

IN VITRO ASSAY BASED ON cAMP LEVELS MODULATED BY Gi-COUPLED RECEPTORS

5 FIELD OF THE INVENTION

The present invention relates to an in vitro assay based on cAMP levels modulated by Gi-coupled receptors.

10 DESCRIPTION OF THE PRIOR ART

G-protein-coupled receptors (GPCR) represent one of the largest super families in the human genome. They are involved in a large number of physiological disorders and diseases and therefore represent a significant target for pharmaceuticals and
15 drug discovery. Approximately 60% of all drugs currently in development target GPCRs (Lundstrom et al., *Trends Biotechnol.*, 2005, 23, 103-108).

GPCRs, characterised by their 7 transmembrane spanning domains, can be activated by a wide variety of extracellular stimuli, such as neurotransmitters,
20 hormones, light and odorants. Heterotrimeric G proteins consist of 3 subunits: the α subunit (35kDa) family, the β subunit (35–36kDa) family and the γ (6–10kDa) subunit family. Agonist binding leads to active conformational adaptation of the receptor and subsequent activation of G proteins through the association of guanosine-5'-triphosphate (GTP) to the $G\alpha$ subunit in exchange for guanosine-5'-diphosphate
25 (GDP) (Kristiansen, K., *Pharmacol Ther*, 2004, 103, 21-81). Then, dissociation of the $G\alpha$ from the $G\beta\gamma$ dimer leads to activation, by $G\alpha$ or $G\beta\gamma$ dimer, of downstream effectors such as adenylyl cyclases, ion channels and phospholipases.

A diverse array of adapted assays is available, covering exploitation of the three
30 main steps in the GPCR signalling process, including ligand binding and functional assays (for review, Greasley P.J. and Jansen, F.P., *Drug Discovery Today: Technologies*, 2005, 2, 2, 163-169). These steps include the binding of the ligand to the receptor, the ligand-mediated stabilisation of the receptor in an active conformation resulting in nucleotidic exchange (GTP for GDP in the $G\alpha$ subunit) and

finally, the subsequent coupling to different intracellular secondary messenger pathways.

G proteins coupled to GPCRs include four families Gq, Gs, Gi and G_{12/13} (for review, Wong, S.K.-F., *Neurosignals*, 2003, 12, 1-12) and coupling to a specific secondary messenger pathway will depend on the G-protein preference of the receptor and of the nature of the agonist used (Akam, E.C. et al., *Br J Pharmacol.*, 2001, 132, 4, 950-8; Wong, S. K.-F., *Neurosignals*, 2003, 12, 1-12) .

10 Activation of the Gq-coupled receptors leads to the activation of phospholipase C inducing release of calcium stores via the generation of inositol 1,4,5-triphosphate (IP3). Functional assays of this pathway are mainly cell-based and exploit calcium and IP3 signals.

15 Activation of Gs and Gi-coupled receptors lead to activation (Gs) or inhibition (Gi) of adenylyl cyclase, resulting in increased or decreased intracellular cAMP levels, respectively. Many types of assay formats using fluorescence or luminescence readout can be applied to the measurement of intracellular cAMP (For review, Williams, C. et al., *Nat Rev Drug Discov.*, 2004, 3, 2, 125-35). They provide good
20 results for the Gs-coupled receptors.

Nevertheless, it is more challenging to obtain a workable assay for the Gi-coupled receptors using these technologies. Detection of the effects induced by Gi-coupled receptors requires pre-activation by forskolin to stimulate adenylyl cyclase and to produce cAMP and inhibition of cAMP production mediated by binding of an agonist
25 on a Gi-coupled receptor rarely exceeds 60% of the stimulation by forskolin. Furthermore, measuring the reversal by an antagonist of the cAMP decrease mediated by a Gi-receptor agonist as a result of prior forskolin stimulation of the adenylyl cyclase activity yet increases the complexity (Greasley P.J. and Jansen, F.P., *Drug Discovery Today: Technologies*, 2005, 2, 2, 163-169).

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One way to overcome these limitations is to transform normal pharmacological effector of Gi-coupled receptors from adenylyl cyclase to PLC β pathway by using chimeric (Gqi5) or promiscuous G proteins (G_{16/15}), (Lui, A.M., et al., *J. Biomol. Screen*, 2003, 8, 39-49). However, the use of non-cognate G proteins has been

shown to induce significant modifications of the receptor / G-protein interactions thereby decreasing the relevance of the assay.

Other approaches for assaying cAMP concentration include reporter gene assays (Kent, T.C et al., *Journal of Biomolecular Screening*, 2005, 10, 5, 437-446). However

5 in this case, measurement of a secondary event, here the activation or inhibition of a reporter gene, favours the generation of false positive and negative hits in a screening.

After the original cloning of the mammalian adenylyl cyclase by Krupinski et al (Krupinski et al., *Science*, 244, 1558-1564), at least nine isoforms of mammalian
10 adenylyl cyclases have been cloned. Several adenylyl cyclase families have been identified based on sequence similarities and regulatory properties, revealing diverse regulation by G proteins, calcium and phosphorylation (Sunahara et al., *Ann. Rev. Pharmacol. Toxicol.*, 1996, 36, 461-480). These intracellular modulators affect the
15 adenylyl cyclase isoenzymes differently. Therefore, the cAMP response upon the activation of a receptor is dependent on the cellular adenylyl cyclase isoenzymes pool present in a given cell.

Mammalian cell lines have a cellular machinery adapted for folding and
20 posttranslational modification of expressed receptor proteins. Therefore, cell lines as chinese hamster ovary (CHO) and human embryonic kidney (HEK) are extensively used as tools for screening and identifying chemical entities that can be developed into drugs. However, specificity of the coupling properties of GPCR expressed in a cell line are often less effective than in their native environment (Kenaskin, T.,
25 *Pharmacol. Rev.*, 1996, 48, 413-463). In addition, the outcome of each step in the signal transduction cascade is dependent on the regulatory properties of the particular isoenzyme pool present in the cells (Varga E.V. et al., *European Journal of Pharmacology*, 1998,348, R1-R2).

30 In mammalian cells, different isoforms of adenylyl cyclases have been identified, which are tissue-specifically expressed. The first study to identify the role of adenylyl cyclase isoforms in mammalian cell lines used in the signaling pathways of GPCRs was performed by Varga E.V. et al., in 1998. They used reverse transcriptase polymerase chain reaction (RT-PCR) to amplify and then identify and sequence the

adenylyl cyclase cDNA in CHO and B12 cells. They identified adenylyl cyclase isoforms VI and VII in CHO and adenylyl cyclase isoforms VI and IX in B12 cells (Varga E.V. et al., *European Journal of Pharmacology*, 1998,348, R1-R2). Adenylyl cyclase isoforms II, III, VI and IX were also reported in HEK-293 cells (Helleveo, M. et al., *Biochem. Biophys. Res. Commun.*, 1993, 192, 311).

Transfection studies on the sensitivity of adenylyl cyclase isoforms to activation by $G_{s\alpha}$ or to inhibition by $G_{i\alpha}$ provided precious informations concerning the integration of signalling at the effector level (Sunahara, R.K., et al., *Ann. Rev. Pharmacol. Toxicol.* 1996, 36, 461-480; Cooper, D.M. et al., *Adv. Sec. Mess. Phosph. Res.*, 1998, 32, 23-51; Simonds, W.F, *Trends Pharmacol Sci.*, 1999, 20, 2, 66-73. 1999). At least 18 different subtypes of $G\alpha$ subunits have been identified. In particular, the G_i/o subfamily that inhibits adenylyl cyclase and regulates ion channels includes G_{i1} , G_{i2} , G_{i3} , G_{o1} , G_{o2} , G_{o3} , G_z , G_{t1} , G_{t2} and G_{gust} .

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Noteworthy, it was shown that G_{ai} does not inhibit all the adenylyl cyclase isoforms and that only adenylyl cyclase isoforms I, V and VI are sensitive to the G_i family.

Another information concerns the impact of the pre-required forskolin activation on the measurement of the G_i effect in a cAMP based GPCR cellular assay. This molecule is widely used as an in vitro activator of cAMP formation. It has been shown to directly activate almost all the adenylyl cyclase isoforms and to raise cyclic AMP levels in a wide variety of cell lines (Seamon, K.B. et al., *J Cyclic Nucleotide Res.*, 1981, 7, 4, 201-24). At present, only adenylyl cyclase isoform IX has been identified not to be stimulated by forskolin.

As set forth above, many types of technologies are applied for measuring changes in cAMP concentration providing useful tools for studying G_s -coupled receptors. However, the need for pre-activation by forskolin which directly activates adenylyl cyclase to detect the effect of G_i limits the development of a relevant, working cAMP functional assay for screening and profiling G_i -coupled receptors. The non homogeneous expression pattern of the nine principal identified adenylyl cyclase isoforms accounting for distinctive cell- and tissue-specific patterns of adenylyl cyclase responsiveness, the sensitivity of only certain adenylyl cyclase isoforms to

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inhibition by Gi proteins as well as multiple G protein coupling subtypes of a significant number of GPCRs (Wong, S.,K.-F, *Neurosignals*, 2003, 12, 1-12) added to the pre-required forskolin activation to detect the Gi effect, all significantly contribute to the encountered difficulties in the development of a working cAMP functional screening assay dedicated to Gi-coupled receptors.

The difficulties encountered in the development of a relevant, working cAMP functional screening and profiling assay dedicated to selective Gi-coupled receptors represents today a breakthrough in the successful identification of new drug candidates for these receptors. Development of methods allowing overcoming these limitations is of strategic importance to the identification of hits and leads for Gi-coupled receptors.

SUMMARY OF THE INVENTION

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It is an objective of the present invention to solve the aforementioned problems in the art.

Accordingly, in a first aspect, the present invention provides a method for screening, identifying or selecting test compounds using an in vitro assay based on cAMP levels modulated by Gi-coupled receptors expressed in a cell comprising the steps of:

- (a) optionally identifying, in a selected cell, the G α i and adenylyl cyclase isoforms which are involved in the modulation of the intracellular cAMP levels,
- 25 (b) favouring the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to inhibition by G α i relative to adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 but not sensitive to inhibition by G α i in said selected cell;
- (c) contacting said cell with forskolin or NKH-477 and a test compound;
- 30 (d) measuring cAMP levels resulting from contacting said test compound with said cell.

In a second aspect, the invention provides an assay system or a kit for measuring the modulation of cAMP levels by test compounds that specifically interact with and modulate the activity of a cellular receptor, said assay system or kit comprising cells which mainly express adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477
5 and sensitive to inhibition by G α i.

In another aspect, the invention provides a recombinant cell engineered from a selected cell expressing both adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to G α i and adenylyl cyclase isoform(s) stimulated by forskolin
10 or NKH-477 and not sensitive to G α i, wherein the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to G α i is favoured relative to the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and not sensitive to G α i.

15 The invention further provides the use of a recombinant cell as defined above as a tool for screening, identifying or selecting potential ligands of Gi-coupled receptors.

DESCRIPTION OF THE INVENTION

20 Before further description of the invention, certain terms employed in the specification, examples and claims are defined below.

Definitions

25 A "DNA construct" as used herein means a polynucleotide which contains an open reading frame which encodes one or more protein(s) and includes any promoter, transcription initiation, transcription termination, or other nucleotide sequences operatively linked to said polynucleotide which might facilitate expression of the encoded protein(s).

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The term "heterologous DNA", "heterologous nucleic acid" or "foreign DNA" relates to DNA that does not occur naturally as part of the genome in which it is present. Generally, although not necessarily, such DNA encodes proteins that are not normally produced by the cell in which it is expressed. Heterologous DNA can be

from the same species, although in preferred embodiments, it is from a different species. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed is herein encompassed by the term heterologous DNA.

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The term "cells" as used herein refers to all normal (natural) and transformed cells derived from, for example eukaryotes such as mammalian cells including hybridoma cells, insect cells, plant cells, yeast cells. Examples of mammalian species include primates (e.g., humans, monkeys, chimpanzees, baboons), rodents (e.g., rats, mice, guinea pigs, hamsters) and ruminants (e.g., cows, horses). Mammalian cells may be derived from any recognized source with respect to species (e. g. human, rodent), tissue source (brain, liver, lung, heart, kidney, skin, muscle) and cell type (e. g. epithelial, endothelial). In addition, transfected cells expressing recombinant genes may also be used in the present invention.

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Cells to be used in the practice of the methods of the present invention may be primary cells, secondary cells or immortalized cells (i.e., established cell lines). They may be prepared by techniques well known in the art or purchased from immunological and microbiological commercial sources (for example, from the American Type Culture Collection, Manassas, VA). Alternatively or additionally, cells may be genetically engineered to contain, for example, a gene of interest such as a gene expressing an adenylyl cyclase isoform, a Gai protein or a receptor.

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Non-limiting examples of useful mammalian cell lines include animal and human cell lines such as Chinese hamster ovary (CHO) cells, Chinese hamster lung (CHL) cells, baby hamster kidney (BHK) cells, COS cells, HeLa cells, THP cell lines, TAg Jurkat cells, hybridoma cells, carcinoma cell lines, hepatocytes and the like.

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"Stably transfected" as used herein means the introduction and maintenance of exogenous DNA into a cell, or cell line, by integration into the genome of the host cell, e.g. into the host chromosomes or by episomal transfection for at least 2 weeks and preferably more than 4 months.

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"Transiently transfected" as used herein means that the transfected exogenous DNA is not integrated in the genome of the host cell.

DNA constructs that express siRNAs can be transiently transfected into mammalian cells using standard techniques. However, cells vary greatly with respect to their capacity to be transfected. Hence, different transfection reagents and cell culture conditions need to be tested for each cell type.

The term "modulation" as in "modulation of the intracellular cAMP levels" is intended to encompass modulation in its various grammatical forms (e.g., "modulated", "modulation", "modulating", etc.) and encompass induction, stimulation, potentiation, inhibition of one or more signal transduction pathways downstream of a receptor.

The term "inactivation" or "inactivate" relates to the inhibition, either total or partial, of the expression of a gene (also termed downregulation or knockdown) or of the activity of a protein. For example, this term means that production of a functional gene product is prevented or inhibited. Inactivation may be achieved, for example, either by deletion of the gene (also termed knockout) or mutation of the promoter controlling a gene so that expression does not occur, or mutation of the coding sequence so that the gene product is inactive, or by "RNAi" technique.

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The term "RNAi" or "RNA interference" technique refers to the post-transcriptional process of gene silencing mediated by double stranded RNA (dsRNA) that is homologous in sequence to a RNA to be silenced and is observed in a wide variety of eukaryotic cells such as animal and plant cells. Depending on the cell type, dsRNA may be processed into 21-23 nucleotides (nts) molecules, called small interfering RNAs (siRNAs), which guide the sequence-specific degradation of the target RNA (Sharp, 2001). Furthermore, transfection of siRNA specific to a target gene of which expression is to be inactivated, or of a vector allowing the expression of such a siRNA in a cell, in particular in mammalian cells, has been proven to be a potent tool in mammalian genetic studies as such specific siRNA induce the downregulation of the expression of the target gene (Elbashir et al., Nature. 2001 May 24;411(6836):494-8; "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells."). Furthermore, RNAi can be used for therapeutic

applications, diagnostic and target validation (EP Patent n°1144623 ; EP Patent 1214945; EP Patent 1352061; US Patent 5898031; US Patent 6107094).

The term "siRNA" as used herein means a double stranded short interfering RNA molecule, designed in vitro, not larger than about 23 nucleotides in length, and
5 preferably of 19-23 nucleotides in length. The scientific literature describes siRNA as mediating the sequence specific degradation of a target mRNA.

The term "miRNA" as used herein means endogenous 15-35 nucleotides, preferably 19-30 nucleotides, most preferably 21-24 nucleotides small RNAs processed from
10 stem-loop RNA precursors (pre-miRNA). miRNA can downregulate gene expression by two posttranscriptional mechanisms: mRNA cleavage or translational repression.

The term "Stem-loop" as used herein means a single stranded polynucleotide including two complementary base sequences separated by a sequence with no
15 complementary base sequence thereof and that allow formation of a duplex structure in the corresponding polyribonucleotide, with a single-stranded loop sequence linking two annealed base sequences. The complementary base sequences forming the stem portion of the stem-loop consist of at least two and more preferably at least three base pairs in length. In particular embodiments, where the stem base pair(s)
20 correspond to the complementing sequence of the first and third sequences, one or more of the second sequence complementing stem base pairs can double as a first and third sequence complementing base pair. Under these special circumstances, the stem portion of the second sequence would be considered to contain only one complementing base pair, or no complementing base pairs.

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Preferred stem-loop sequences are based on stem-loop regions known to those persons skilled in the art to be present in RNA molecules such as tRNA, snRNA, rRNA, mtRNA, or structural RNA sequences. One skilled in the art can readily identify stem-loop RNA structures using predictive computer modelling programs such as
30 Mfold (M. Zuker, D. H. Mathews & D. H. Turner Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide In RNA Biochemistry and Biotechnology, 11-43, J. Barciszewski & B. F. C. Clark, eds, NATO ASI Series, Kluwer Academic Publishers, (1999)), RNAstructure (Mathews, D. H.; Sabina, J.; Zuker, M.; and Turner, D. H. , "expanded sequence dependence of thermodynamic

parameters improves prediction of RNA secondary structures", Journal of Molecular Biology, 1999,288, 911-940), RNAfold in the Vienna RNA Package (Ivo Hofacker, Institut fur theoretische Chemie,Wahringerstr. 17, A-1090 Wien, Austria), Tinoco plot (Tinoco, I. Jr. , Uhlenbeck,O. C. & Levine, M. D. (1971) Nature 230, 363-367),

5 ConStruct, which seeks conserved secondary structures (Luck, R. , Steger, G. & Riesner, D. (1996), Thermodynamic prediction of conserved secondary structure: Application to RRE-element of HIV, tRNA-like element of CMV, and mRNA of prion protein.J. Mol. Biol. 258, 813-826; and Luck, R. , Graf, S. & Steger, G. (1999), ConStruct : A tool for thermodynamic controlled prediction of conserved secondary

10 structure. Nucleic Acids Res. 21, 4208- 4217), FOLDALIGN, (J. Gorodkin, L. J. Heyer and G. D. Stormo. Nucleic Acids Research, Vol. 25, no. 18 pp 3724-3732,1997a ; and J. Gorodkin, L. J. Heyer, and G. D. Stormo. ISMB5; 120- 123,1997b), and RNAdraw (Ole Matzura and Anders Wennborg Computer Applications in the Biosciences (CABIOS), Vol. 12 no. 3 1996, 247-249).

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The term "compound" as used herein (e.g., as in "test compound") is meant to include exogenously added test compounds. The test compound may be contacted with a cell. Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules and

20 natural product extract libraries. Both agonizing and antagonizing compounds are intended to be screened, identified or selected in the present invention.

The term "active compounds" as used herein refers to pharmaceutically effective compounds that specifically interact with, and modulate, the activity of a cellular

25 receptor. This term is equivalent to ligand.

The term "adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477" denotes adenylyl cyclase isoform(s) that can be potentially stimulated by forskolin or NKH-477.

30 The term "in vitro activator of cAMP formation" as used herein refers to forskolin that directly activates almost all the adenylyl cyclase isoforms and forskolin derivatives, in particular, colforsin daropate hydrochloride (6-(3-dimethylaminopropionyl) forskolin hydrochloride also named NKH-477 that is a selective potent activator of adenylyl cyclase isoforms V and VI.

As used herein, the term "effective amount" refers to an amount of a modulator compound, or pharmaceutical composition comprising at least one such compound, that is sufficient to achieve an intended purpose. For example, the intended purpose
5 may be to inhibit or enhance the cAMP accumulation or adenylyl cyclase activity in the whole organism of a subject or in (s) particular tissue(s).

BRIEF DESCRIPTION OF THE DRAWINGS

10 Figure 1 shows Western Blots characterising Gai protein in CHO cell.

Figure 2 shows an example of a targeted sequence of SEQ ID N° 10 (ACVII_miR_173) and the miRNA construction to be inserted into the plasmid.

15 Figure 3 depicts blots that show the suppressive effect of different miRNAs on the expression of adenylyl cyclase VII in CHO-K1 cells.

Figure 4 confirms the suppressive effect of different miRNAs on the expression of adenylyl cyclase VII by using confocal fluorescence microscopy.

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Figure 5 shows the dose-response curves characterising the measurement of cAMP induced by acetylcholine and quimpirole.

Figure 6 illustrates the effect of Oxotremorine on the production of cAMP induced by
25 Forskolin in two cell lines (M4/CHO & M4/CHO/miRNA)

Figure 7 illustrates the dose dependent signal of the production of cAMP induced by Forskolin or NKH-477

30 DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a reliable and effective in vitro cAMP based assay for Gi-coupled receptors expressed in a selected cell for screening and identifying

pharmaceutically effective compounds that specifically interact with and modulate the activity of a cellular receptor.

As set forth above, observation of the modulation of cAMP levels in a cell by Gi-coupled receptors requires traditionally the prior treatment of the cell with forskolin, an activator of adenylyl cyclase isoenzymes. Taking into account that the cAMP response upon activation is dependent on the cellular isoenzyme pool present in a particular cell or cell line, non specificity of the forskolin mediated adenylyl cyclase isoforms stimulation, required to detect the G α i effect, may significantly contribute to the lack of efficient inhibition of cAMP production mediated by agonists of Gi-coupled receptors. As a consequence, the resulting agonist mediated inhibition rarely exceeds 60 % of the stimulation by forskolin. This renders difficult to then measure the reversal by an antagonist of the cAMP decrease mediated by a receptor agonist.

The applicant has shown that one way to efficiently improve the agonist inhibition of the cAMP production mediated by an in vitro activator of cAMP formation, and therefore to favour easy quantification of an antagonist activity through the reversal of an agonist effect in a particular cell expressing Gi-coupled receptors, is to favour the influence of adenylyl cyclase stimulated by an in vitro activator of cAMP formation and sensitive to G α i protein.

Accordingly, resulting detection of the Gi effect is mainly associated to the specific inhibition, induced by the interaction with an agonist, of the pre-stimulated adenylyl cyclase isoform activity sensitive to the G α i family.

Accordingly, in a first aspect, the invention provides a method for screening, identifying or selecting test compounds using an in vitro assay based on cAMP levels modulated by Gi-coupled receptors expressed in a cell comprising the steps of:

- (a) optionally identifying, in a selected cell, the G α i and adenylyl cyclase isoforms which are involved in the modulation of the intracellular cAMP levels,
- (b) favouring the influence of adenylyl cyclase isoform(s) (A) stimulated by forskolin or NKH-477 and sensitive to inhibition by G α i relative to

- adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 but not sensitive to inhibition by Gai in said selected cell,
- (c) contacting said cell with forskolin or NKH-477 and a test compound; and
 - 5 (d) measuring cAMP levels resulting from contacting said test compound with said cell.

Selection of the most appropriate cells comprises both identification of Gai protein and adenylyl cyclase isoforms which are involved in the modulation of intracellular
10 cAMP levels. Indeed, it has been shown that the expression pattern of the different adenylyl cyclase isoforms is cell- or tissue-specific. Accordingly, for the cells for which no data is available in the literature on the expression of adenylyl cyclase isoforms, a first step of identification of the adenylyl cyclase isoforms expressed in the specific cell type selected for use in the invention must be implemented.

15

To date, among the cloned mammalian adenylyl cyclase, all except isoform IX have been identified as stimulated by forskolin. Furthermore, it has been shown that NKH-477 is a selective potent activator of adenylyl cyclase isoforms V and VI. In addition, only adenylyl cyclase I, V and VI are sensitive to the Gai family.

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The choice of an appropriate cell line is influenced by the presence or absence of particular adenylyl cyclase. The selected cell should express at least one adenylyl cyclase isoform shown to be activated by forskolin or NKH-477 and sensitive to inhibition by Gai proteins. In another embodiment, the cell used in the method of the
25 present invention contains only adenylyl cyclase isoforms activated by forskolin or NKH-477 and sensitive to inhibition by Gai proteins, and no adenylyl cyclase isoform activated by forskolin or NKH-477 and insensitive to inhibition by Gai proteins.

An embodiment of the invention consists in selecting cells from the group consisting
30 of natural cells and established cell lines expressing endogenous Gai protein and adenylyl cyclase isoforms.

In another embodiment of the invention, the established cell lines express cloned Gai protein and/or adenylyl cyclase isoforms.

In a particular embodiment, the host cell is a eukaryotic cell selected among a mammalian cell, a yeast cell or an established cell line selected among CHO, HEK or the insect SF9 lines. In a preferred embodiment the host cell is the CHO-K1 or CHO-S line.

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In a preferred embodiment, the cells express a number of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 but not sensitive to inhibition by Gai protein comprised between 1 and 4, more preferably 4, more preferably 3, more preferably 2 and most preferably 1.

- 10 In case adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 but not sensitive to inhibition by Gai are expressed in the selected cell, they must be inactivated in order to overcome the problem encountered in the methods described in the state of the art, i.e. the poor induction of inhibition of cAMP production mediated by Gi-coupled receptors as a consequence of the activation by forskolin or
- 15 NKH-477 of a number of adenylyl cyclases, including those adenylyl cyclases which are stimulated by forskolin or NKH-477 but insensitive to inhibition by Gai proteins.

Alternatively, the selected cells may correspond to a cell type in which no Gai protein and/or no adenylyl cyclase isoform is naturally expressed. In this case, exogenous

20 expression of given isoform(s) of the aforementioned isoforms can be obtained by, for example, transient or stable transfection of vectors comprising polynucleotides coding for the specific protein of interest to express.

- In a particular embodiment of the invention, step b) consists of favouring the
- 25 influence of adenylyl cyclase isoform(s) (A) stimulated by forskolin and sensitive to inhibition by Gai relative to adenylyl cyclase isoform(s) (B) stimulated by forskolin but not sensitive to inhibition by Gai.

- In another particular embodiment, favouring the influence of adenylyl cyclase
- 30 isoform(s) (A) stimulated by forskolin or NKH-477 and sensitive to inhibition by Gai relative to adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 but not sensitive to inhibition by Gai is achieved by contacting the selected cell with NKH-477. In this embodiment, step c) is achieved by contacting said cell with a test compound, but not with forskolin. Indeed, adenylyl cyclase isoform(s) (A) stimulated by NKH-477

and sensitive to inhibition by Gai have already been stimulated in step b), as NKH-477 is a selective potent activator of specific adenylyl cyclase isoforms, as mentioned above, such that stimulation of the production of cAMP in the selected cell achieved in step c) is no more necessary.

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Accordingly, when step b) is achieved by contacting the selected cell with NKH-477, step b) and c) may be performed either successively or simultaneously.

The applicant has shown that the use of the compound NHK-477 instead of forskolin,
10 as an in vitro activator of cAMP formation, has surprisingly resulted in the increase of the production of cAMP up to 20% in the cells expressing the adenylyl cyclase isoform V and/or VI.

Accordingly, in a particular embodiment, step b) of the method of the invention is
15 achieved by specifically stimulating adenylyl cyclase isoform(s) V and/or VI with NKH-477.

In another particular embodiment, favouring the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to inhibition by Gai
20 relative to adenylyl cyclase isoform(s) sensitive to forskolin or NKH-477 but not sensitive to inhibition by Gai is achieved by specifically inactivating the adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 but not sensitive to inhibition by Gai protein. Inactivation of such Gai-insensitive adenylyl isoform(s) can be achieved, for example, by inhibiting specifically their catalytic activity or preferably by
25 downregulating their expression.

The term "specifically inactivating the adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 but not sensitive to inhibition by Gai protein" denotes the preferential, and preferentially exclusive, inactivation of the adenylyl cyclase
30 isoform(s) stimulated by forskolin or NKH-477 but not sensitive to inhibition by Gai protein relative to the adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to inhibition by Gai.

While modifying gene expression in a cell in the method of the present invention, one must take care to carry out such modification without affecting cell viability.

The nucleic acid molecules can be inserted into a construct which can, optionally,
5 replicate and/or integrate into the mammalian cell, by known methods to those skilled in this art. In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA can be used. Such techniques are well known and are explained in details in, for example, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring
10 Harbor Laboratory Press, Cold Spring Harbor, New York; DNA Cloning : A Practical Approach, Volumes I and II, 1985 (D. N. Glover ed.) ; Oligonucleotide Synthesis, 1984, (M. L. Gait ed.) ; Nucleic Acid Hybridization, 1985, (Hames and Higgins); Transcription and Translation, 1984 (Hames and Higgins eds.) ; Animal Cell Culture, 1986 (R. I. Freshney ed.) ; Immobilized Cells and Enzymes, 1986 (IRL Press); Perbal,
15 1984, A Practical Guide to Molecular Cloning; the series, Methods in Enzymology (Academic Press, Inc.) ; Gene Transfer Vectors for Mammalian Cells, 1987 (J. H. Miller and M. P. Calos eds., Cold Spring Harbor Laboratory); and Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively).

20 Step (b) can be achieved in various ways. In an exemplary embodiment, favouring the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to inhibition by Gai relative to adenylyl cyclase isoform(s) sensitive to forskolin or NKH-477 but not sensitive to inhibition by Gai is achieved by increasing the expression of adenylyl cyclase isoform(s) gene(s) stimulated by forskolin or NKH-
25 477 and sensitive to inhibition by Gai protein.

In a further embodiment of the invention, any type of genes from the natural cells or established cell lines identified as interfering with Gi-coupled receptor specific pathways can be upregulated or downregulated to favour these specific pathways. In
30 addition, any exogenous gene not present in the selected cell line expressing Gi-coupled receptor(s) of interest and susceptible to enhance the adenylyl cyclase isoforms response to Gi in response to a test compound can be expressed or co-expressed with other endogenous selected genes or any type of oligonucleotides including siRNA or miRNA in order to improve the assay.

It is well known that certain agonists can demonstrate high affinity for G-coupling receptor states due to the promotion of G protein coupling. In many natural receptors systems, the amount of G protein available for formation of such complexes is not
5 limiting and the complete receptor population can be converted into a high affinity ternary complex by an efficacious agonist (Kenanin, T., *TiPS*, 1997, 18, 456-464). However, in some systems including, for example mammalian cell lines, the availability of the required G proteins as Gi proteins subtypes is limiting. On the other hand, in the context of the invention, the pre-required stimulation of the endogeneous
10 identified adenylyl cyclase isoforms by forskolin or NKH-477 may limits the extent of inhibition by Gai (Dessauer, C., W., *The Journal of Biological Chemistry*, 2002, 277, 32, 28823-28829). In other hands, according to the invention, one can develop an established stable host cell line in which it is possible to co-express in the same plasmid, specific miRNAs and a specific Gi subtype protein in order to re-enforce the
15 Gai inhibition of the forskolin or NKH-477 and/or Gas stimulated adenylyl cyclases. To illustrate this, CHO-K1 and CHO-S stably expressing adenylyl cyclase VII specific miRNAs can be used to develop relevant and robust cAMP based cellular assays expressing GPCR coupling the specific Gi protein by co-expressing, preferably in the same plasmid, the specific adenylyl cyclase isoform VII miRNA and a specific Gai
20 protein specific subtype.

In one embodiment of the present invention the selected cells express the Gi protein of interest endogenously. In yet other embodiments, the cells are engineered to express a heterologous Gi protein. In both cases, it may be desirable to inactivate
25 one or more endogenous genes of the host cells.

According to the method of the present invention Gai proteins are selected among the $G_{i/0}$ subfamily that inhibits adenylyl cyclase and include G_{i1} , G_{i2} , G_{i3} , G_{01} , G_{02} , G_{03} , G_Z , G_{t1} , G_{t2} and G_{gust} .

30

A large number of well-known vectors can be used in the invention for expression in a variety of eukaryotic hosts. For example, the Gai protein or Gi-coupled receptors may be expressed by transfecting well-known mammalian expression vectors.

One skilled in the art has at his disposal a number of techniques for identifying the expression of a given protein. For example, the identification of adenylyl cyclase isoforms expressed in the selected cell can be performed by PCR, preferably RT-PCR amplification using degenerate primers. Alternatively, one can use western-blot analysis to identify which adenylyl cyclase isoform(s) are expressed in the selected cell by using antibodies specific of each isoform.

The different types of adenylyl cyclase identified to date share a common structure. Each has two domains, consisting of six putative membrane-spanning regions separated by a large cytoplasmic loop, as well as a large cytoplasmic loop at the carboxyl terminus. The different isoforms of adenylyl cyclase are reviewed in Taussig R, Gilman AG. (1995) Mammalian membrane-bound adenylyl cyclases. *J Biol Chem* 270 and Sunahara RK, Dessauer CW, Gilman AG. (1996) Complexity and diversity of mammalian adenylyl cyclases. *Annu Rev Pharmacol Toxicol* 36, 461-80.

Various methods are known in the art for identification of nucleic acids having sequence similarity, based on the ability of single stranded nucleic acids, e.g. DNA or RNA oligo- or polynucleotides, to form a double stranded complex by complementarity. A sample may be screened for the presence of specific target nucleic acids capable of forming double stranded complexes with, for example, an adenylyl cyclase probe, in particular under low stringency conditions. "Sample" as used herein includes but is not limited to genomic libraries, cDNA libraries, nucleic acid molecule extracts from tissue, or nucleic acid molecule extracts from cell culture.

After complex formation, the target nucleic acid molecule is isolated by methods known in the art. Methods include the polymerase chain reaction (PCR) or reverse transcriptase PCR (RT-PCR), where two probes specific of a target nucleic acids sequence are used to amplify a region of the sample DNA or cDNA. PCR may also be performed with a single adenylyl cyclase probe, and a second oligonucleotide complementary to a known genomic repeat sequence.

Generally the adenylyl cyclase probe will be labelled with a detectable marker, e.g. ^{32}P , ^{35}S , biotin, FITC, etc. when used in a northern or southern blot.

Nucleic acids used in the invention may be isolated directly from well-known cells. Alternatively, the polymerase chain reaction (PCR) method can be used to produce the nucleic acids of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the
5 sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

Multiple sequences from a target mRNA can be selected in order to identify a target
10 sequence which is specific for a particular mRNA. Sequence alignments with other mRNA sequences that have high homology to the target mRNA are used. If the goal is to identify in a selected cell, Gai or adenylyl cyclase isoforms, gene homology technique can be used. In the particular case of the present invention, sequences of Gi proteins or adenylyl cyclase isoforms are available from Genbank. For example
15 the mouse adenylyl cyclase VI gene (NM_007405) can be used to identify the homologue hamster gene in CHO cells.

The invention comprises the use of natural cells or established cell lines in which the expression of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 but not
20 sensitive to inhibition by Gai can be down regulated by any type of approaches. Particularly preferred cells are those in which RNAi technique can be implemented in order to downregulate the expression of given gene. This technique includes the design, the selection and then the use of adapted RNAi as microRNA (miRNA).

25 Inactivation by RNAi may be obtained either by transiently transfecting adapted RNAi such as siRNA or miRNA specific for a given target gene to be inactivated or by transfecting, either transiently or stably, an expression vector coding such siRNA or miRNA.

30 In a particular embodiment, expression of a miRNA is desired. The expression of miRNA may be obtained with DNA-based vectors including engineered pre-miRNA primary transcripts. Designed polynucleotide sequences can be inserted for expression in an eukaryotic cell vectors such as, for example, pSilencer 4.1-CMV (Ambion), pRc/RSV, pCMV5 or pRc/CMV2, pRNA-CMV3.1 (GenScript Corporation),

or U6 promoter derived vectors such pRNA-U6.1 (GenScript Corporation) or pSilencer 2.0-U6 (Ambion), or Hi promoter derived vectors such pRNA-H1.1 (GenScript Corporation) and pSilencer 3.0- H1 (Ambion).

- 5 In a particular embodiment of the invention, the designed polynucleotides for expressing miRNA includes a Drosha cleavage site to the hairpin construct, which has been shown to greatly increase knockdown efficiency (Boden *et al* 2004, Silva *et al* 2005).
- 10 Advantageously, the hairpin may consist of 19 to 22-nt of dsRNA and a 19-nt loop from human miR30. Furthermore, in another embodiment of the invention, the miR30 loop and/or 125-nt of miR30 flanking sequence on either side of the hairpin results in greater than 10-fold increase in Drosha and Dicer processing of the expressed hairpins when compared with conventional shRNA designs without miRNA
- 15 adaptations (Silva *et al* 2005). This technique will be further detailed in the examples section.

In certain embodiments the method described herein is used for identifying active compounds for "orphan receptors" for which no ligand is known.

20

In a particular embodiment of the invention, the method enables rapid screening or profiling of large numbers of test compounds to ascertain their agonist or antagonist activity with respect to Gi-coupled receptors expressed in a cell.

- 25 Test compounds are screened, identified or selected on the basis of the result of step d) of the method of the invention. Specifically, test compounds which are screened, identified or selected by the method of the invention are those that result in the modulation of cAMP levels in the selected cell, relative to a measure performed according to the method of the invention in the absence of said test compound.

30

The test compound can interact with a receptor protein which can be any receptor which interacts with said test compound to modulate a signal in the cell. For example, the target receptor may be a cell surface receptor. It must be understood that the method of the invention is a screening method which allow the rapid a reliable

identification of compounds potentially having the ability to modulate Gi-coupled receptors such as:

[alpha]2-adrenergic receptor, [alpha]2B-adrenergic receptor, [beta]2-adrenergic
5 receptor, m2 AChR, m4 AChR, D2 dopamine receptor, D3 dopamine receptor, D4
dopamine receptor, A1 adenosine receptor, 5-HT1a receptor, 5-HT1b receptor,
5HT1-like receptor, 5-HT1d receptor, 5HT1d-like receptor, 5HT1d beta receptor,
fMLP receptor, fMLP-like receptor, somatostatin SSTR1 and SSTR2, SSTR3,
cannabinoid receptor (CB1/CB2), C5a anaphylatoxin receptor, Interleukin 8 (IL-8) IL-
10 8RA, IL-8RB, Delta Opioid receptor, Kappa Opioid receptor, mip-1/RANTES receptor,
-Rhodopsin, Red opsin, Green opsin, Blue opsin, metabotropic glutamate mGluR1-6,
ATP receptor, neuropeptide Y receptor.

In a particular embodiment, the invention provides a method for screening, identifying
15 or selecting test compounds potentially having a Gi-coupled receptor modulating
activity in a selected cell by implementing the above described method steps. All Gi-
coupled receptors must physically couple to Gi proteins in order to transduce
extracellular stimuli into intracellular signals, i.e. the modulation of cAMP levels that
lead to functional responses. Accordingly, as to further determine which compounds
20 identified in the method of the invention are ligands of Gi-coupled receptors, further
characterisation may be necessary for eliminating those compounds which act on the
level of cAMP in the cell by secondary interfering pathways. This characterisation can
be achieved, for example, by competitive assays on known Gi-coupled receptors in
order to determine which receptor the identified test compounds bind.

25

According to the present invention, screening or profiling can be performed in a high
throughput mode.

In a particular embodiment of the method of the invention, the potential effects of the
30 test compound on Gi-coupled receptors is carried out by comparing cAMP levels in
cells treated by forskolin or NKH-477 and either treated or untreated by the test
compound.

Measurement of intracellular cAMP may be accomplished by well-known in the art detection assays. Among them one can use, for example, the most common automatable technologies used for HTS as SPA (Amersham Biosciences), Flashplates and AlphaScreen (Perkin Elmer life and Analytical Sciences),
5 fluorescence polarisation (Perkin Elmer lifesciences, Amersham Biosciences), DiscovereX/HitHunter, β -Gal EFC (DiscoverX/GE healthcare), Time resolved-FRET (Cisbio International, Perkin Elmer Lifesciences), Electrochemiluminescence (Meso Scale Discovery), CatchPoint (Molecular Devices) ACTOne cAMP assay (BD Biosciences).

10

Said assays may be, for example, homogeneous or heterogeneous assays including sandwich assays.

In another embodiment of the present invention, measurement of intracellular cAMP
15 level modulation induced by test compounds can be performed by measurement of the changes in intracellular cAMP levels through technologies using radioactivity, colorimetry, fluorescence or luminescence.

In a particular embodiment of the invention, selected cells are disposed in a plurality
20 of wells of a multi-well assay plate. The cells may be, for example, incubated in some wells of the assay plate with a test compound under conditions and for a time sufficient to allow equilibration. The test is then read, for example, with a labelling approach that combines the time-resolved fluorescence (TRF) technique and the fluorescence resonance energy transfer (FRET) technique. FRET refers to a non-
25 radiative energy transfer of excitation energy between suitable donor and acceptor fluorophores.

The amount of fluorescence read is proportional to the adenylyl cyclase activity induced by active compounds, in effective amount, that specifically interact with and modulate the activity of a cellular receptor.

30

Cells to be used in the inventive assays may be cultured according to standard cell culture techniques. For example, cells are often grown in a suitable vessel in a sterile environment at 37°C in an incubator containing a humidified 95% air - 5% CO₂ atmosphere. Vessels may contain stirred or stationary cultures. Various cell culture

media may be used including media containing undefined biological fluids such as fetal calf serum, as well as media which are fully defined, such as 293 SFM serum free medium (Invitrogen Corp., Carlsbad, CA). Cell culture techniques are well known in the art and established protocols are available for the culture of diverse cell types
5 (see, for example, R.I. Freshney, "Culture of Animal Cells: A Manual of Basic Technique", 2nd Edition, 1987, Alan R. Liss, Inc.)

In a preferred embodiment, the invention comprises implementing CHO-S or CHO-K1 cells in which adenylyl cyclase VII expression is knocked-down in the method
10 described above for screening, selecting or identifying test compounds.

A further aspect of the invention relates to an assay system or a kit for measuring the modulation of cAMP levels by test compounds that specifically interact with and modulate the activity of a cellular receptor, said assay system or kit comprising cells
15 which mainly express adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to inhibition by Gai.

The term "mainly express" as used herein denotes the more prevalent influence of Adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to
20 inhibition by Gai relative to Adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 but not sensitive to inhibition by Gai, in selected cells, as set forth above.

In a particular embodiment, the assay system or kit of the invention comprises cells in which the expression of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-
25 477 but not sensitive to inhibition by Gai has been inactivated by specific targeting by miRNA.

The assay system or kit of the present invention is implemented by contacting the cells thereof with forskolin or NKH-477 and a test compound and then measuring the
30 levels of cAMP resulting from said contacting.

In a particular embodiment, the invention provides an assay system or kit wherein said cell stably co-express i) nucleic acid sequences which code for specific Gai protein or specific adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and

sensitive to inhibition by Gα i protein (ii) and specific miRNA that target adenylyl cyclase transcripts coding for adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and not sensitive to inhibition by Gα i protein.

- 5 In another particular embodiment, the assay system or kit of the invention, comprises the recombinant cells described hereinabove.

In another aspect, the invention provides a recombinant cell engineered from a selected cell expressing both adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to Gα i and adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and not sensitive to Gα i, wherein the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to Gα i is favoured relative to the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and not sensitive to Gα i.

15

According to a particular embodiment, expression of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to Gα i is upregulated in the recombinant cell of the invention.

- 20 In another particular embodiment, adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and not sensitive to Gα i are inactivated in the recombinant cell of the invention, for example by deletion of the gene(s) coding said isoform(s) or by downregulation by RNAi.

- 25 Additionally, different ways to achieve a favoured influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to Gα i relative to the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and not sensitive to Gα i have been described hereinabove.

- 30 In a particular embodiment of the recombinant cell of the invention, said recombinant cell is transfected, preferably stably, with at least one recombinant expression vector coding for one or more miRNA specific for Adenylyl Cyclase isoform(s) expressed in the cell from which said cell is engineered. In another embodiment, said recombinant expression vector coding for said at least one or more miRNA is co-transfected with a

recombinant expression vector comprising one or more nucleic acid sequences coding for specific Gai protein or specific adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to inhibition by Gai protein.

- 5 The recombinant expression vectors transfected in the recombinant cell of the invention can be an appropriate eukaryotic expression vector, including an engineered pre-miRNA structure. Advantageously, the recombinant expression vectors may further comprise a Drosha cleavage site in the hairpin construct. The hairpin stem preferably consists of 21-22-nt of dsRNA (mature miRNA) derived from
10 the target gene and the hairpin loop may comprise, in a preferred embodiment, 19-nt.

In a particular embodiment of the present invention, a recombinant cell line is engineered which co-express a heterologous Gai protein and specific miRNA that target adenylyl cyclase transcripts coding for adenylyl cyclase isoform(s) stimulated
15 by forskolin or NKH-477 and not sensitive to inhibition by Gai protein.

In a particular embodiment, the recombinant cell of the invention is a cell stably transformed with a recombinant DNA vector comprising (i) engineered pre-miRNA primary transcripts, ii) nucleic acid sequences which code for a specific Gai protein or
20 a specific adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to inhibition by G α i protein, stably co-expressed in said recombinant cell.

In a particular embodiment, the recombinant cell of the invention is a cell engineered to co-express a specific adenylyl cyclase isoform(s) stimulated by forskolin or NKH-
25 477 and sensitive to inhibition by G α i protein and specific miRNA that target adenylyl cyclase transcripts coding for adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and not sensitive to inhibition by G α i protein.

In a particular embodiment the recombinant cell is a mammalian cell, for example of
30 the CHO cell line.

The recombinant cell according to the invention can be used as a tool in an in vitro assay based on cAMP levels modulated by Gi-coupled receptors. Accordingly, the

invention further provides the use of a recombinant cell as defined above as a tool for screening, identifying or selecting potential ligands of Gi-coupled receptors.

The use of CHO-K1 or CHO-S cells is particularly preferred. These cells are available
5 from the European Collection of Cell Cultures (ECACC), Porton Down, UK Accession Number 85051005. HEK-293 cells are available under Accession Number 85120602 (ECACC) and sf9 cells under Accession Number 89070101 (ECACC)

The invention now being generally described will be more readily understood by
10 reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

EXAMPLES

15

As example and to illustrate the invention, CHO cells (CHO-K1 and CHO-S) were selected as appropriate cell lines to establish robust and relevant cAMP based cellular assays for the Gi-coupled receptors. Characterisation of these cell lines has confirmed that only the adenylyl cyclase isoforms VI and VII are expressed in CHO.

20 As already mentioned both isoforms are activated by forskolin but only adenylyl cyclase isoform VI is sensitive to G_{ai}, adenylyl cyclase isoform VII being activated by G_{as}.

The adenylyl cyclase isoform VI is predominantly expressed in CHO cell lines (CHO-K1 or CHO-S) favouring the approach of using miRNA to knock-down adenylyl
25 cyclase VII which is stimulated by forskolin but not inhibited by activated Gi protein in these cell lines.

As illustrated herein, design of miRNA for knock-down of the expression of adenylyl
30 cyclase VII in CHO cell lines (CHO-K1 & CHO-S) has been performed. Identification of the miRNA constructs which suppress the expression of adenylyl cyclase VII in CHO cells was performed by transiently transfecting cultured CHO-K1 and CHO-S cells with simultaneously one to three different miRNA constructs and by measuring gene expression (QPCR, Western Blot) and the production of cAMP induced by

Forskolin or NKH-477 in cultured cells transfected with miRNAs. After selection of appropriate miRNA we could establish stable cell lines (CHO-K1 and CHO-S) in which the expression of adenylyl cyclase VII is suppressed by miRNA.

- 5 According to the invention, establishment of conditions in which Gi-coupled receptor specific pathways are favoured through up- or downregulation of the expression of one or more identified endogenous genes of the cell lines interfering with these specific pathways include control of the non or limited influence of the up- or downregulation of the targeted genes on the cell viability.

10

As illustrated herein for the CHO-K1 and CHO-S cell lines, characterisation of the relative abundance of the adenylyl cyclases VI and VII has been determined showing predominant expression of the isoform VI in CHO cell line. This favours the approach using siRNA or miRNA to knock-down adenylyl cyclase VII which is stimulated by

- 15 forskolin but not inhibited by activated Gi protein.

1. Characterisation of Gai proteins in CHO cell by Immunoblot

Membrane Preparation and Western Blotting. CHO-S and CHO-K1 cells were

- 20 washed in PBS 37°C, harvested in a total volume of 5 ml of PBS, 0.5mM EDTA, 0.2M NaOH. Cells were pelleted 5min at 500g at 4°C and resuspended in 3mL of a buffer containing Tris/HCl 50mM, EGTA/Tris 1mM, EDTA 1mM, Trypsin 10µg/mL, Leupeptin 1µg/mL, PMSF 75µg/mL, Pepstatin 1µM. Cells were frozen at -80°C, thawed at 37°C, and ruptured on ice with by 10 passages through a 26-gauge
- 25 needle. Membranes were centrifuged at 48000g during 20min at 4°C and resuspended in 20mM Hepes/NaOH, 150mM NaCl, Trypsin 10µg/mL, Leupeptin 1µg/mL, PMSF 75µg/mL, Pepstatin 1µM. Proteins were quantified with the Bio-Rad Protein Assay kit using bovine serum albumin (BSA) as a standard. Samples (20–40 µg of membrane protein) were resolved by SDS-polyacrylamide gel electrophoresis
- 30 (4-12%), transferred to PVDF membranes, and incubated O/N at 4°C with rabbit polyclonal anti-αi1, mouse monoclonal anti-αi2 or rabbit polyclonal anti-αi3 antibodies (Santa Cruz) diluted 1/1000 in blocking reagent 2% (manufacturer recommended conditions) (ECL Advance kit from Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Membrane was probed with secondary anti-rabbit

horseradish peroxidase conjugate (BioRad) or anti-mouse peroxylase conjugate (BioRad) diluted 1/200000 in blocking reagent 2% (manufacturer recommended conditions) (ECL Advance kit from Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Immunoreactive proteins were visualized with the ECL
5 Advance kit from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK).

Results are shown in figure 1.

Real-time PCR. Total RNA was purified from cultured CHO-K1 and CHO-S cells
10 using Tri-Reagent (Sigma). Using total RNA as template for reverse transcriptase, the first-strand cDNA was synthesized using RT-PCR kit (BD Bioscience Clontech) and random hexamers according to the manufacturer's instructions.

Real-time PCR was performed using Mix Sybr Green (Bio-Rad) in a iQ5 Multicolor
15 Real-Time PCR Detection System (Bio-Rad).

The primers used for real-time PCR are as follows:

Mm_Gnai1 QT01058687 (Qiagen) for Gai1
20 Mm_Gnai2 QT00140469 (Qiagen) for Gai2
Mm_Gnai3 QT01062278 (Qiagen) for Gai3
Mm_Gnao QT00171108 (Qiagen) for Gao
Mm_Gapd QT00309099 (Qiagen) for GAPDH

25 Thermocycling conditions consisted of denaturation at 95°C for 3 min followed by 40 cycles comprising a denaturation step at 95°C for 15 s, an annealing step at 55°C for 15 s and an extension step at 72°C for 20 s. Melting curve analysis was performed with data collection from a temperature ramp from 60°C to 95°C.

30 Results

GAPDH was used as an internal control gene for normalizing the PCRs.

The threshold cycle (Ct) was chosen as a point where amplification was in an exponential phase. ΔCt being the normalized Ct (Ct gene of interest – Ct control gene) value was used to determine relative abundance of gene expression.

- 5 There was no Gai1 amplification from neither CHO-K1 nor CHO-S cells, which does correspond to the immunoblot results.

Gai2, Gai3 and Gao gene expression was detected by real-time PCR in CHO-K1 and CHO-S cells. Table 1 shows the relative abundance of the expression of the Gai
10 protein isoforms.

Table 1

Cell lines	ΔCt (Ct gene of interest – Ct control gene)		
	Gai3	Gai2	Gao
CHO-K1	6.71	14.81	15.72
CHO-S	7.21	16.2	17.73

- 15 2. Identification of adenylyl cyclase isoenzymes expressed in CHO cell lines by PCR amplification using degenerate primers

Total RNA was purified from culture CHO-K1 and CHO-S cell using Tri-Reagent (Sigma). Using total RNA as template for reverse transcriptase, the first-strand cDNA
20 was synthesized using QRT-PCR SYBR Green kit (Abgen) and random hexamers according to the manufacture's instructions.

The degenerate primers (SEQ ID N° 1) were designed by matching conserved regions of the 9 families of mouse adenylyl cyclase as reported by Richard T.
25 Premont (Methods Enzymol. 238, 116-127)

5'-CGGCAGCTCGAGAA(A/G)AT(A/C/T)AA(A/G)AC(I)AT(A/C/T)GG-3'
(SEQ ID NO:1)

5'-CCGGGACTCGAGAC(A/G)TT(I)AC(I)GT(I)T(C/T)(I)CCCCA-3'
30 (SEQ ID NO:2)

PCR amplification of the cDNA was performed with Advantage-HF 2 PCR kit (*BD Science*) in Thermocycle Perkin-Elmer 2400.

- 5 The PCR amplification conditions consisted of denaturation at 94°C for 1 min followed by 40 cycles comprising a denaturation step at 94°C for 15 s, an annealing step at 58°C for 30 s and an extension step at 68°C for 1 min. At the completion of the cycling reaction, an additional extension step at 68°C for 7 min was performed.
- 10 PCR products (≈300 bp) were isolated by low-melting agarose gel electrophoresis and purified by GENECLAN SPIN (Bio 101). The purified fragments were ligated into pCR2.1 vector and 4 µl of the ligation mixture were transformed into TOP 10 competent cells using a T/A cloning kit (Invitrogen).
- 15 White plasmid clones were prepared with a Minipreps DNA purification system (Eppendorf) and screened by EcoRI digestion and agarose gel electrophoresis. The positive clones, fourteen clones in CHO-K1 and twelve clones in CHO-S were sequenced.
- 20 The sequences were analysed using the BLAST search. Two different sequences were identified with high homology to published adenylyl cyclases.

Sequences SEQ ID N° 3 and SEQ ID N° 4 were aligned with known adenylyl cyclase sequences and analysed with DNASIS Software (Hitachi).

25

Sequence SEQ ID N° 3 is 89% (29/258 bp) homologous to that of mouse adenylyl cyclase VI (NM_007405). The homology of the deduced amino acid sequence is 99 % (1/85 aa).

- 30 Sequence SEQ ID N° 4 is 90% (27/269 bp) homologous to that of mouse adenylyl cyclase VII (NM_007406). The homology of the deduced amino acid sequence is 95% (4/80 aa).

(SEQ ID N° 3)

AGAAGATCAAAACGATAGGCAGCACCTACATGGCTGCCTCTGGGCTAAATGCCA
 GCACCTATGATCAGGTGGGCCGTTCCCACATCACTGCCCTGGCAGACTATGCC
 ATGAGGCTCATGGAGCAGATGAAACATATCAACGAACACTCTTTCAACAATTTCC
 AGATGAAGATTGGACTGAACATGGGTCCAGTTGTGGCCGGTGTCAATTGGGGCC
 5 CGGAAGCCACAGTATGACATCTGGGGCGACACCGTCAACGTCT

(SEQ ID N° 4)

GAGAAAATAAAAACGATCGGCAGCACCTATATGGCTGCAGCAGGGCTCAGCAT
 CCCCTCAGGACACGAGAACCAGGATCTGGAGCGGCAGCATGCGCACATTGGTG
 10 TCTTGGTGGAGTTCAGCATGGCCCTGATGAGCAAGCTGGATGGCATCAACAGG
 CACTCCTTCAACTCTTTCCGCCTCCGAGTTGGCATAAACCATGGGCCTGTGATT
 GCTGGAGTGATTGGAGCACGAAAGCCTCAGTATGACATCTGGGGCGACACCGT
 CAAC

15 Table 2 shows the relative abundance of adenylyl cyclase isoforms in CHO-K1 and CHO-S cells.

Table 2

	CHO-K1	CHO-S
Number of clone for sequencing	14	12
Number of clone containing adenylyl cyclase isoform VI sequence	13	11
Number of clone containing adenylyl cyclase isoform VII sequence	1	1
VII/VI % (VII %)	1/13 (7%)	1/12 (8%)

Conclusion

Two isoforms of adenylyl cyclases are expressed in CHO cell line (CHO-K1 & CHO-S) as reported in the literature (Eva V. Varga et al, 1998. Euro J Pharmacol.348, R1-R2)

5 Predominant expression of isoform VI in CHO cell line might favour the approach using siRNA to knock-down adenylyl cyclase VII which is stimulated by forskolin but not inhibited by activated Gi protein.

Suppressing the adenylyl cyclase VII whose expression is minor in CHO cells won't influence the cell physiological function.

10

3. Amplification of adenylyl cyclase VII expressed in CHO cell lines by PCR

For siRNA (miRNA) constructions, three sets of primers were designed according to homology study between published mouse (NM_007046) and rat (XM223666)

15 adenylyl cyclase isoform VII sequence.

For amplification of the fragment corresponding to 43-805 nt of mouse adenylyl cyclase isoform VII, primers were as follows (SEQ ID NO:5 and SEQ ID NO:6)

Sense: 5'- ATGCCAGCCAAGGGGCGCTAC-3' (SEQ ID NO:5)

20 Anti-sense: 5'- AGATGCTGACATTCTGGTGC-3 (SEQ ID NO:6)

For amplification of the fragment corresponding to 628-1618 nt of mouse adenylyl cyclase isoform VII, primers were as follows (SEQ ID NO:7 and SEQ ID NO:8)

Sense: 5'-CACAAGCACCAAGCTGCAGGACGC-3' (SEQ ID NO:7)

25 Anti-sense: 5'-AGACTTGGAGCAGCAGGGCCT-3 (SEQ ID NO:8)

For amplification of the fragment corresponding to 1685-2816 nt of mouse adenylyl cyclase isoform VII, primers were as follows (SEQ ID NO:9 and SEQ ID NO:10)

Sense: 5'-TGACGAGATGCTGTCAGCCAT-3' (SEQ ID NO:9)

30 Anti-sense: 5'-TGCCAATGGTCTTGATCTTCTCC-3' (SEQ ID N°:10)

Total RNA was purified from culture CHO-K1 and CHO-S cell using Tri-Reagent (Sigma). Using total RNA as template for reverse transcriptase, the first-strand cDNA

was synthesized using QRT-PCR SYBR Green kit (Abgen) and random hexamers according to the manufacture's instructions.

5 PCR amplification of the cDNA was performed with Phusion High-Fidelity DNA polymerase (Finnzymes) in Thermocycle Perkin-Elmer 2400 as follows: 98°C for 30 sec; 40 cycles of 98°C for 10 sec, 68°C for 30 sec, 72°C for 45 sec; and 72°C for 10 min.

10 PCR products were isolated by low-melting agarose gel electrophoresis, purified by GENECLAN SPIN (Bio 101). The purified fragments were sequenced

Sequencing results:

15 PCR fragment of CHO adenylyl cyclase isoform VII corresponding to 43-805 nt of mouse adenylyl cyclase isoform VII

(SEQ ID N° 11)

GGCCCCAACCAGGCAAAGCTCTATGAGAAGTACCGCCTCACCAGCCAGCATGG
GCCACTGTTGCTCTTGCTCCTCCTGGTGGCTGTGGCCACCTGCATCGCACTCAT
20 CATCATCACCTTCAGCCATGAGGATCCCCCAGACACCAGGCTATCCTGGGCA
CTGCGTTCTTCATGCTGACGCTGTTTGTGGCTCTCTATGTGCTGGTATATGTTGA
GTGCCTGGTGCGGCGATGGCTGTGGGCCTTGGCTCTACTCACCTGGGCCTGCC
TCATGATGCTCGGCTTTGTGCTGGTGTGGGACTCCTGGAAGAATGATGCCTGTG
CGTGGGAGCAGGTGCCTTTCTTCCTGTTCAATTGTCTTTGTGGTGTATGCATTACT
25 GCCCCTCAGCACGAGGGCAGCCATCACAGTGGGGATGGTTTCCACTGTTTCCC
ACCTCCTGATGTTTGGAGCTGTGACAGGAGCCTTCAAGATGTCCGTGTCCGGA
GCACAGCTGGCGCTGCAGCTCCTGGCCAATGCCATTATCCTCCTGGGTGGGAA
CTTCACGGGTGCCTTCCACAAGCACCAAGCTGCAGGATGCTTCCCGGGACCTCT
TCATCTACACAGTCAAGTGCATCCAGATCCGCCGGAAGCTGCGTGTGGAGAAG
30 CGCCAGCAGGAGAACCTGCTGCTTTCGGTGCTCCCAGCTCACATATCCATGGG
CATGAAGCTGGCCATCATTGAGCGCCTCAAAGAGGGTGGTGACCACCGCCACA
TGCCTGACAACAACCT

PCR fragment of CHO adenylyl cyclase isoform VII corresponding to 628-1618 nt of mouse adenylyl cyclase isoform VII

(SEQ ID N° 12)

5 CTCTTCATCTACACAGTCAAGTGCATCCAGATCCGCCGGAAGCTGCGTGTGGAG
AAGCGCCAGCAGGAGAACCTGCTGCTTTTCGGTGCTCCCAGCTCACATATCCAT
GGGCATGAAGCTGGCCATCATTGAGCGCCTCAAAGAGGGTGGTGACCACCGCC
ACATGCCTGACAACAACCTTTCACAGCCTCTATGTCAAGCGGCACCAGAATGTCA
GTATCTTGTATGCAGACATTGTGGGCTTCACACGGCTGGCCAGTGACTGCTCGC
10 CCAAGGAGCTGGTGGTGGTACTCAACGAGCTGTTTCGGGAAATTTGACCAGATT
GCCAAGGCCAATGAGTGCATGCGGATCAAGATCCTGGGTGACTGCTACTACTG
TGTGTCAGGCCTGCCTGTGTCGCTGCCACCCATGCCCGCAACTGCGTGAAGA
TGGGTCTGGACATCTGTGAGGCCATTAAGCAGGTTTCGTGAGGCCACAGGAGTG
GACATCAGCATGCGTGTGGGTATCCACTCCGGGAATGTTCTGTGTGGGGTCAT
15 CGGGCTCCGCAAGTGGCAGTATGATGTGTGGTCCCATGATGTGTCCCTGGCCA
ACAGGATGGAGGCAGCTGGAGTCCCTGGCCGGGTGCACATCACAGAGGCGAC
ACTGAACCACCTGGACAAGGCATACGAGGTGGAGGATGGGAACGGGCAGCAG
CGTGACCCCTATCTGAAAGAGATGAACATCCGAACCTACCTGGTGATCGACCCC
CGGAGCCAGCAGCCGCCTCCACCCAGCCATCACCTCCCCAAGCCCAAGGGGG
20 ATGCAACCCTGAAGATTCGGGCTTCAGTGCGTGTAACGCGCTATCTCGAGTCCT
GGGGGGCAGCACGGCCCTTCGCACACCTCAACCACCGGGAGAGTGTGAGCAG
CAGCGAGACCCCCAGTCCCACTGGACAGAGGCCAAAAGCCACTCCTCTGCGCC
GACACCGTGCCATTGACAGGAGTACATCCCCCAAGGGGCGCTTGGAGGATGAC
TGTGATGACGAGATGCTCTCGGCCAT

25

PCR fragment of CHO adenylyl cyclase isoform VII corresponding to 1685-2816 of mouse adenylyl cyclase isoform VII

(SEQ ID N° 13)

30 CCTGCTGCTCCAAGTCCGATGACTTCCACACCTTTGGTCCCATCTTCCTGGAGA
AGGGCTTTGAGCGTGAGTATCGCCTGGTGCCTATCCCCGGGCTCGCTACGAC
TTTGCCTGTGCCAGCCTTGTCTTCATCTGCATCCTGCTTGTCCACCTTCTTGTGA
TGCCCAGGATGGCCACTCTGGGCGTGTCTTTGGACTGGTGGCCTGCCTGCTG
GGGCTGGTGCTGAGTTTCTGCTTTGCTACTGAGTTCTCAAGGTGCTTTCCAGCC

CGAGGTACACTCCAGGCCATCTCGGAGAGTGTGGAGACACAGCCCCtGCTCAG
 GCTATCCTTGGTTGTGCTGACTGTTGGTAGCCTACTGACTGTTGCCATCATCAA
 CATGCCACTGATGCTTAGCCCAGGCCCAGAGTGGCCAGGAGGCAATGAGACAA
 GCTCGCTGGCTGCAAGGAATAGAGTTGGAATCCCGTGTGAGCTCCTCCTGTACT
 5 ACACCTGCAGCTGCATCCTGGGCTTCATCTCGTGCTCCGTTTTTCCTGCGGATGA
 GCCTGGAAGTGAAGGCCCTGCTGCTGACAGTGGCCTTGGTGGCTTACCTGCTG
 CTCTTCAACCTCTCCCCCTGCTGGCATGTTCCAGGCAACAGCACTGAAACCAAC
 AGTACACACAGGACACAAACACTCCTGCCTGCTGGACAAAGCACACCCAGCCA
 CACCCTTGCTCTGGGGGCTCAGGTGACTGCCTCCTCCCCTGGCAGATGTTTAG
 10 AGAAAGATCTGAAGACCATGATCAACTTCTACCTGGTCCTGTTCTATGCCACCCT
 CATCTTGCTGTCTAGACAGATTGACTACTATTGCCGCTTGGATTGTCTGTGGAAG
 AAGAAGTTCAAGAAAGAGCATGAGGAGTTTGAAACAATGGAGAATGTGAACCGC
 CTCCTCCTGGAAAATGTTCTGCCAGCGCATGTGGCTGCCCACTTCATTGGCGAC
 AAGGCGGCTGAGGACTGGTACCATCAGTCTTATGACTGTGTCTGTGTCATGTTT
 15 GCCTCGGTTCCGGACTTCAAAGTGTTCTACACTGAGTGCGATGTCAACAAAGAA
 GGGTTGGAGTGCCTTCGCCTGCTGAATGAGATCATTGCTGATTTtGACGAGCTG
 CT

The sequences SEQ ID N° 11, SEQ ID N° 12 and SEQ ID N° 13 were aligned with
 20 known adenylyl cyclase sequences and analysed with DNASIS Software (Hitachi)
 and used as the templates for siRNA(miRNA) design.

4. miRNA design

25 Using the Invitrogen BLOCK-iT™ RNAi Designer, eight miRNAs (SEQ ID N° 14 to 21)
 were synthesized and cloned into pcDNA6.2-GW/miR vector (Invitrogen). Figure 2
 shows an example of the construction.

In order to demonstrate reduction of the target RNA, CHO-K1 cells were transfected
 30 with an appropriate vector.

The miRNA targeted sequences for knock-downing CHO- adenylyl cyclase isoform
 VII (noted ACVII_miR_NNN) are as follows:

- ACVII_miR_173 : ACAACTTTCAGCCTCTATG (SEQ ID N° 14)
- ACVII_miR_220 : TGGTGCTGTTTCTGCTTTG (SEQ ID N° 15)
- 5 ACVII_miR_456 : TGACAGGACTTCAAGATGT (SEQ ID N° 16)
- ACVII_miR_553 : TATGATGTGGTCCCATGAT (SEQ ID N° 17)
- ACVII_miR_619 : TTCCAGGCCAGCACTGAAA (SEQ ID N° 18)
- 10 ACVII_miR_1071 : AAAGAAGGTGGAGTGCCTT (SEQ ID N° 19)
- ACVII_miR_2102 : TCCTCCTGCTACACCTGCA (SEQ ID N° 20)
- 15 ACVII_miR_2260 : AGCACTGACCAACAGTACA (SEQ ID N° 21)

5. Identification of miRNA which suppress the expression of adenylyl cyclase VII in CHO cells.

20 Transfection

CHO-K1 cells were plated in a 6 wells plate, 500 000 cells per well. 24 hours after plating, the cells were washed by serum-free DMEM medium. The ratio between miRNA and Lipofectamine 2000 for constructing miRNA/lipid complex was 1:2.5. The

25 quantity of miRNA used for transfection was 1.0 µg per well for each construct and miRNA negative control prepared from Block-iT Pol II miRNAi kit (invitrogen). 6 hours after transfection, the medium was changed to DMEM with 5% dialysed serum. 48 hours later, total RNA was prepared using TriReagent (Sigma). cDNA was synthesized by RT-PCR kit (Clontech).

30

Using conventional polymerase chain reaction to quantify adenylyl cyclase VII messenger RNA in the cultured cells treated by miRNAs.

Figure 3 shows the suppressive effect of different miRNAs on the expression of adenylyl cyclase VII by Polymerase Chain Reaction (PCR) at 48 hours after transient transfection in CHO-K1 cells and the morphologies of CHO-K1 cells transfected by miRNA 173 and control miRNA.

5

Figure 4 confirms the suppressive effect of different miRNAs on the expression of adenylyl cyclase VII by using confocal fluorescence microscopy.

Conclusion:

10

1: miRNA 173 inhibits more than 90% of expression of adenylyl cyclase VII in CHO-K1 cells after 48 hours of transient transfection.

2: No significant morphologic difference between the cells treated by miRNA 173 or negative miRNA has been observed.

15

6. cAMP assay

cAMP measurements on CHO-K1 cells that stably expressed the human muscarinic M4 or the human dopamine D4.4 receptor were performed via the HTRF (homogeneous time-resolved fluorescence) technology (CIS Bio, France). The principle of these cAMP assays is based on a competition between cAMP produced by cells and cAMP-XL665 conjugate for the binding with monoclonal anti-cAMP-cryptate conjugate. The HTRF® conjugates are prepared in lysis buffer so that the cell lysis and the detection step can be performed simultaneously.

25

Preparation of cells and compounds. CHO-K1 stably expressing the human muscarinic M4 or the human dopamine D4.4 receptor were cultured under standard conditions. Cells were harvested by EDTA, washed once in phosphate buffered saline and resuspended in assay buffer (HBSS containing 0.1% glucose and 0.5 mM IBMX). The cells were dispensed into a black 96 half-well plate at a cell density of 10,000 – 15,000 cells. After incubation for 30 min at room temperature or at 37°C, an equal volume of assay buffer containing forskolin, then acetylcholine or quimpirole was added to the cells. After incubation for 30 min at room temperature or 37°C, cAMP detection was performed following the protocol described above. The plate

30

was incubated for one more hour at room temperature and then measured on a RUBYstar^a (BMG Labtechnologies) time-resolved fluorescence reader.

Experimental conditions

5 To measure the production of cAMP from cells, cells were harvested and resuspended with HBSS Buffer. Varying concentrations of cells were plated in a 96-well plate. Serial dilutions of agonists (Dopamine or Epinephrine) were added and incubated for 10' at room temperature with the cells. Forskolin or NKH477 were
10 added, and the plate was incubated at 37°C for 10 minutes. After cell induction, the assay reagents were added as outlined in the assay procedure.

For the D4 – CHO K1 cell line (D4 stably transfected in CHO K1 host cell line) 0.7µM of NKH477 and Forskolin were used with or without 1µM of Dopamine

15 For the alpha2c – CHO S cell line (alpha 2c stably transfected in CHO S host cell line) was incubate with 5µM of NKH477 and Forskolin 10' at 37°C without or with 1µM of Epinephrine

20 Results:

a) The EC50 values for acetylcholine or quimpirole were calculated from the dose response curves by Hill as shown in figure 5. CHO-K1 cells were exposed to increasing concentrations of acetylcholine or quimpirole ranging from 0,01 nM to 10
25 µM in the presence of 30 µM forskolin.

b) Functional assay based on the measurement of cAMP by HTRF (Cisbio) was performed in order to compare the effect of Oxotremorine on the production of cAMP induced by Forskolin in two cell lines (M4/CHO & M4/CHO/miRNA).

30

Figure 6 illustrates the dose dependent signal in response to Forskolin.

More than 85 % of inhibition of Forskolin stimulated cAMP production by 1µM of Oxotremorine in M4/CHO/miRNA cell line was obtained. The inhibitory potency of

Oxotremorine on Forskolin induced cAMP production increases 25-30% as compared to the results shown in the wild CHO cells expressing M4 receptor.

- c) CHO-K1 cells expressing D4.4, α_2c H3 and D2S receptors were exposed to increasing concentrations of ligands, respectively dopamine, epinephrine, histamine and dopamine ranging from 0,01 nM to 10 μ M in the presence of 30 μ M forskolin or 10 μ M NKH-477.

Figure 7 illustrates the difference in dose dependent signal in response to Forskolin or NKH-477.

On the D4.4 receptor stably expressed in CHO-K1 and α_2c receptor stably expressed in CHO-S, NKH477 gives a greater signal window than Forskolin at 1 μ M of agonist (The use of NKH477 instead of forskolin enhanced the cAMP production inhibition up to 20%).

CLAIMS

1. A method for screening, identifying or selecting test compounds using an in vitro
5 assay based on cAMP levels modulated by Gi-coupled receptors expressed in a cell comprising the steps of:
- (a) optionally identifying, in a selected cell, the G α i and adenylyl cyclase isoforms which are involved in the modulation of the intracellular cAMP levels,
 - 10 (b) favouring the influence of adenylyl cyclase isoform(s) (A) stimulated by forskolin or NKH-477 and sensitive to inhibition by G α i relative to adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 but not sensitive to inhibition by G α i in said selected cell,
 - (c) contacting said cell with forskolin or NKH-477 and a test compound;
15 and
 - (d) measuring cAMP levels resulting from contacting said test compound with said cell.
2. The method according to claim 1, wherein step b) is achieved by contacting said
20 cell with NKH-477.
3. The method according to claim 1 or 2, wherein favouring the influence of adenylyl cyclase isoform(s) (A) stimulated by forskolin or NKH-477 and sensitive to inhibition by G α i relative to adenylyl cyclase isoform(s) (B) sensitive to forskolin or NKH-477
25 but not sensitive to inhibition by G α i is achieved by specifically inactivating the adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 but not sensitive to inhibition by G α i protein.
4. The method according to claim 3, wherein said inactivation is obtained by
30 downregulating the expression of said adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 but not sensitive to inhibition by G α i protein.

5. The method according to claim 4, wherein said adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 but not sensitive to inhibition by G α i expressed in said selected cell are downregulated by specific RNAi.
- 5 6. The method according to claim 5, wherein said specific RNAi are specific miRNA.
7. The method according to claim 6, wherein the adenylyl cyclase VII isoform is knocked-down by using specific miRNA.
- 10 8. The method according to any one of claims 1 to 7, wherein said selected cell is a eukaryotic cell selected from a mammalian cell or a yeast cell or an established cell line selected from CHO lines, HEK lines or the insect SF9 lines.
9. The method according to claim 8, wherein said selected cell is the CHO-K1 or
15 CHO-S cell line.
10. The method according to any one of claim 1-9, wherein measurement of cAMP levels resulting from contacting said test compound with said cell is performed via technologies using read out as fluorescence or luminescence.
- 20 11. An assay system or kit for measuring the modulation of cAMP levels by test compounds that specifically interact with and modulate the activity of a cellular receptor, said assay system or kit comprising cells which mainly express adenylyl cyclase isoform(s) (A) stimulated by forskolin or NKH-477 and sensitive to inhibition
25 by G α i.
12. The assay system or kit according to claim 11, wherein said cell stably co-express i) nucleic acid sequences which code for a specific G α i protein or a specific adenylyl cyclase isoform(s) (A) stimulated by forskolin or NKH-477 and sensitive to
30 inhibition by G α i protein (ii) and specific miRNA that target adenylyl cyclase transcripts coding for adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 and not sensitive to inhibition by G α i protein.

13. A recombinant cell engineered from a selected cell expressing both adenylyl cyclase isoform(s) (A) stimulated by forskolin or NKH-477 and sensitive to Gai and adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 and not sensitive to Gai, wherein the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to Gai is favoured relative to the influence of adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and not sensitive to Gai.
14. The recombinant cell according to claim 13, wherein expression of adenylyl cyclase isoform(s) (A) stimulated by forskolin or NKH-477 and sensitive to Gai is upregulated.
15. The recombinant cell according to claim 14, wherein adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 and not sensitive to Gai are inactivated.
16. The recombinant cell according to claim 15, wherein the gene(s) coding for adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and not sensitive to Gai are deleted.
17. The recombinant cell according to claim 16, wherein the expression of adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 and not sensitive to Gai is downregulated by RNAi.
18. The recombinant cell according to claim 17, wherein said cell is stably transformed with a recombinant DNA vector comprising (i) engineered pre-miRNA primary transcripts, and ii) nucleic acid sequences which code for a specific Gai protein or a specific adenylyl cyclase isoform(s) stimulated by forskolin or NKH-477 and sensitive to inhibition by Gai protein, stably co-expressed in said recombinant cell.
19. The recombinant cell according to claim 18, which is engineered to co-express a specific adenylyl cyclase isoform(s) (A) stimulated by forskolin or NKH-477 and sensitive to inhibition by Gai protein and specific miRNA that target adenylyl cyclase transcripts coding for adenylyl cyclase isoform(s) (B) stimulated by forskolin or NKH-477 and not sensitive to inhibition by Gai protein.

20. The recombinant cell according to any one of claims 13 to 19, which is a mammalian cell.
- 5 21. The recombinant cell according to claim 20, which is a CHO cell line.
22. A use of a recombinant cell as defined in any one claims 13 to 21, as a tool for screening, identifying or selecting potential ligands of Gi-coupled receptors.
- 10 23. The assay system or kit as defined in claim 11 or 12, wherein said cells are as defined in anyone of claims 13-21.