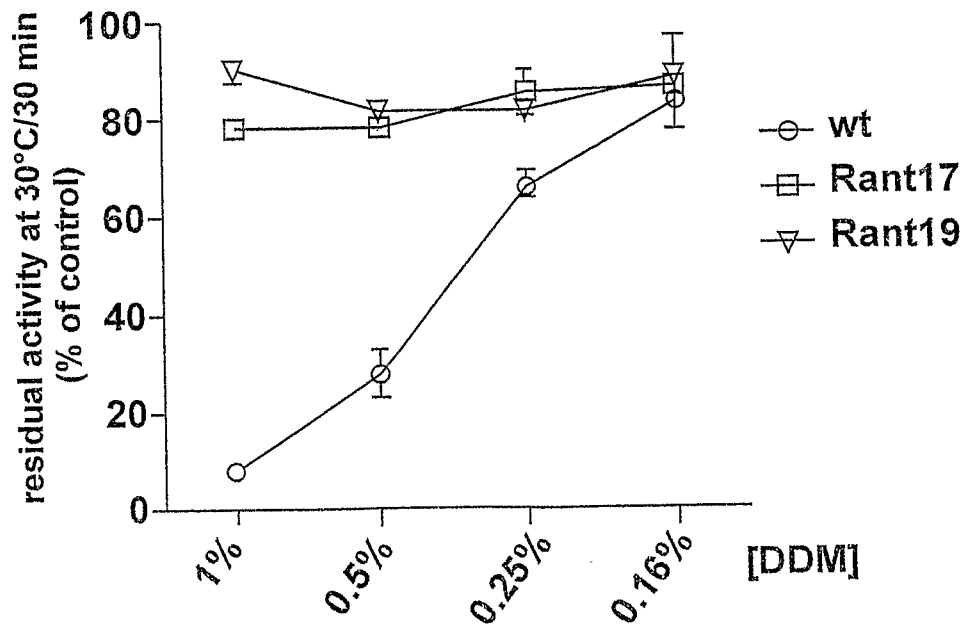
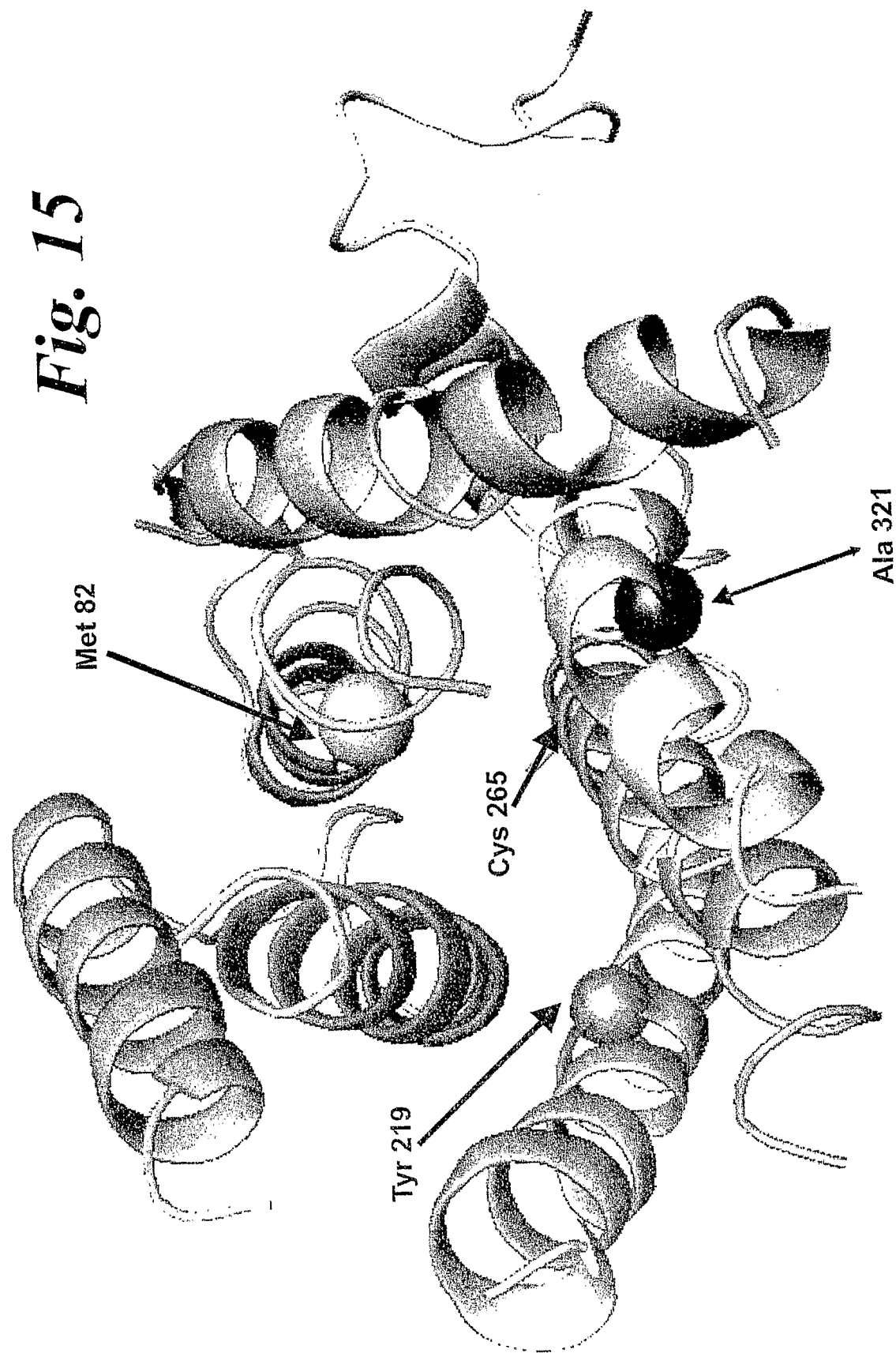


Fig. 12

*Fig. 13A**Fig. 13B**Fig. 13C*

*Fig. 13D**Fig. 13E**Fig. 13F*

*Fig. 14A**Fig. 14B*



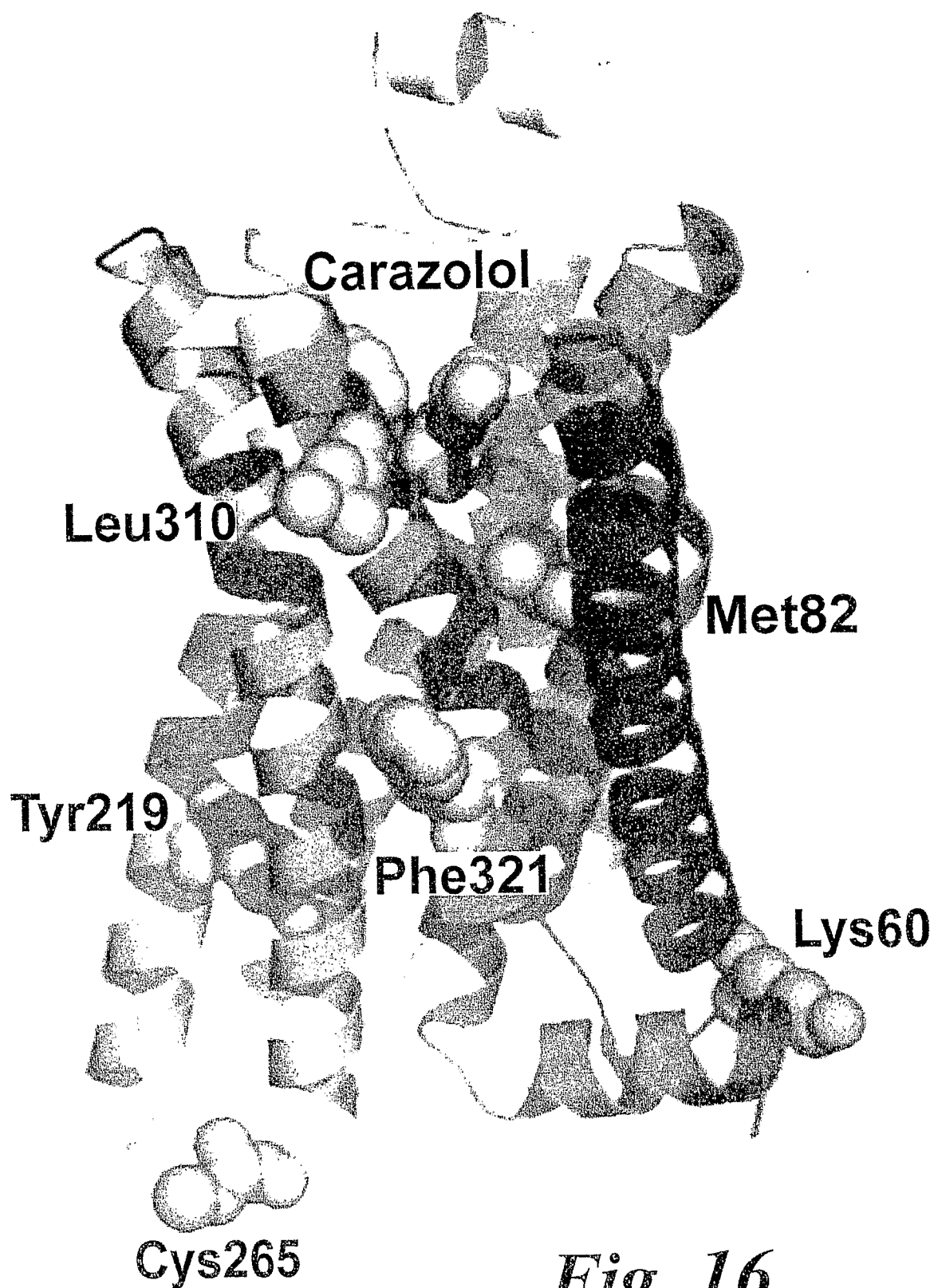


Fig. 16

adrB2_human	237	---	GRHH	---	VQMSQVEG	25
NTR1_rat	274	---	GRVCH	---	VGTHNGIHRSTNNHLEEC	29
muscm1_human	234	---	CCCHSSSRHQP	---	GAAGSPETPPRCRCGRAPPI	26
adrB1_melga	245	---	GAFLGSOE	---	OFFEFCOR	26
ADORA2A	215	---	---	---	---	21
adrB2_human	250	---	---	---	---	25
NTR1_rat	293	---	---	---	---	29
muscm1_human	269	---	WNTTETD	---	PC	30
adrB1_melga	265	---	---	---	SEVA	26
ADORA2A	215	---	---	---	---	21
adrB2_human	250	---	---	---	---	25
NTR1_rat	298	---	---	---	---	29
muscm1_human	302	---	MDPFAQAPTKOE	---	RSPPATVRRPKK	33
adrB1_melga	265	---	---	---	---	26
ADORA2A	215	---	---	---	---	21
adrB2_human	250	---	---	---	---	27
NTR1_rat	298	---	---	---	---	31
muscm1_human	332	---	CHPACGCKRERGRSQAARKTE	---	RVQALRRHEVLELRALV	37
adrB1_melga	265	---	---	---	---	29
ADORA2A	215	---	---	---	---	23

Fig. 17 (Page 2 of 3)

adrB2_human	280	CHRMNIGQPHETPTVNTVHTVETQD	---	WLLTRRVALELLNMRKAVANISCPNPTREACRS	32
NTR1_rat	315	LEEDVNGRDEGEAPRNTGATGCEQNNM	---	ELRDEEAFATWMLDWALEEEENETRN	37
musCM1_human	372	PAFTLIMMPPVYRQITGSHPPQPR	---	CVPRNLEHLLGRMMBCVANSULNEMQSLATG	42
adrB1_melga	297	GVHLLMMPVYRQITGSHPPQPR	---	ELRDEEAFATWMLDWALEEEENETRN	34
ADORA2A	240	GVHLLMMPVYRQITGSHPPQPR	---	ELRDEEAFATWMLDWALEEEENETRN	29
adrB2_human	329	EDDRTATQELLICRRSS	---	ELRDEEAFATWMLDWALEEEENETRN	37
NTR1_rat	373	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	41
musCM1_human	422	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	46
adrB1_melga	346	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	40
ADORA2A	292	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	35
adrB2_human	372	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	41
NTR1_rat	419	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	42
musCM1_human	460	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	46
adrB1_melga	406	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	46
ADORA2A	352	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	41
adrB2_human	413	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	413
NTR1_rat	424	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	424
musCM1_human	460	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	460
adrB1_melga	466	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	483
ADORA2A	412	ELRDEEAFATWMLDWALEEEENETRN	---	ELRDEEAFATWMLDWALEEEENETRN	412

Fig. 17 (Page 3 of 3)

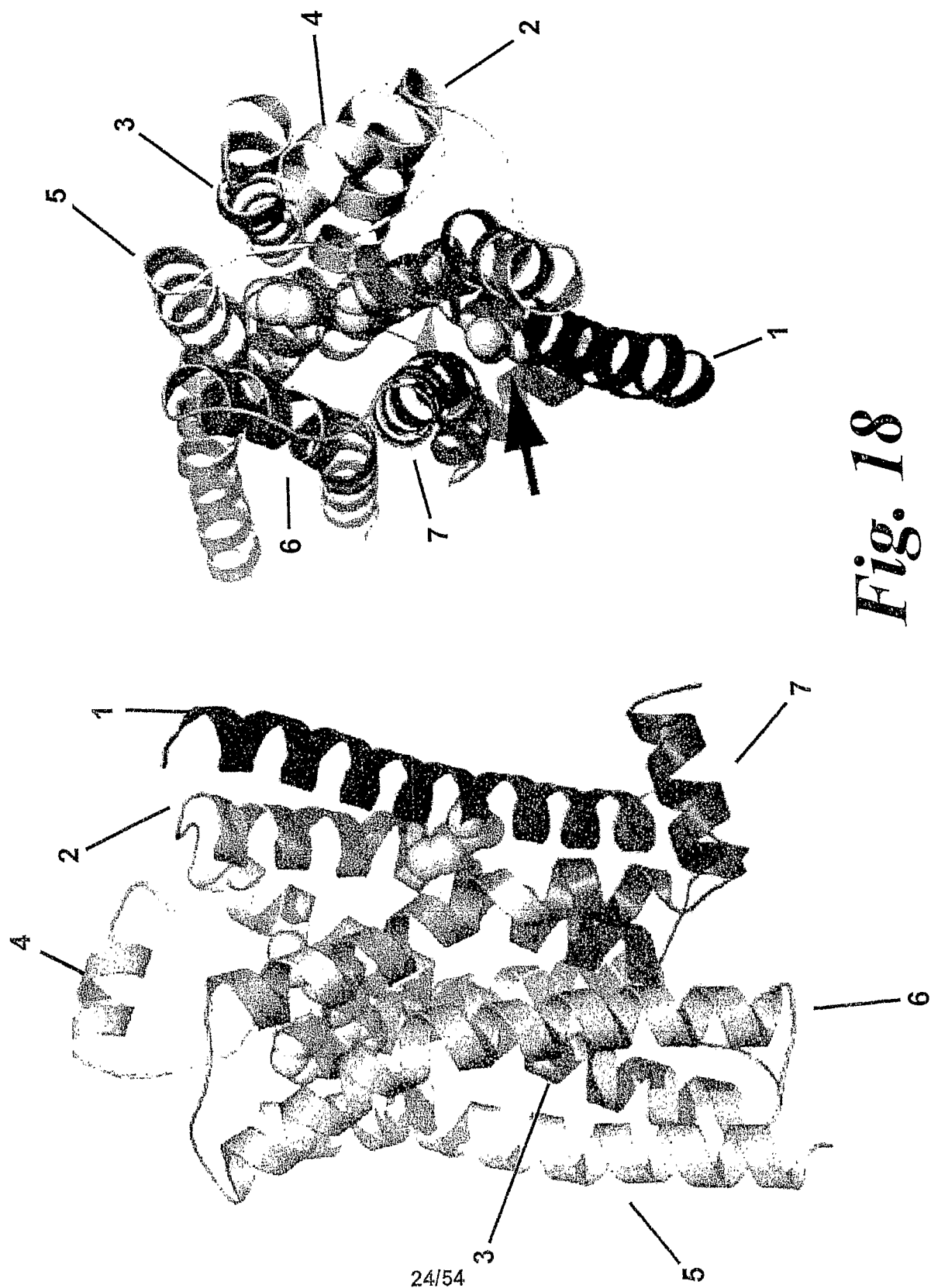


Fig. 18

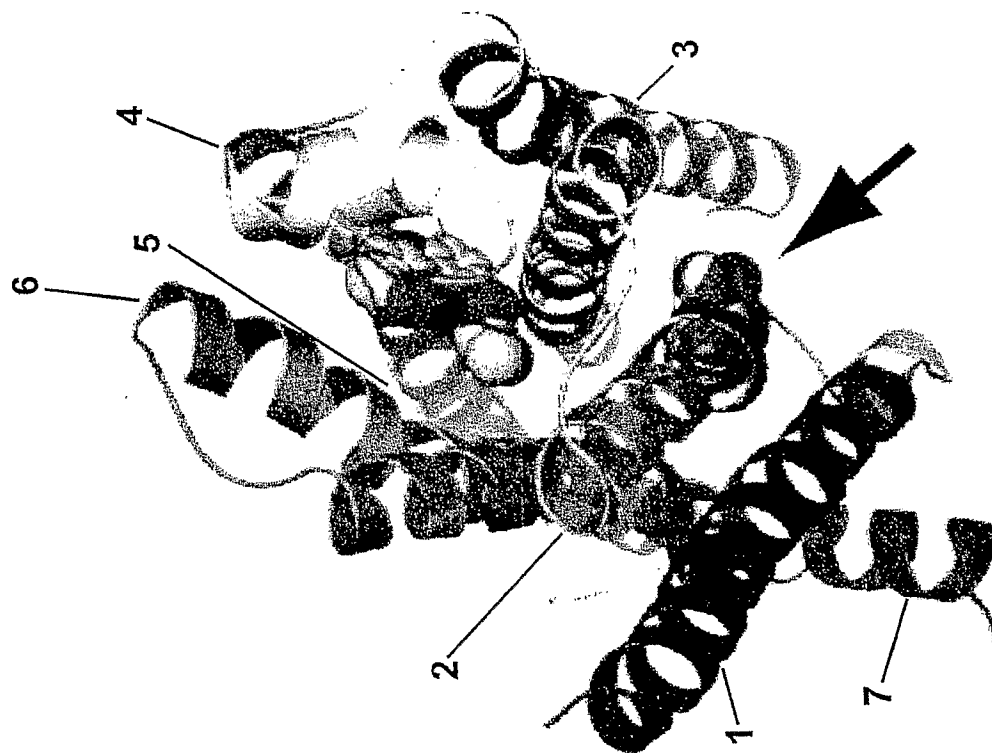
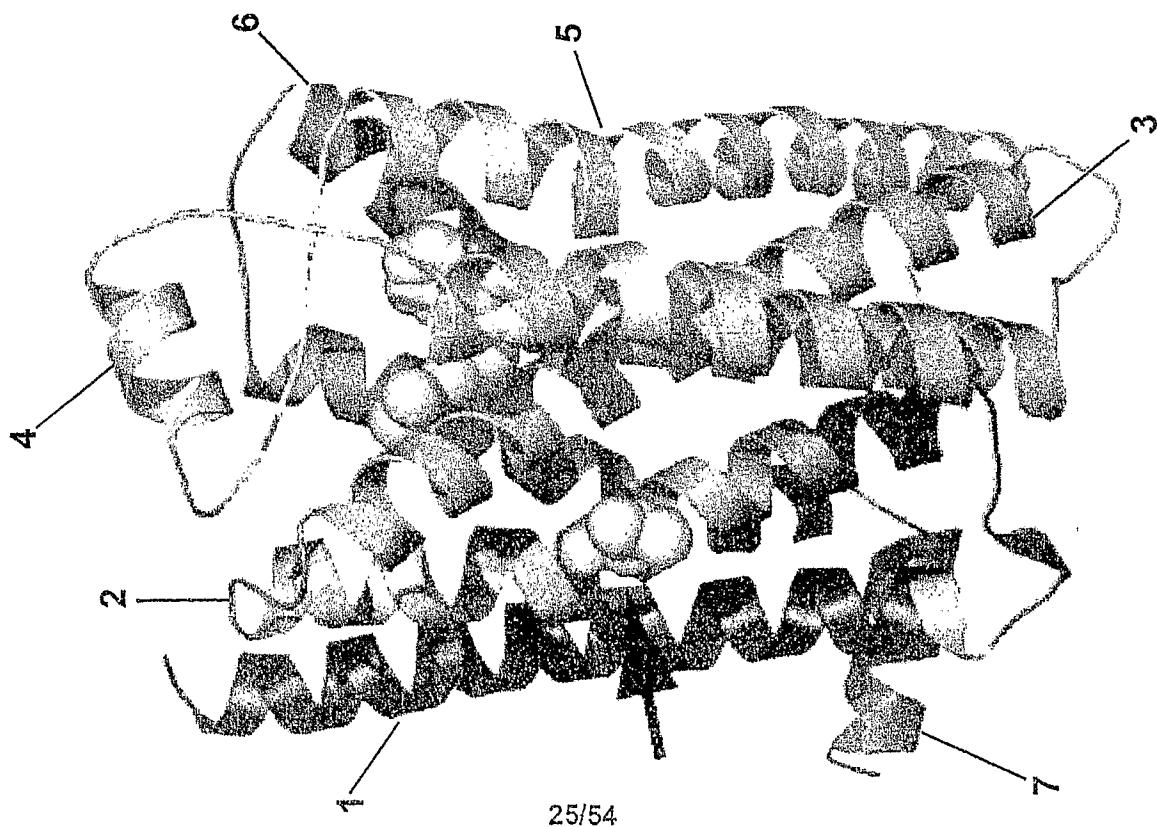


Fig. 19



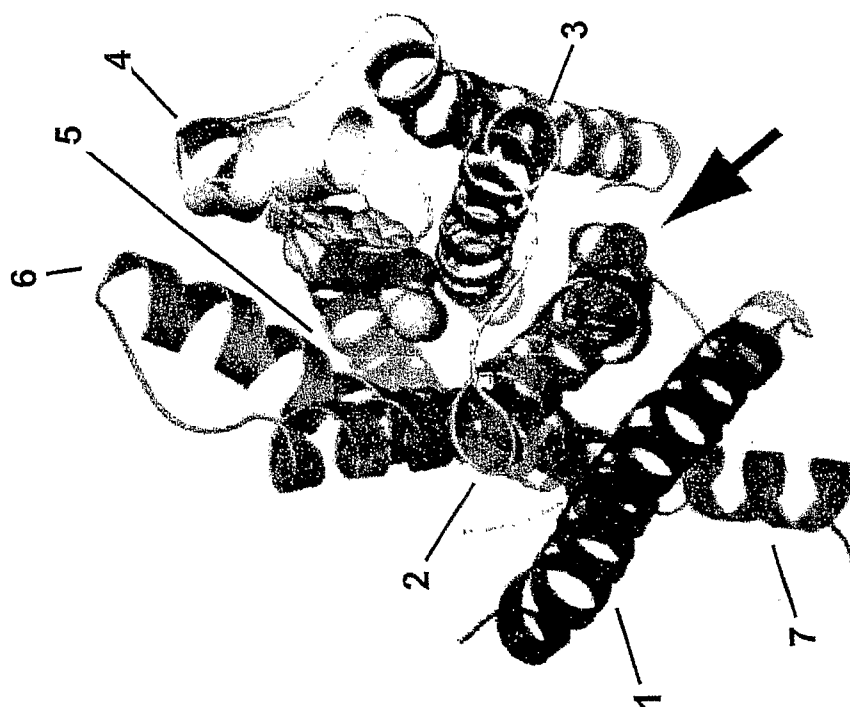
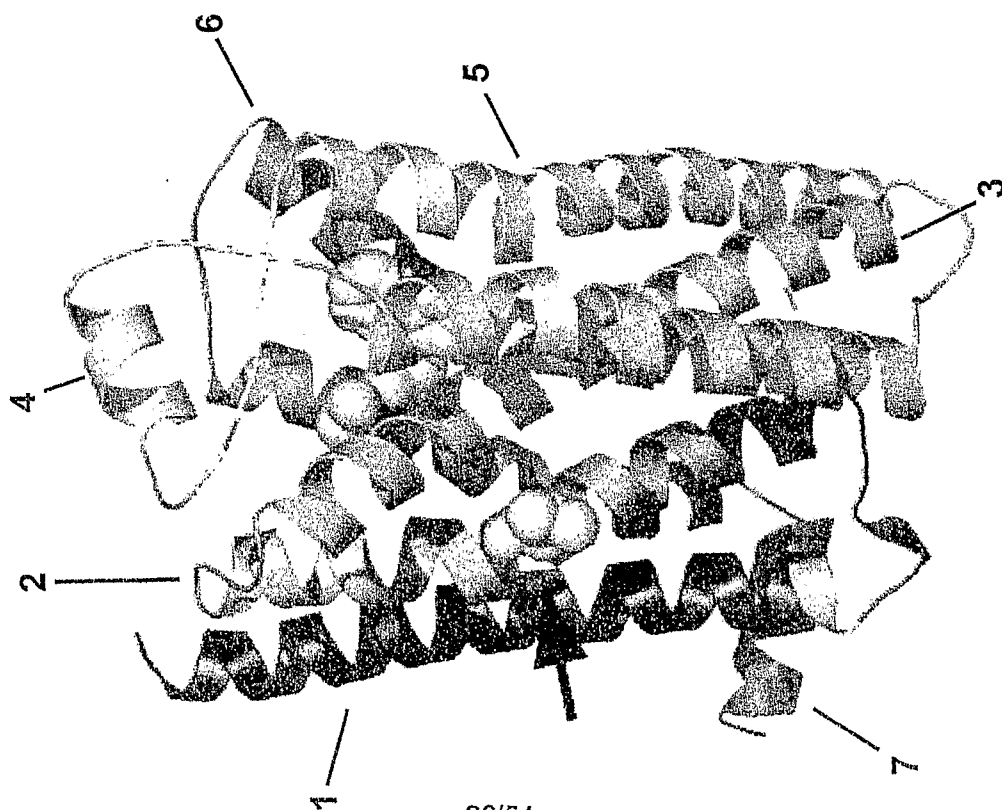


Fig. 20



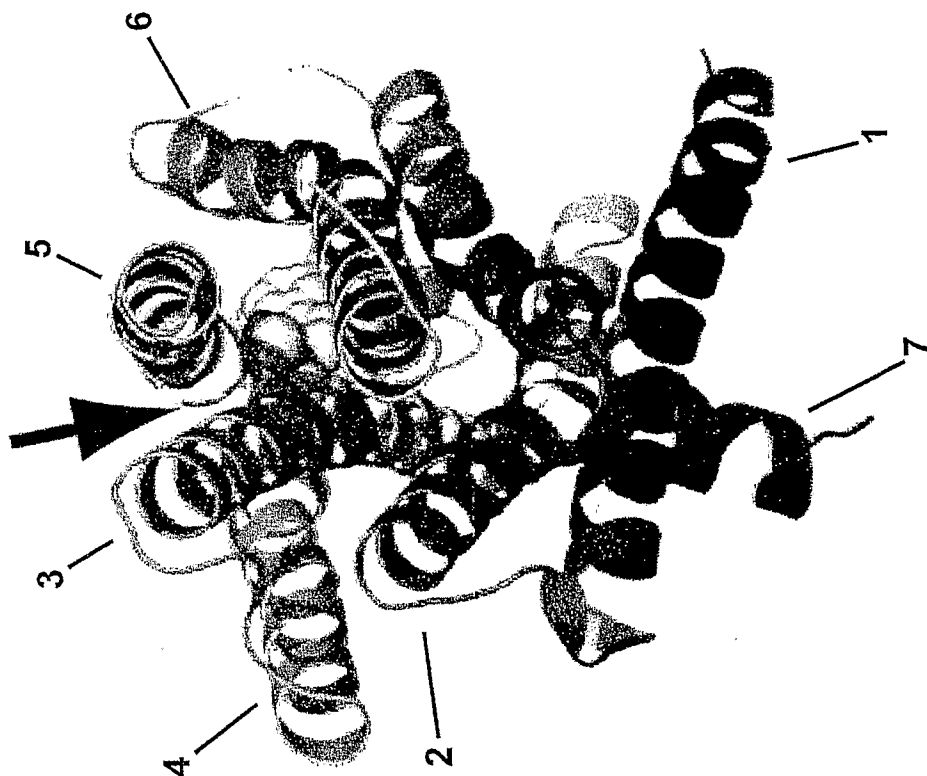
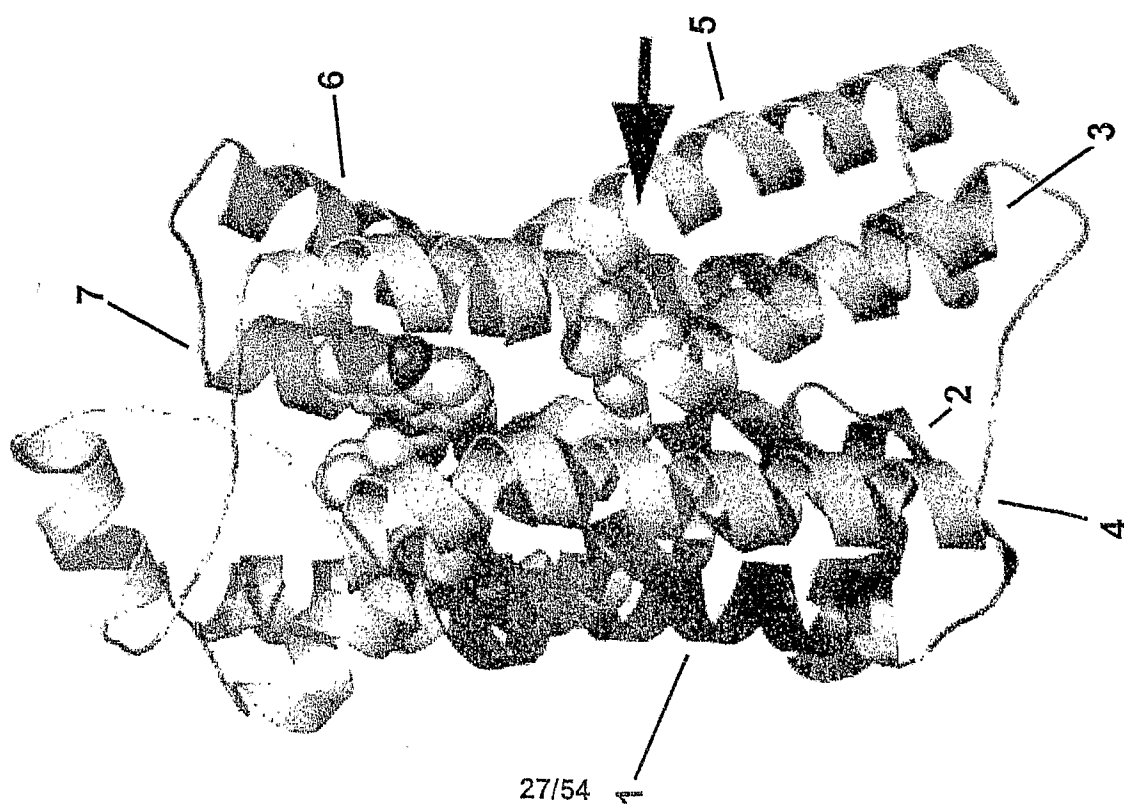


Fig. 21



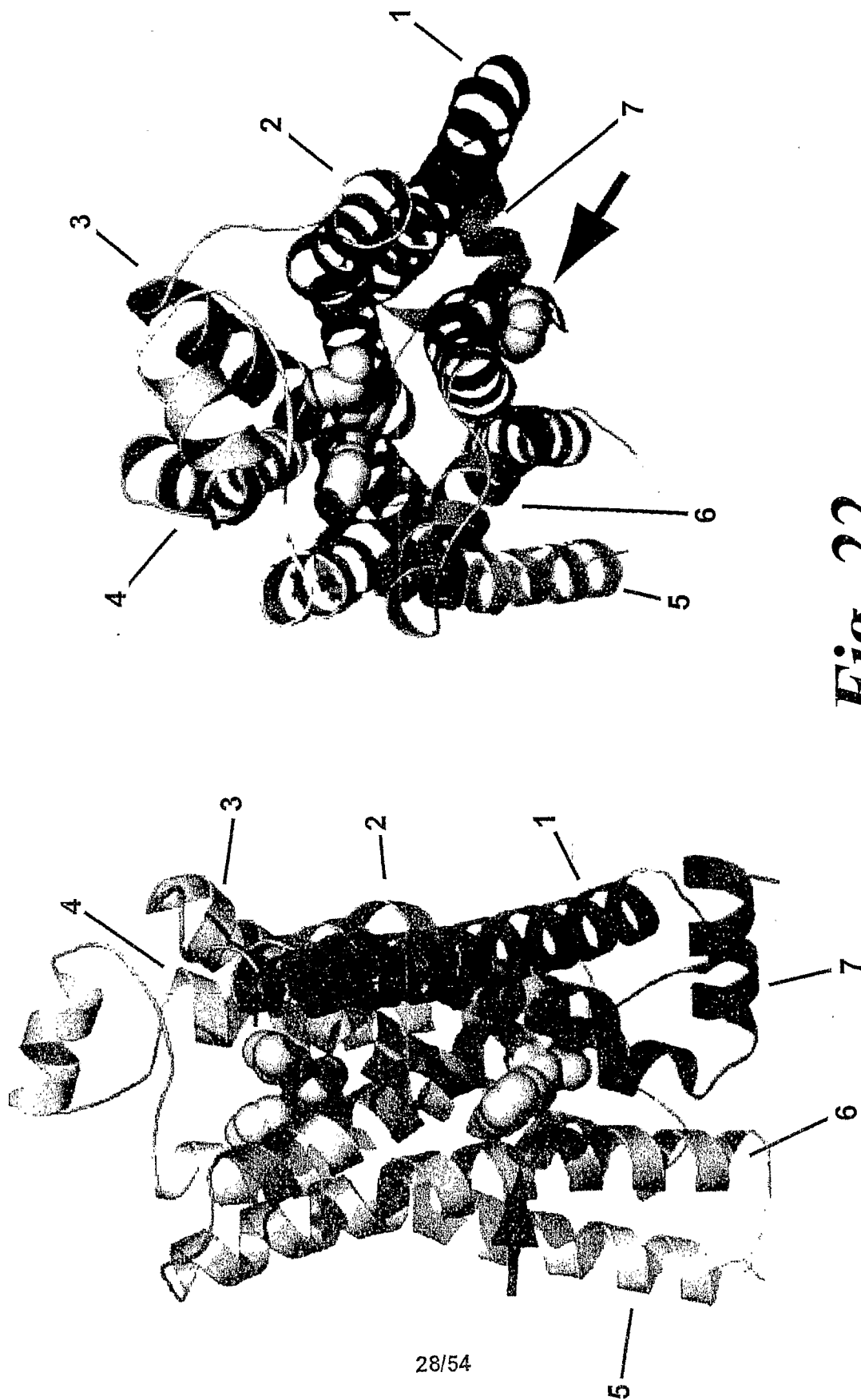
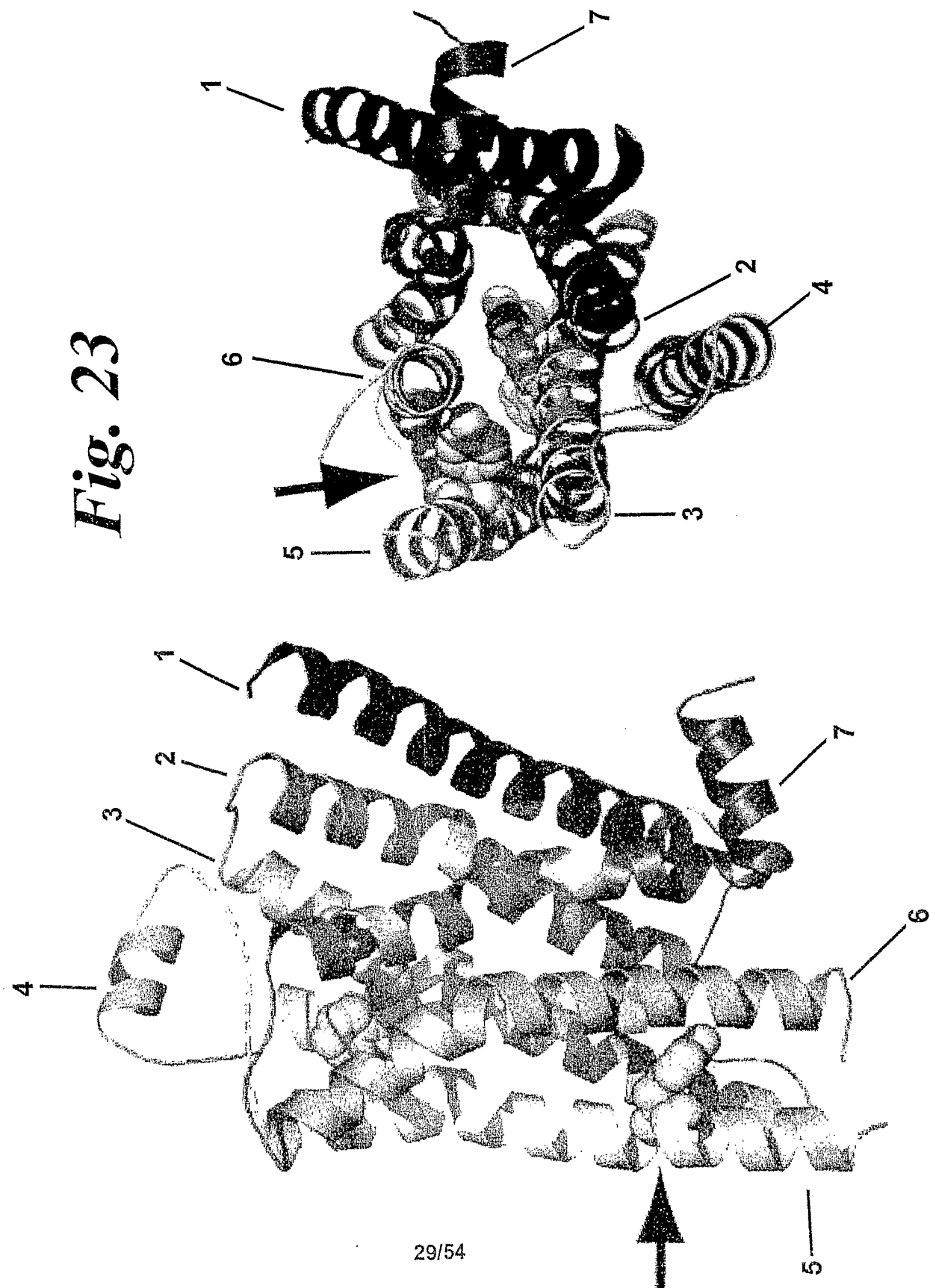


Fig. 22

Fig. 23



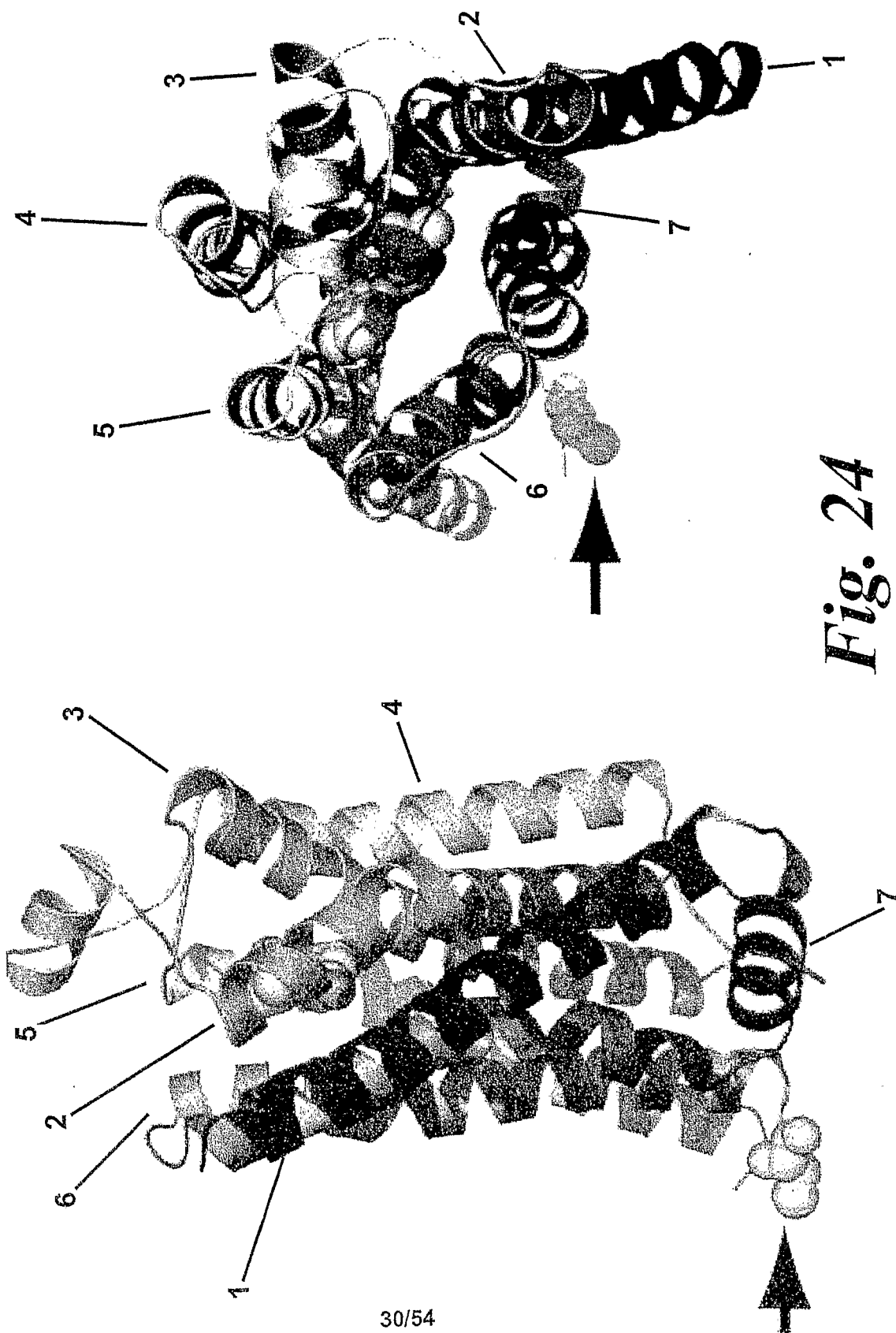


Fig. 24

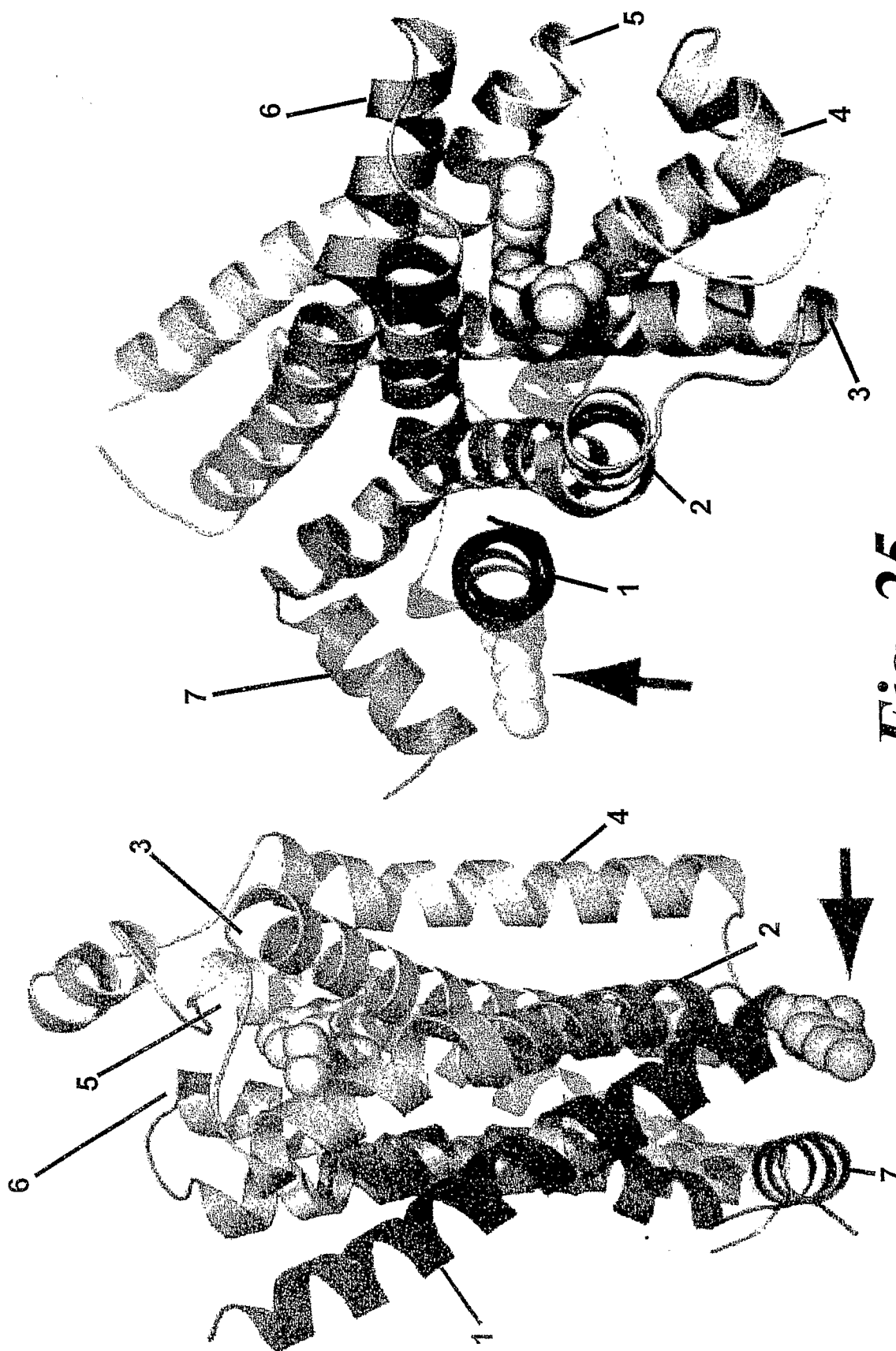


Fig. 25

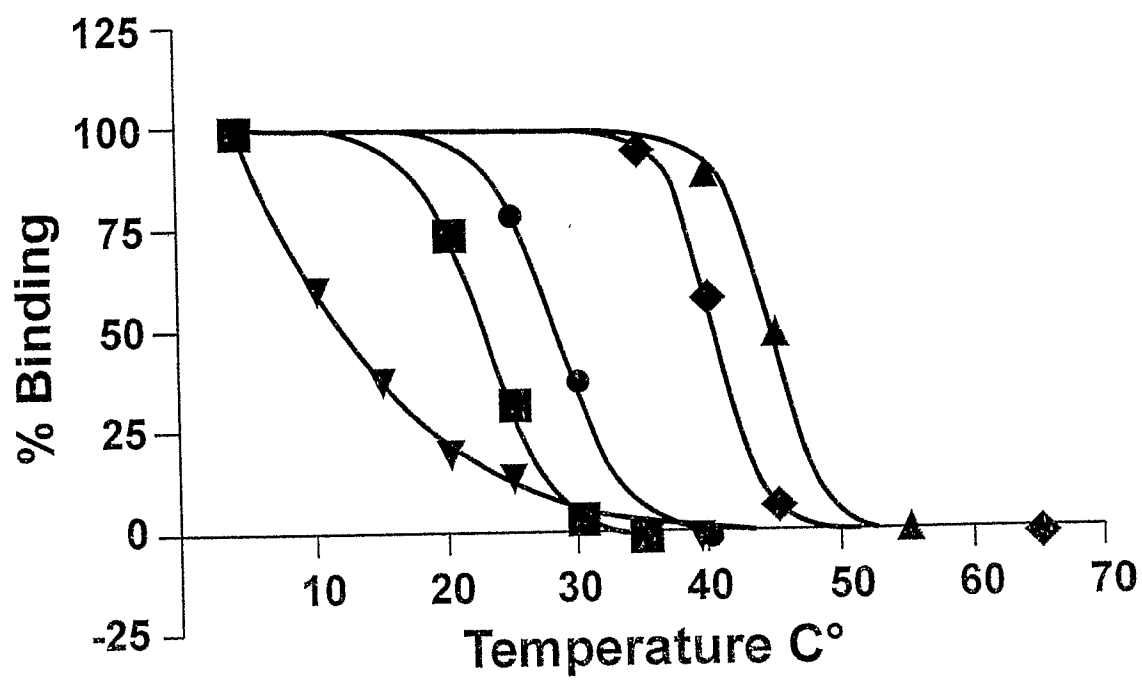


Fig. 26

Turkey β 1	Human β 1	Human β 2	Human β 3	
100%	76%	59%	54%	Turkey β 1
	100%	60%	59%	Human β 1
		100%	50%	Human β 2
			100%	Human β 3

Human AA2a	Human AA2b	Human AA1	Human AA3	
100%	58%	48%	39%	Human AA2a
	100%	43%	35%	Human AA2b
		100%	47%	Human AA1
			100%	Human AA3




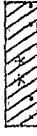
Rat NTR1	Human NTR1	Human NTR2	
100%	91%	44%	Rat NTR1
	100%	42%	Human NTR1
		100%	Human NTR2






Fig. 27

Fig. 28 (Page 1 of 2)

Alignment of neurotensin receptors

NTR1_rat	1	MHLNSSVPQGTGPEPDAQFSGPQSEMEATFLALSLSNGSGNTSESDTAG	50
NTR1_human	1	MRLNSSAP-GTPGTPAADPFQRAQAGLEEAALLAPGFGNASGNASERVIAA	49
NTR2_human	1	METSSP--RPSPSSNPG-----LS	18
		. . * * * * *	
NTR1_rat	51	PNSDLDVNTDIYSKVLVTALFVVGTVGNSVTATLARKKSLQSLQS	100
NTR1_human	50	PSSELDVNTDIYSKVLVTAVYLALEFVVGTVGNTVTAFTLARKKSLQSLQS	99
NTR2_human	19	LDARLGVDTRLWAKVLEFTALYALIWIWALGAAGNALSVHVVLKAR--AGRAG	66
		. * * * * .	
NTR1_rat	101	TVHYHLGSLALSDLLTLLAMPVELYNFIWVHHPWAFGAGCGRYFELRD	150
NTR1_human	100	TVHYHLGSLALSDLLTLLAMPVELYNFIWVHHPWAFGAGCGRYFELRD	149
NTR2_human	67	RLRHVLSLALAGLLLLLVGPVELYSFVWFHYPWVFGDLGCRGYFVHE	116
		*** **	
NTR1_rat	151	ACTYATALNVASLSVERYLAIChPFAKTLMSRSRTKKFI SAIWLASALL	200
NTR1_human	150	ACTYATALNVASLSVERYLAIChPFAKTLMSRSRTKKFI SAIWLASALL	199
NTR2_human	117	LCAYATVLSVAGLSAERCLAVCQPLRARSLLTPRRTRWLVALSWAASLGL	166
		*** **	
NTR1_rat	201	AIPMEFMGLQNR--SGDG-THPGGLVCTPIMDTATVKVVIQVNTFMSEFL	247
NTR1_human	200	AVPMLFTMGEQNR--SADG-QHAGGLVCTPTIHTATVKVVIQVNTFMSEFI	246
NTR2_human	167	ALPMAVIMGQKHELETADGEPEPASRVCTVLVSR TALQVFIQVNVLVSEV	216
		*** **	

NTR1_rat	248	FPMVLSISLNTVIAKLTVMHQAAEQ---G-----RVCIVGTHNGLEHS	289
NTR1_human	247	FPMVISVINTIIANKLTVMVRQAAEQ---G-----QVCTVGG-----EHS	284
NTR2_human	217	IPLALTAFNGVTVSHLLALCSQVPSTSTPGSSTPSRLELLSEGLLSFI	266
		 . * . . *	
NTR1_rat	290	TENNTIE-----PGRVQALRHGVETIRAVVIAFVVCWLPHYHVR	327
NTR1_human	285	TFSMAIE-----PGRVQALRHGVRLRAVVIAFVVCWLPHYHVR	322
NTR2_human	267	VWKKTFIQGGQVSLVRHKDVRRIQSVQVLRIVVMYVICWLPHYHAR	316
		 *	
NTR1_rat	328	RLMFCYISDEQWTFELFHYFYMVMTNALTYSSTAINPILYNILVSANER	377
NTR1_human	323	RLMFCYISDEQWTFELYDFHYFYMTNALTYSSTINPILYNILVSANER	372
NTR2_human	317	RLMYCVDPDDAWTDPLYNFHYFYMTNTLFYVSSAVTPLLYNAVSSSER	366
		 *	
NTR1_rat	378	QVELSTLACLCHGVPRPKKRRPTFSRKPNSSMSSNHAFSTSATRETTY	424
NTR1_human	373	HIFLATLACLCPVWRRRRK-RPAFSRKADSVSSNHTLSSNATRETTY	418
NTR2_human	367	KLFLEAVSSLC-GEHHPMKRLPPKPPQSPTLMDTASGFGD--PPETR	409
		 ** . . * * . . *	

 Mutations determined by heating in the absence of neurotensin
 Mutations determined by heating in the presence of neurotensin
 Mutations that significantly improve expression levels in E. coli
 Position of transmembrane domains
 Position of helix 8

(a) H103:Thermostability obtained with A, N, S, V, L, M
Only H103N and H103S gave wt levels of expression

Fig. 28 (Page 2 of 2)

Figure 29

Schematic representation of typical lead isolation process for the identification of inhibitory scFv binders

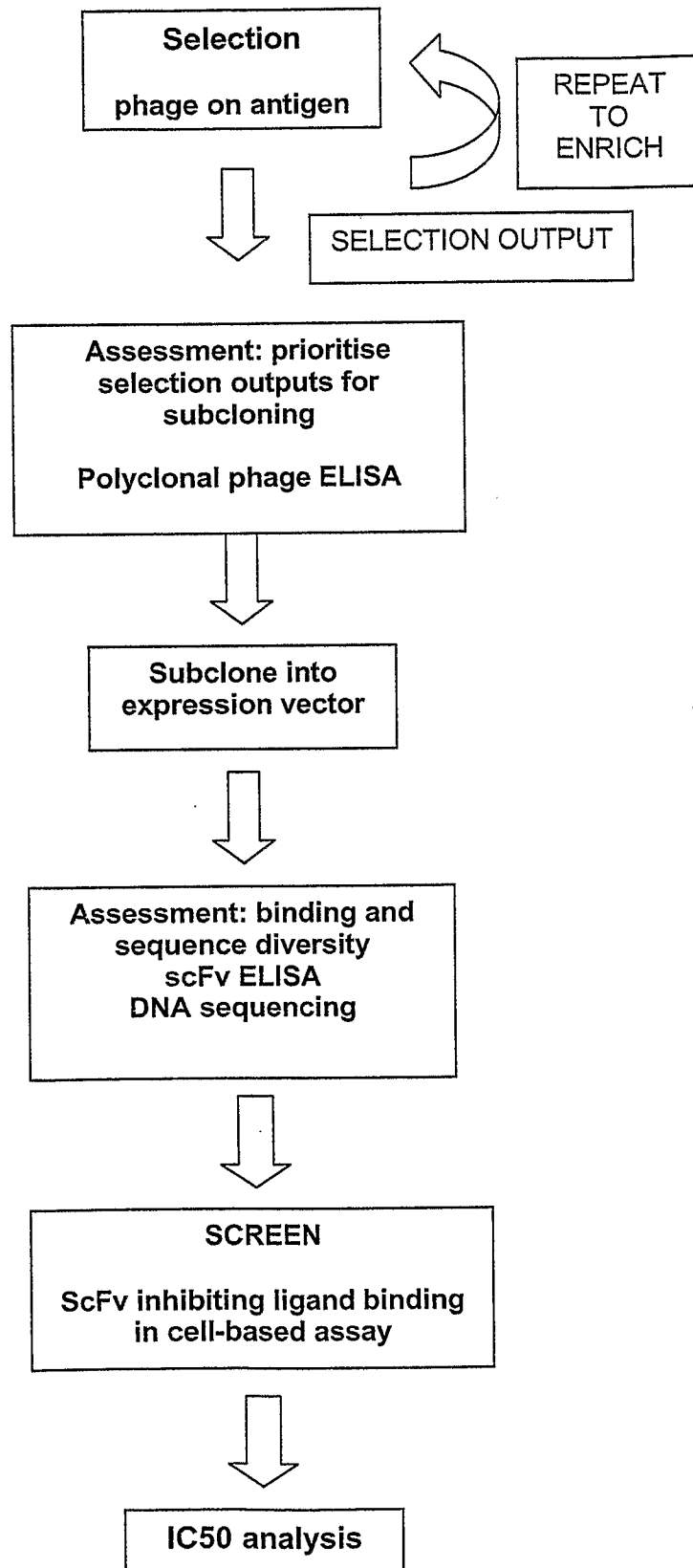


Figure 30 (Page 1 of 2)

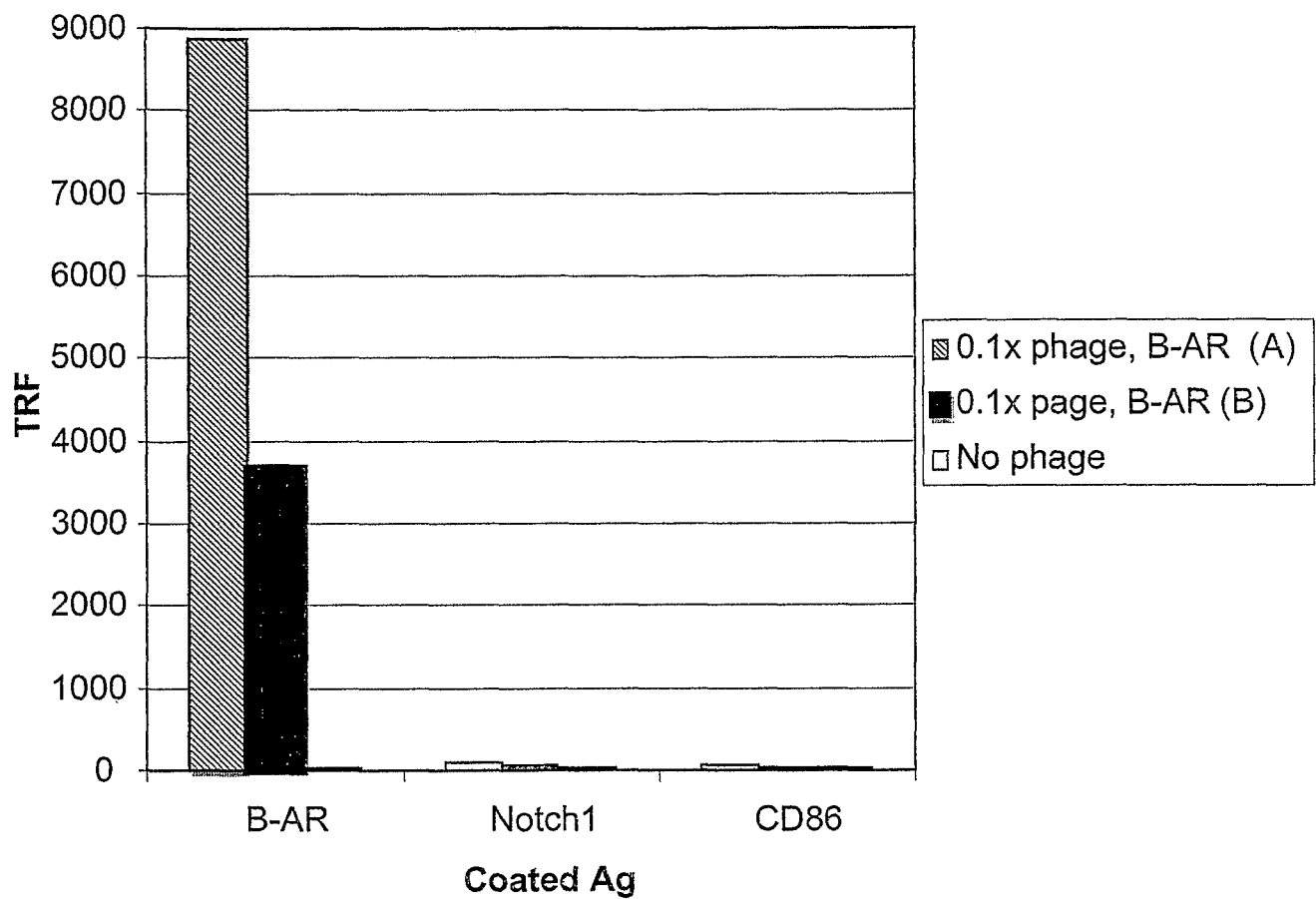
A **B- adrenergic receptor, Polyclonal ELISA**

Figure 30 (Page 2 of 2)

B

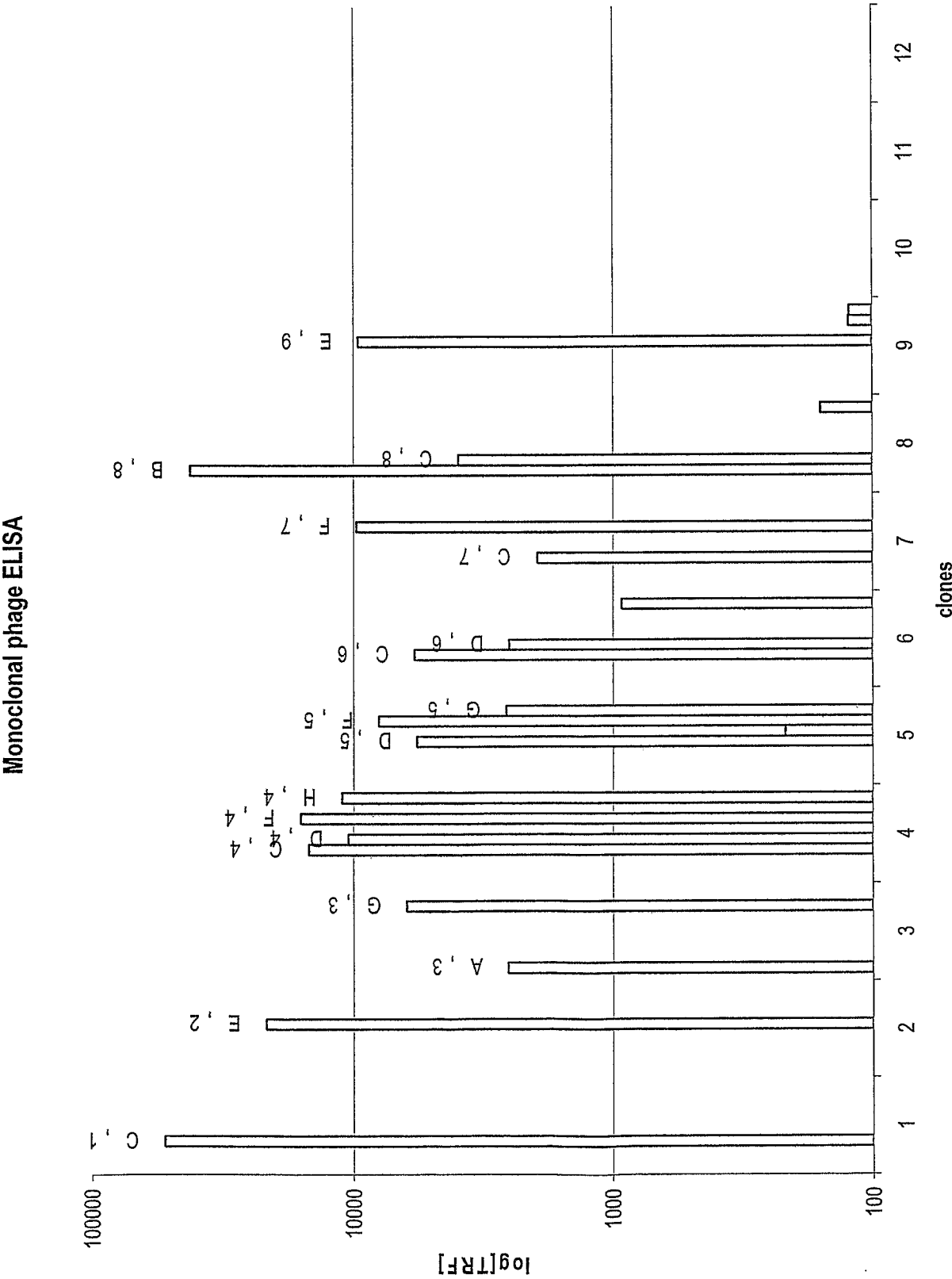
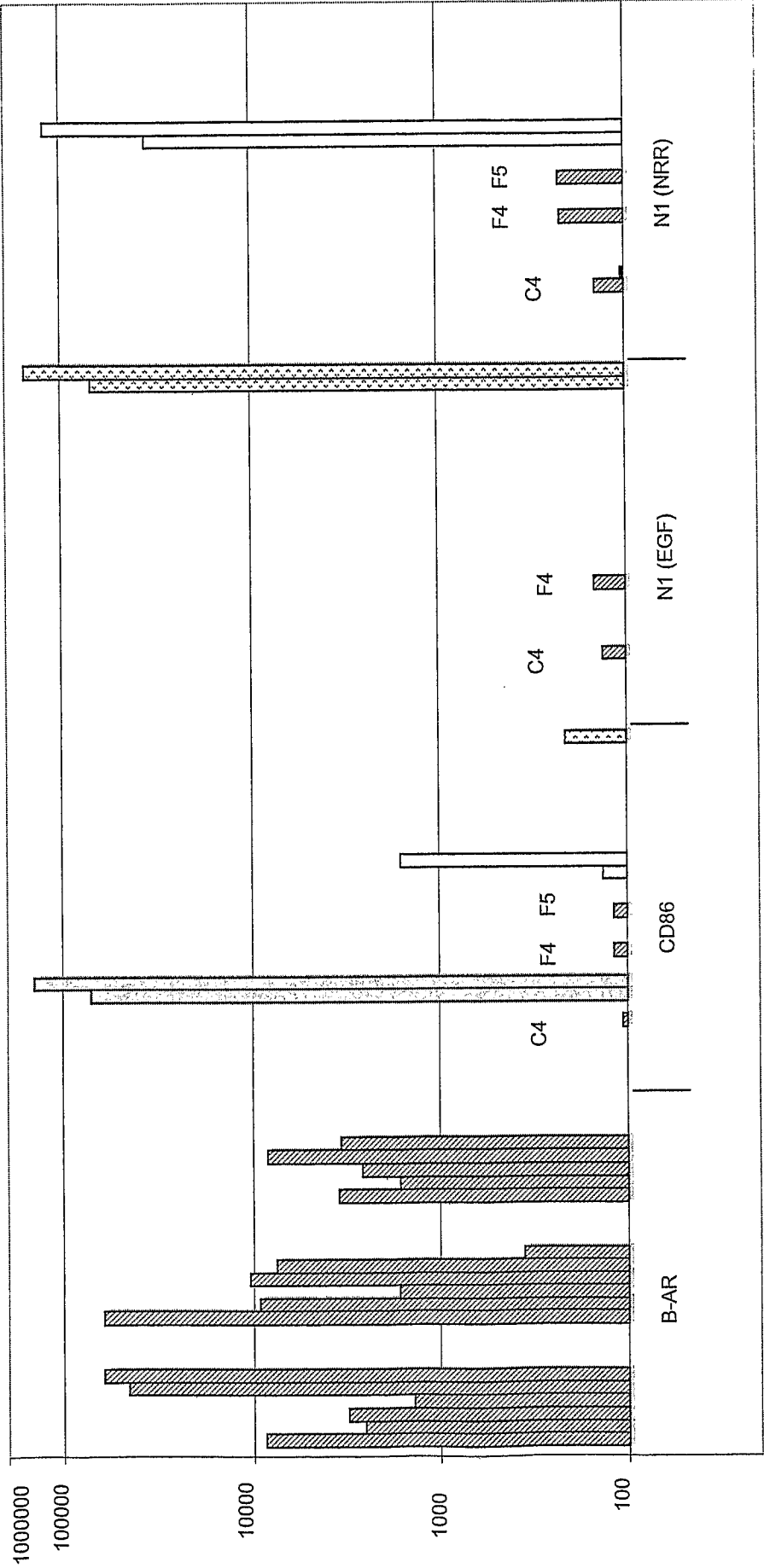


Figure 31 (Page 1 of 2)
26/9/8 B-AR mono phage (amino-plate)

A



B

Figure 31 (Page 2 of 2)
26/9/8 B-AR mono phage ELISA (His-plate)

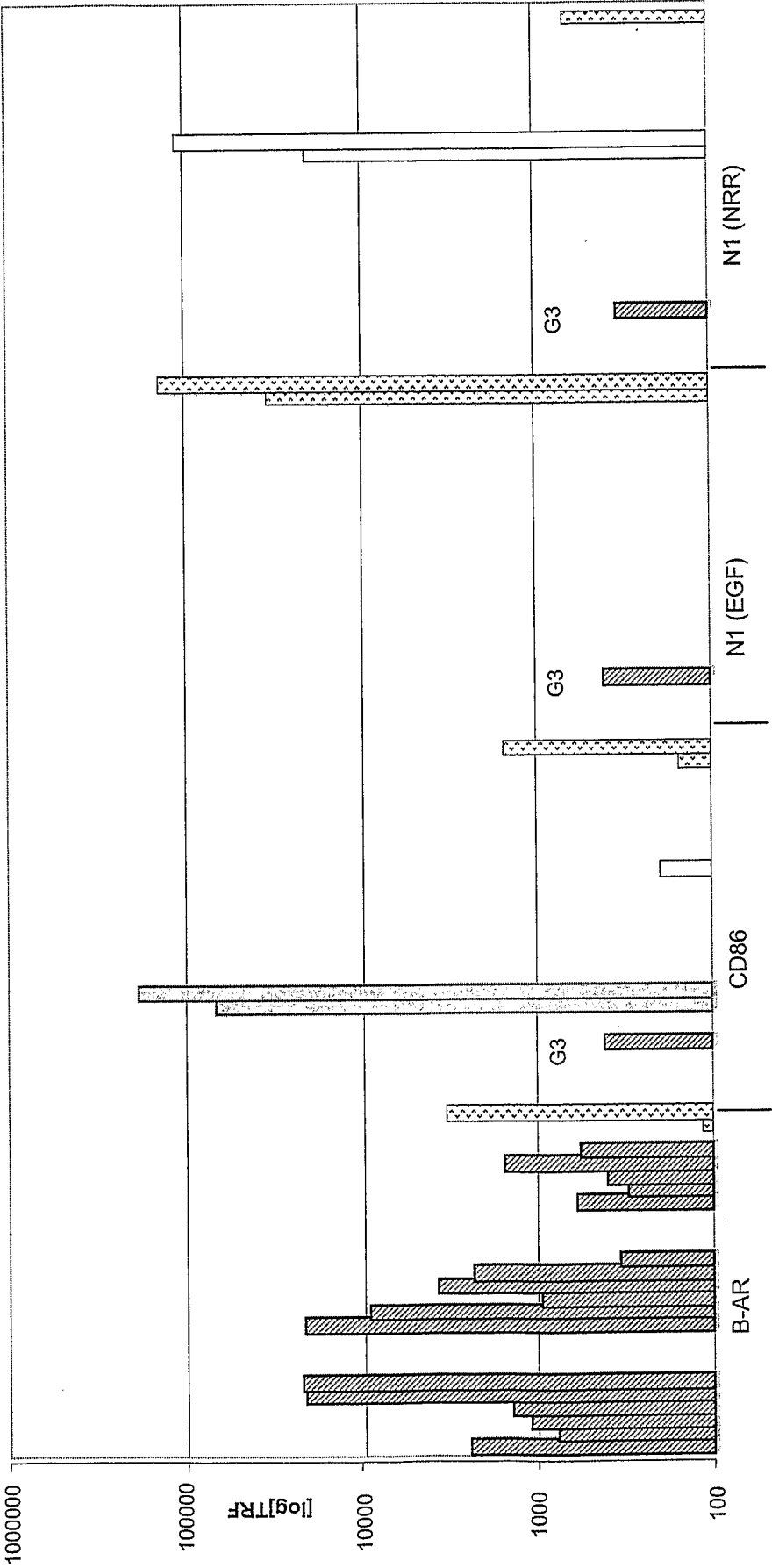


Figure 32

Beta-AR scFv monoclonal ELISA

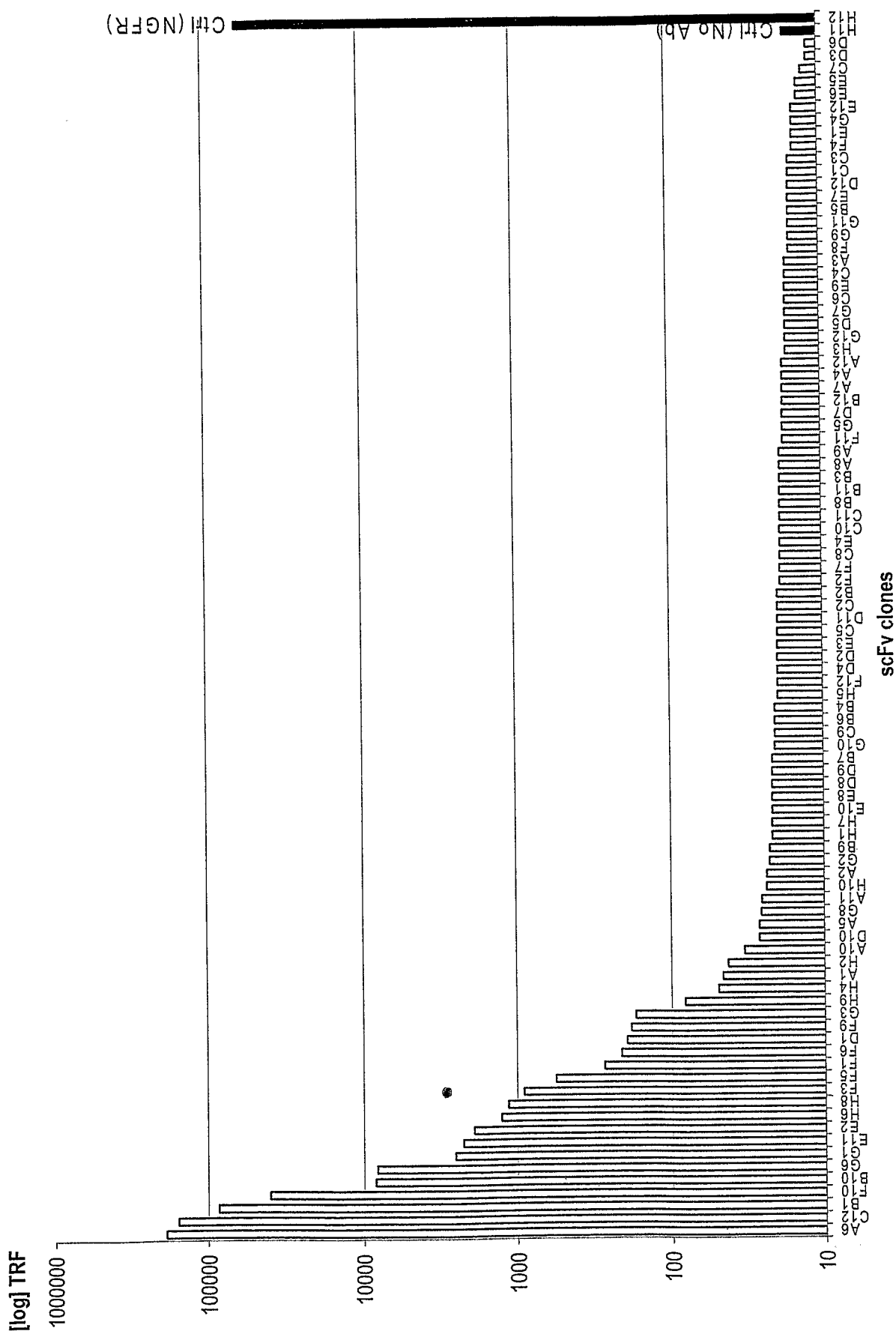


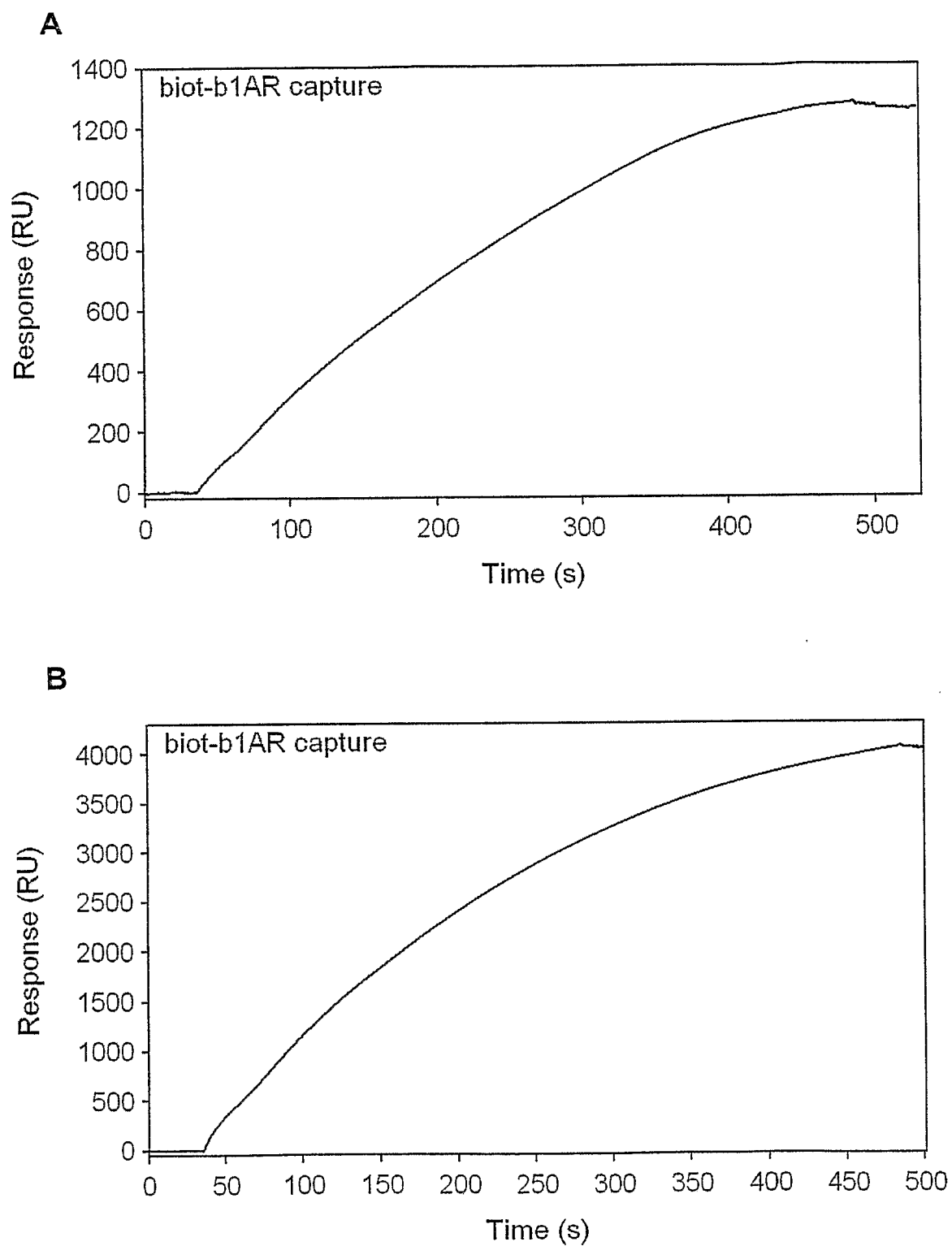
Figure 33

Figure 34

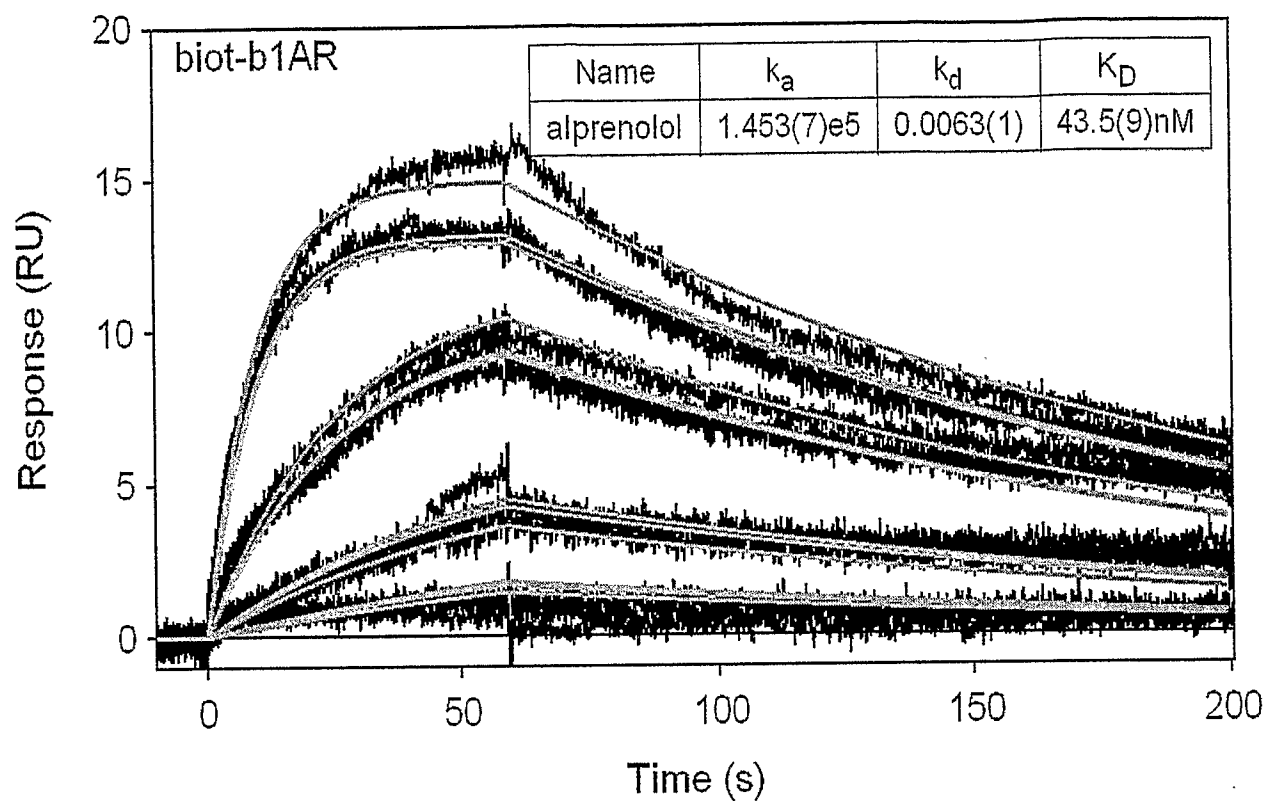


Figure 35

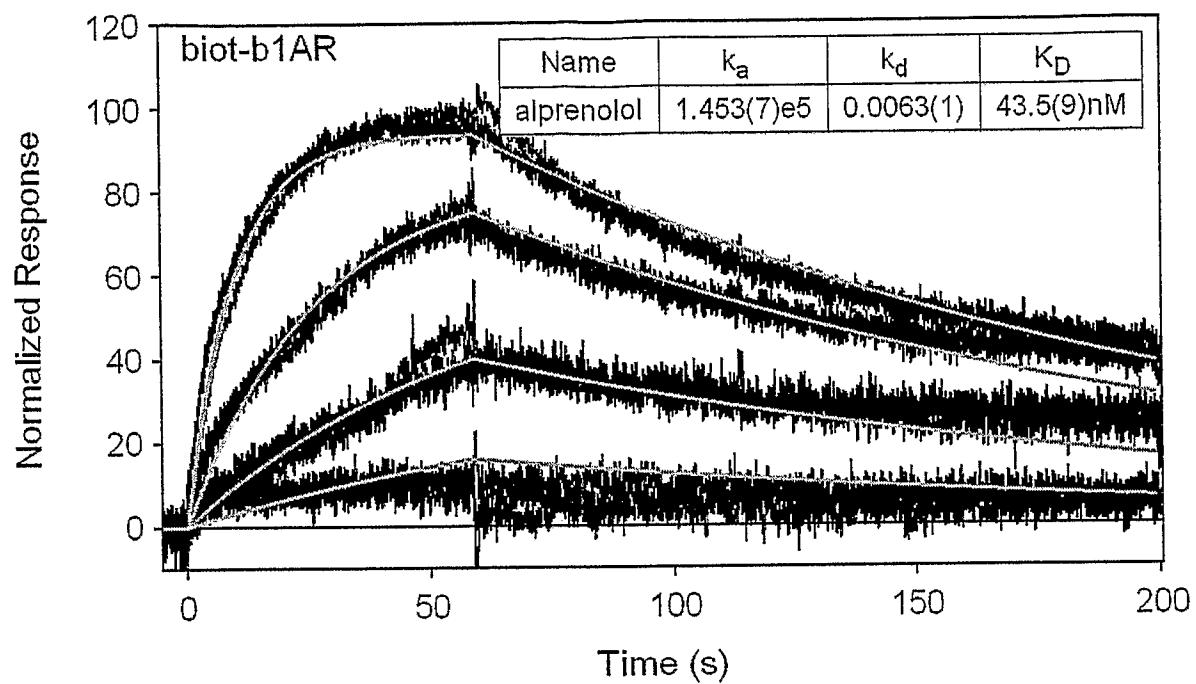


Figure 36

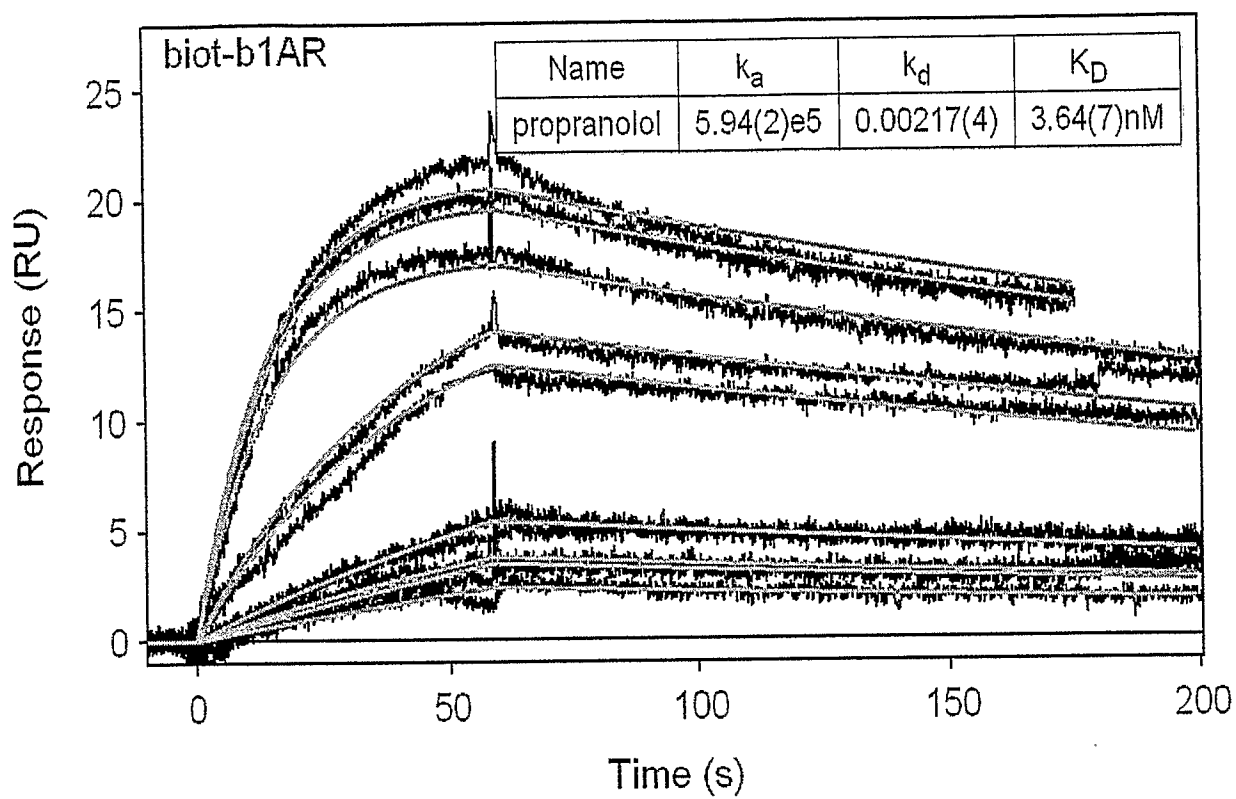


Figure 37

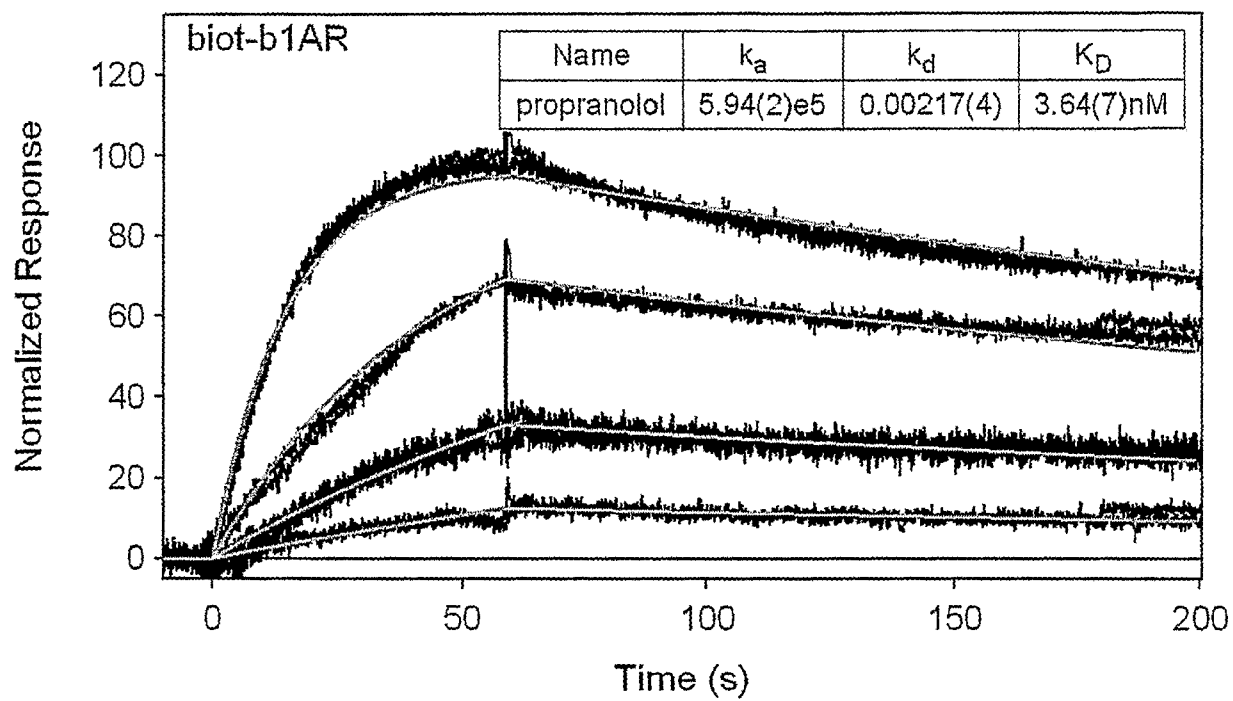


Figure 38

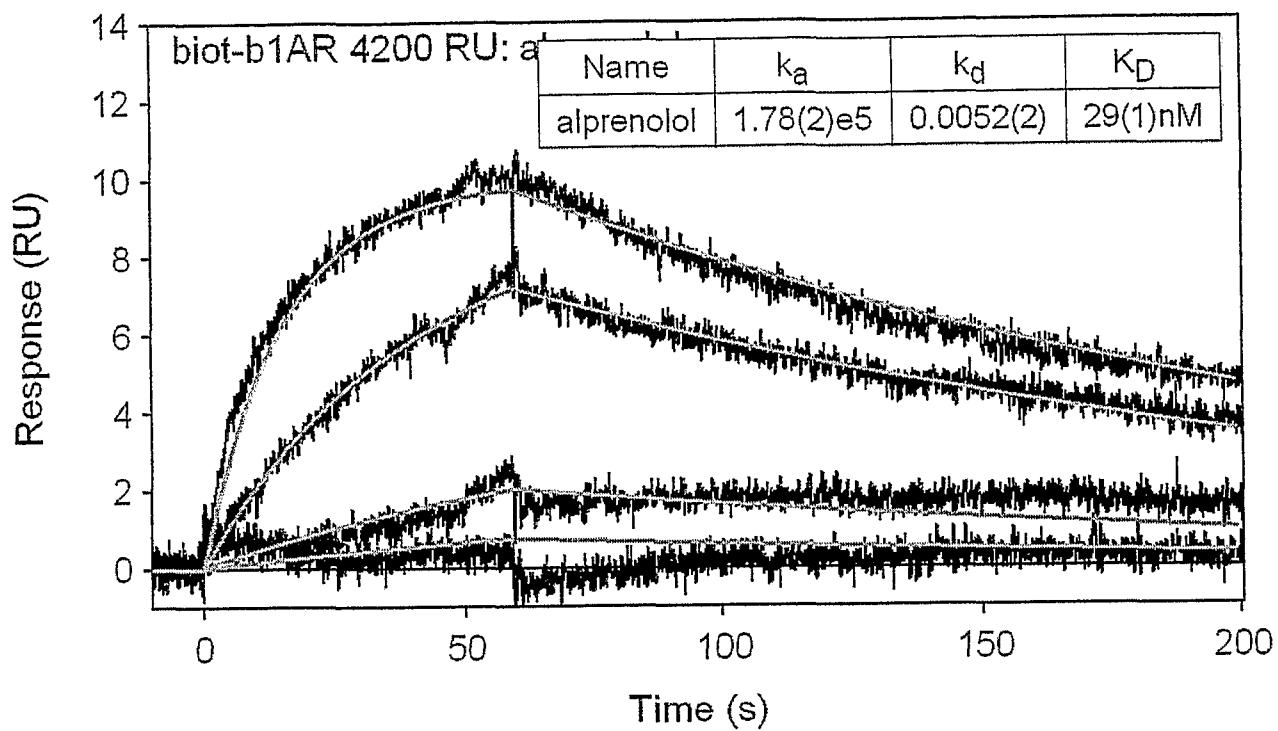


Figure 39

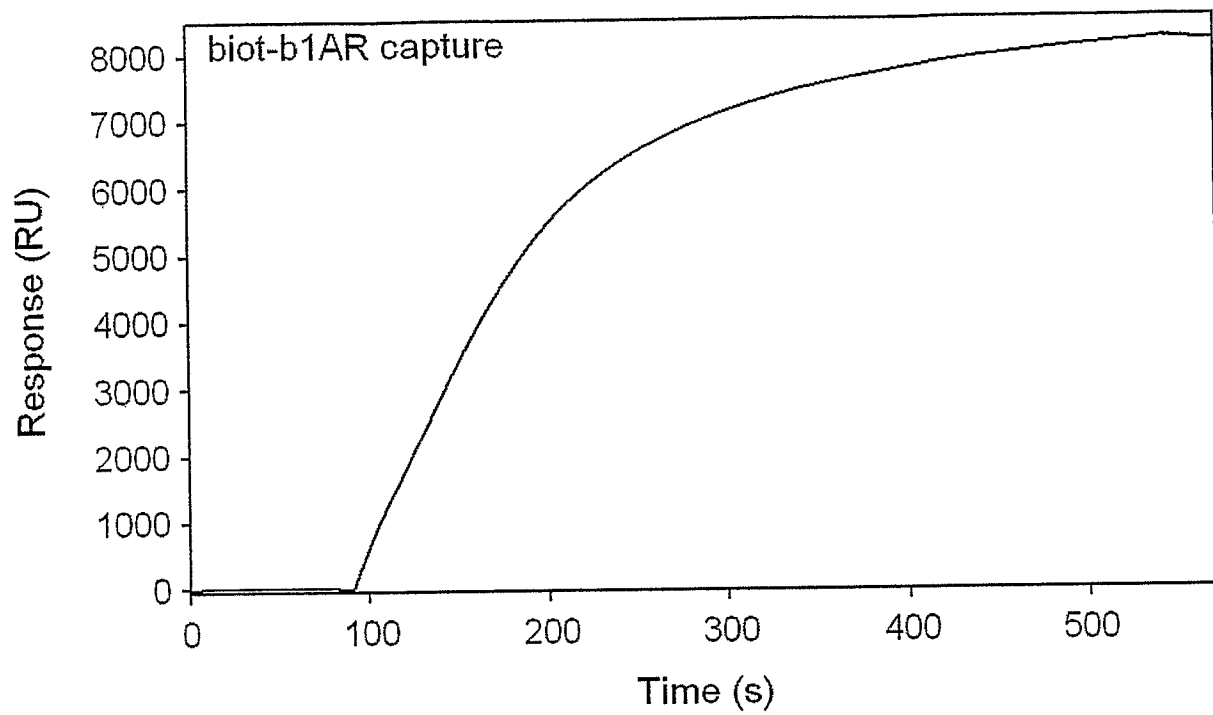


Figure 40

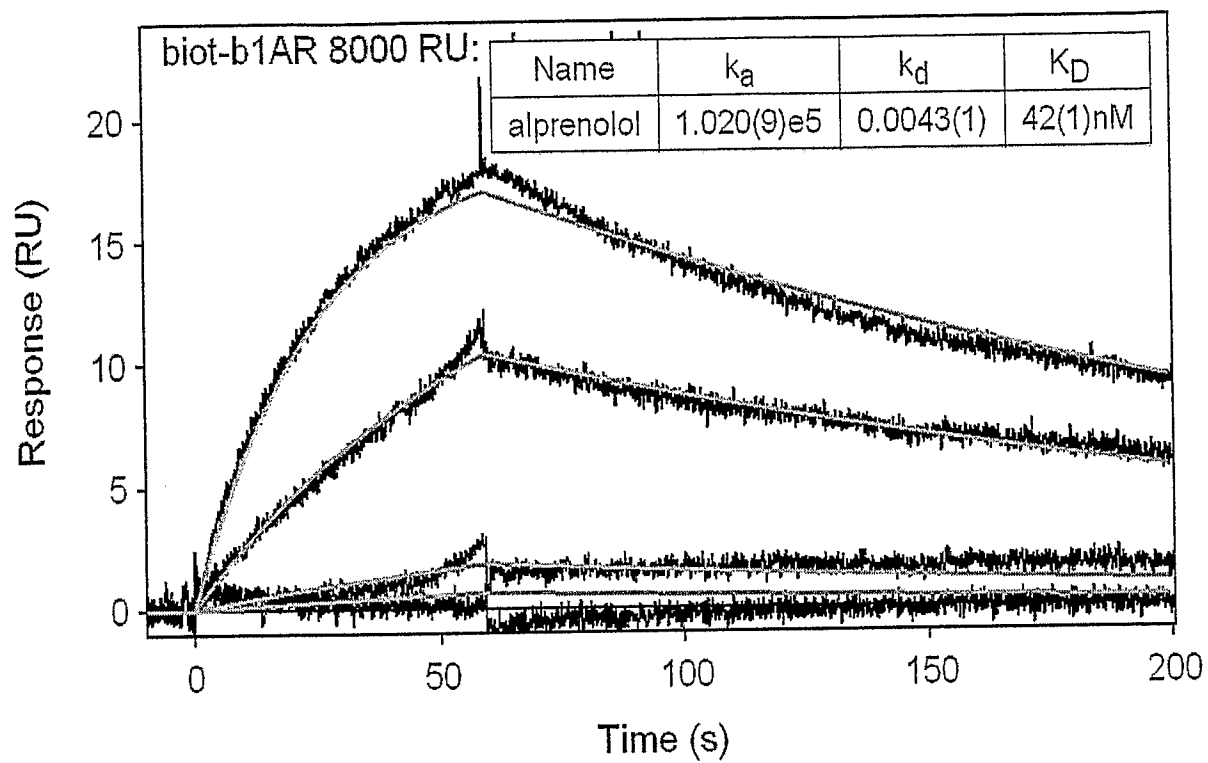


Figure 41

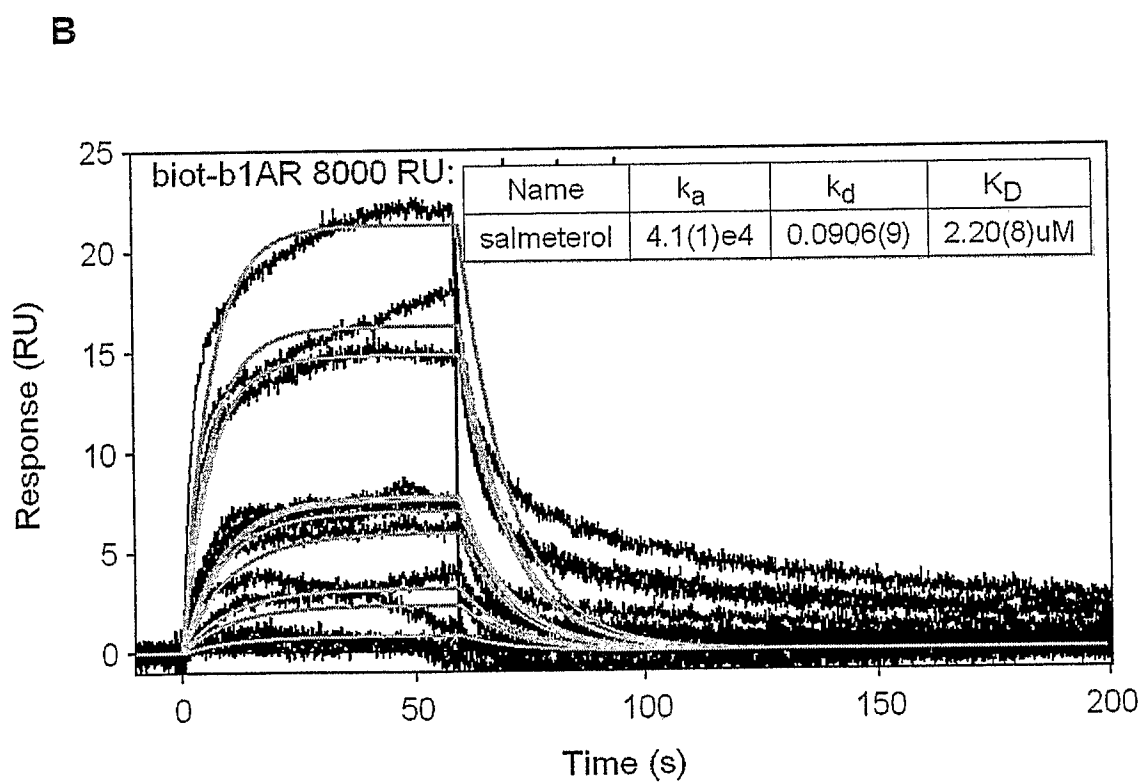
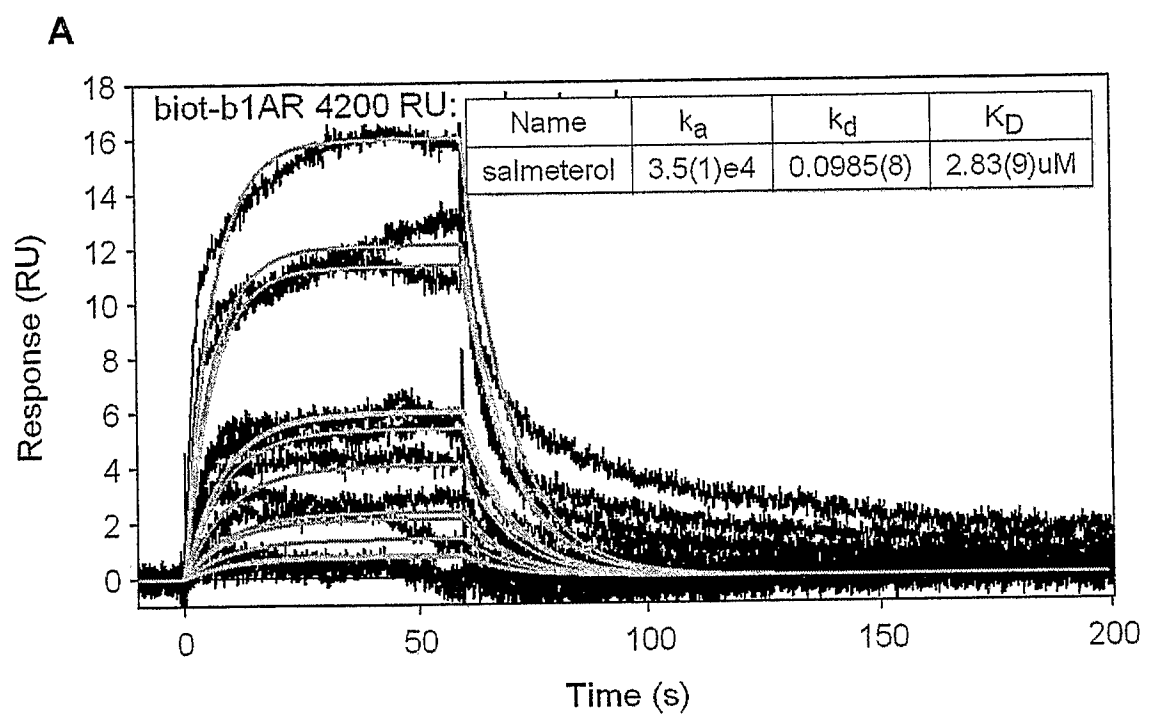
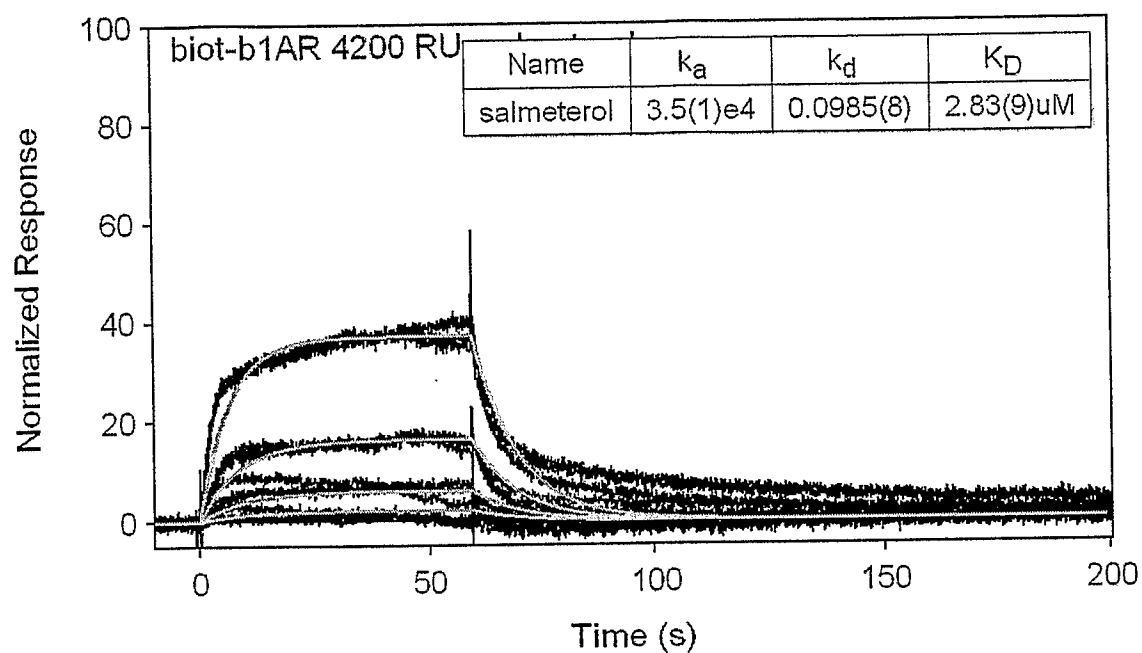


Figure 42

A



B

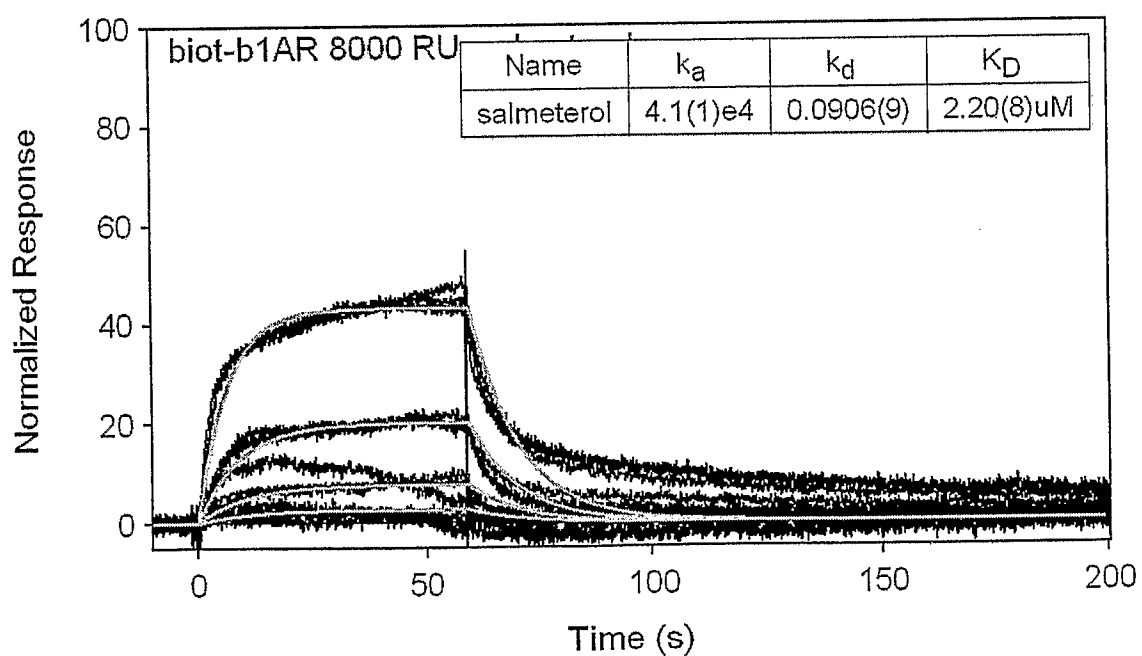
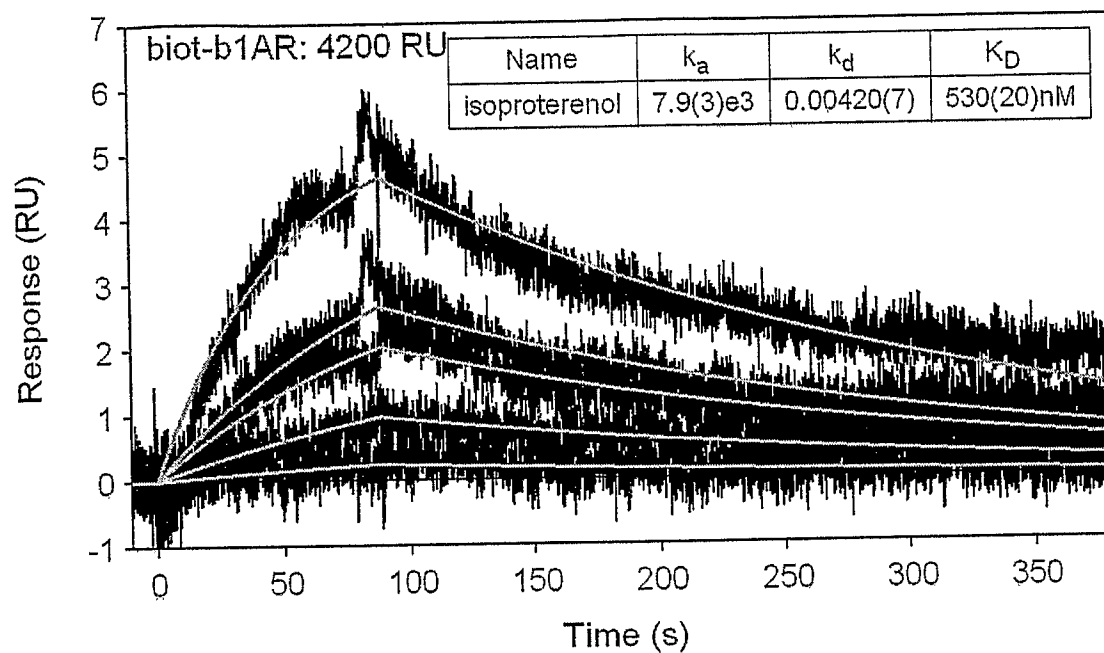


Figure 43

A



B

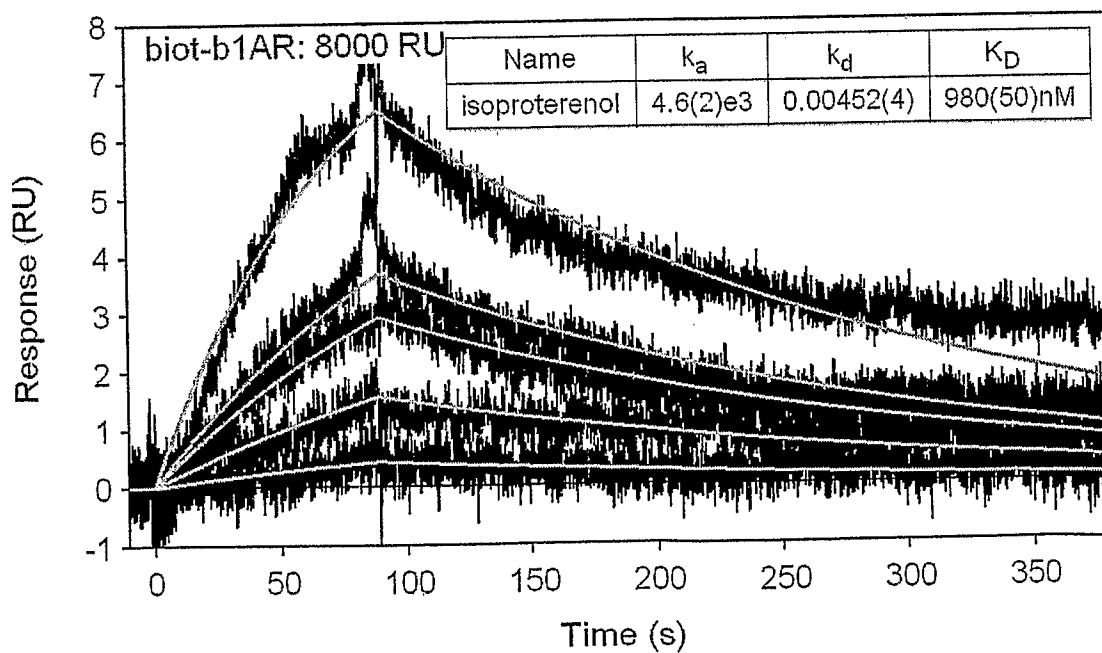
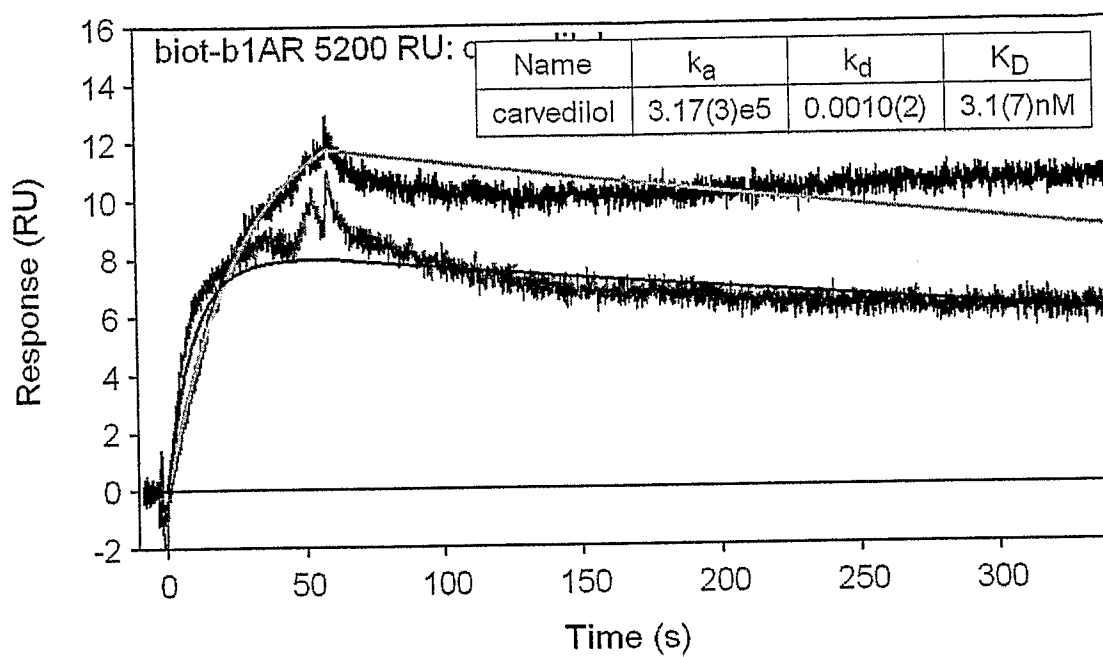


Figure 44

A



B

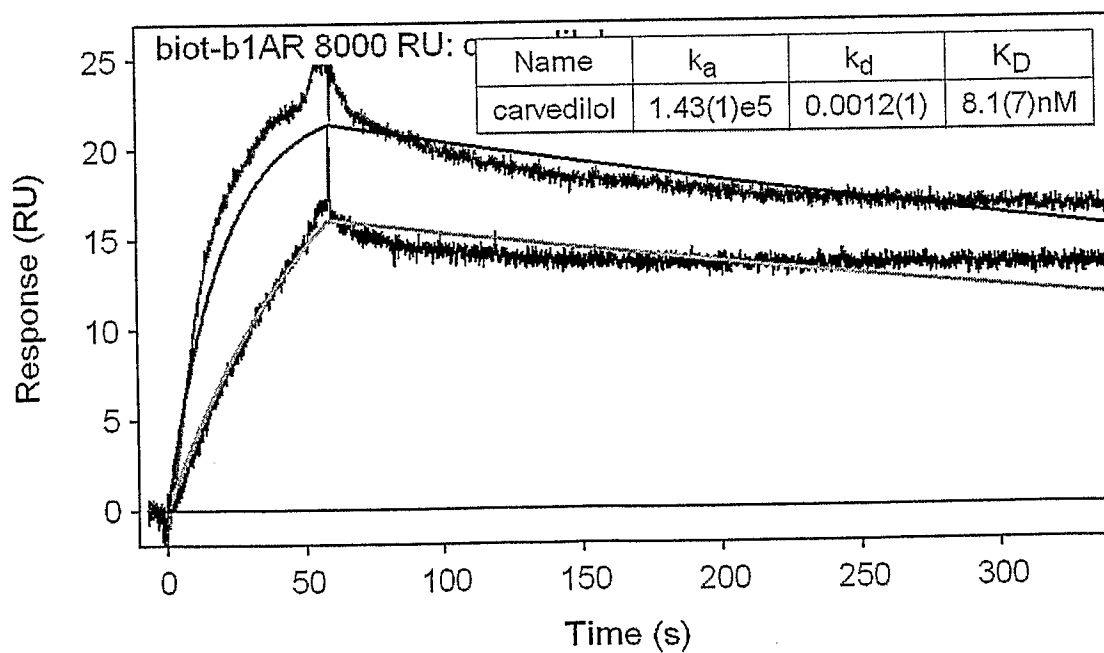


Figure 45

Rant 22 Trial Screen HTL0245-HTL0252 17/11/08

