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(54) Title: REGULATION OF HUMAN PDE1A

(57) Abstract: Reagents that regulate human PDE1A and reagents which bind to human PDE1A gene products can play a role in preventing, ameliorating, or correcting dysfunctions or diseases including, but not limited to, cancer, diabetes, CNS disorders, asthma, obesity, and cardiovascular disorders.



## REGULATION OF HUMAN PDE1A

This application incorporates by reference co-pending provisional application Serial  
5 No. 60/414,641 filed October 1, 2002.

### FIELD OF THE INVENTION

The invention relates to the regulation of human phosphodiesterase 1A (PDE1A).  
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### BACKGROUND OF THE INVENTION

3',5' cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of 3',5'-  
cyclic nucleotides to their respective nucleoside 5'-monophosphates. Because of the  
15 importance of these enzymes, there is a need in the art to identify additional members  
of this enzyme family, which can be regulated to provide therapeutic effects.

It is an object of the invention to provide reagents and methods of regulating a  
human PDE1A. This and other objects of the invention are provided by one or more  
20 of the embodiments described below.

### BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the DNA-sequence encoding a PDE1A Polypeptide (SEQ ID NO: 1).  
25

Fig. 2 shows the amino acid sequence deduced from the DNA-sequence of Fig.1  
(SEQ ID NO: 2).

### DETAILED DESCRIPTION OF THE INVENTION

30 The invention relates to an isolated polynucleotide from the group consisting of:

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- a) a polynucleotide encoding a PDE1A polypeptide comprising an amino acid sequence selected from the group consisting of:
- amino acid sequences which are at least about 20% identical to  
5 the amino acid sequence shown in SEQ ID NO: 2; and  
the amino acid sequence shown in SEQ ID NO: 2.
- b) a polynucleotide comprising the sequence of SEQ ID NO: 1;
- 10 c) a polynucleotide which hybridizes under stringent conditions to a polynucleotide specified in (a) and (b) and encodes a PDE1A polypeptide;
- d) a polynucleotide the sequence of which deviates from the polynucleotide sequences specified in (a) to (c) due to the degeneration of  
15 the genetic code and encodes a PDE1A polypeptide; and
- e) a polynucleotide which represents a fragment, derivative or allelic variation of a polynucleotide sequence specified in (a) to (d) and  
20 encodes a PDE1A polypeptide.

The human PDE1A of the invention is a splice variant of the human calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1A (swissnew|P54750|CN1A\_HUMAN and trembl|AB038224|AB038224\_2). Its amino  
25 acid sequence is shown in SEQ ID NO: 2; a coding sequence is shown in SEQ ID NO: 1. This novel sequence has 1 extra exon in C terminus. This gene maps to chromosome 2q32.1. The protein is identical to human calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1A except that it contains one extra exon at N terminus. The 3D structure identifies to a phosphodiesterase 4b  
30 fragment: catalytic domain;(pde4b) mutant. The protein is a 3'5'-cyclic nucleotide

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phosphodiesterase. A PDEASE\_I and RGD regions are identified. Related EST (AI745620) is expressed in adult prostate.

Human PDE1A of the invention is expected to be useful for the same purposes as previously identified PDE1A enzymes. Human PDE1A is believed to be useful in therapeutic methods to treat disorders such as cancer, diabetes, CNS disorders, asthma, obesity, and cardiovascular disorders. Human PDE1A also can be used to screen for human PDE1A activators and inhibitors.

One embodiment of the present invention is an expression vector containing any polynucleotide of the present invention.

Yet another embodiment of the present invention is a host cell containing any expression vector of the present invention.

Still another embodiment of the present invention is a substantially purified PDE1A polypeptide encoded by any polynucleotide of the present invention.

Even another embodiment of the present invention is a method of producing a PDE1A polypeptide of the present invention, wherein the method comprises the following steps:

- a. culturing the host cells of the present invention under conditions suitable for the expression of the PDE1A polypeptide; and
- b. recovering the PDE1A polypeptide from the host cell culture.

Yet another embodiment of the present invention is a method for detecting a polynucleotide encoding a PDE1A polypeptide in a biological sample comprising the following steps:

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- a. hybridizing any polynucleotide of the present invention to a nucleic acid material of a biological sample, thereby forming a hybridization complex; and
- b. detecting said hybridization complex.

5

Still another embodiment of the present invention is a method for detecting a polynucleotide of the present invention or a PDE1A polypeptide of the present invention comprising the steps of:

- 40 a. contacting a biological sample with a reagent which specifically interacts with the polynucleotide or the PDE1A polypeptide and
- b. detecting the interaction

15 Even another embodiment of the present invention is a diagnostic kit for conducting any method of the present invention.

Yet another embodiment of the present invention is a method of screening for agents which decrease the activity of a PDE1A, comprising the steps of:

20

- a. contacting a test compound with a PDE1A polypeptide encoded by any polynucleotide of the present invention;
- b. detecting binding of the test compound to the PDE1A polypeptide, wherein a  
25 test compound which binds to the polypeptide is identified as a potential therapeutic agent for decreasing the activity of a PDE1A.

Still another embodiment of the present invention is a method of screening for agents which regulate the activity of a PDE1A, comprising the steps of:

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- 5 -

- a. contacting a test compound with a PDE1A polypeptide encoded by any polynucleotide of the present invention; and
- b. detecting a PDE1A activity of the polypeptide, wherein a test compound which increases the PDE1A activity is identified as a potential therapeutic agent for increasing the activity of the PDE1A, and wherein a test compound which decreases the PDE1A activity of the polypeptide is identified as a potential therapeutic agent for decreasing the activity of the PDE1A.

Even another embodiment of the present invention is a method of screening for agents which decrease the activity of a PDE1A, comprising the step of:

contacting a test compound with any polynucleotide of the present invention and detecting binding of the test compound to the polynucleotide, wherein a test compound which binds to the polynucleotide is identified as a potential therapeutic agent for decreasing the activity of PDE1A.

Yet another embodiment of the present invention is a method of reducing the activity of a PDE1A, comprising the step of:

contacting a cell with a reagent which specifically binds to any polynucleotide of the present invention or any PDE1A polypeptide of the present invention, whereby the activity of PDE1A is reduced.

Still another embodiment of the present invention is a reagent that modulates the activity of a PDE1A polypeptide or a polynucleotide wherein said reagent is identified by any methods of the present invention.

Even another embodiment of the present invention is a pharmaceutical composition, comprising:

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an expression vector of the present invention or a reagent of the present invention and a pharmaceutically acceptable carrier.

Yet another embodiment of the present invention is the use of an expression vector of the present invention or a reagent of the present invention for modulating the activity of a PDE1A in a disease, preferably cancer, diabetes, a CNS disorder, asthma, obesity or a cardiovascular disorder.

The invention thus provides a human PDE1A that can be used to identify test compounds that may act, for example, as activators or inhibitors at the enzyme's active site. Human PDE1A and fragments thereof also are useful in raising specific antibodies that can block the enzyme and effectively reduce its activity.

#### *Polypeptides*

Human PDE1A polypeptides according to the invention comprise at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 400, 500, 550, or 553 contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof, as defined below. A PDE1A polypeptide of the invention therefore can be a portion of a PDE1A protein, a full-length PDE1A protein, or a fusion protein comprising all or a portion of a PDE1A protein.

#### *Biologically active variants*

Human PDE1A polypeptide variants that are biologically active, e.g., retain enzymatic activity, also are human PDE1A polypeptides. Preferably, naturally or non-naturally occurring human PDE1A polypeptide variants have amino acid sequences which are at least about 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, or 70, preferably about 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to the amino acid sequence shown in SEQ ID NO: 2 or a fragment thereof. Percent identity between a

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putative human PDE1A polypeptide variant and an amino acid sequence of SEQ ID NO: 2 is determined by conventional methods. See, for example, Altschul *et al.*, *Bull. Math. Bio.* 48:603 (1986), and Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM62" scoring matrix of Henikoff & Henikoff, 1992.

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson & Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative variant. The FASTA algorithm is described by Pearson & Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444(1988), and by Pearson, *Meth. Enzymol.* 183:63 (1990). Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (*e.g.*, SEQ ID NO: 2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then rescored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman & Wunsch, *J. Mol. Biol.* 48:444 (1970); Sellers, *SIAM J. Appl. Math.* 26:787 (1974)), which allows for amino acid insertions and deletions. Preferred parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring

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matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol.* 183:63 (1990).

5 "% identity" of a first sequence towards a second sequence, within the meaning of the invention, means the % identity which is calculated as follows: First the optimal global alignment between the two sequences is determined with the CLUSTALW algorithm [Thomson JD, Higgins DG, Gibson TJ. 1994. ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, 22: 10 4673-4680], Version 1.8, applying the following command line syntax: `./clustalw -infile=./infile.txt -output= -outorder=aligned -pwmatrix=gonnet -pwnamatrix=clustalw -pwgapopen=10.0 -pwgapext=0.1 -matrix=gonnet -gapopen=10.0 -gapext=0.05 -gapdist=8 -hgapresidues=GPSNDQERK -maxdiv=40`. Implementations of the CLUSTAL W algorithm are readily available 15 at numerous sites on the internet, including, e.g., <http://www.ebi.ac.uk>. Thereafter, the number of matches in the alignment is determined by counting the number of identical nucleotides (or amino acid residues) in aligned positions. Finally, the total number of matches is divided by the number of nucleotides (or amino acid residues) of the longer of the two sequences, and multiplied by 100 to yield the % identity of 20 the first sequence towards the second sequence.

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, most 25 preferably three, with other parameters set as default.

Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid 30 has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological or immunological activity of a human PDE1A polypeptide can be found  
5 using computer programs well known in the art, such as DNASTAR software.

The invention additionally, encompasses PDE1A polypeptides that are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation,  
10 phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications can be carried out by known techniques including, but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH<sub>4</sub>, acetylation, formylation,  
15 oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

Additional post-translational modifications encompassed by the invention include, for example, *e.g.*, N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid  
20 backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of prokaryotic host cell expression. The PDE1A polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

25

The invention also provides chemically modified derivatives of PDE1A polypeptides that may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U.S. Patent No. 4,179,337). The chemical moieties for derivitization can be selected from water  
30 soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol, and the like. The

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polypeptides can be modified at random or predetermined positions within the molecule and can include one, two, three, or more attached chemical moieties.

Whether an amino acid change or a polypeptide modification results in a biologically active PDE1A polypeptide can readily be determined by assaying for enzymatic activity, as described for example, in Example 4.

### *Fusion proteins*

Fusion proteins are useful for generating antibodies against PDE1A polypeptide amino acid sequences and for use in various assay systems. For example, fusion proteins can be used to identify proteins that interact with portions of a human PDE1A polypeptide. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

A human PDE1A polypeptide fusion protein comprises two polypeptide segments fused together by means of a peptide bond. The first polypeptide segment comprises a PDE1A polypeptide, such as those described above. The first polypeptide segment also can comprise full-length PDE1A protein.

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include  $\beta$ -galactosidase,  $\beta$ -glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horseradish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Additionally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose binding protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4

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DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. A fusion protein also can be engineered to contain a cleavage site located between the PDE1A polypeptide-encoding sequence and the heterologous protein sequence, so that the PDE1A polypeptide can be cleaved and purified away from the  
5 heterologous moiety.

A fusion protein can be synthesized chemically, as is known in the art. Preferably, a fusion protein is produced by covalently linking two polypeptide segments or by standard procedures in the art of molecular biology. Recombinant DNA methods  
10 can be used to prepare fusion proteins, for example, by making a DNA construct which comprises coding sequences selected from SEQ ID NO: 1 in proper reading frame with nucleotides encoding the second polypeptide segment and expressing the DNA construct in a host cell, as is known in the art. Many kits for constructing fusion proteins are available from companies such as Promega Corporation  
15 (Madison, WI), Stratagene (La Jolla, CA), CLONTECH (Mountain View, CA), Santa Cruz Biotechnology (Santa Cruz, CA), MBL International Corporation (MIC; Watertown, MA), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

#### 20 *Identification of species homologs*

Species homologs of human PDE1A polypeptide can be obtained using PDE1A polypeptide polynucleotides (described below) to make suitable probes or primers for screening cDNA expression libraries from other species, such as mice, monkeys, or  
25 yeast, identifying cDNAs which encode homologs of PDE1A polypeptide, and expressing the cDNAs as is known in the art.

#### *Polynucleotides*

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A human PDE1A polynucleotide can be single- or double-stranded and comprises a coding sequence or the complement of a coding sequence for a PDE1A polypeptide. A coding sequence for human PDE1A is shown in SEQ ID NO: 1.

5 Degenerate nucleotide sequences encoding human PDE1A polypeptides, as well as homologous nucleotide sequences which are at least about 50, 55, 60, 65, 70, preferably about 75, 90, 96, 98, or 99% identical to the nucleotide sequence shown in SEQ ID NO: 1 or its complement also are PDE1A polynucleotides. Percent  
10 sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of PDE1A polynucleotides that encode biologically active PDE1A polypeptides also are PDE1A polynucleotides. Polynucleotide fragments comprising at least 8, 9, 10, 11, 12, 15,  
15 20, or 25 contiguous nucleotides of SEQ ID NO: 1 or its complement also are PDE1A polynucleotides. These fragments can be used, for example, as hybridization probes or as antisense oligonucleotides.

*Identification of polynucleotide variants and homologs*

20 Variants and homologs of the PDE1A polynucleotides described above also are PDE1A polynucleotides. Typically, homologous PDE1A polynucleotide sequences can be identified by hybridization of candidate polynucleotides to known PDE1A polynucleotides under stringent conditions, as is known in the art. For example,  
25 using the following wash conditions--2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50 °C once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes each--homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain  
30 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

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Species homologs of the PDE1A polynucleotides disclosed herein also can be identified by making suitable probes or primers and screening cDNA expression libraries from other species, such as mice, monkeys, or yeast. Human variants of PDE1A polynucleotides can be identified, for example, by screening human cDNA expression libraries. It is well known that the  $T_m$  of a double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner *et al.*, *J. Mol. Biol.* 81, 123 (1973). Variants of human PDE1A polynucleotides or PDE1A polynucleotides of other species can therefore be identified by hybridizing a putative homologous PDE1A polynucleotide with a polynucleotide having a nucleotide sequence of SEQ ID NO: 1 or the complement thereof to form a test hybrid. The melting temperature of the test hybrid is compared with the melting temperature of a hybrid comprising polynucleotides having perfectly complementary nucleotide sequences, and the number or percent of basepair mismatches within the test hybrid is calculated.

Nucleotide sequences which hybridize to PDE1A polynucleotides or their complements following stringent hybridization and/or wash conditions also are PDE1A polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., 1989, at pages 9.50-9.51.

Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20°C below the calculated  $T_m$  of the hybrid under study. The  $T_m$  of a hybrid between a PDE1A polynucleotide having a nucleotide sequence shown in SEQ ID NO: 1 or the complement thereof and a polynucleotide sequence which is at least about 50, preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences can be calculated, for example, using the equation of Bolton and McCarthy, *Proc. Natl. Acad. Sci. U.S.A.* 48, 1390 (1962):

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$$T_m = 81.5^{\circ}\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G + C) - 0.63(\%\text{formamide}) - 600/l,$$

where  $l$  = the length of the hybrid in basepairs.

Stringent wash conditions include, for example, 4X SSC at 65°C, or 50% formamide,  
5 4X SSC at 42°C, or 0.5X SSC, 0.1% SDS at 65°C. Highly stringent wash conditions include, for example, 0.2X SSC at 65°C.

#### *Preparation of polynucleotides*

10 A human PDE1A polynucleotide can be isolated free of other cellular components such as membrane components, proteins, and lipids. Polynucleotides can be made by a cell and isolated using standard nucleic acid purification techniques, or synthesized using an amplification technique, such as the polymerase chain reaction (PCR), or by using an automatic synthesizer. Methods for isolating polynucleotides are routine  
15 and are known in the art. Any such technique for obtaining a polynucleotide can be used to obtain isolated PDE1A polynucleotides. For example, restriction enzymes and probes can be used to isolate polynucleotide fragments, which comprise PDE1A nucleotide sequences. Isolated polynucleotides are in preparations that are free or at least 70, 80, or 90% free of other molecules.

20 Human PDE1A cDNA molecules can be made with standard molecular biology techniques, using PDE1A mRNA as a template. Human PDE1A cDNA molecules can thereafter be replicated using molecular biology techniques known in the art and disclosed in manuals such as Sambrook *et al.* (1989). An amplification technique,  
25 such as PCR, can be used to obtain additional copies of polynucleotides of the invention, using either human genomic DNA or cDNA as a template.

Alternatively, synthetic chemistry techniques can be used to synthesize PDE1A polynucleotides. The degeneracy of the genetic code allows alternate nucleotide  
30 sequences to be synthesized which will encode a human PDE1A polypeptide having,

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for example, an amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof.

*Extending polynucleotides*

5 Various PCR-based methods can be used to extend the nucleic acid sequences disclosed herein to detect upstream sequences such as promoters and regulatory elements. For example, restriction-site PCR uses universal primers to retrieve unknown sequence adjacent to a known locus. Sarkar, *PCR Methods Applic.* 2, 318-322, 1993; Triglia *et al.*, *Nucleic Acids Res.* 16, 8186, 1988; Lagerstrom *et al.*,  
10 *PCR Methods Applic.* 1, 111-119, 1991; Parker *et al.*, *Nucleic Acids Res.* 19, 3055-3060, 1991). Additionally, PCR, nested primers, and PROMOTERFINDER libraries (CLONTECH, Palo Alto, Calif.) can be used to walk genomic DNA (CLONTECH, Palo Alto, Calif.). See WO 01/98340

15 *Obtaining Polynucleotides*

Human PDE1A polypeptides can be obtained, for example, by purification from human cells, by expression of PDE1A polynucleotides, or by direct chemical synthesis.

20

*Protein purification*

Human PDE1A polypeptides can be purified from any human cell which expresses the receptor, including host cells which have been transfected with PDE1A  
25 polynucleotides. A purified PDE1A polypeptide is separated from other compounds that normally associate with the PDE1A polypeptide in the cell, such as certain proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and  
30 preparative gel electrophoresis.

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A preparation of purified PDE1A polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis.

#### *Expression of polynucleotides*

5

To express a human PDE1A polynucleotide, the polynucleotide can be inserted into an expression vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding PDE1A polypeptides and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook *et al.* (1989) and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, N.Y., 1989.

15

A variety of expression vector/host systems can be utilized to contain and express sequences encoding a human PDE1A polypeptide. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (*e.g.*, baculovirus), plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids), or animal cell systems. See WO 01/98340.

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#### *Host cells*

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed PDE1A polypeptide in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the polypeptide also

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can be used to facilitate correct insertion, folding and/or function. Different host cells that have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38) are available from the American Type Culture Collection (ATCC; 10801 University  
5 Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein. See WO 01/98340.

Alternatively, host cells which contain a human PDE1A polynucleotide and which express a human PDE1A polypeptide can be identified by a variety of procedures  
10 known to those of skill in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). Hampton *et al.*, SEROLOGICAL METHODS: A LABORATORY MANUAL, APS Press, St. Paul, Minn., 1990) and Maddox *et al.*, *J. Exp. Med.* 158, 1211-1216, 1983). See WO 01/98340.

15 A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding PDE1A polypeptides include oligolabeling, nick trans-  
20 lation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, sequences encoding a human PDE1A polypeptide can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These  
25 procedures can be conducted using a variety of commercially available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include radionuclides, enzymes, and fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

*Expression and purification of polypeptides*

Host cells transformed with nucleotide sequences encoding a human PDE1A polypeptide can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode PDE1A polypeptides can be designed to contain signal sequences which direct secretion of soluble PDE1A polypeptides through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound PDE1A polypeptide. See WO 01/98340.

*Chemical synthesis*

Sequences encoding a human PDE1A polypeptide can be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers *et al.*, *Nucl. Acids Res. Symp. Ser.* 215-223, 1980; Horn *et al.* *Nucl. Acids Res. Symp. Ser.* 225-232, 1980). Alternatively, a human PDE1A polypeptide itself can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc.* 85, 2149-2154, 1963; Roberge *et al.*, *Science* 269, 202-204, 1995). Protein synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of PDE1A polypeptides can be separately synthesized and combined using chemical methods to produce a full-length molecule. See WO 01/98340.

As will be understood by those of skill in the art, it may be advantageous to produce PDE1A polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or

eukaryotic host can be selected to increase the rate of protein expression or to produce an RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

5 The nucleotide sequences disclosed herein can be engineered using methods generally known in the art to alter PDE1A polypeptide-encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic  
10 oligonucleotides can be used to engineer the nucleotide sequences. For example, site-directed mutagenesis can be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

### 15 *Antibodies*

Any type of antibody known in the art can be generated to bind specifically to an epitope of a human PDE1A polypeptide. "Antibody" as used herein includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab, F(ab')<sub>2</sub>, and  
20 Fv, which are capable of binding an epitope of a human PDE1A polypeptide. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, *e.g.*, at least 15, 25, or 50 amino acids.

25 An antibody which specifically binds to an epitope of a human PDE1A polypeptide can be used therapeutically, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art. Various immunoassays can be used to identify antibodies having the desired specificity. Numerous  
30 protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex

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formation between an immunogen and an antibody that specifically binds to the immunogen.

Typically, an antibody that specifically binds to a human PDE1A polypeptide provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay. Preferably, antibodies that specifically bind to PDE1A polypeptides do not detect other proteins in immunochemical assays and can immunoprecipitate a human PDE1A polypeptide from solution. See WO 01/98340.

10

#### *Antisense oligonucleotides*

Antisense oligonucleotides are nucleotide sequences that are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes and block either transcription or translation. Preferably, an antisense oligonucleotide is at least 11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences also can be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease the level of PDE1A gene products in the cell.

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such as alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. See Brown, *Meth. Mol. Biol.* 20, 1-8, 1994; Sonveaux, *Meth. Mol. Biol.* 26, 1-72, 1994; Uhlmann *et al.*, *Chem. Rev.* 90, 543-583, 1990.

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Modifications of PDE1A gene expression can be obtained by designing antisense oligonucleotides that will form duplexes to the control, 5', or regulatory regions of the PDE1A gene. Oligonucleotides derived from the transcription initiation site, *e.g.*, between positions -10 and +10 from the start site, are preferred. Similarly, inhibition  
5 can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature (*e.g.*, Gee *et al.*, in Huber & Carr, MOLECULAR AND IMMUNOLOGIC APPROACHES, Futura  
10 Publishing Co., Mt. Kisco, N.Y., 1994). An antisense oligonucleotide also can be designed to block translation of mRNA by preventing the transcript from binding to ribosomes. See WO 01/98340.

### *Ribozymes*

15 Ribozymes are RNA molecules with catalytic activity. *See, e.g.*, Cech, *Science* 236, 1532-1539; 1987; Cech, *Ann. Rev. Biochem.* 59, 543-568; 1990, Cech, *Curr. Opin. Struct. Biol.* 2, 605-609; 1992, Couture & Stinchcomb, *Trends Genet.* 12, 510-515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence,  
20 as is known in the art (*e.g.*, Haseloff *et al.*, U.S. Patent 5,641,673). The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide  
25 sequences.

The coding sequence of a human PDE1A polynucleotide can be used to generate ribozymes that will specifically bind to mRNA transcribed from the PDE1A polynucleotide. Methods of designing and constructing ribozymes which can cleave  
30 other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (*see* Haseloff *et al.* *Nature* 334, 585-591, 1988).

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For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach *et al.*, EP 321,201). See WO  
5 01/98340.

*Differentially expressed genes*

Described herein are methods for the identification of genes whose products interact  
10 with human PDE1A. Such genes may represent genes that are differentially expressed in disorders including, but not limited to, cancer, diabetes, CNS disorders, asthma, obesity, and cardiovascular disorders. Further, such genes may represent genes that are differentially regulated in response to manipulations relevant to the progression or treatment of such diseases. Additionally, such genes may have a  
15 temporally modulated expression, increased or decreased at different stages of tissue or organism development. A differentially expressed gene may also have its expression modulated under control versus experimental conditions. In addition, the human PDE1A gene or gene product may itself be tested for differential expression.

20 The degree to which expression differs in a normal versus a diseased state need only be large enough to be visualized via standard characterization techniques such as differential display techniques. Other such standard characterization techniques by which expression differences may be visualized include but are not limited to, quantitative RT (reverse transcriptase), PCR, and Northern analysis.

25 To identify differentially expressed genes total RNA or, preferably, mRNA is isolated from tissues of interest. For example, RNA samples are obtained from tissues of experimental subjects and from corresponding tissues of control subjects. Any RNA isolation technique that does not select against the isolation of mRNA may  
30 be utilized for the purification of such RNA samples. See, for example, Ausubel *et al.*, ed., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, Inc.

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New York, 1987-1993. Large numbers of tissue samples may readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski, U.S. Patent 4,843,155.

5 Transcripts within the collected RNA samples that represent RNA produced by differentially expressed genes are identified by methods well known to those of skill in the art. They include, for example, differential screening (Tedder *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 85, 208-12, 1988), subtractive hybridization (Hedrick *et al.*, *Nature* 308, 149-53; Lee *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 88, 2825, 1984), and, preferably, differential display (Liang & Pardee, *Science* 257, 967-71, 1992; U.S. Patent 5,262,311).

The differential expression information may itself suggest relevant methods for the treatment of disorders involving the human PDE1A. For example, treatment may include a modulation of expression of the differentially expressed genes and/or the gene encoding the human PDE1A. The differential expression information may indicate whether the expression or activity of the differentially expressed gene or gene product or the human PDE1A gene or gene product are up-regulated or down-regulated.

20

#### *Screening methods*

The invention provides assays for screening test compounds that bind to or modulate the activity of a human PDE1A polypeptide or a human PDE1A polynucleotide. A test compound preferably binds to a human PDE1A polypeptide or polynucleotide. More preferably, a test compound decreases or increases enzymatic activity by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the test compound.

30

#### *Test compounds*

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Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced re-combinantly, or synthesized by chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds. See Lam, *Anticancer Drug Des.* 12, 145, 1997.

Methods for the synthesis of molecular libraries are well known in the art (see, for example, DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90, 6909, 1993; Erb *et al.* *Proc. Natl. Acad. Sci. U.S.A.* 91, 11422, 1994; Zuckermann *et al.*, *J. Med. Chem.* 37, 2678, 1994; Cho *et al.*, *Science* 261, 1303, 1993; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33, 2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33, 2061; Gallop *et al.*, *J. Med. Chem.* 37, 1233, 1994). Libraries of compounds can be presented in solution (see, e.g., Houghten, *BioTechniques* 13, 412-421, 1992), or on beads (Lam, *Nature* 354, 82-84, 1991), chips (Fodor, *Nature* 364, 555-556, 1993), bacteria or spores (Ladner, U.S. Patent 5,223,409), plasmids (Cull *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89, 1865-1869, 1992), or phage (Scott & Smith, *Science* 249, 386-390, 1990; Devlin, *Science* 249, 404-406, 1990); Cwirla *et al.*, *Proc. Natl. Acad. Sci.* 97, 6378-6382, 1990; Felici, *J. Mol. Biol.* 222, 301-310, 1991; and Ladner, U.S. Patent 5,223,409).

#### *High throughput screening*

Test compounds can be screened for the ability to bind to PDE1A polypeptides or polynucleotides or to affect PDE1A activity or PDE1A gene expression using high

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throughput screening. Using high throughput screening, many discrete compounds can be tested in parallel so that large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96-well microtiter plates. The wells of the microtiter plates typically require assay volumes that range from 50  
5 to 500  $\mu$ l. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, and plate readers are commercially available to fit the 96-well format.

Alternatively, "free format assays," or assays that have no physical barrier between samples, can be used. For example, an assay using pigment cells (melanocytes) in a  
10 simple homogeneous assay for combinatorial peptide libraries is described by Jayawickreme *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 19, 1614-18 (1994). The cells are placed under agarose in petri dishes, then beads that carry combinatorial compounds are placed on the surface of the agarose. The combinatorial compounds are partially released the compounds from the beads. Active compounds can be visualized as  
15 dark pigment areas because, as the compounds diffuse locally into the gel matrix, the active compounds cause the cells to change colors.

Another example of a free format assay is described by Chelsky, "Strategies for Screening Combinatorial Libraries: Novel and Traditional Approaches," reported at  
20 the First Annual Conference of The Society for Biomolecular Screening in Philadelphia, Pa. (Nov. 7-10, 1995). Chelsky placed a simple homogenous enzyme assay for carbonic anhydrase inside an agarose gel such that the enzyme in the gel would cause a color change throughout the gel. Thereafter, beads carrying combinatorial compounds via a photolinker were placed inside the gel and the compounds  
25 were partially released by UV-light. Compounds that inhibited the enzyme were observed as local zones of inhibition having less color change.

Yet another example is described by Salmon *et al.*, *Molecular Diversity* 2, 57-63 (1996). In this example, combinatorial libraries were screened for compounds that  
30 had cytotoxic effects on cancer cells growing in agar.

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Another high throughput screening method is described in Beutel *et al.*, U.S. Patent 5,976,813. In this method, test samples are placed in a porous matrix. One or more assay components are then placed within, on top of, or at the bottom of a matrix such as a gel, a plastic sheet, a filter, or other form of easily manipulated solid support.

5 When samples are introduced to the porous matrix they diffuse sufficiently slowly, such that the assays can be performed without the test samples running together.

#### *Binding assays*

10 For binding assays, the test compound is preferably a small molecule that binds to and occupies, for example, the active site of the PDE1A polypeptide, such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules.

15 In binding assays, either the test compound or the PDE1A polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label, such as horseradish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound that is bound to the PDE1A polypeptide can then be accomplished, for example, by direct counting of radioemmission, by scintillation

20 counting, or by determining conversion of an appropriate substrate to a detectable product.

Alternatively, binding of a test compound to a human PDE1A polypeptide can be determined without labeling either of the interactants. For example, a micro-

25 physiometer can be used to detect binding of a test compound with a human PDE1A polypeptide. A microphysiometer (*e.g.*, Cytosensor™) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a test compound and a human PDE1A

30 polypeptide (McConnell *et al.*, *Science* 257, 1906-1912, 1992).

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Determining the ability of a test compound to bind to a human PDE1A polypeptide also can be accomplished using a technology such as real-time Bimolecular Interaction Analysis (BIA) (Sjolander & Urbaniczky, *Anal. Chem.* 63, 2338-2345, 1991, and Szabo *et al.*, *Curr. Opin. Struct. Biol.* 5, 699-705, 1995). BIA is a  
5 technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore<sup>TM</sup>). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

10 In yet another aspect of the invention, a human PDE1A polypeptide can be used as a "bait protein" in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent 5,283,317; Zervos *et al.*, *Cell* 72, 223-232, 1993; Madura *et al.*, *J. Biol. Chem.* 268, 12046-12054, 1993; Bartel *et al.*, *BioTechniques* 14, 920-924, 1993; Iwabuchi *et al.*, *Oncogene* 8, 1693-1696, 1993; and Brent W094/10300), to identify other proteins  
15 which bind to or interact with the PDE1A polypeptide and modulate its activity.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. For example, in one construct, polynucleotide  
20 encoding a human PDE1A polypeptide can be fused to a polynucleotide encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct a DNA sequence that encodes an unidentified protein ("prey" or "sample") can be fused to a polynucleotide that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact *in vivo*  
25 to form an protein-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ), which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected, and cell colonies containing the functional transcription factor  
30 can be isolated and used to obtain the DNA sequence encoding the protein that interacts with the PDE1A polypeptide.

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It may be desirable to immobilize either the PDE1A polypeptide (or polynucleotide) or the test compound to facilitate separation of bound from unbound forms of one or both of the interactants, as well as to accommodate automation of the assay. Thus, either the PDE1A polypeptide (or polynucleotide) or the test compound can be bound to a solid support. Suitable solid supports include, but are not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach the polypeptide (or polynucleotide) or test compound to a solid support, including use of covalent and non-covalent linkages, passive absorption, or pairs of binding moieties attached respectively to the polypeptide (or polynucleotide) or test compound and the solid support. Test compounds are preferably bound to the solid support in an array, so that the location of individual test compounds can be tracked. Binding of a test compound to a human PDE1A polypeptide (or polynucleotide) can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

In one embodiment, the PDE1A polypeptide is a fusion protein comprising a domain that allows the PDE1A polypeptide to be bound to a solid support. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and the non-adsorbed PDE1A polypeptide; the mixture is then incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as described above. Alternatively, the complexes can be dissociated from the solid support before binding is determined.

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Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used in the screening assays of the invention. For example, either a human PDE1A polypeptide (or polynucleotide) or a test compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated PDE1A polypeptides  
5 (or polynucleotides) or test compounds can be prepared from biotin-NHS(N-hydroxysuccinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which specifically bind to a PDE1A polypeptide, polynucleotide, or a test compound, but which do not  
10 interfere with a desired binding site, such as the active site of the PDE1A polypeptide, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

Methods for detecting such complexes, in addition to those described above for the  
15 GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to the PDE1A polypeptide or test compound, enzyme-linked assays which rely on detecting an activity of the PDE1A polypeptide, and SDS gel electrophoresis under non-reducing conditions.

20 Screening for test compounds which bind to a human PDE1A polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises a PDE1A polypeptide or polynucleotide can be used in a cell-based assay system. A PDE1A polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Binding of the test compound to a  
25 PDE1A polypeptide or polynucleotide is determined as described above.

#### *Enzymatic activity*

Test compounds can be tested for the ability to increase or decrease the enzymatic  
30 activity of a human PDE1A polypeptide. Enzymatic activity can be measured, for example, as described in Example 4.

- 30 -

Enzyme assays can be carried out after contacting either a purified PDE1A polypeptide, a cell membrane preparation, or an intact cell with a test compound. A test compound that decreases enzymatic activity of a human PDE1A polypeptide by  
5 at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for decreasing PDE1A activity. A test compound which increases enzymatic activity of a human PDE1A polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for increasing human PDE1A activity.

10

#### *Gene expression*

In another embodiment, test compounds that increase or decrease PDE1A gene expression are identified. A PDE1A polynucleotide is contacted with a test  
15 compound, and the expression of an RNA or polypeptide product of the PDE1A polynucleotide is determined. The level of expression of appropriate mRNA or polypeptide in the presence of the test compound is compared to the level of expression of mRNA or polypeptide in the absence of the test compound. The test compound can then be identified as a modulator of expression based on this  
20 comparison. For example, when expression of mRNA or polypeptide is greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator or enhancer of the mRNA or polypeptide expression. Alternatively, when expression of the mRNA or polypeptide is less in the presence of the test compound than in its absence, the test compound is identified as an inhibitor of the  
25 mRNA or polypeptide expression.

The level of PDE1A mRNA or polypeptide expression in the cells can be determined by methods well known in the art for detecting mRNA or polypeptide. Either qualitative or quantitative methods can be used. The presence of polypeptide  
30 products of a human PDE1A polynucleotide can be determined, for example, using a variety of techniques known in the art, including immunochemical methods such as

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radioimmunoassay, Western blotting, and immunohistochemistry. Alternatively, polypeptide synthesis can be determined *in vivo*, in a cell culture, or in an *in vitro* translation system by detecting incorporation of labeled amino acids into a human PDE1A polypeptide.

5

Such screening can be carried out either in a cell-free assay system or in an intact cell. Any cell that expresses a human PDE1A polynucleotide can be used in a cell-based assay system. The PDE1A polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Either a  
10 primary culture or an established cell line, such as CHO or human embryonic kidney 293 cells, can be used.

#### *Pharmaceutical compositions*

15 The invention also provides pharmaceutical compositions that can be administered to a patient to achieve a therapeutic effect. Pharmaceutical compositions of the invention can comprise, for example, a human PDE1A polypeptide, PDE1A polynucleotide, ribozymes or antisense oligonucleotides, antibodies which specifically bind to a PDE1A polypeptide, or mimetics, activators, or inhibitors of a human  
20 PDE1A polypeptide activity. The compositions can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or  
25 hormones.

In addition to the active ingredients, these pharmaceutical compositions can contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be  
30 used pharmaceutically. Pharmaceutical compositions of the invention can be administered by any number of routes including, but not limited to, oral, intravenous,

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intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means. Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores can be used in conjunction with suitable coatings, such as concentrated sugar solutions, which also can contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, *i.e.*, dosage.

Pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds can be

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dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration can be formulated  
5 in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions can contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds can be prepared as appropriate  
10 oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers also can be used for delivery. Optionally, the suspension also can contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated  
15 solutions. For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention can be manufactured in a  
20 manner that is known in the art, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. The pharmaceutical composition can be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in  
25 aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation can be a lyophilized powder which can contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

30 Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co.,

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Easton, Pa.). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.

5

*Therapeutic indications and methods*

Human PDE1A can be regulated to treat cancer, diabetes, CNS disorders, asthma, obesity, and cardiovascular disorders.

10

*Cancer*

Cancer is a disease fundamentally caused by oncogenic cellular transformation. There are several hallmarks of transformed cells that distinguish them from their normal counterparts and underlie the pathophysiology of cancer. These include uncontrolled cellular proliferation, unresponsiveness to normal death-inducing signals (immortalization), increased cellular motility and invasiveness, increased ability to recruit blood supply through induction of new blood vessel formation (angiogenesis), genetic instability, and dysregulated gene expression. Various combinations of these aberrant physiologies, along with the acquisition of drug-resistance frequently lead to an intractable disease state in which organ failure and patient death ultimately ensue.

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Most standard cancer therapies target cellular proliferation and rely on the differential proliferative capacities between transformed and normal cells for their efficacy. This approach is hindered by the facts that several important normal cell types are also highly proliferative and that cancer cells frequently become resistant to these agents. Thus, the therapeutic indices for traditional anti-cancer therapies rarely exceed 2.0.

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The advent of genomics-driven molecular target identification has opened up the possibility of identifying new cancer-specific targets for therapeutic intervention that will provide safer, more effective treatments for cancer patients. Thus, newly discovered tumor-associated genes and their products can be tested for their role(s) in disease and used as tools to discover and develop innovative therapies. Genes playing important roles in any of the physiological processes outlined above can be characterized as cancer targets.

Genes or gene fragments identified through genomics can readily be expressed in one or more heterologous expression systems to produce functional recombinant proteins. These proteins are characterized *in vitro* for their biochemical properties and then used as tools in high-throughput molecular screening programs to identify chemical modulators of their biochemical activities. Agonists and/or antagonists of target protein activity can be identified in this manner and subsequently tested in cellular and *in vivo* disease models for anti-cancer activity. Optimization of lead compounds with iterative testing in biological models and detailed pharmacokinetic and toxicological analyses form the basis for drug development and subsequent testing in humans.

## Diabetes

Diabetes mellitus is a common metabolic disorder characterized by an abnormal elevation in blood glucose, alterations in lipids and abnormalities (complications) in the cardiovascular system, eye, kidney and nervous system. Diabetes is divided into two separate diseases: type 1 diabetes (juvenile onset), which results from a loss of cells which make and secrete insulin, and type 2 diabetes (adult onset), which is caused by a defect in insulin secretion and a defect in insulin action.

Type I diabetes is initiated by an autoimmune reaction that attacks the insulin secreting cells (beta cells) in the pancreatic islets. Agents that prevent this reaction from occurring or that stop the reaction before destruction of the beta cells has been

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accomplished are potential therapies for this disease. Other agents that induce beta cell proliferation and regeneration also are potential therapies.

5 Type II diabetes is the most common of the two diabetic conditions (6% of the population). The defect in insulin secretion is an important cause of the diabetic condition and results from an inability of the beta cell to properly detect and respond to rises in blood glucose levels with insulin release. Therapies that increase the response by the beta cell to glucose would offer an important new treatment for this disease.

10 The defect in insulin action in Type II diabetic subjects is another target for therapeutic intervention. Agents that increase the activity of the insulin receptor in muscle, liver, and fat will cause a decrease in blood glucose and a normalization of plasma lipids. The receptor activity can be increased by agents that directly stimulate  
15 the receptor or that increase the intracellular signals from the receptor. Other therapies can directly activate the cellular end process, *i.e.* glucose transport or various enzyme systems, to generate an insulin-like effect and therefore a produce beneficial outcome. Because overweight subjects have a greater susceptibility to Type II diabetes, any agent that reduces body weight is a possible therapy.

20 Both Type I and Type II diabetes can be treated with agents that mimic insulin action or that treat diabetic complications by reducing blood glucose levels. Likewise, agents that reduces new blood vessel growth can be used to treat the eye complications that develop in both diseases.

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#### *CNS disorders*

Central and peripheral nervous system disorders also can be treated, such as primary and secondary disorders after brain injury, disorders of mood, anxiety disorders,  
30 disorders of thought and volition, disorders of sleep and wakefulness, diseases of the motor unit, such as neurogenic and myopathic disorders, neurodegenerative disorders

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such as Alzheimer's and Parkinson's disease, and processes of peripheral and chronic pain.

Pain that is associated with CNS disorders also can be treated by regulating the activity of human PDE1A. Pain which can be treated includes that associated with central nervous system disorders, such as multiple sclerosis, spinal cord injury, sciatica, failed back surgery syndrome, traumatic brain injury, epilepsy, Parkinson's disease, post-stroke, and vascular lesions in the brain and spinal cord (e.g., infarct, hemorrhage, vascular malformation). Non-central neuropathic pain includes that associated with post mastectomy pain, reflex sympathetic dystrophy (RSD), trigeminal neuralgia, radioculopathy, post-surgical pain, HIV/AIDS related pain, cancer pain, metabolic neuropathies (e.g., diabetic neuropathy, vasculitic neuropathy secondary to connective tissue disease), paraneoplastic polyneuropathy associated, for example, with carcinoma of lung, or leukemia, or lymphoma, or carcinoma of prostate, colon or stomach, trigeminal neuralgia, cranial neuralgias, and post-herpetic neuralgia. Pain associated with cancer and cancer treatment also can be treated, as can headache pain (for example, migraine with aura, migraine without aura, and other migraine disorders), episodic and chronic tension-type headache, tension-type like headache, cluster headache, and chronic paroxysmal hemicrania.

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### *Asthma*

Allergy is a complex process in which environmental antigens induce clinically adverse reactions. The inducing antigens, called allergens, typically elicit a specific IgE response and, although in most cases the allergens themselves have little or no intrinsic toxicity, they induce pathology when the IgE response in turn elicits an IgE-dependent or T cell-dependent hypersensitivity reaction. Hypersensitivity reactions can be local or systemic and typically occur within minutes of allergen exposure in individuals who have previously been sensitized to an allergen. The hypersensitivity reaction of allergy develops when the allergen is recognized by IgE antibodies bound to specific receptors on the surface of effector cells, such as mast cells, basophils, or eosinophils, which causes the activation of the effector cells and

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the release of mediators that produce the acute signs and symptoms of the reactions. Allergic diseases include asthma, allergic rhinitis (hay fever), atopic dermatitis, and anaphylaxis.

5 Asthma is thought to arise as a result of interactions between multiple genetic and environmental factors and is characterized by three major features: 1) intermittent and reversible airway obstruction caused by bronchoconstriction, increased mucus production, and thickening of the walls of the airways that leads to a narrowing of the airways, 2) airway hyperresponsiveness caused by a decreased control of airway  
10 caliber, and 3) airway inflammation. Certain cells are critical to the inflammatory reaction of asthma and they include T cells and antigen presenting cells, B cells that produce IgE, and mast cells, basophils, eosinophils, and other cells that bind IgE. These effector cells accumulate at the site of allergic reaction in the airways and release toxic products that contribute to the acute pathology and eventually to the  
15 tissue destruction related to the disorder. Other resident cells, such as smooth muscle cells, lung epithelial cells, mucus-producing cells, and nerve cells may also be abnormal in individuals with asthma and may contribute to the pathology. While the airway obstruction of asthma, presenting clinically as an intermittent wheeze and shortness of breath, is generally the most pressing symptom of the disease requiring  
20 immediate treatment, the inflammation and tissue destruction associated with the disease can lead to irreversible changes that eventually make asthma a chronic disabling disorder requiring long-term management.

25 Despite recent important advances in our understanding of the pathophysiology of asthma, the disease appears to be increasing in prevalence and severity (Gergen and Weiss, *Am. Rev. Respir. Dis.* 146, 823-24, 1992). It is estimated that 30-40% of the population suffer with atopic allergy, and 15% of children and 5% of adults in the population suffer from asthma (Gergen and Weiss, 1992). Thus, an enormous burden is placed on our health care resources. However, both diagnosis and treatment of  
30 asthma are difficult. The severity of lung tissue inflammation is not easy to measure and the symptoms of the disease are often indistinguishable from those of respiratory

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infections, chronic respiratory inflammatory disorders, allergic rhinitis, or other respiratory disorders. Often, the inciting allergen cannot be determined, making removal of the causative environmental agent difficult. Current pharmacological treatments suffer their own set of disadvantages. Commonly used therapeutic agents, such as beta agonists, can act as symptom relievers to transiently improve pulmonary function, but do not affect the underlying inflammation. Agents that can reduce the underlying inflammation, such as anti-inflammatory steroids, can have major drawbacks that range from immunosuppression to bone loss (Goodman and Gilman's THE PHARMACOLOGIC BASIS OF THERAPEUTICS, Seventh Edition, MacMillan Publishing Company, NY, USA, 1985). In addition, many of the present therapies, such as inhaled corticosteroids, are short-lasting, inconvenient to use, and must be used often on a regular basis, in some cases for life, making failure of patients to comply with the treatment a major problem and thereby reducing their effectiveness as a treatment.

Because of the problems associated with conventional therapies, alternative treatment strategies have been evaluated. Glycophorin A (Chu and Sharom, *Cell. Immunol.* 145, 223-39, 1992), cyclosporin (Alexander *et al.*, *Lancet* 339, 324-28, 1992), and a nonapeptide fragment of IL-2 (Zav'yalov *et al.*, *Immunol. Lett.* 31, 285-88, 1992) all inhibit interleukin-2 dependent T lymphocyte proliferation; however, they are known to have many other effects. For example, cyclosporin is used as a immunosuppressant after organ transplantation. While these agents may represent alternatives to steroids in the treatment of asthmatics, they inhibit interleukin-2 dependent T lymphocyte proliferation and potentially critical immune functions associated with homeostasis. Other treatments that block the release or activity of mediators of bronchoconstriction, such as cromones or anti-leukotrienes, have recently been introduced for the treatment of mild asthma, but they are expensive and not effective in all patients and it is unclear whether they have any effect on the chronic changes associated with asthmatic inflammation. What is needed in the art is the identification of a treatment that can act in pathways critical to the development of asthma

that both blocks the episodic attacks of the disorder and preferentially dampens the hyperactive allergic immune response without immunocompromising the patient.

### *Obesity*

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Obesity and overweight are defined as an excess of body fat relative to lean body mass. An increase in caloric intake or a decrease in energy expenditure or both can bring about this imbalance leading to surplus energy being stored as fat. Obesity is associated with important medical morbidities and an increase in mortality. The causes of obesity are poorly understood and may be due to genetic factors, environmental factors or a combination of the two to cause a positive energy balance. In contrast, anorexia and cachexia are characterized by an imbalance in energy intake versus energy expenditure leading to a negative energy balance and weight loss. Agents that either increase energy expenditure and/or decrease energy intake, absorption or storage would be useful for treating obesity, overweight, and associated comorbidities. Agents that either increase energy intake and/or decrease energy expenditure or increase the amount of lean tissue would be useful for treating cachexia, anorexia and wasting disorders.

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This gene, translated proteins and agents which modulate this gene or portions of the gene or its products are useful for treating obesity, overweight, anorexia, cachexia, wasting disorders, appetite suppression, appetite enhancement, increases or decreases in satiety, modulation of body weight, and/or other eating disorders such as bulimia. Also this gene, translated proteins and agents which modulate this gene or portions of the gene or its products are useful for treating obesity/overweight-associated comorbidities including hypertension, type 2 diabetes, coronary artery disease, hyperlipidemia, stroke, gallbladder disease, gout, osteoarthritis, sleep apnea and respiratory problems, some types of cancer including endometrial, breast, prostate, and colon cancer, thrombotic disease, polycystic ovarian syndrome, reduced fertility, complications of pregnancy, menstrual irregularities, hirsutism, stress incontinence, and depression.

*Cardiovascular disorders*

Cardiovascular diseases include the following disorders of the heart and the vascular system: congestive heart failure, myocardial infarction, ischemic diseases of the heart, all kinds of atrial and ventricular arrhythmias, hypertensive vascular diseases, and peripheral vascular diseases.

Heart failure is defined as a pathophysiologic state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirement of the metabolizing tissue. It includes all forms of pumping failure, such as high-output and low-output, acute and chronic, right-sided or left-sided, systolic or diastolic, independent of the underlying cause.

Myocardial infarction (MI) is generally caused by an abrupt decrease in coronary blood flow that follows a thrombotic occlusion of a coronary artery previously narrowed by arteriosclerosis. MI prophylaxis (primary and secondary prevention) is included, as well as the acute treatment of MI and the prevention of complications.

Ischemic diseases are conditions in which the coronary flow is restricted resulting in a perfusion which inadequate to meet the myocardial requirement for oxygen. This group of diseases includes stable angina, unstable angina, and asymptomatic ischemia.

Arrhythmias include all forms of atrial and ventricular tachyarrhythmias (atrial tachycardia, atrial flutter, atrial fibrillation, atrio-ventricular reentrant tachycardia, preexcitation syndrome, ventricular tachycardia, ventricular flutter, and ventricular fibrillation), as well as bradycardic forms of arrhythmias.

Vascular diseases include primary as well as all kinds of secondary arterial hypertension (renal, endocrine, neurogenic, others). The disclosed gene and its product

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may be used as drug targets for the treatment of hypertension as well as for the prevention of all complications. Peripheral vascular diseases are defined as vascular diseases in which arterial and/or venous flow is reduced resulting in an imbalance between blood supply and tissue oxygen demand. It includes chronic peripheral  
5 arterial occlusive disease (PAOD), acute arterial thrombosis and embolism, inflammatory vascular disorders, Raynaud's phenomenon, and venous disorders.

This invention further pertains to the use of novel agents identified by the screening assays described above. Accordingly, it is within the scope of this invention to use a  
10 test compound identified as described herein in an appropriate animal model. For example, an agent identified as described herein (e.g., a modulating agent, an antisense nucleic acid molecule, a specific antibody, ribozyme, or a human PDE1A polypeptide binding molecule) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an  
15 agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

20 A reagent which affects PDE1A activity can be administered to a human cell, either *in vitro* or *in vivo*, to reduce PDE1A activity. The reagent preferably binds to an expression product of a human PDE1A gene. If the expression product is a protein, the reagent is preferably an antibody. For treatment of human cells *ex vivo*, an antibody can be added to a preparation of stem cells that have been removed from the  
25 body. The cells can then be replaced in the same or another human body, with or without clonal propagation, as is known in the art.

In one embodiment, the reagent is delivered using a liposome. Preferably, the liposome is stable in the animal into which it has been administered for at least about  
30 30 minutes, more preferably for at least about 1 hour, and even more preferably for at least about 24 hours. A liposome comprises a lipid composition that is capable of

targeting a reagent, particularly a polynucleotide, to a particular site in an animal, such as a human. Preferably, the lipid composition of the liposome is capable of targeting to a specific organ of an animal, such as the lung, liver, spleen, heart brain, lymph nodes, and skin.

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A liposome useful in the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver its contents to the cell. Preferably, the transfection efficiency of a liposome is about 0.5  $\mu\text{g}$  of DNA per 16 nmole of liposome delivered to about  $10^6$  cells, more preferably about 1.0  $\mu\text{g}$  of DNA per 16 nmole of liposome delivered to about  $10^6$  cells, and even more preferably about 2.0  $\mu\text{g}$  of DNA per 16 nmol of liposome delivered to about  $10^6$  cells. Preferably, a liposome is between about 100 and 500 nm, more preferably between about 150 and 450 nm, and even more preferably between about 200 and 400 nm in diameter.

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Suitable liposomes for use in the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes include liposomes having a polycationic lipid composition and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol. Optionally, a liposome comprises a compound capable of targeting the liposome to a particular cell type, such as a cell-specific ligand exposed on the outer surface of the liposome.

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Complexing a liposome with a reagent such as an antisense oligonucleotide or ribozyme can be achieved using methods that are standard in the art (see, for example, U.S. Patent 5,705,151). Preferably, from about 0.1  $\mu\text{g}$  to about 10  $\mu\text{g}$  of polynucleotide is combined with about 8 nmol of liposomes, more preferably from about 0.5  $\mu\text{g}$  to about 5  $\mu\text{g}$  of polynucleotides are combined with about 8 nmol liposomes, and even more preferably about 1.0  $\mu\text{g}$  of polynucleotides is combined with about 8 nmol liposomes.

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In another embodiment, antibodies can be delivered to specific tissues *in vivo* using receptor-mediated targeted delivery. Receptor-mediated DNA delivery techniques are taught in, for example, Findeis *et al.* *Trends in Biotechnol.* 11, 202-05 (1993); Chiou *et al.*, GENE THERAPEUTICS: METHODS AND APPLICATIONS OF DIRECT GENE TRANSFER (J.A. Wolff, ed.) (1994); Wu & Wu, *J. Biol. Chem.* 263, 621-24 (1988); Wu *et al.*, *J. Biol. Chem.* 269, 542-46 (1994); Zenke *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87, 3655-59 (1990); Wu *et al.*, *J. Biol. Chem.* 266, 338-42 (1991).

#### *Determination of a therapeutically effective dose*

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The determination of a therapeutically effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient which increases or decreases enzymatic activity relative to the enzymatic activity which occurs in the absence of the therapeutically effective dose.

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For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model also can be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

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Therapeutic efficacy and toxicity, *e.g.*, ED<sub>50</sub> (the dose therapeutically effective in 50% of the population) and LD<sub>50</sub> (the dose lethal to 50% of the population), can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD<sub>50</sub>/ED<sub>50</sub>.

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Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED<sub>50</sub> with

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little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

5 The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Factors that can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and  
10 tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

15 Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions,  
20 locations, etc.

If the reagent is a single-chain antibody, polynucleotides encoding the antibody can be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques including, but not limited to, transferrin-polycation-mediated  
25 DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun," and DEAE- or calcium phosphate-mediated transfection.

30 Effective *in vivo* dosages of an antibody are in the range of about 5  $\mu\text{g}$  to about 50  $\mu\text{g/kg}$ , about 50  $\mu\text{g}$  to about 5  $\text{mg/kg}$ , about 100  $\mu\text{g}$  to about 500  $\mu\text{g/kg}$  of patient

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body weight, and about 200 to about 250 µg/kg of patient body weight. For administration of polynucleotides encoding single-chain antibodies, effective *in vivo* dosages are in the range of about 100 ng to about 200 ng, 500 ng to about 50 mg, about 1 µg to about 2 mg, about 5 µg to about 500 µg, and about 20 µg to about 100 µg of DNA.

If the expression product is mRNA, the reagent is preferably an antisense oligonucleotide or a ribozyme. Polynucleotides that express antisense oligonucleotides or ribozymes can be introduced into cells by a variety of methods, as described above.

Preferably, a reagent reduces expression of a human PDE1A gene or the activity of a PDE1A polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the reagent. The effectiveness of the mechanism chosen to decrease the level of expression of a human PDE1A gene or the activity of a human PDE1A polypeptide can be assessed using methods well known in the art, such as hybridization of nucleotide probes to PDE1A-specific mRNA, quantitative RT-PCR, immunologic detection of a human PDE1A polypeptide, or measurement of enzymatic activity.

In any of the embodiments described above, any of the pharmaceutical compositions of the invention can be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy can be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents can act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

Any of the therapeutic methods described above can be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

*Diagnostic methods*

Human PDE1A also can be used in diagnostic assays for detecting diseases and abnormalities or susceptibility to diseases and abnormalities related to the presence of mutations in the nucleic acid sequences that encode the enzyme. For example, differences can be determined between the cDNA or genomic sequence encoding PDE1A in individuals afflicted with a disease and in normal individuals. If a mutation is observed in some or all of the afflicted individuals but not in normal individuals, then the mutation is likely to be the causative agent of the disease.

Sequence differences between a reference gene and a gene having mutations can be revealed by the direct DNA sequencing method. In addition, cloned DNA segments can be employed as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer can be used with a double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures using radiolabeled nucleotides or by automatic sequencing procedures using fluorescent tags.

Genetic testing based on DNA sequence differences can be carried out by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized, for example, by high resolution gel electrophoresis. DNA fragments of different sequences can be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (*see, e.g., Myers et al., Science 230, 1242, 1985*). Sequence changes at specific locations can also be revealed by nuclease protection assays, such as RNase and S 1 protection or the chemical cleavage method (*e.g., Cotton et al., Proc. Natl. Acad. Sci. USA 85, 4397-4401, 1985*). Thus, the detection of a specific DNA sequence can be performed

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by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes and Southern blotting of genomic DNA. In addition to direct methods such as gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

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Altered levels of PDE1A also can be detected in various tissues. Assays used to detect levels of the receptor polypeptides in a body sample, such as blood or a tissue biopsy, derived from a host are well known to those of skill in the art and include radioimmunoassays, competitive binding assays, Western blot analysis, and ELISA assays.

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All patents and patent applications cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples, which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

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### EXAMPLE 1

#### *Detection of PDE1A activity*

5 The polynucleotide of SEQ ID NO: 1 is inserted into the expression vector pCEV4 and the expression vector pCEV4-PDE1A polypeptide obtained is transfected into human embryonic kidney 293 cells. From these cells extracts are obtained, and PDE1A assays are performed in duplicate as follows: 0.5-1 µg of the cell extract is incubated for 15 min at 30°C with appropriate concentrations of either 2,8-  
10 [3H]cAMP or 8-[3H]cGMP in a buffer containing 40 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, and 0.1 unit of snake venome nucleotidase. Reactions are terminated by the addition of EDTA, AMP, and cAMP to final concentrations of 10, 1.25, and 1.25 mM, respectively, and transfer to 4°C. Reaction mixtures are applied to AG-1-X8 anion exchange columns (Bio-Rad) and the hydrolyzed products are eluted with  
15 50% ethanol. The 3H content of the eluate is determined using a liquid scintillation counter. Analysis and plotting of kinetic data is performed by using the computer program Kaleidagraph. The contributions of the high and low affinities cGMP PDE1As of peak I to total PDE activity of this peak at a given substrate concentration are calculated using the formula:  $V = V_1 + V_2 = V_{m1} \cdot S / (K_{m1} + S) + V_{m2} \cdot S / (K_{m1} + S)$ . It is shown that the polypeptide of SEQ ID NO: 2 has a PDE1A  
20 activity.

### EXAMPLE 2

#### *Expression of recombinant human PDE1A*

The *Pichia pastoris* expression vector pPICZB (Invitrogen, San Diego, CA) is used to produce large quantities of recombinant human PDE1A polypeptides in yeast. The PDE1A-encoding DNA sequence is derived from SEQ ID NO: 1. Before insertion  
30 into vector pPICZB, the DNA sequence is modified by well known methods in such a way that it contains at its 5'-end an initiation codon and at its 3'-end an

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enterokinase cleavage site, a His6 reporter tag and a termination codon. Moreover, at both termini recognition sequences for restriction endonucleases are added and after digestion of the multiple cloning site of pPICZ B with the corresponding restriction enzymes the modified DNA sequence is ligated into pPICZB. This expression vector  
5 is designed for inducible expression in *Pichia pastoris*, driven by a yeast promoter.

The resulting pPICZ/md-His6 vector is used to transform the yeast.

The yeast is cultivated under usual conditions in 5 liter shake flasks and the  
10 recombinantly produced protein isolated from the culture by affinity chromatography (Ni-NTA-Resin) in the presence of 8 M urea. The bound polypeptide is eluted with buffer, pH 3.5, and neutralized. Separation of the polypeptide from the His6 reporter tag is accomplished by site-specific proteolysis using enterokinase (Invitrogen, San Diego, CA) according to manufacturer's instructions. Purified human PDE1A poly-  
15 peptide is obtained.

### **EXAMPLE 3**

#### *Identification of test compounds that bind to PDE1A polypeptides*

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Purified PDE1A polypeptides comprising a glutathione-S-transferase protein and absorbed onto glutathione-derivatized wells of 96-well microtiter plates are contacted with test compounds from a small molecule library at pH 7.0 in a physiological buffer solution. Human PDE1A polypeptides comprise the amino acid sequence  
25 shown in SEQ ID NO: 2. The test compounds comprise a fluorescent tag. The samples are incubated for 5 minutes to one hour. Control samples are incubated in the absence of a test compound.

The buffer solution containing the test compounds is washed from the wells.  
30 Binding of a test compound to a human PDE1A polypeptide is detected by fluorescence measurements of the contents of the wells. A test compound that

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increases the fluorescence in a well by at least 15% relative to fluorescence of a well in which a test compound is not incubated is identified as a compound which binds to a human PDE1A polypeptide.

#### 5     **EXAMPLE 4**

##### *Identification of a test compound which decreases PDE1A gene expression*

10     A test compound is administered to a culture of human cells transfected with a PDE1A expression construct and incubated at 37°C for 10 to 45 minutes. A culture of the same type of cells that have not been transfected is incubated for the same time without the test compound to provide a negative control.

15     RNA is isolated from the two cultures as described in Chirgwin *et al.*, *Biochem.* 18, 5294-99, 1979). Northern blots are prepared using 20 to 30 µg total RNA and hybridized with a <sup>32</sup>P-labeled PDE1A-specific probe at 65°C in Express-hyb (CLONTECH). The probe comprises at least 11 contiguous nucleotides selected from the complement of SEQ ID NO: 1. A test compound that decreases the PDE1A-specific signal relative to the signal obtained in the absence of the test  
20     compound is identified as an inhibitor of PDE1A gene expression.

#### **EXAMPLE 5**

##### *Identification of a test compound which decreases PDE1A activity*

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A test compound is administered to a culture of human cells transfected with a PDE1A expression construct and incubated at 37°C for 10 to 45 minutes. A culture of the same type of cells that have not been transfected is incubated for the same time without the test compound to provide a negative control. Enzymatic activity can be  
30     measured as described in U.S. Patent 6,350,603.

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A test compound which decreases the enzymatic activity of the PDE1A relative to the enzymatic activity in the absence of the test compound is identified as an inhibitor of PDE1A activity.

## 5     EXAMPLE 6

### *Tissue-specific expression of PDE1A*

10     The qualitative expression pattern of PDE1A in various tissues is determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

### *Quantitative expression profiling*

15     To demonstrate that PDE1A is involved in cancer, expression is determined in the following tissues: adrenal gland, bone marrow, brain, cerebellum, colon, fetal brain, fetal liver, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle, small intestine, spinal cord, spleen, stomach, testis, thymus, thyroid, trachea, uterus, and peripheral blood lymphocytes. Expression in the following cancer cell lines also is determined: DU-145 (prostate), NCI-H125  
20     (lung), HT-29 (colon), COLO-205 (colon), A-549 (lung), NCI-H460 (lung), HT-116 (colon), DLD-1 (colon), MDA-MD-231 (breast), LS174T (colon), ZF-75 (breast), MDA-MN-435 (breast), HT-1080, MCF-7 (breast), and U87. Matched pairs of malignant and normal tissue from the same patient also are tested.

25     To demonstrate that PDE1A is involved in the disease process of diabetes, the following whole body panel is screened to show predominant or relatively high expression: subcutaneous and mesenteric adipose tissue, adrenal gland, bone marrow, brain, colon, fetal brain, heart, hypothalamus, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle, small intestine, spleen,  
30     stomach, testis, thymus, thyroid, trachea, and uterus. Human islet cells and an islet cell library also are tested. As a final step, the expression of PDE1A in cells derived

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from normal individuals with the expression of cells derived from diabetic individuals is compared.

To demonstrate that PDE1A is involved in the disease process of obesity, expression is determined in the following tissues: subcutaneous adipose tissue, mesenteric adipose tissue, adrenal gland, bone marrow, brain (cerebellum, spinal cord, cerebral cortex, caudate, medulla, substantia nigra, and putamen), colon, fetal brain, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle small intestine, spleen, stomach, testes, thymus, thyroid trachea, and uterus. Neuroblastoma cell lines SK-Nr-Be (2), Hr, Sk-N-As, HTB-10, IMR-32, SNSY-5Y, T3, SK-N-D2, D283, DAOY, CHP-2, U87MG, BE(2)C, T986, KANTS, MO59K, CHP234, C6 (rat), SK-N-F1, SK-PU-DW, PFSK-1, BE(2)M17, and MCIXC also are tested for PDE1A expression. As a final step, the expression of PDE1A in cells derived from normal individuals with the expression of cells derived from obese individuals is compared.

To demonstrate that PDE1A is involved in CNS disorders, the following tissues are screened: fetal and adult brain, muscle, heart, lung, kidney, liver, thymus, testis, colon, placenta, trachea, pancreas, kidney, gastric mucosa, colon, liver, cerebellum, skin, cortex (Alzheimer's and normal), hypothalamus, cortex, amygdala, cerebellum, hippocampus, choroid, plexus, thalamus, and spinal cord.

To demonstrate that PDE1A is involved in the disease process of asthma, the following whole body panel is screened to show predominant or relatively high expression in lung or immune tissues: brain, heart, kidney, liver, lung, trachea, bone marrow, colon, small intestine, spleen, stomach, thymus, mammary gland, skeletal muscle, prostate, testis, uterus, cerebellum, fetal brain, fetal liver, spinal cord, placenta, adrenal gland, pancreas, salivary gland, thyroid, peripheral blood leukocytes, lymph node, and tonsil. Once this is established, the following lung and immune system cells are screened to localize expression to particular cell subsets: lung microvascular endothelial cells, bronchial/tracheal epithelial cells, bronchial/-

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tracheal smooth muscle cells, lung fibroblasts, T cells (T helper 1 subset, T helper 2 subset, NKT cell subset, and cytotoxic T lymphocytes), B cells, mononuclear cells (monocytes and macrophages), mast cells, eosinophils, neutrophils, and dendritic cells. As a final step, the expression of PDE1A in cells derived from normal  
5 individuals with the expression of cells derived from asthmatic individuals is compared.

Quantitative expression profiling is performed by the form of quantitative PCR analysis called "kinetic analysis" firstly described in Higuchi *et al.*, *BioTechnology*  
10 10, 413-17, 1992, and Higuchi *et al.*, *BioTechnology* 11, 1026-30, 1993. The principle is that at any given cycle within the exponential phase of PCR, the amount of product is proportional to the initial number of template copies.

If the amplification is performed in the presence of an internally quenched  
15 fluorescent oligonucleotide (TaqMan probe) complementary to the target sequence, the probe is cleaved by the 5'-3' endonuclease activity of Taq DNA polymerase and a fluorescent dye released in the medium (Holland *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 88, 7276-80, 1991). Because the fluorescence emission will increase in direct proportion to the amount of the specific amplified product, the exponential growth  
20 phase of PCR product can be detected and used to determine the initial template concentration (Heid *et al.*, *Genome Res.* 6, 986-94, 1996, and Gibson *et al.*, *Genome Res.* 6, 995-1001, 1996).

The amplification of an endogenous control can be performed to standardize the  
25 amount of sample RNA added to a reaction. In this kind of experiment, the control of choice is the 18S ribosomal RNA. Because reporter dyes with differing emission spectra are available, the target and the endogenous control can be independently quantified in the same tube if probes labeled with different dyes are used. All "real time PCR" measurements of fluorescence are made in the ABI Prism 7700.

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*RNA extraction and cDNA preparation.* Total RNA from the tissues listed above are used for expression quantification. RNAs labeled "from autopsy" were extracted from autaptic tissues with the TRIzol reagent (Life Technologies, MD) according to the manufacturer's protocol.

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Fifty  $\mu\text{g}$  of each RNA were treated with DNase I for 1 hour at 37°C in the following reaction mix: 0.2 U/ $\mu\text{l}$  RNase-free DNase I (Roche Diagnostics, Germany); 0.4 U/ $\mu\text{l}$  RNase inhibitor (PE Applied Biosystems, CA); 10 mM Tris-HCl pH 7.9; 10mM  $\text{MgCl}_2$ ; 50 mM NaCl; and 1 mM DTT.

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After incubation, RNA is extracted once with 1 volume of phenol:chloroform:isoamyl alcohol (24:24:1) and once with chloroform, and precipitated with 1/10 volume of 3 M sodium acetate, pH5.2, and 2 volumes of ethanol.

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Fifty  $\mu\text{g}$  of each RNA from the autaptic tissues are DNase treated with the DNA-free kit purchased from Ambion (Ambion, TX). After resuspension and spectrophotometric quantification, each sample is reverse transcribed with the TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA) according to the manufacturer's protocol. The final concentration of RNA in the reaction mix is 200 ng/ $\mu\text{L}$ . Reverse transcription is carried out with 2.5  $\mu\text{M}$  of random hexamer primers.

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*TaqMan quantitative analysis.* Specific primers and probe are designed according to the recommendations of PE Applied Biosystems; the probe can be labeled at the 5' end FAM (6-carboxy-fluorescein) and at the 3' end with TAMRA (6-carboxy-tetramethyl-rhodamine). Quantification experiments are performed on 10 ng of reverse transcribed RNA from each sample. Each determination is done in triplicate.

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Total cDNA content is normalized with the simultaneous quantification (multiplex PCR) of the 18S ribosomal RNA using the Pre-Developed TaqMan Assay Reagents (PDAR) Control Kit (PE Applied Biosystems, CA).

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The assay reaction mix is as follows: 1X final TaqMan Universal PCR Master Mix (from 2X stock) (PE Applied Biosystems, CA); 1X PDAR control – 18S RNA (from 20X stock); 300 nM forward primer; 900 nM reverse primer; 200 nM probe; 10 ng  
5 cDNA; and water to 25 µl.

Each of the following steps are carried out once: pre PCR, 2 minutes at 50°C, and 10 minutes at 95°C. The following steps are carried out 40 times: denaturation, 15 seconds at 95°C, annealing/extension, 1 minute at 60°C.

10

The experiment is performed on an ABI Prism 7700 Sequence Detector (PE Applied Biosystems, CA). At the end of the run, fluorescence data acquired during PCR are processed as described in the ABI Prism 7700 user's manual in order to achieve better background subtraction as well as signal linearity with the starting target  
15 quantity.

### EXAMPLE 7

*Proliferation inhibition assay: Antisense oligonucleotides suppress the growth of  
20 cancer cell lines*

The cell line used for testing is the human colon cancer cell line HCT116. Cells are cultured in RPMI-1640 with 10-15% fetal calf serum at a concentration of 10,000 cells per milliliter in a volume of 0.5 ml and kept at 37°C in a 95% air/5%CO<sub>2</sub>  
25 atmosphere.

Phosphorothioate oligoribonucleotides are synthesized on an Applied Biosystems Model 380B DNA synthesizer using phosphoroamidite chemistry. A sequence of 24 bases complementary to the nucleotides at position 1 to 24 of SEQ ID NO: 1 is used  
30 as the test oligonucleotide. As a control, another (random) sequence is used: 5'-TCA ACT GAC TAG ATG TAC ATG GAC-3'. Following assembly and deprotection,

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oligonucleotides are ethanol-precipitated twice, dried, and suspended in phosphate buffered saline at the desired concentration. Purity of the oligonucleotides is tested by capillary gel electrophoresis and ion exchange HPLC. The purified oligonucleotides are added to the culture medium at a concentration of 10  $\mu$ M once per day for seven days.

The addition of the test oligonucleotide for seven days results in significantly reduced expression of human PDE1A as determined by Western blotting. This effect is not observed with the control oligonucleotide. After 3 to 7 days, the number of cells in the cultures is counted using an automatic cell counter. The number of cells in cultures treated with the test oligonucleotide (expressed as 100%) is compared with the number of cells in cultures treated with the control oligonucleotide. The number of cells in cultures treated with the test oligonucleotide is not more than 30% of control, indicating that the inhibition of human PDE1A has an anti-proliferative effect on cancer cells.

### **EXAMPLE 8**

#### *In vivo testing of compounds/target validation*

#### *Acute Mechanistic Assays*

#### *Reduction in Mitogenic Plasma Hormone Levels*

This non-tumor assay measures the ability of a compound to reduce either the endogenous level of a circulating hormone or the level of hormone produced in response to a biologic stimulus. Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.). At a predetermined time after administration of test compound, blood plasma is collected. Plasma is assayed for levels of the hormone of interest. If the normal circulating levels of the hormone are too low and/or variable to provide consistent results, the level of the hormone may be elevated by a pre-treatment with a

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biologic stimulus (i.e., LHRH may be injected i.m. into mice at a dosage of 30 ng/mouse to induce a burst of testosterone synthesis). The timing of plasma collection would be adjusted to coincide with the peak of the induced hormone response. Compound effects are compared to a vehicle-treated control group. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test. Significance is  $p \leq 0.05$  compared to the vehicle control group.

#### *Hollow Fiber Mechanism of Action Assay*

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Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Fibers are harvested in accordance with specific readout assay protocol, these may include assays for gene expression (bDNA, PCR, or Taqman), or a specific biochemical activity (i.e., cAMP levels. Results are analyzed by Student's t-test or Rank Sum test after the variance between groups is compared by an F-test, with significance at  $p \leq 0.05$  as compared to the vehicle control group.

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#### *Subacute Functional In Vivo Assays*

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##### *Reduction in Mass of Hormone Dependent Tissues*

This is another non-tumor assay that measures the ability of a compound to reduce the mass of a hormone dependent tissue (i.e., seminal vesicles in males and uteri in females). Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.) according to a predetermined schedule and for a predetermined duration (i.e., 1 week). At termination of the study, animals are weighed, the target organ is excised, any fluid is expressed, and the weight of the organ is recorded. Blood plasma may also be collected. Plasma may be assayed for levels of a hormone of interest or for levels of test agent. Organ weights may be directly compared or they may be normalized for the body weight of the animal. Compound effects are

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compared to a vehicle-treated control group. An F-test is performed to determine if the variance is equal or unequal followed by a Student's t-test. Significance is p value  $\leq 0.05$  compared to the vehicle control group.

5     *Hollow Fiber Proliferation Assay*

Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Fibers are harvested in accordance with specific readout assay protocol. Cell proliferation is determined by measuring a marker of cell number (i.e., MTT or LDH). The cell number and change in cell number from the starting inoculum are analyzed by Student's t-test or Rank Sum test after the variance between groups is compared by an F-test, with significance at  $p \leq 0.05$  as compared to the vehicle control group.

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*Anti-angiogenesis Models*

*Corneal Angiogenesis*

20     Hydron pellets with or without growth factors or cells are implanted into a micropocket surgically created in the rodent cornea. Compound administration may be systemic or local (compound mixed with growth factors in the hydron pellet). Corneas are harvested at 7 days post implantation immediately following intracardiac infusion of colloidal carbon and are fixed in 10% formalin. Readout is qualitative scoring and/or image analysis. Qualitative scores are compared by Rank Sum test. Image analysis data is evaluated by measuring the area of neovascularization (in pixels) and group averages are compared by Student's t-test (2 tail). Significance is  $p \leq 0.05$  as compared to the growth factor or cells only group.

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*Matrigel Angiogenesis*

Matrigel, containing cells or growth factors, is injected subcutaneously. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Matrigel plugs are harvested at  
5 predetermined time point(s) and prepared for readout. Readout is an ELISA-based assay for hemoglobin concentration and/or histological examination (i.e. vessel count, special staining for endothelial surface markers: CD31, factor-8). Readouts are analyzed by Student's t-test, after the variance between groups is compared by an F-test, with significance determined at  $p \leq 0.05$  as compared to the vehicle control  
10 group.

*Primary Antitumor Efficacy**Early Therapy Models**15 Subcutaneous Tumor*

Tumor cells or fragments are implanted subcutaneously on Day 0. Vehicle and/or compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule starting at a time, usually on Day 1, prior to the ability to measure the tumor  
20 burden. Body weights and tumor measurements are recorded 2-3 times weekly. Mean net body and tumor weights are calculated for each data collection day. Anti-tumor efficacy may be initially determined by comparing the size of treated (T) and control (C) tumors on a given day by a Student's t-test, after the variance between groups is compared by an F-test, with significance determined at  $p \leq 0.05$ . The  
25 experiment may also be continued past the end of dosing in which case tumor measurements would continue to be recorded to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-  
30 Meier curves from the times for individual tumors to attain the evaluation size. Significance is  $p \leq 0.05$ .

*Intraperitoneal/Intracranial Tumor Models*

5 Tumor cells are injected intraperitoneally or intracranially on Day 0. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule starting on Day 1. Observations of morbidity and/or mortality are recorded twice daily. Body weights are measured and recorded twice weekly. Morbidity/mortality data is expressed in terms of the median time of survival and the number of long-term survivors is indicated separately. Survival times are used to generate Kaplan-Meier curves. Significance is  $p \leq 0.05$  by a log-rank test compared to the control group in the experiment.

*Established Disease Model*

15 Tumor cells or fragments are implanted subcutaneously and grown to the desired size for treatment to begin. Once at the predetermined size range, mice are randomized into treatment groups. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Tumor and body weights are measured and recorded 2-3 times weekly. Mean tumor weights of all groups over days post inoculation are graphed for comparison. An F-test is performed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is  $p \leq 0.05$  as compared to the control group. Tumor measurements may be recorded after dosing has stopped to monitor tumor growth delay. Tumor growth delays are expressed as  
25 the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is  $p$  value  $\leq 0.05$  compared to the vehicle control group.

*Orthotopic Disease Models**Mammary Fat Pad Assay*

5 Tumor cells or fragments, of mammary adenocarcinoma origin, are implanted directly into a surgically exposed and reflected mammary fat pad in rodents. The fat pad is placed back in its original position and the surgical site is closed. Hormones may also be administered to the rodents to support the growth of the tumors. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined  
10 schedule. Tumor and body weights are measured and recorded 2-3 times weekly. Mean tumor weights of all groups over days post inoculation are graphed for comparison. An F-test is performed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is  $p \leq 0.05$  as compared to the control  
15 group.

Tumor measurements may be recorded after dosing has stopped to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the  
20 median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is  $p \text{ value} \leq 0.05$  compared to the vehicle control group. In addition, this model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the  
25 study by counting the number of visible foci per target organ, or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at  $p \leq 0.05$  compared to the control group in the experiment.

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*Intraprostatic Assay*

Tumor cells or fragments, of prostatic adenocarcinoma origin, are implanted directly  
5 into a surgically exposed dorsal lobe of the prostate in rodents. The prostate is  
externalized through an abdominal incision so that the tumor can be implanted  
specifically in the dorsal lobe while verifying that the implant does not enter the  
seminal vesicles. The successfully inoculated prostate is replaced in the abdomen  
and the incisions through the abdomen and skin are closed. Hormones may also be  
10 administered to the rodents to support the growth of the tumors. Compounds are  
administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body  
weights are measured and recorded 2-3 times weekly. At a predetermined time, the  
experiment is terminated and the animal is dissected. The size of the primary tumor  
is measured in three dimensions using either a caliper or an ocular micrometer  
15 attached to a dissecting scope. An F-test is performed to determine if the variance is  
equal or unequal followed by a Student's t-test to compare tumor sizes in the treated  
and control groups at the end of treatment. Significance is  $p \leq 0.05$  as compared to  
the control group. This model provides an opportunity to increase the rate of  
spontaneous metastasis of this type of tumor. Metastasis can be assessed at  
20 termination of the study by counting the number of visible foci per target organ (i.e.,  
the lungs), or measuring the target organ weight (i.e., the regional lymph nodes). The  
means of these endpoints are compared by Student's t-test after conducting an F-test,  
with significance determined at  $p \leq 0.05$  compared to the control group in the  
experiment.

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*Intrabronchial Assay*

Tumor cells of pulmonary origin may be implanted intrabronchially by making an  
incision through the skin and exposing the trachea. The trachea is pierced with the  
30 beveled end of a 25 gauge needle and the tumor cells are inoculated into the main  
bronchus using a flat-ended 27 gauge needle with a 90° bend. Compounds are

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administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is performed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is  $p \leq 0.05$  as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the contralateral lung), or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at  $p \leq 0.05$  compared to the control group in the experiment.

#### *Intracecal Assay*

Tumor cells of gastrointestinal origin may be implanted intracurally by making an abdominal incision through the skin and externalizing the intestine. Tumor cells are inoculated into the cecal wall without penetrating the lumen of the intestine using a 27 or 30 gauge needle. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is performed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is  $p \leq 0.05$  as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the liver), or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test,

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with significance determined at  $p \leq 0.05$  compared to the control group in the experiment.

#### Secondary (Metastatic) Antitumor Efficacy

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##### *Spontaneous Metastasis*

Tumor cells are inoculated s.c. and the tumors allowed to grow to a predetermined range for spontaneous metastasis studies to the lung or liver. These primary tumors are then excised. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule which may include the period leading up to the excision of the primary tumor to evaluate therapies directed at inhibiting the early stages of tumor metastasis. Observations of morbidity and/or mortality are recorded daily. Body weights are measured and recorded twice weekly. Potential endpoints include survival time, numbers of visible foci per target organ, or target organ weight. When survival time is used as the endpoint the other values are not determined. Survival data is used to generate Kaplan-Meier curves. Significance is  $p \leq 0.05$  by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance determined at  $p \leq 0.05$  compared to the control group in the experiment for both of these endpoints.

##### *Forced Metastasis*

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Tumor cells are injected into the tail vein, portal vein, or the left ventricle of the heart in experimental (forced) lung, liver, and bone metastasis studies, respectively. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Observations of morbidity and/or mortality are recorded daily. Body weights are measured and recorded twice weekly. Potential endpoints include survival time, numbers of visible foci per target organ, or target organ weight. When

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survival time is used as the endpoint the other values are not determined. Survival data is used to generate Kaplan-Meier curves. Significance is  $p \leq 0.05$  by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance at  $p \leq 0.05$  compared to the vehicle control group in the experiment for both endpoints.

### EXAMPLE 9

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*Diabetes: In vivo testing of compounds/target validation*

#### Glucose Production

Over-production of glucose by the liver, due to an enhanced rate of gluconeogenesis, is the major cause of fasting hyperglycemia in diabetes. Overnight fasted normal rats or mice have elevated rates of gluconeogenesis as do streptozotocin-induced diabetic rats or mice fed ad libitum. Rats are made diabetic with a single intravenous injection of 40 mg/kg of streptozotocin while C57BL/KsJ mice are given 40-60 mg/kg i.p. for 5 consecutive days. Blood glucose is measured from tail-tip blood and then compounds are administered via different routes (p.o., i.p., i.v., s.c.). Blood is collected at various times thereafter and glucose measured. Alternatively, compounds are administered for several days, then the animals are fasted overnight, blood is collected and plasma glucose measured. Compounds that inhibit glucose production will decrease plasma glucose levels compared to the vehicle-treated control group.

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#### Insulin Sensitivity

Both ob/ob and db/db mice as well as diabetic Zucker rats are hyperglycemic, hyperinsulinemic and insulin resistant. The animals are pre-bled, their glucose levels

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measured, and then they are grouped so that the mean glucose level is the same for each group. Compounds are administered daily either q.d. or b.i.d. by different routes (p.o., i.p., s.c.) for 7-28 days. Blood is collected at various times and plasma glucose and insulin levels determined. Compounds that improve insulin sensitivity in these models will decrease both plasma glucose and insulin levels when compared to the vehicle-treated control group.

#### Insulin Secretion

Compounds that enhance insulin secretion from the pancreas will increase plasma insulin levels and improve the disappearance of plasma glucose following the administration of a glucose load. When measuring insulin levels, compounds are administered by different routes (p.o., i.p., s.c. or i.v.) to overnight fasted normal rats or mice. At the appropriate time an intravenous glucose load (0.4 g/kg) is given, blood is collected one minute later. Plasma insulin levels are determined. Compounds that enhance insulin secretion will increase plasma insulin levels compared to animals given only glucose. When measuring glucose disappearance, animals are bled at the appropriate time after compound administration, then given either an oral or intraperitoneal glucose load (1g/kg), bled again after 15, 30, 60 and 90 minutes and plasma glucose levels determined. Compounds that increase insulin levels will decrease glucose levels and the area-under-the glucose curve when compared to the vehicle-treated group given only glucose.

Compounds that enhance insulin secretion from the pancreas will increase plasma insulin levels and improve the disappearance of plasma glucose following the administration of a glucose load. When measuring insulin levels, test compounds which regulate PDE1A are administered by different routes (p.o., i.p., s.c., or i.v.) to overnight fasted normal rats or mice. At the appropriate time an intravenous glucose load (0.4 g/kg) is given, blood is collected one minute later. Plasma insulin levels are determined. Test compounds that enhance insulin secretion will increase plasma insulin levels compared to animals given only glucose. When measuring glucose

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disappearance, animals are bled at the appropriate time after compound administration, then given either an oral or intraperitoneal glucose load (1 g/kg), bled again after 15, 30, 60, and 90 minutes and plasma glucose levels determined. Test compounds that increase insulin levels will decrease glucose levels and the area-under-the glucose curve when compared to the vehicle-treated group given only glucose.

### **EXAMPLE 10**

#### *In vivo testing of compounds/target validation*

##### **Pain**

*Acute pain.* Acute pain is measured on a hot plate mainly in rats. Two variants of hot plate testing are used: In the classical variant animals are put on a hot surface (52 to 56°C) and the latency time is measured until the animals show nocifensive behavior, such as stepping or foot licking. The other variant is an increasing temperature hot plate where the experimental animals are put on a surface of neutral temperature. Subsequently this surface is slowly but constantly heated until the animals begin to lick a hind paw. The temperature which is reached when hind paw licking begins is a measure for pain threshold.

Compounds are tested against a vehicle treated control group. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

*Persistent pain.* Persistent pain is measured with the formalin or capsaicin test, mainly in rats. A solution of 1 to 5% formalin or 10 to 100 µg capsaicin is injected into one hind paw of the experimental animal. After formalin or capsaicin application the animals show nocifensive reactions like flinching, licking and biting of the affected paw. The number of nocifensive reactions within a time frame of up to 90 minutes is a measure for intensity of pain.

Compounds are tested against a vehicle treated control group. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to formalin or capsaicin administration.

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*Neuropathic pain.* Neuropathic pain is induced by different variants of unilateral sciatic nerve injury mainly in rats. The operation is performed under anesthesia. The first variant of sciatic nerve injury is produced by placing loosely constrictive ligatures around the common sciatic nerve. The second variant is the tight ligation of about the half of the diameter of the common sciatic nerve. In the next variant, a group of models is used in which tight ligations or transections are made of either the L5 and L6 spinal nerves, or the L<sub>5</sub> spinal nerve only. The fourth variant involves an axotomy of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact whereas the last variant comprises the axotomy of only the tibial branch leaving the sural and common nerves uninjured. Control animals are treated with a sham operation.

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Postoperatively, the nerve injured animals develop a chronic mechanical allodynia, cold allodynia, as well as a thermal hyperalgesia. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA; Electronic von Frey System, Somedic Sales AB, Hörby, Sweden). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy), or by means of a cold plate of 5 to 10°C where the nocifensive reactions of the affected hind paw are counted as a measure of pain intensity. A further test for cold induced pain is the counting of nocifensive reactions, or duration of nocifensive responses after plantar administration of acetone to the affected hind limb. Chronic pain in general is assessed by registering the circadian rhythms in activity (Surjo and Arndt, Universität zu Köln, Cologne, Germany), and by scoring differences in gait (foot print patterns; FOOTPRINTS program, Klapdor et al., 1997. A low cost method to analyze footprint patterns. J. Neurosci. Methods 75, 49-54).

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Compounds are tested against sham operated and vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

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*Inflammatory Pain.* Inflammatory pain is induced mainly in rats by injection of 0.75 mg carrageenan or complete Freund's adjuvant into one hind paw. The animals develop an edema with mechanical allodynia as well as thermal hyperalgesia. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy, Paw thermal stimulator, G. Ozaki, University of California, USA). For edema measurement two methods are being used. In the first method, the animals are sacrificed and the affected hindpaws sectioned and weighed. The second method comprises differences in paw volume by measuring water displacement in a plethysmometer (Ugo Basile, Comerio, Italy).

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Compounds are tested against uninflamed as well as vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

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*Diabetic neuropathic pain.* Rats treated with a single intraperitoneal injection of 50 to 80 mg/kg streptozotocin develop a profound hyperglycemia and mechanical allodynia within 1 to 3 weeks. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA).

30

Compounds are tested against diabetic and non-diabetic vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

# Parkinson's disease

5        *6-Hydroxydopamine (6-OH-DA) Lesion.* Degeneration of the dopaminergic nigrostriatal and striatopallidal pathways is the central pathological event in Parkinson's disease. This disorder has been mimicked experimentally in rats using single/sequential unilateral stereotaxic injections of 6-OH-DA into the medium forebrain bundle (MFB).

10        Male Wistar rats (Harlan Winkelmann, Germany), weighing 200±250 g at the beginning of the experiment, are used. The rats are maintained in a temperature- and humidity-controlled environment under a 12 h light/dark cycle with free access to food and water when not in experimental sessions. The following in vivo protocols are approved by the governmental authorities. All efforts are made to minimize  
15        animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

Animals are administered pargyline on the day of surgery (Sigma, St. Louis, MO, USA; 50 mg/kg i.p.) in order to inhibit metabolism of 6-OHDA by monoamine  
20        oxidase and desmethylinipramine HCl (Sigma; 25 mg/kg i.p.) in order to prevent uptake of 6-OHDA by noradrenergic terminals. Thirty minutes later the rats are anesthetized with sodium pentobarbital (50 mg/kg) and placed in a stereotaxic frame. In order to lesion the DA nigrostriatal pathway 4 µl of 0.01% ascorbic acid-saline containing 8 µg of 6-OHDA HBr (Sigma) are injected into the left medial fore-brain  
25        bundle at a rate of 1 µl/min (2.4 mm anterior, 1.49 mm lateral, -2.7 mm ventral to Bregma and the skull surface). The needle is left in place an additional 5 min to allow diffusion to occur.

*Stepping Test.* Forelimb akinesia is assessed three weeks following lesion placement  
30        using a modified stepping test protocol. In brief, the animals are held by the experimenter with one hand fixing the hindlimbs and slightly raising the hind part

above the surface. One paw is touching the table, and is then moved slowly sideways (5 s for 1 m), first in the forehand and then in the backhand direction. The number of adjusting steps is counted for both paws in the backhand and forehand direction of movement. The sequence of testing is right paw forehand and backhand adjusting stepping, followed by left paw forehand and backhand directions. The test is repeated three times on three consecutive days, after an initial training period of three days prior to the first testing. Forehand adjusted stepping reveals no consistent differences between lesioned and healthy control animals. Analysis is therefore restricted to backhand adjusted stepping.

10

*Balance Test.* Balance adjustments following postural challenge are also measured during the stepping test sessions. The rats are held in the same position as described in the stepping test and, instead of being moved sideways, tilted by the experimenter towards the side of the paw touching the table. This maneuver results in loss of balance and the ability of the rats to regain balance by forelimb movements is scored on a scale ranging from 0 to 3. Score 0 is given for a normal forelimb placement. When the forelimb movement is delayed but recovery of postural balance detected, score 1 is given. Score 2 represents a clear, yet insufficient, forelimb reaction, as evidenced by muscle contraction, but lack of success in recovering balance, and score 3 is given for no reaction of movement. The test is repeated three times a day on each side for three consecutive days after an initial training period of three days prior to the first testing.

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*Staircase Test (Paw Reaching).* A modified version of the staircase test is used for evaluation of paw reaching behavior three weeks following primary and secondary lesion placement. Plexiglass test boxes with a central platform and a removable staircase on each side are used. The apparatus is designed such that only the paw on the same side at each staircase can be used, thus providing a measure of independent forelimb use. For each test the animals are left in the test boxes for 15 min. The double staircase is filled with 7 x 3 chow pellets (Precision food pellets, formula: P, purified rodent diet, size 45 mg; Sandown Scientific) on each side. After each test the

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number of pellets eaten (successfully retrieved pellets) and the number of pellets taken (touched but dropped) for each paw and the success rate (pellets eaten/pellets taken) are counted separately. After three days of food deprivation (12 g per animal per day) the animals are tested for 11 days. Full analysis is conducted only for the last five days.

*MPTP treatment.* The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes degeneration of mesencephalic dopaminergic (DAergic) neurons in rodents, non-human primates, and humans and, in so doing, reproduces many of the symptoms of Parkinson's disease. MPTP leads to a marked decrease in the levels of dopamine and its metabolites, and in the number of dopaminergic terminals in the striatum as well as severe loss of the tyrosine hydroxylase (TH)-immunoreactive cell bodies in the substantia nigra, pars compacta.

In order to obtain severe and long-lasting lesions, and to reduce mortality, animals receive single injections of MPTP, and are then tested for severity of lesion 7–10 days later. Successive MPTP injections are administered on days 1, 2 and 3. Animals receive application of 4 mg/kg MPTP hydrochloride (Sigma) in saline once daily. All injections are intraperitoneal (i.p.) and the MPTP stock solution is frozen between injections. Animals are decapitated on day 11.

*Immunohistology.* At the completion of behavioral experiments, all animals are anaesthetized with 3 ml thiopental (1 g/40 ml i.p., Tyrol Pharma). The mice are perfused transcardially with 0.01 M PBS (pH 7.4) for 2 min, followed by 4% paraformaldehyde (Merck) in PBS for 15 min. The brains are removed and placed in 4% paraformaldehyde for 24 h at 4°C. For dehydration they are then transferred to a 20% sucrose (Merck) solution in 0.1 M PBS at 4°C until they sink. The brains are frozen in methylbutan at -20°C for 2 min and stored at -70°C. Using a sledge microtome (mod. 3800-Frigocut, Leica), 25 µm sections are taken from the genu of the corpus callosum (AP 1.7 mm) to the hippocampus (AP 21.8 mm) and from AP 24.16 to AP 26.72. Forty-six sections are cut and stored in assorters in 0.25 M Tris buffer (pH 7.4) for immunohistochemistry.

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A series of sections is processed for free-floating tyrosine hydroxylase (TH) immunohistochemistry. Following three rinses in 0.1 M PBS, endogenous peroxidase activity is quenched for 10 min in 0.3% H<sub>2</sub>O<sub>2</sub> ±PBS. After rinsing in  
5 PBS, sections are preincubated in 10% normal bovine serum (Sigma) for 5 min as blocking agent and transferred to either primary anti-rat TH rabbit antiserum (dilution 1:2000).

Following overnight incubation at room temperature, sections for TH immuno-  
40 reactivity are rinsed in PBS (2 x10 min) and incubated in biotinylated anti-rabbit immunoglobulin G raised in goat (dilution 1:200) (Vector) for 90 min, rinsed repeatedly and transferred to Vectastain ABC (Vector) solution for 1 h. 3,3'-Diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.1 M PBS, supplemented with 0.005% H<sub>2</sub>O<sub>2</sub>, serves as chromogen in the subsequent visualization reaction.  
15 Sections are mounted on to gelatin-coated slides, left to dry overnight, counter-stained with hematoxylin, dehydrated in ascending alcohol concentrations and cleared in butylacetate. Coverslips are mounted on entellan.

*Rotarod Test.* We use a modification of the procedure described by Rozas and  
20 Labandeira-Garcia (1997), with a CR-1 Rotamex system (Columbus Instruments, Columbus, OH) comprising an IBM-compatible personal computer, a CIO-24 data acquisition card, a control unit, and a four-lane rotarod unit. The rotarod unit consists of a rotating spindle (diameter 7.3 cm) and individual compartments for each mouse. The system software allows preprogramming of session protocols with varying  
25 rotational speeds (0–80 rpm). Infrared beams are used to detect when a mouse has fallen onto the base grid beneath the rotarod. The system logs the fall as the end of the experiment for that mouse, and the total time on the rotarod, as well as the time of the fall and all the set-up parameters, are recorded. The system also allows a weak current to be passed through the base grid, to aid training.

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## Dementia

*The object recognition task.* The object recognition task has been designed to assess the effects of experimental manipulations on the cognitive performance of rodents. A  
5 rat is placed in an open field, in which two identical objects are present. The rat inspects both objects during the first trial of the object recognition task. In a second trial, after a retention interval of for example 24 hours, one of the two objects used in the first trial, the 'familiar' object, and a novel object are placed in the open field. The inspection time at each of the objects is registered. The basic measures in the OR  
10 task is the time spent by a rat exploring the two object the second trial. Good retention is reflected by higher exploration times towards the novel than the 'familiar' object.

Administration of the putative cognition enhancer prior to the first trial pre-  
15 dominantly allows assessment of the effects on acquisition, and eventually on consolidation processes. Administration of the testing compound after the first trial allows to assess the effects on consolidation processes, whereas administration before the second trial allows to measure effects on retrieval processes.

20 *The passive avoidance task.* The passive avoidance task assesses memory performance in rats and mice. The inhibitory avoidance apparatus consists of a two-compartment box with a light compartment and a dark compartment. The two compartments are separated by a guillotine door that can be operated by the experimenter. A threshold of 2 cm separates the two compartments when the  
25 guillotine door is raised. When the door is open, the illumination in the dark compartment is about 2 lux. The light intensity is about 500 lux at the center of the floor of the light compartment.

Two habituation sessions, one shock session, and a retention session are given,  
30 separated by inter-session intervals of 24 hours. In the habituation sessions and the retention session the rat is allowed to explore the apparatus for 300 sec. The rat is

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placed in the light compartment, facing the wall opposite to the guillotine door. After an accommodation period of 15 sec. the guillotine door is opened so that all parts of the apparatus can be visited freely. Rats normally avoid brightly lit areas and will enter the dark compartment within a few seconds.

5

In the shock session the guillotine door between the compartments is lowered as soon as the rat has entered the dark compartment with its four paws, and a scrambled 1 mA footshock is administered for 2 sec. The rat is removed from the apparatus and put back into its home cage. The procedure during the retention session is identical to that of the habituation sessions.

10

The step-through latency, that is the first latency of entering the dark compartment (in sec.) during the retention session is an index of the memory performance of the animal; the longer the latency to enter the dark compartment, the better the retention is. A testing compound is given half an hour before the shock session, together with 1 mg\*kg<sup>-1</sup> scopolamine. Scopolamine impairs the memory performance during the retention session 24 hours later. If the test compound increases the enter latency compared with the scopolamine-treated controls, is likely to possess cognition enhancing potential.

15

20

*The Morris water escape task.* The Morris water escape task measures spatial orientation learning in rodents. It is a test system that has extensively been used to investigate the effects of putative therapeutic on the cognitive functions of rats and mice. The performance of an animal is assessed in a circular water tank with an escape platform that is submerged about 1 cm below the surface of the water. The escape platform is not visible for an animal swimming in the water tank. Abundant extra-maze cues are provided by the furniture in the room, including desks, computer equipment, a second water tank, the presence of the experimenter, and by a radio on a shelf that is playing softly.

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The animals receive four trials during five daily acquisition sessions. A trial is started by placing an animal into the pool, facing the wall of the tank. Each of four starting positions in the quadrants north, east, south, and west is used once in a series of four trials; their order is randomized. The escape platform is always in the same position.

5 A trial is terminated as soon as the animal had climbs onto the escape platform or when 90 seconds have elapsed, whichever event occurs first. The animal is allowed to stay on the platform for 30 seconds. Then it is taken from the platform and the next trial is started. If an animal did not find the platform within 90 seconds it is put on the platform by the experimenter and is allowed to stay there for 30 seconds. After  
40 the fourth trial of the fifth daily session, an additional trial is given as a probe trial: the platform is removed, and the time the animal spends in the four quadrants is measured for 30 or 60 seconds. In the probe trial, all animals start from the same start position, opposite to the quadrant where the escape platform had been positioned during acquisition.

15 Four different measures are taken to evaluate the performance of an animal during acquisition training: escape latency, traveled distance, distance to platform, and swimming speed. The following measures are evaluated for the probe trial: time (s) in quadrants and traveled distance (cm) in the four quadrants. The probe trial  
20 provides additional information about how well an animal learned the position of the escape platform. If an animal spends more time and swims a longer distance in the quadrant where the platform had been positioned during the acquisition sessions than in any other quadrant, one concludes that the platform position has been learned well.

25 In order to assess the effects of putative cognition enhancing compounds, rats or mice with specific brain lesions which impair cognitive functions, or animals treated with compounds such as scopolamine or MK-801, which interfere with normal learning, or aged animals which suffer from cognitive deficits, are used.

30 *The T-maze spontaneous alternation task.* The T-maze spontaneous alternation task (TeMCAT) assesses the spatial memory performance in mice. The start arm and the

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two goal arms of the T-maze are provided with guillotine doors which can be operated manually by the experimenter. A mouse is put into the start arm at the beginning of training. The guillotine door is closed. In the first trial, the 'forced trial', either the left or right goal arm is blocked by lowering the guillotine door. After the mouse has been released from the start arm, it will negotiate the maze, eventually enter the open goal arm, and return to the start position, where it will be confined for 5 seconds, by lowering the guillotine door. Then, the animal can choose freely between the left and right goal arm (all guillotine-doors opened) during 14 'free choice' trials. As soon as the mouse has entered one goal arm, the other one is closed.

The mouse eventually returns to the start arm and is free to visit whichever goal arm it wants after having been confined to the start arm for 5 seconds. After completion of 14 free choice trials in one session, the animal is removed from the maze. During training, the animal is never handled.

The percent alternations out of 14 trials is calculated. This percentage and the total time needed to complete the first forced trial and the subsequent 14 free choice trials (in s) is analyzed. Cognitive deficits are usually induced by an injection of scopolamine, 30 min before the start of the training session. Scopolamine reduced the per-cent alternations to chance level, or below. A cognition enhancer, which is always administered before the training session, will at least partially, antagonize the scopolamine-induced reduction in the spontaneous alternation rate.

### **EXAMPLE 11**

*In vivo testing of compounds/target validation*

*Tests for activity of T cells*

*Mouse anti-CD3 induced cytokine production model*

BALB/c mice are injected with a single intravenous injection of 10 µg of 145-2C11 (purified hamster anti-mouse CD3ε monoclonal antibodies, PHARMINGEN). A test compound is administered intraperitoneally 60 min prior to the anti-CD3 mAb

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injection. Blood is collected 90 minutes after the antibody injection. Serum is obtained by centrifugation at 3000 r.p.m. for 10 min. IL-2 and IL-4 levels in the serum are determined by an ELISA.

5     *Tests for activity of B cells*

*Mouse anti-IgD induced IgE production model*

BALB/c mice are injected intravenously with 0.8 mg of purified goat anti-mouse IgD antibody or PBS (defined as day 0). Compound is administered intraperitoneally  
10     from day 0 to day 6. On day 7 blood is collected and serum is obtained by centrifugation at 3000 r.p.m. for 10 min. Serum total levels of IgE are determined by YAMASA's ELISA kit and their Ig subtypes are done by an Ig ELISA KIT (Rougier Biotech's, Montreal, Canada).

15     *Tests for activity of monocytes/macrophages*

*Mouse LPS-induced TNF- $\alpha$  production model*

BALB/c mice are injected intraperitoneally with LPS (200  $\mu$ g/mouse). Compound is administered intraperitoneally 1 hr before the LPS injection. Blood is collected at  
20     90 min post-LPS injection and plasma is obtained. TNF- $\alpha$  concentration in the sample is determined using an ELISA kit.

*Tests of eosinophil activation*

*Mouse eotaxin-induced eosinophilia model*

25

BALB/c mice are injected intradermally with a 2.5 ml of air on days -6 and -3 to prepare airpouch. On day 0 compound is administered intraperitoneally 60 min before eotaxin injection (3  $\mu$ g/mouse, i.d.). IL-5 (300 ng/mouse) is injected intravenously 30 min before the eotaxin injection. After 4 hr of the eotaxin injection  
30     leukocytes in exudate is collected and the number of total cells is counted. The

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differential cell counts in the exudate are performed by staining with May-Grunwald Gimsa solution.

*Tests for activation of Th2 cells*

5 *Mouse D10 cell transfer model*

D10.G4.1 cells ( $1 \times 10^7$  cells/mouse) containing 2 mg of conalbumin in saline is administered i.v. to AKR mice. After 6 hr blood is collected and serum is obtained by centrifugation at 3000 r.p.m. for 10min. IL-4 and IL-5 level in serum are determined  
10 by ELISA kits. Compound is administered intraperitoneally at -4 and +1 hr after these cells injection.

*Passive cutaneous anaphylaxis (PCA) test in rats*

15 6 Weeks old male Wistar rats are sensitized intradermally (i.d.) on their shaved backs with 50  $\mu$ l of 0.1  $\mu$ g/ml mouse anti-DNP IgE monoclonal antibody (SPE-7) under a light anesthesia. After 24 hours, the rats are challenged intravenously with 1 ml of saline containing 0.6 mg DNP-BSA (30) (LSL CO., LTD) and 0.005 g of Evans blue. Compounds are injected intraperitoneally (i.p.) 0.5 hr prior to antigen injection. Rats  
20 without the sensitization, challenge, and compound treatment are used for a blank (control) and rats with sensitization, challenge and vehicle treatment are used to determine a value without inhibition. Thirty min after the challenge, the rats are killed, and the skin of the back is removed. Evans blue dye in the skin is extracted in formamide overnight at 63°C. Then an absorbance at 620 nm is measured to obtain  
25 the optical density of the leaked dye.

Percent inhibition of PCA with a compound is calculated as follows:

30 
$$\% \text{ inhibition} = \{(\text{mean vehicle value} - \text{sample value}) / (\text{mean vehicle value} - \text{mean control value})\} \times 100$$

*Anaphylactic bronchoconstriction in rats*

6 Weeks old male Wistar rats are sensitized intravenously (i.v.) with 10 µg mouse anti-DNP IgE, SPE-7, and 1 days later, the rats are challenged intravenously with 0.3 ml of saline containing 1.5 mg DNP-BSA (30) under anesthesia with urethane (1000 mg/kg, i.p.) and gallamine (50 mg/kg, i.v.). The trachea is cannulated for artificial respiration (2 ml / stroke, 70 strokes / min). Pulmonary inflation pressure (PIP) is recorded through a side-arm of cannula connected to pressure transducer. Change in PIP reflects change of both resistance and compliance of the lungs. To evaluate the drugs, each drug is given i.v. 5 min before challenge.

**EXAMPLE 12***In vivo testing of compounds/target validation*

*Evaluation of Compound's Efficacy on the Reduction of Body Weight and Food and Water Consumption in Obese Zucker fa/fa Rats*

The purpose of this protocol is to determine the effect of chronic administration of an unknown compound on body weight and food and water consumption in obese Zucker fa/fa rats. Obese Zucker fa/fa rats are frequently used in the determination of compound efficacy in the reduction of body weight. This animal model has been successfully used in the identification and characterization of the efficacy profile of compounds that are or have been used in the management of body weight in obese humans (Al-Barazanji *et al.*, *Obes. Res.* 8, 317-23, 2000; Assimacopoulos-Jeannet *et al.*, *Am. J. Physiol.* 260 (2 Pt 2):R278-83, 1991; Dryden *et al.*, *Horm. Metab. Res.* 31 (6), 363-6, 1999; Edwards & Stevens, *Pharmacol. Biochem. Behav.* 47, 865-72, 1994; Grinker *et al.*, *Pharmacol. Biochem Behav.* 12 (2), 265-75, 1980).

A typical study includes 60-80 male Zucker fa/fa, (n=10/treatment group) with an average body weight of approximately 550g. Rats are kept in standard animal rooms

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under controlled temperature and humidity and a 12/12 light dark cycle. Water and food are continuously available. Rats are single housed in large rat shoeboxes containing grid floor. Animals are adapted to the grid floors and sham dosed with study vehicle for at least four days before the recording of two-days baseline measurement of body weight and 24 hr food and water consumption. Rats are assigned to one of 6-8 treatment groups based upon their body weight on baseline. The groups are set up so that the mean and standard error of the mean of body weight were similar.

Animals are orally gavaged (2ml/kg) daily before the dark phase of the LD/cycle for a pre-determined number of days (typically 8-14 days) with their assigned dose/-compound. At this time, body weight, food and water consumption are measured. On the final day, animals are euthanized using CO<sub>2</sub> inhalation.

#### *Evaluation of Compound's Efficacy on the Reduction of Body Weight in Diet-Induced Obese Mice*

The purpose of this protocol is to determine the effect of chronic administration of an unknown compound on body weight of mice made obese by exposure to a 45% kcal/g high fat diet during more than 10 weeks. The body weight of mice selected for the studies is higher than three standard deviations from the weight of a control group of mice fed standard low fat (5-6% fat) mouse chow. Diet-induced obese (DIO) animals are frequently used in the determination of compound efficacy in the reduction of body weight (Brown *et al.*, *Br. J. Pharmacol.* 132, 1898-1904, 2001; Guerre-Millom *et al.*, *J. Biol. Chem.* 275 (22), 16638-42, 2000; Han *et al.*, *Int. J. Obes. Relat. Metab. Disord.* 23, 174-9, 1999; Surwit *et al.*, *Endocrinology* 141 (10), 3630-37, 2000).

This animal model has been successfully used in the identification and characterization of the efficacy profile of compounds that are or have been used in the management of body weight in obese humans (Brown *et al.*, *Br. J. Pharmacol.* 132,

1898-1904, 2001; Guerre-Millom *et al.*, *J. Biol. Chem.* 275 (22), 16638-42, 2000; Han *et al.*, *Int. J. Obes. Relat. Metab. Disord.* 23(2), 174-9, 1999).

5 A typical study includes 60-80 male C57bl/J6 mice (n=10/treatment group) with an average body weight of approximately 45 g. Mice are kept in standard animal rooms under controlled temperature and humidity and a 12/12 light dark cycle. Water and food are continuously available. Mice are single housed in shoeboxes. Animals are sham dosed with study vehicle for at least four days before the recording of two-days baseline measurement of body weight and 24 hr food and water consumption. Mice  
10 are assigned to one of 6-8 treatment groups based upon their body weight on baseline. The groups are set up so that the mean and standard error of the mean of body weight were similar.

15 Animals are orally gavaged (5ml/kg) daily before the dark phase of the LD/cycle for a pre-determined number of days (typically 8-14 days) with their assigned dose/-compound. At this time, body weight, food and water consumption are measured. Data is analyzed using appropriate statistics following the research design. On the final day, animals are euthanized using CO<sub>2</sub> inhalation.

20 *Evaluation of Compound's Efficacy on the Reduction of Food Intake in Lean Overnight Fasted Rats*

The purpose of this protocol is to determine the effect of a single dose of an unknown compound on food consumption of lean overnight fasted rats. The fasted-refed rat  
25 model is frequently used in the field of obesity to identify compounds with potential for anorectic effects. This animal model has been successfully used in the identification and characterization of the efficacy profile of compounds that are or have been used in the management of body weight in obese humans.

30 A typical study includes 60-80 male rats (n=10/treatment group) with an average body weight of approximately 280 g. Rats are kept in standard animal rooms under

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controlled temperature and humidity and a 12/12 light dark cycle. Rats are single housed in suspended cages with a mesh floor. Water and food are continuously available unless the animals are being fasted for the study.

5 The efficacy test: The rats are fasted overnight during the dark phase (total of approx. 16-18 hrs). The animal is dosed orally with his assigned treatment (2 mg/ml). One hour after dosing, pre-weighed food jars are returned to the cage. Food intake is recorded 30, 60, 90, 180, 240 minutes post food return. At each time point, spillage is returned to the food jar and then the food jars are weighed. The amount of food  
10 consumed is determined for each time point. Difference between treatment group is determined using appropriate statistical analysis. Blavet *et al.*, *Gen Pharmacology* 13, 293-97, 1982; Grignaschi *et al.*, *Br. J. Pharmacol.* 127, 1190-94, 1999; McTavish & Heel, *Drug.* 43, 713-33, 1992; Rowland *et al.*, *Life Science* 36, 2295-300, 1985.

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### EXAMPLE 13

#### *In vivo target validation*

20 Effects on plasma cholesterol levels including HDL cholesterol are typically assessed in humanized apo-AI transgenic mice. Modulation of human target proteins can be determined in corresponding transgenic mice (*e.g.*, CETP transgenic mice). Triglyceride-lowering is usually evaluated in ob/ob mice or Zucker rats. Animals are fed with normal diets or modified diets (*e.g.*, enriched by 0.5% cholesterol 20%  
25 coconut oil). Standard protocols consist of oral applications once daily for 7 to 10 days at doses ranging from 0,1 to 100 mg/kg. The compounds are dissolved (*e.g.*, in Solutol/Ethanol/saline mixtures) and applied by oral gavage or intravenous injection. Before and at the end of the application period, blood samples are typically drawn by retroorbital punctation. Plasma cholesterol and triglyceride levels are determined  
30 with standardized clinical diagnostic kits (*e.g.*, INFINITY™ cholesterol reagent and INFINITY™ triglyceride reagent; Sigma, St. Louis). HDL cholesterol is determined

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after phosphotungstic acid precipitation of non-HDL lipoproteins or FPLC gel filtration with post-column derivatization of cholesterol using the reagents mentioned above. Plasma levels of human apolipoprotein-AI in relevant humanized transgenic mice are measured by immunoturbidimetry (Sigma).

5

Long-term anti-atherosclerotic potency of drug candidates are evaluated in Apo E-knockout mice. Therefore, animals are fed a standard chow diet (4.5% fat) or a Western diet (20% fat) containing 1 to 100 mg/kg of the respective compounds for 3 to 5 month. Arterial lesions are quantified in serial cryosections of the proximal aorta  
10 by staining with Oil Red O and counterstaining with hematoxylin. Lesion area size is determined using a digital imaging system.

#### **EXAMPLE 14**

15 *In vivo testing of cardiovascular effects of test compounds*  
*Hemodynamics in anesthetized rats*

Male Wistar rats weighing 300-350 g (Harlan Winkelmann, Borcheln, Germany) are anesthetized with thiopental "Nycomed" (Nycomed, Munich, Germany) 100 mg kg<sup>-1</sup>  
20 i.p. A tracheotomy is performed, and catheters are inserted into the femoral artery for blood pressure and heart rate measurements (Gould pressure transducer and recorder, model RS 3400) and into the femoral vein for substance administration. The animals are ventilated with room air and their body temperature is controlled. Test compounds are administered orally or intravenously.

25

*Hemodynamics in conscious SHR*

Female conscious SHR (Moellegaard/Denmark, 220 – 290 g) are equipped with implantable radiotelemetry, and a data aquisition system (Data Sciences, St. Paul,  
30 MN, USA), comprising a chronically implantable transducer/transmitter unit

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equipped with a fluid-filled catheter is used. The transmitter is implanted into the peritoneal cavity, and the sensing catheter is inserted into the descending aorta.

Single administration of test compounds is performed as a solution in Transcutol®/  
5 Cremophor®/ H<sub>2</sub>O (10/20/70 = v/v/v) given orally by gavage. The animals of control groups only receive the vehicle. Before treatment, mean blood pressure and heart rate of treated and untreated control groups are measured.

#### *Hemodynamics in anesthetized dogs*

10

Studies are performed on anesthetized dogs of either sex (body weight between 20-30 kg). Anesthesia is initiated by slow intravenous injection of 25 mg kg<sup>-1</sup> sodium thiopental (Trapanal®, Byk Gulden, Konstanz, Germany). The anesthesia is continued and maintained throughout the experiment by continuous infusion of  
15 0.04 mg kg<sup>-1</sup> h<sup>-1</sup> fentanyl (Fentanyl®, Janssen, Neuss, Germany) and 0.25 mg kg<sup>-1</sup> h<sup>-1</sup> droperidol (DihydrobenzperidolR, Janssen, Neuss, Germany). During this anaesthesia, heart rate is as low as 35-40 bpm due to increased vagal tone. Therefore, a parasympathetic blockade is achieved by intermittent injections of atropine (0.1 mg per animal) (AtropinsulfatR, Eifelfango, Bad Neuenahr, Germany). After intubation  
20 the animals are artificially ventilated at constant volume (EngströmR 300, Engström, Sweden) with room air enriched with 30% oxygen to maintain an end-tidal CO<sub>2</sub> concentration of about 5% (NormocapR, Datex, Finland).

The following catheters are implanted for measurement of cardiovascular parameters:  
25 a tip catheter for recording of left ventricular pressure is inserted into the ventricle via the carotid artery (PC350, Millar Instruments, Houston, TX, USA), a hollow catheter is inserted into the femoral artery and connected to a strain gauge (type 4-327-1, Telos Medical, Upland, CA, USA for recording of arterial blood pressure, two venous catheters are inserted into either femoral vein and one additional catheter into  
30 a forearm vein for application of the anesthetic and drugs, respectively, and an

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oxymetry catheter for recording of oxygen saturation is inserted into the coronary sinus via the jugular vein (Schwarzer IVH4, München, Germany).

After a left-sided thoracotomy the ramus circumflexus of the left coronary artery  
5 (LCX) is freed from connective tissue, and an electromagnetic flow probe (Gould  
Statham, Oxnard, CA, USA) is applied for measurement of coronary blood flow.  
Arterial blood pressure, electrocardiogram (lead II), left ventricular pressure, first  
derivative of left ventricular pressure (dP/dt), heart rate, coronary blood flow, and  
oxygen saturation in the coronary sinus are continuously recorded on a pen recorder  
10 (Brush, Gould, Cleveland, OH, USA). The maximum of dP/dt is used as measure of  
left ventricular contractility (dP/dtmax). After completion of the instrumentation, an  
interval of 60 min is allowed for stabilization before the test compound is  
intravenously applied as bolus injections. Care is taken that all measured cardio-  
vascular parameters have returned to control level before injection of the next dose.  
15 Each dose of the test compound is tested at least three times in different animals.  
The order of injection of the different doses is randomized in each animal.

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CLAIMS

1. An isolated polynucleotide being selected from the group consisting of:
  - 5 a. a polynucleotide encoding a PDE1A polypeptide comprising an amino acid sequence selected from the group consisting of:
    - i. amino acid sequences which are at least about 20% identical to the amino acid sequence shown in SEQ ID NO: 2; and
    - 10 ii. the amino acid sequence shown in SEQ ID NO: 2.
  - b. a polynucleotide comprising the sequence of SEQ ID NO: 1;
  - c. a polynucleotide which hybridizes under stringent conditions to a polynucleotide specified in (a) and (b) and encodes a PDE1A polypeptide;
  - 15 d. a polynucleotide the sequence of which deviates from the polynucleotide sequences specified in (a) to (c) due to the degeneration of the genetic code and encodes a PDE1A polypeptide; and
  - 20 e. a polynucleotide which represents a fragment, derivative or allelic variation of a polynucleotide sequence specified in (a) to (d) and encodes a PDE1A polypeptide.
- 25 2. An expression vector containing any polynucleotide of claim 1.
3. A host cell containing the expression vector of claim 2.
- 30 4. A substantially purified PDE1A polypeptide encoded by a polynucleotide of claim 1.

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5. A method for producing a PDE1A polypeptide, wherein the method comprises the following steps:
- 5 a. culturing the host cell of claim 3 under conditions suitable for the expression of the PDE1A polypeptide; and
- b. recovering the PDE1A polypeptide from the host cell culture.
- 10 6. A method for detection of a polynucleotide encoding a PDE1A polypeptide in a biological sample comprising the following steps:
- a. hybridizing any polynucleotide of claim 1 to a nucleic acid material of a biological sample, thereby forming a hybridization complex; and
- 15 b. detecting said hybridization complex.
7. The method of claim 6, wherein before hybridization, the nucleic acid material of the biological sample is amplified.
- 20 8. A method for the detection of a polynucleotide of claim 1 or a PDE1A polypeptide of claim 4 comprising the steps of:
- a. contacting a biological sample with a reagent which specifically
- 25 interacts with the polynucleotide or the PDE1A polypeptide and
- b. detecting the interaction
9. A diagnostic kit for conducting the method of any one of claims 6 to 8.
- 30

- 90 -

10. A method of screening for agents which decrease the activity of a PDE1A, comprising the steps of:
- 5 a. contacting a test compound with any PDE1A polypeptide encoded by any polynucleotide of claim 1;
- b. detecting binding of the test compound to the PDE1A polypeptide, wherein a test compound which binds to the polypeptide is identified as a potential therapeutic agent for decreasing the activity of a PDE1A.
- 40 11. A method of screening for agents which regulate the activity of a PDE1A, comprising the steps of:
- 15 a. contacting a test compound with a PDE1A polypeptide encoded by any polynucleotide of claim 1; and
- b. detecting a PDE1A activity of the polypeptide, wherein a test compound which increases the PDE1A activity is identified as a potential therapeutic agent for increasing the activity of the PDE1A, and wherein a test compound which decreases the PDE1A activity of the polypeptide is identified as a potential therapeutic agent for decreasing the activity of the PDE1A.
- 20 12. A method of screening for agents which decrease the activity of a PDE1A, comprising the step of:
- 25 contacting a test compound with any polynucleotide of claim 1 and detecting binding of the test compound to the polynucleotide, wherein a test compound which binds to the polynucleotide is identified as a potential therapeutic agent for decreasing the activity of PDE1A.
- 30

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13. A method of reducing the activity of PDE1A, comprising the step of:
- 5                   contacting a cell with a reagent which specifically binds to any polynucleotide of claim 1 or any PDE1A polypeptide of claim 4, whereby the activity of PDE1A is reduced.
14. A reagent that modulates the activity of a PDE1A polypeptide or a polynucleotide wherein said reagent is identified by the method of any of the
- 10                   claim 10 to 12.
15. A pharmaceutical composition, comprising:
- 15                   the expression vector of claim 2 or the reagent of claim 14 and a pharmaceutically acceptable carrier.
16. Use of the expression vector of claim 2 or the reagent of claim 14 in the preparation of a medicament for modulating the activity of a PDE1A in a disease.
- 20                   17. Use of claim 16 wherein the disease is cancer, diabetes, a CNS disorder, asthma, obesity or a cardiovascular disorder.

Fig. 1

|              |             |             |             |             |             |      |
|--------------|-------------|-------------|-------------|-------------|-------------|------|
| atggggtctta  | gtgccaccaga | gattgaagaa  | teggaaaaca  | ccacttttaa  | gtatccttaca | 60   |
| ggagaacacaga | ctgaaaaaat  | gtggcagcgc  | ctgaaagгаа  | tactaagatg  | cttggtgaaг  | 120  |
| cagctggaaa   | gagtgatgt   | taacgtcgtc  | gacttaagaa  | agaatatgя   | atatgcggca  | 180  |
| tctgtgctgg   | aagcagttta  | tatcgatgaa  | acaaгaaгac  | ttctggatac  | tgaagatgag  | 240  |
| ctcagtgaca   | ttcagactga  | ctcagtccca  | tctgaaгtc   | gggactggt   | ggcttctacc  | 300  |
| tttacacgga   | aaatggggat  | gacaaaaaag  | aaactgagг   | aaaaaccaaa  | atttcggagc  | 360  |
| attgtgcatg   | ctgttccaagc | tggaatttt   | gtggaaaгaa  | tgtaaccгaa  | aacatatacat | 420  |
| atggttggt    | tggcataatcc | agcagctgtc  | atcgtaaacat | taaaggatgt  | tgataaatgг  | 480  |
| tctttcgatg   | tatttgccct  | aaatgaaгca  | agtgгagagc  | atagttctgaa | gtttatgat   | 540  |
| tatgaaactgt  | ttaccagata  | tgatcttatac | aaccgtttca  | agattcctgt  | ttcttgccca  | 600  |
| atcacctttg   | cagaaгcctt  | agaaгttggt  | tacagccaagt | acaaaaaatcc | atatccaaat  | 660  |
| ttgatctcatg  | cagctgatgt  | cactcaaaact | gtgcattaca  | taatgcttca  | tacaggtatc  | 720  |
| atgcactggc   | tcactgaaact | gгaaatttta  | gcaatggtct  | ttgctgtgc   | catctcatgat | 780  |
| tatgagcata   | cagggacaac  | aaacaaactt  | cacattcagя  | caaggtcagя  | tgttgccatt  | 840  |
| ttgtataatg   | atcgctctgt  | ccttgagaaт  | caccacgtgя  | gtgcagctta  | tcgacttatg  | 900  |
| caagaagaaг   | aaatgaaтat  | cttgataaat  | ttatccaagг  | atgactggag  | ggatcttcgg  | 960  |
| aaacctagtgя  | ttgaaatggt  | tttatctaca  | gacatgtcag  | gtcacttcca  | gcaaattaaa  | 1020 |
| aatataagaa   | acagtttgca  | gcaгcctgaa  | gggattgaca  | gagccaaaaac | catgtccctg  | 1080 |
| attctccacг   | cagcagacat  | ggttttcctg  | cagggagata  | aaгaaгctgca | ttatcggtgг  | 1140 |
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Fig. 2

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|         |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met Gly | Ser | Ser | Ala | Thr | Glu | Ile | Glu | Glu | Leu | Glu | Asn | Thr | Thr | Phe |
| 1       |     |     | 5   |     |     |     | 10  |     |     |     |     | 15  |     |     |
| Lys Tyr | Leu | Thr | Gly | Glu | Gln | Thr | Glu | Lys | Met | Trp | Gln | Arg | Leu | Lys |
|         |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |
| Gly Ile | Leu | Arg | Cys | Leu | Val | Lys | Gln | Leu | Glu | Arg | Gly | Asp | Val | Asn |
|         | 35  |     |     |     |     | 40  |     |     |     |     | 45  |     |     |     |
| Val Val | Asp | Leu | Lys | Lys | Asn | Ile | Glu | Tyr | Ala | Ala | Ser | Val | Leu | Glu |
| 50      |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     |
| Ala Val | Tyr | Ile | Asp | Glu | Thr | Arg | Arg | Leu | Leu | Asp | Thr | Glu | Asp | Glu |
| 65      |     |     |     | 70  |     |     |     |     | 75  |     |     |     |     | 80  |
| Leu Ser | Asp | Ile | Gln | Thr | Asp | Ser | Val | Pro | Ser | Glu | Val | Arg | Asp | Trp |
|         |     |     | 85  |     |     |     |     | 90  |     |     |     |     | 95  |     |
| Leu Ala | Ser | Thr | Phe | Thr | Arg | Lys | Met | Gly | Met | Thr | Lys | Lys | Lys | Pro |
|         |     | 100 |     |     |     |     | 105 |     |     |     |     | 110 |     |     |
| Glu Glu | Lys | Pro | Lys | Phe | Arg | Ser | Ile | Val | His | Ala | Val | Gln | Ala | Gly |
|         | 115 |     |     |     |     | 120 |     |     |     |     | 125 |     |     |     |
| Ile Phe | Val | Glu | Arg | Met | Tyr | Arg | Lys | Thr | Tyr | His | Met | Val | Gly | Leu |
|         | 130 |     |     |     |     | 135 |     |     |     | 140 |     |     |     |     |
| Ala Tyr | Pro | Ala | Ala | Val | Ile | Val | Thr | Leu | Lys | Asp | Val | Asp | Lys | Trp |
| 145     |     |     |     | 150 |     |     |     |     | 155 |     |     |     |     | 160 |
| Ser Phe | Asp | Val | Phe | Ala | Leu | Asn | Glu | Ala | Ser | Gly | Glu | His | Ser | Leu |
|         |     |     | 165 |     |     |     |     | 170 |     |     |     |     | 175 |     |
| Lys Phe | Met | Ile | Tyr | Glu | Leu | Phe | Thr | Arg | Tyr | Asp | Leu | Ile | Asn | Arg |
|         |     | 180 |     |     |     |     | 185 |     |     |     |     | 190 |     |     |
| Phe Lys | Ile | Pro | Val | Ser | Cys | Leu | Ile | Thr | Phe | Ala | Glu | Ala | Leu | Glu |
|         | 195 |     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |
| Val Gly | Tyr | Ser | Lys | Tyr | Lys | Asn | Pro | Tyr | His | Asn | Leu | Ile | His | Ala |
| 210     |     |     |     |     | 215 |     |     |     |     |     | 220 |     |     |     |

Fig. 2 (continued)

|                                                                 |     |     |     |     |
|-----------------------------------------------------------------|-----|-----|-----|-----|
| Ala Asp Val Thr Gln Thr Val His Tyr Ile Met Leu His Thr Gly Ile | 225 | 230 | 235 | 240 |
| Met His Trp Leu Thr Glu Leu Glu Ile Leu Ala Met Val Phe Ala Ala | 245 | 250 | 255 |     |
| Ala Ile His Asp Tyr Glu His Thr Gly Thr Thr Asn Asn Phe His Ile | 260 | 265 | 270 |     |
| Gln Thr Arg Ser Asp Val Ala Ile Leu Tyr Asn Asp Arg Ser Val Leu | 275 |     |     |     |
| Glu Asn His His Val Ser Ala Ala Tyr Arg Leu Met Gln Glu Glu Glu | 290 | 295 | 300 |     |
| Met Asn Ile Leu Ile Asn Leu Ser Lys Asp Asp Trp Arg Asp Leu Arg | 305 | 310 | 315 | 320 |
| Asn Leu Val Ile Glu Met Val Leu Ser Thr Asp Met Ser Gly His Phe | 325 | 330 |     |     |
| Gln Gln Ile Lys Asn Ile Arg Asn Ser Leu Gln Gln Pro Glu Gly Ile | 340 | 345 | 350 |     |
| Asp Arg Ala Lys Thr Met Ser Leu Ile Leu His Ala Ala Asp Ile Ser | 355 | 360 | 365 |     |
| His Pro Ala Lys Ser Trp Lys Leu His Tyr Arg Trp Thr Met Ala Leu | 370 | 375 | 380 |     |
| Met Glu Glu Phe Phe Leu Gln Gly Asp Lys Glu Ala Glu Leu Gly Leu | 385 | 390 | 395 | 400 |
| Pro Phe Ser Pro Leu Cys Asp Arg Lys Ser Thr Met Val Ala Gln Ser | 405 | 410 | 415 |     |
| Gln Ile Gly Phe Ile Asp Phe Ile Val Glu Pro Thr Phe Ser Leu Leu | 420 | 425 | 430 |     |
| Thr Asp Ser Thr Glu Lys Ile Val Ile Pro Leu Ile Glu Glu Ala Ser | 435 | 440 | 445 |     |
| Lys Ala Glu Thr Ser Ser Tyr Val Ala Ser Ser Ser Thr Thr Ile Val | 450 | 455 | 460 |     |

Fig. 2 (continued)

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Leu | His | Ile | Ala | Asp | Ala | Leu | Arg | Arg | Ser | Asn | Thr | Lys | Gly | Ser |
| 465 |     |     |     |     | 470 |     |     |     |     | 475 |     |     |     | 480 |     |
| Met | Ser | Asp | Gly | Ser | Tyr | Ser | Pro | Asp | Tyr | Ser | Leu | Ala | Ala | Val | Asp |
|     |     |     |     | 485 |     |     |     |     | 490 |     |     |     |     | 495 |     |
| Leu | Lys | Ser | Phe | Lys | Asn | Asn | Leu | Val | Asp | Ile | Ile | Gln | Gln | Asn | Lys |
|     |     |     | 500 |     |     |     |     | 505 |     |     |     |     | 510 |     |     |
| Glu | Arg | Trp | Lys | Glu | Leu | Ala | Ala | Gln | Glu | Ala | Arg | Thr | Ser | Ser | Gln |
|     |     | 515 |     |     |     |     | 520 |     |     |     |     | 525 |     |     |     |
| Lys | Cys | Glu | Ser | Asp | Leu | His | Lys | Asn | Ser | Glu | Asp | Leu | Val | Asn | Ala |
|     | 530 |     |     |     |     | 535 |     |     |     |     | 540 |     |     |     |     |
| Glu | Glu | Lys | His | Asp | Glu | Thr | His | Ser |     |     |     |     |     |     |     |
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## SEQUENCE LISTING

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 210 215 220  
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 245 250 255  
 Ala Ile His Asp Tyr Glu His Thr Gly Thr Thr Asn Asn Phe His Ile  
 260 265 270  
 Gln Thr Arg Ser Asp Val Ala Ile Leu Tyr Asn Asp Arg Ser Val Leu  
 275 280 285  
 Glu Asn His His Val Ser Ala Ala Tyr Arg Leu Met Gln Glu Glu Glu  
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 325 330 335  
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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 03/10832

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/55 C12N9/16 C12Q1/68 G01N33/68 A61K31/713

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, SEQUENCE SEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages            | Relevant to claim No. |
|------------|-----------------------------------------------------------------------------------------------|-----------------------|
| X          | WO 92 18541 A (UNIV WASHINGTON)<br>29 October 1992 (1992-10-29)                               | 1-14                  |
| Y          | SEQ ID NO. 48 and 49<br>abstract; claims 3,13,14,16,24,26,31;<br>example 7<br><br>---<br>-/-- | 15-17                 |

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

☒ Patent family members are listed in annex.

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- \* & \* document member of the same patent family

Date of the actual completion of the international search

8 January 2004

Date of mailing of the international search report

27/01/2004

Name and mailing address of the ISA

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Huse, I

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International Application No  
PCT/EP 03/10832

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| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                      | Relevant to claim No. |
|------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X          | <p>MICHIBATA A H ET AL: "Human Ca/calmodulin-dependent phosphodiesterase PDE1A: novel splice variants, their specific expression, genomic organization, and chromosomal localization" BIOCHIMICA ET BIOPHYSICA ACTA. GENE STRUCTURE AND EXPRESSION, ELSEVIER, AMSTERDAM, NL, vol. 1517, no. 2, 26 January 2001 (2001-01-26), pages 278-287, XP004248813 ISSN: 0167-4781 cited in the application the whole document</p> | 1-9                   |
| X          | <p>-----</p> <p>DATABASE EMBL SEQUENCE 'Online! 6 February 2002 (2002-02-06) STRAUSBERG R.L. ET AL.: "Homo sapiens phosphodiesterase 1A, calmodulin-dependent, mRNA (cds)" retrieved from EBI Database accession no. BC022480 XP002265470 abstract</p>                                                                                                                                                                  | 1-4                   |
| X          | <p>-----</p> <p>DATABASE EMBL SEQUENCE 'Online! 1 October 1996 (1996-10-01) LOUGHNEY K. ET AL.: "Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1A" retrieved from EBI Database accession no. P54750 XP002265471 cited in the application abstract</p>                                                                                                                                          | 1-4                   |
| Y          | <p>-----</p> <p>US 2002/086810 A1 (BEASLEY ELLEN M ET AL) 4 July 2002 (2002-07-04) page 3, paragraph 29 page 8, paragraph 78 page 6, paragraph 64 claims 17,18</p> <p>-----</p>                                                                                                                                                                                                                                         | 15-17                 |

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## Continuation of Box I.1

As far as claims 11 and 12 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

As far as claim 13 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

## Continuation of Box I.2

Claims Nos.: 14, 15-17 (partially)

Present claim 14 and in part claims 15-17 relate to a reagent defined by reference to a desirable characteristic or property, namely a reagent that modulates the activity of a PDE1A polypeptide or a polynucleotide.

The claims cover all reagents having this characteristic or property. However, no such reagents are defined in the application. Therefore the claims lack support and the application lacks disclosure (Article 5 and 6 PCT), that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the reagents by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out only for reagents which inevitably derive from the polypeptide and/or polynucleotide sequences they refer to, namely antibodies, antisense oligonucleotides and ribozymes.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 03/10832

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☒ Claims Nos.: 14, 15-17 (partially)  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

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

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 03/10832

| Patent document<br>cited in search report |    | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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