

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
8 May 2003 (08.05.2003)

PCT

(10) International Publication Number  
WO 03/037254 A2

(51) International Patent Classification<sup>7</sup>:

A61K

(21) International Application Number: PCT/US02/34567

(22) International Filing Date: 28 October 2002 (28.10.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/335,078 31 October 2001 (31.10.2001) US

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(81) Designated States (national): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

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

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/037254 A2

(54) Title: METHODS AND COMPOSITIONS FOR THE TREATMENT AND DIAGNOSIS OF PAIN DISORDERS USING 46566

(57) Abstract: The present invention relates to methods and compositions for the treatment and diagnosis of pain disorders, including, but not limited to, inflammatory pain, chronic pain and/or neuropathic pain. The invention further provides methods for identifying a compound capable of treating a pain disorder or modulating pain and/or inflammation response. The invention further provides a method for modulating pain and/or inflammation in a subject. In addition, the invention provides a method for treating a subject having a pain disorder characterized by aberrant 46566 polypeptide activity or aberrant 46566 nucleic acid expression.

## METHODS AND COMPOSITIONS FOR THE TREATMENT AND DIAGNOSIS OF PAIN DISORDERS USING 46566

This application claims priority to U.S. provisional application number 60/335,078, 5 filed October 31, 2001, the contents of which are herein incorporated by reference.

Pain is initiated when the peripheral terminals of a subgroup of sensory neurons are activated by noxious chemical, mechanical or thermal stimuli. These neurons, called nociceptors, transmit information regarding tissue damage to pain-processing centers in the spinal cord and brain (Fields, H.L. *Pain*, McGraw-Hill, New York, 1987).

10 Once a nociceptor is activated, a chain of events occur that transmit this sensation to the brain to be perceived as pain. An important step in this process is the generation of an action potential in a neuron. An action potential results in the accumulation of calcium ions in the axon terminal. This accumulation of calcium causes a release of neurotransmitter into the synapse and the propagation, ultimately, of the information 15 regarding pain to the next neuron in the pathway from the nociceptor to the brain.

Calcium homeostasis in neurons is vital for proper control of impulses. When action potentials reach the terminal end of an axon, calcium enters the cell at a rate much faster than it can be removed. This causes the release of neurotransmitter into the synapse. In order to regulate this pathway it is vital that the cell have a mechanism to control the 20 intracellular concentration of calcium ions.

The K<sup>+</sup>-dependent Na<sup>+</sup>/Ca<sup>+2</sup> exchangers are transporters of the plasma membrane of most cell types. This Na<sup>+</sup>/Ca<sup>+2</sup> exchanging activity is particularly important to excitable cells in general and neurons in particular. In these cells, K<sup>+</sup>-dependent Na<sup>+</sup>/Ca<sup>+2</sup> exchangers have a crucial role in the control of the Ca<sup>+2</sup> homeostasis in environments 25 where the Na<sup>+</sup> gradient and/or the membrane potential are lower than normal.

Given the prevalence of pain disorders, and the lack of effective cures and early diagnostics, there currently exists a great need for methods and compositions which can serve as markers before the onset of symptoms and which can serve as a means for identifying therapeutics to treat and/or cure these disorders.

30 The present invention provides methods and compositions for the diagnosis and treatment of pain disorders. The present invention is based, at least in part, on the discovery that 46566 (Na-Ca exchanger SLC8) is predominantly expressed in nervous tissues (the brain, spinal cord, and dorsal root ganglia (DRG)). The present invention is

also based, at least in part, on the discovery that the 46566 gene is down-regulated in the spinal cord of animal models for pain, known as complete Freund's adjuvant (CFA) and axotomy models. In the CFA model, CFA is injected in the rodent paw or the monkey knee joint, thereby inducing an inflammatory response with the development of altered 5 pain responses manifested as reduced threshold to noxious stimuli (hyperalgesia) and lowered thresholds to innocuous stimuli (allodynia). The axotomy model involves severing the sciatic nerve of an animal, thereby inducing neuropathic pain.

In one aspect, the invention provides methods for identifying a compound capable of treating a pain disorder, *e.g.*, inflammatory pain, chronic pain and/or neuropathic pain. 10 The method includes assaying the ability of the compound to modulate 46566 nucleic acid expression or 46566 polypeptide activity. In one embodiment, the ability of the compound to modulate nucleic acid expression or 46566 polypeptide activity is determined by detecting modulation of Na-Ca activity in a cell or by detecting intracellular calcium levels.

In another aspect, the invention provides methods for identifying a compound 15 capable of modulating pain and/or inflammation. The method includes contacting a cell expressing a 46566 nucleic acid or polypeptide, *e.g.*, a neuron, with a test compound and assaying the ability of the test compound to modulate the expression of a 46566 nucleic acid or the activity of a 46566 polypeptide.

In a further aspect, the invention features a method for modulating a pain signaling 20 mechanism in a cell. The method includes contacting a cell, *e.g.*, a neuron, with a an effective amount of 46566 modulator, for example, an anti-46566 antibody, a 46566 polypeptide comprising the amino acid sequence of SEQ ID NO:2, or a fragment thereof, a 46566 polypeptide comprising an amino acid sequence which is at least 90 percent 25 identical to the amino acid sequence of SEQ ID NO:2, an isolated naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:2, a small molecule, an antisense 46566 nucleic acid molecule, a nucleic acid molecule of SEQ ID NO:1, or a fragment thereof, or a ribozyme.

In yet another aspect, the invention features a method for treating a subject having a 30 pain disorder, *e.g.*, a pain disorder characterized by aberrant 46566 polypeptide activity or aberrant 46566 nucleic acid expression. The method includes administering to the subject a therapeutically effective amount of a 46566 modulator, *e.g.*, in a pharmaceutically acceptable formulation or by using a gene therapy vector. In one embodiment, the 46566 modulator may be a small molecule, an anti-46566 antibody, a 46566 polypeptide

comprising the amino acid sequence of SEQ ID NO:2, or a fragment thereof, a 46566 polypeptide comprising an amino acid sequence which is at least 90 percent identical to the amino acid sequence of SEQ ID NO:2, an isolated naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:2, an antisense 46566 nucleic acid molecule, a nucleic acid molecule of SEQ ID NO:1, or a fragment thereof, or a 5 ribozyme.

In one embodiment, the pain disorder is inflammatory pain, chronic pain and/or neuropathic pain.

Other features and advantages of the invention will be apparent from the following 10 detailed description and claims.

*Figures 1A-1B* depict the cDNA sequence (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) of 46566.

*Figure 2* depicts a hydrophobicity analysis of the 46566 polypeptide.

The present invention provides methods and compositions for the diagnosis and 15 treatment of pain disorders. The present invention is based, at least in part, on the discovery that 46566 is predominantly expressed in nervous tissues (the brain, spinal cord, and dorsal root ganglia (DRG)). The present invention is also based, at least in part, on the discovery that the 46566 gene is down-regulated in the spinal cord of animal models for pain, known as complete Freund's adjuvant (CFA) and axotomy models. In the CFA 20 model, CFA is injected in the rodent paw or the monkey knee joint, thereby inducing an inflammatory response with the development of altered pain responses manifested as reduced threshold to noxious stimuli (hyperalgesia) and lowered thresholds to innocuous stimuli (allodynia). The axotomy model involves severing the sciatic nerve of an animal, thereby inducing neuropathic pain.

Without intending to be limited by theory, it is believed that the 46566 molecule 25 may be critical for regulating the physiology of neurons involved in nociceptive pathways. Evidence demonstrating that 46566 is downregulated in the spinal cord in the inflammatory model of pain and after nerve injury suggests a significant role of this exchanger in pain signaling mechanisms. Thus, the 46566 molecules, by participating in 30 pain signaling mechanisms, can modulate pain elicitation and provide diagnostic targets and therapeutic agents to control pain and treat pain disorders.

As used herein, the term "pain signaling mechanisms" includes the cellular mechanisms involved in the development and regulation of pain, *e.g.*, pain elicited by

noxious chemical, mechanical, or thermal stimuli, in a subject, *e.g.*, a mammal such as a human. In mammals, the initial detection of noxious chemical, mechanical, or thermal stimuli, a process referred to as "nociception", occurs predominantly at the peripheral terminals of specialized, small diameter primary afferent neurons, called polymodal nociceptors. These afferent neurons transmit the information to the central nervous system, evoking a perception of pain or discomfort and initiating appropriate protective reflexes.

As used herein, the term "pain disorder" includes a disease, and disorder or condition associated with or caused by pain. Examples of pain disorders include, arthritis, 10 allodynia, atypical trigeminal neuralgia, trigeminal neuralgia, somatoform disorder, hypoesthesia, hypealgesia, neuralgia, heuritis, neurogenic pain, analgesia, anesthesia dolorosa, causalgia, sciatic nerve pain disorder, degenerative joint disorder, fibromyalgia, visceral disease, chronic pain disorders, migraine/headache pain, chronic fatigue syndrome, complex regional pain syndrome, neurodystrophy, plantar fasciitis or pain associated with 15 cancer.

The term pain disorder, as used herein, also includes conditions or disorders which are secondary to disorders such as chronic pain and/or neuropathic pain, *i.e.*, are influenced or caused by a disorder such as chronic pain and/or neuropathic pain. Examples of such conditions include, vasodilation and hypotension; conditions which are behavioral, *e.g.*, 20 alcohol dependence (see, *e.g.*, Hungund and Basavarajappa, (2000) *Alcohol and Alcoholism* 35:126-133); or conditions in which detrimental effect(s) are the result of separate disorders or injuries, *e.g.*, multiple sclerosis or spinal cord injury.

As used herein, the term "pain" is art recognized and includes a bodily sensation elicited by noxious chemical, mechanical, or thermal stimuli, in a subject, *e.g.*, a mammal 25 such as a human. Pain is initiated when the peripheral terminals of a subgroup of sensory neurons are activated by noxious chemical, mechanical or thermal stimuli. These neurons, called nociceptors, transmit information regarding tissue damage to pain-processing centres in the spinal chord and brain (Fields, H.L. *Pain*, McGraw-Hill, New York, 1987). The term "pain" includes chronic pain, such as lower back pain; pain due to arthritis, *e.g.*, 30 osteoarthritis; joint pain, *e.g.*, knee pain or carpal tunnel syndrome; myofascial pain, and neuropathic pain. The term "pain" further includes acute pain, such as pain associated with muscle strains and sprains; tooth pain; headaches; pain associated with surgery; or pain associated with various forms of tissue injury, *e.g.*, inflammation, infection, and ischemia.

As used interchangeably herein, the terms “46566 activity,” “biological activity of 46566” or “functional activity of 46566,” include an activity exerted by a 46566 protein, polypeptide or nucleic acid molecule on a 46566 responsive cell or tissue or on a 46566 protein substrate, as determined *in vivo*, or *in vitro*, according to standard techniques.

5 46566 activity can be a direct activity, such as an association with a 46566-target molecule. As used herein, a “substrate” or “target molecule” or “binding partner” is a molecule with which a 46566 protein binds or interacts in nature, such that 46566-mediated function, *e.g.*, modulation of a pain signaling mechanism, is achieved. A 46566 target molecule can be a non-46566 molecule (*e.g.*, NAD<sup>+</sup>, NADP<sup>+</sup>, or other cofactor, or a biochemical molecule

10 involved in a pain signaling mechanism), or a 46566 protein or polypeptide. Examples of such target molecules include proteins in the same signaling path as the 46566 protein, *e.g.*, proteins which may function upstream (including both stimulators and inhibitors of activity) or downstream of the 46566 protein in a pain signaling pathway. Alternatively, a 46566 activity is an indirect activity, such as a cellular signaling activity mediated by

15 interaction of the 46566 protein with a 46566 target molecule. The biological activities of 46566 are described herein. For example, the 46566 proteins have one or more of the following activities: (1) regulation of Ca<sup>2+</sup> production in a cell, *e.g.*, to be used as a second messenger in a signal transduction cascade; (2) modulation of a pain signaling mechanism; (3) modulation of neurotransmitter release; (4) modulation of synaptic, *e.g.*, spontaneous

20 synaptic, activity; (5) regulation of sodium exchange in a cell to be used as a second messenger in a signal transduction cascade.

Various aspects of the invention are described in further detail in the following subsections:

25 I. Screening Assays:

The invention provides methods (also referred to herein as “screening assays”) for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules, ribozymes, or 46566 antisense molecules) which bind to 46566 proteins, have a stimulatory or inhibitory effect on 46566 expression or 46566 activity, or have a stimulatory or inhibitory effect on the expression or activity of a 46566 target molecule. Compounds identified using the assays described herein may be useful for treating pain disorders.

Candidate/test compounds include, for example, 1) peptides such as soluble peptides, including Ig-tailed fusion peptides and members of random peptide libraries (see, e.g., Lam, K.S. *et al.* (1991) *Nature* 354:82-84; Houghten, R. *et al.* (1991) *Nature* 354:84-86) and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids; 2) phosphopeptides (e.g., members of random and partially degenerate, directed phosphopeptide libraries, see, e.g., Songyang, Z. *et al.* (1993) *Cell* 72:767-778); 3) antibodies (e.g., polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, and single chain antibodies as well as Fab, F(ab')<sub>2</sub>, Fab expression library fragments, and epitope-binding fragments of antibodies); and 4) small organic and inorganic molecules (e.g., molecules obtained from combinatorial and natural product libraries).

The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: 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 peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994) *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:1865-1869) or phage (Scott and Smith (1990) *Science* 249:386-390; Devlin (1990) *Science* 249:404-406; Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici (1991) *J. Mol. Biol.* 222:301-310; Ladner *supra*).

In one aspect, an assay is a cell-based assay in which a cell which expresses a 46566 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate 46566 activity is determined. In a preferred embodiment, the biologically active portion of the 46566 protein includes a domain or 5 motif that can modulate pain and/or inflammation. Determining the ability of the test compound to modulate 46566 activity can be accomplished by monitoring, for example, modulation of pain and/or inflammation. The cell, for example, can be of mammalian origin.

The ability of the test compound to modulate 46566 binding to a substrate can also 10 be determined. Determining the ability of the test compound to modulate 46566 binding to a substrate can be accomplished, for example, by coupling the 46566 substrate with a radioisotope, fluorescent, or enzymatic label such that binding of the 46566 substrate to 46566 can be determined by detecting the labeled 46566 substrate in a complex.

Alternatively, 46566 could be coupled with a radioisotope or enzymatic label to monitor 15 the ability of a test compound to modulate 46566 binding to a 46566 substrate in a complex. Determining the ability of the test compound to bind 46566 can be accomplished, for example, by coupling the compound with a radioisotope or enzymatic label such that binding of the compound to 46566 can be determined by detecting the labeled 46566 compound in a complex. For example, 46566 substrates can be labeled with 20  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^3\text{H}$ , either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

25 It is also within the scope of this invention to determine the ability of a compound to interact with 46566 without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a compound with 46566 without the labeling of either the compound or the 46566 (McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912). As used herein, a "microphysiometer" (e.g., Cytosensor) is an analytical 30 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 compound and 46566.

Because 46566 expression is downregulated in dorsal root ganglia (DRG) after axotomy, compounds which modulate pain and/or inflammation can be identified by the ability to modulate 46566 expression. To determine whether a test compound modulates 46566 expression, a cell which expresses 46566 is contacted with a test compound, and the 5 ability of the test compound to modulate 46566 expression can be determined by measuring 46566 mRNA by, *e.g.*, Northern Blotting, quantitative PCR (*e.g.*, TaqMan), or *in vitro* transcriptional assays. To perform an *in vitro* transcriptional assay, the full length promoter and enhancer of 46566 can be linked to a reporter gene such as chloramphenicol acetyltransferase (CAT) or luciferase and introduced into host cells. The same host cells 10 can then be transfected with or contacted with the test compound. The effect of the test compound can be measured by reporter gene activity and comparison to reporter gene activity in cells which do not contain the test compound. An increase or decrease in reporter gene activity indicates a modulation of 46566 expression and is, therefore, an indicator of the ability of the test compound to modulate pain and/or inflammation .

15 Assays that may be used to identify compounds that modulate 46566 activity also include assays that test for the ability of a compound to modulate pain and/or inflammation . The ability of a test compound to modulate pain and/or inflammation can be measured by its ability to modulate inflammation of the tissues surrounding the site of injury.

In yet another embodiment, an assay of the present invention is a cell-free assay in 20 which a 46566 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to or to modulate (*e.g.*, stimulate or inhibit) the activity of the 46566 protein or biologically active portion thereof is determined. Preferred biologically active portions of the 46566 proteins to be used in assays of the present invention include fragments that participate in interactions with non- 25 46566 molecules, *e.g.*, fragments with high surface probability scores. Binding of the test compound to the 46566 protein can be determined either directly or indirectly as described above. Determining the ability of the 46566 protein to bind to a test compound can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345; Szabo *et al.* 30 (1995) *Curr. Opin. Struct. Biol.* 5:699-705). As used herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIACore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In yet another embodiment, the cell-free assay involves contacting a 46566 protein or biologically active portion thereof with a known compound which binds the 46566 protein to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with the 46566 protein, wherein

5 determining the ability of the test compound to interact with the 46566 protein comprises determining the ability of the 46566 protein to preferentially bind to or modulate the activity of a 46566 target molecule (e.g., a 46566 substrate). The cell-free assays of the present invention are amenable to use of both soluble and/or membrane-bound forms of isolated proteins (e.g., 46566 proteins or biologically active portions thereof). In the

10 case of cell-free assays in which a membrane-bound form of an isolated protein is used it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the isolated protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-15 114, Thesit®, Isotridecy poly(ethylene glycol ether)<sub>n</sub>, 3-[(3-

cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-

cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one embodiment of the above assay methods of the present invention, 20 it may be desirable to immobilize either 46566 or a 46566 target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to a 46566 protein, or interaction of a 46566 protein with a 46566 target molecule in the presence and absence of a test compound, can be accomplished in any vessel suitable for containing the 25 reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/46566 fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, 30 MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or 46566 protein, and the mixture incubated under conditions conducive to complex formation (e.g.,

at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix is immobilized in the case of beads, and complex formation is determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the 5 matrix, and the level of 46566 binding or activity determined using standard techniques.

Other techniques for immobilizing proteins or cell membrane preparations on 10 matrices can also be used in the screening assays of the invention. For example, either a 46566 protein or a 46566 target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated 46566 protein or target molecules can be prepared 15 from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which are reactive with 46566 protein or target molecules but which do not interfere with binding of the 46566 protein to its target molecule can be derivatized to the wells of the plate, and 20 unbound target or 46566 protein is trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive 25 with the 46566 protein or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the 46566 protein or target molecule.

20 In yet another aspect of the invention, the 46566 protein or fragments thereof can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300) to identify other proteins which bind to 25 or interact with 46566 ("46566-binding proteins" or "46566-bp) and are involved in 46566 activity. Such 46566-binding proteins are also likely to be involved in the propagation of signals by the 46566 proteins or 46566 targets as, for example, downstream elements of a 46566-mediated signaling pathway. Alternatively, such 46566-binding proteins are likely 30 to be 46566 inhibitors.

30 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. In one construct, the gene that codes for a 46566 protein is fused to a gene encoding the DNA binding domain of a known transcription

factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo*, forming a 46566-dependent complex, the 5 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 can be isolated and used to obtain the cloned gene that encodes the 10 protein that interacts with the 46566 protein.

In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a modulating agent can be identified using a cell-based or a cell-free assay, and the ability of the agent to modulate the activity of a 46566 protein can be confirmed *in vivo*, e.g., in an animal such as an animal model for chronic 15 pain and/or neuropathic pain.

Moreover, a 46566 modulator identified as described herein (e.g., an antisense 46566 nucleic acid molecule, a 46566-specific antibody, or a small molecule) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such a modulator. Alternatively, a 46566 modulator identified as described herein can be 20 used in an animal model to determine the mechanism of action of such a modulator.

The ability of a given modulating agent to modulate pain can be quantitated by using any one of the following tests: tight ligation of L6 and L7, as a model of neuropathic pain; complete Freund's adjuvant into knee joint or hind paw as a model of Long term inflammatory pain (Palecek, J. (1992) *Neurophysiol* 68:1951-66); nerve ligation (CCI); 25 thermal hyperalgesia, tactile allodynia and cold allodynia (Carlton, S.M. *et al.* (1994) *Pain* 56:155-66); thermal paw withdrawal latency (Hargreaves test); von Frey mechanical withdrawal threshold; the hot-plate latency test; the tail flick test (Stone, L.S., *et al.* (1997) *NeruroReport* 8:3131-3135); the warm-water immersion tail flick assay (Stone, L.S., *et al.* (1997) *NeruroReport* 8:3131-3135); the crush injury to the sciatic nerve test (De Konig, *et* 30 *al.* (1986) *J. Neurol. Sci.* 74:237-246); the cold water allodynia test (Hunter, *et al.* (1997) *Pain* 69:317-322; the paw pressure latency assay (Hakki-Onen, S., *et al.* (2001) *Brain Research* 900(2):261-7; or the radiant heat test (Yoshimura, M., (2001) *Pharm. Research* 44(2):105-11.

Briefly, the tail flick latency test involves projecting a beam of light to the tail of an animal. The time is measured from the onset of the tail heating and stops at the moment of the tail flick. Typically, five tail flick latency (TFL) measurements are made per rat per session with 5-10 minutes between trials.

5 The thermal paw withdrawal latency test, also known as the Hargreaves test, consists of directing a light beam onto the ventral surface of the rats' left hindpaw from below and measuring the time until the paw is reflexively moved away from the light.

10 The von Frey mechanical withdrawal threshold involves placing the rat on a screen surface and attaching a von Frey filament to a force transducer. The filament is pressed upward against the ventral right hindpaw of the animal to measure the force at the instant of paw withdrawal.

The hot-plate latency test involves placing a rat onto a heated surface and measuring the time it takes the animal to jump or to lick a hindpaw.

15 Animal models for pain or inflammation may also be produced by the following methods: subcutaneous injection of formalin, lambda-carrageenan, Mustard oil, or complete Freund's adjuvant (CFA) into the right hind paw or knee of an animal, which causes inflammatory pain; chronic constriction of the sciatic nerve of an animal, which induces neuropathic pain; dibutylin dichloride injection in an animal, which causes chronic pancreatic inflammation; axotomy of the sciatic nerve or the tibial nerve of an animal; or 20 chronic constriction of the spinal nerves of an animal which induces neuropathic pain.

The ability of a given modulating agent to moderate the Na-Ca exchange can be quantitated by using a calcium uptake assay. The assay is performed with adult rat dorsal root ganglion cells and tests the exchange of radioactive Ca, as described by Wood *et al.* (1988) *J. Neurosci.* 8:3208-3220, herein incorporated by reference in its entirety.

25

## II. Predictive Medicine:

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one 30 aspect of the present invention relates to diagnostic assays for determining 46566 protein and/or nucleic acid expression as well as 46566 activity, in the context of a biological sample (*e.g.*, blood, serum, cells, or tissue, *e.g.*, neural tissue) to thereby determine whether an individual is afflicted with a pain disorder. The invention also provides for prognostic

(or predictive) assays for determining whether an individual is at risk of developing a pain disorder. For example, mutations in a 46566 gene can be assayed for in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a pain disorder.

5 Another aspect of the invention pertains to monitoring the influence of 46566 modulators (*e.g.*, anti-46566 antibodies or 46566 ribozymes) on the expression or activity of 46566 in clinical trials.

These and other agents are described in further detail in the following sections.

10 **A. Diagnostic Assays For Pain Disorders**

To determine whether a subject is afflicted with a pain disorder, a biological sample may be obtained from a subject and the biological sample may be contacted with a compound or an agent capable of detecting a 46566 protein or nucleic acid (*e.g.*, mRNA or genomic DNA) that encodes a 46566 protein, in the biological sample. A preferred agent 15 for detecting 46566 mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to 46566 mRNA or genomic DNA. The nucleic acid probe can be, for example, the 46566 nucleic acid set forth in SEQ ID NO:1, or a portion thereof, such as an oligonucleotide of at least 15, 20, 25, 30, 25, 40, 45, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to 46566 mRNA 20 or genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein.

A preferred agent for detecting 46566 protein in a sample is an antibody capable of binding to 46566 protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof 25 (*e.g.*, Fab or F(ab')2) can be used. The term “labeled”, with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of direct substances that can be coupled to an antibody or a nucleic acid probe 30 include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-

labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin.

The term "biological sample" is intended to include tissues, cells, and biological fluids isolated from a subject, as well as tissues, cells, and fluids present within a subject.

5 That is, the detection method of the invention can be used to detect 46566 mRNA, protein, or genomic DNA in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of 46566 mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of 46566 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and 10 immunofluorescence. *In vitro* techniques for detection of 46566 genomic DNA include Southern hybridizations. Furthermore, *in vivo* techniques for detection of 46566 protein include introducing into a subject a labeled anti-46566 antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

15 In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting 46566 protein, mRNA, or genomic DNA, such that the presence of 46566 protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of 46566 protein, mRNA or genomic DNA in the control sample 20 with the presence of 46566 protein, mRNA or genomic DNA in the test sample.

#### B. Prognostic Assays For Pain Disorders

The present invention further pertains to methods for identifying subjects having or at risk of developing a pain disorder, *e.g.*, a pain disorder associated with aberrant 46566 expression or activity.

25 As used herein, the term "aberrant" includes a 46566 expression or activity that deviates from the wild type 46566 expression or activity. Aberrant expression or activity includes increased or decreased expression or activity, as well as expression or activity that does not follow the wild type developmental pattern of expression or the subcellular 30 pattern of expression. For example, aberrant 46566 expression or activity is intended to include the cases in which a mutation in the 46566 gene causes the 46566 gene to be under-expressed or over-expressed and situations in which such mutations result in a non-functional 46566 protein or a protein which does not function in a wild-type fashion, *e.g.*, a

protein which does not interact with a 46566 substrate, or one which interacts with a non-46566 substrate.

The assays described herein, such as the preceding diagnostic assays or the following assays, can be used to identify a subject having or at risk of developing a pain disorder, *e.g.*, inflammatory pain, chronic pain and/or neuropathic pain. A biological sample may be obtained from a subject and tested for the presence or absence of a genetic alteration. For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a 46566 gene, 2) an addition of one or more nucleotides to a 46566 gene, 3) a substitution of one or more nucleotides of a 46566 gene, 4) a chromosomal rearrangement of a 46566 gene, 5) an alteration in the level of a messenger RNA transcript of a 46566 gene, 6) aberrant modification of a 46566 gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a 46566 gene, 8) a non-wild type level of a 46566-protein, 9) allelic loss of a 46566 gene, and 10) 15) inappropriate post-translational modification of a 46566-protein.

As described herein, there are a large number of assays known in the art that can be used for detecting genetic alterations in a 46566 gene. For example, a genetic alteration in a 46566 gene may be detected using a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, 20) or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:360-364), the latter of which can be particularly useful for detecting point mutations in a 46566 gene (see Abravaya *et al.* (1995) *Nucleic Acids Res.* 23:675-682). This method includes collecting a biological sample from a subject, isolating nucleic acid (*e.g.*, genomic DNA, 25) mRNA or both) from the sample; contacting the nucleic acid sample with one or more primers which specifically hybridize to a 46566 gene under conditions such that hybridization and amplification of the 46566 gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or 30) LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self sustained sequence replication (Guatelli, J.C. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional

amplification system (Kwoh, D.Y. *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi, P.M. *et al.* (1988) *Bio-Technology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially 5 useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a 46566 gene from a biological sample can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more 10 restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

15 In other embodiments, genetic mutations in 46566 can be identified by hybridizing biological sample derived and control nucleic acids, *e.g.*, DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotide probes (Cronin, M.T. *et al.* (1996) *Hum. Mutat.* 7:244-255; Kozal, M.J. *et al.* (1996) *Nat. Med.* 2:753-759). For example, genetic mutations in 46566 can be identified in two dimensional arrays 20 containing light-generated DNA probes as described in Cronin, M.T. *et al.* (1996) *supra*. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential, overlapping probes. This step allows for the identification of point mutations. This step is followed by a second hybridization array that allows for the 25 characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

30 In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the 46566 gene in a biological sample and detect mutations by comparing the sequence of the 46566 in the biological sample with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert (1977) *Proc. Natl. Acad. Sci.*

USA 74:560) or Sanger (1977) *Proc. Natl. Acad. Sci. USA* 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays (Naeve, C. W. (1995) *Biotechniques* 19:448-53), including sequencing by mass spectrometry (see, e.g., PCT International Publication No.

5 WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in the 46566 gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) *Science* 230:1242). In general, the art 10 technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type 46566 sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample 15 strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated 20 by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4397 and Saleeba *et al.* (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more 25 proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in 46566 cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 15:1657-1662). 30 According to an exemplary embodiment, a probe based on a 46566 sequence, e.g., a wild-type 46566 sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if

any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in 46566 genes. For example, single strand conformation 5 polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci USA*: 86:2766; see also Cotton (1993) *Mutat. Res.* 285:125-144 and Hayashi (1992) *Genet. Anal. Tech. Appl.* 9:73-79). Single-stranded DNA fragments of sample and control 46566 nucleic acids will be denatured and allowed to renature. The secondary structure of single-10 stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes 15 heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet.* 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing 20 gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA will be modified to ensure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample 25 DNA (Rosenbaum and Reissner (1987) *Biophys. Chem.* 265:12753).

Examples of other techniques for detecting point mutations include, but are not 30 limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature* 324:163); Saiki *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different 35 mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention.

Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential 5 hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based 10 detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

15 Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered a 46566 modulator (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, or small molecule) to effectively treat a pain.

20 **C. Monitoring of Effects During Clinical Trials**

The present invention further provides methods for determining the effectiveness of a 46566 modulator (*e.g.*, a 46566 modulator identified herein) in treating a pain disorder in a subject. For example, the effectiveness of a 46566 modulator in increasing 46566 gene expression, protein levels, or in upregulating 46566 activity, can be monitored in clinical 25 trials of subjects exhibiting decreased 46566 gene expression, protein levels, or downregulated 46566 activity. Alternatively, the effectiveness of a 46566 modulator in decreasing 46566 gene expression, protein levels, or in downregulating 46566 activity, can be monitored in clinical trials of subjects exhibiting increased 46566 gene expression, protein levels, or 46566 activity. In such clinical trials, the expression or activity of a 30 46566 gene, and preferably, other genes that have been implicated in, for example, a pain disorder can be used as a "read out" or marker of the phenotype of a particular cell.

For example, and not by way of limitation, genes, including 46566, that are modulated in cells by treatment with an agent which modulates 46566 activity (*e.g.*,

identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents which modulate 46566 activity on subjects suffering from a pain disorder in, for example, a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of 46566 and other genes implicated in the pain disorder. The 5 levels of gene expression (*e.g.*, a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods described herein, or by measuring the levels of activity of 46566 or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent which modulates 10 46566 activity. This response state may be determined before, and at various points during treatment of the individual with the agent which modulates 46566 activity.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent that modulates 46566 activity (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, or small 15 molecule identified by the screening assays described herein) including the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a 46566 protein, mRNA, or genomic DNA in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the 46566 protein, mRNA, 20 or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the 46566 protein, mRNA, or genomic DNA in the pre-administration sample with the 46566 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression 25 or activity of 46566 to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of 46566 to lower levels than detected, *i.e.*, to decrease the effectiveness of the agent. According to such an embodiment, 46566 expression or activity may be used as an indicator of the effectiveness of an agent, even in the absence of an 30 observable phenotypic response.

III. Methods of Treatment of Subjects Suffering From Pain Disorders:

The present invention provides for both prophylactic and therapeutic methods of treating a subject, *e.g.*, a human, at risk of (or susceptible to) a pain disorder such as inflammatory pain, chronic pain and/or neuropathic pain, for example, chronic pain disorders, fibromyalgia, migraine/headache pain, cancer pain, chronic fatigue syndrome, arthritis, complex regional pain syndrome, causalgia, neurodystrophy, or plantar fasciitis.

5      disorders, fibromyalgia, migraine/headache pain, cancer pain, chronic fatigue syndrome, arthritis, complex regional pain syndrome, causalgia, neurodystrophy, or plantar fasciitis.

As used herein, “treatment” of a subject includes the application or administration of a therapeutic agent to a subject, or application or administration of a therapeutic agent to a cell or tissue from a subject, who has a disease or disorder, has a symptom of a disease or

10     disorder, or is at risk of (or susceptible to) a disease or disorder, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease or disorder, the symptom of the disease or disorder, or the risk of (or susceptibility to) the disease or disorder. As used herein, a “therapeutic agent” includes, but is not limited to, small molecules, peptides, polypeptides, antibodies, ribozymes, and antisense oligonucleotides.

15     With regard to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. “Pharmacogenomics,” as used herein, refers to the application of genomics technologies such as gene sequencing, statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More specifically,

20     the term refers to the study of how a patient's genes determine his or her response to a drug (*e.g.*, a patient's “drug response phenotype”, or “drug response genotype”).

Thus, another aspect of the invention provides methods for tailoring a subject's prophylactic or therapeutic treatment with either the 46566 molecules of the present invention or 46566 modulators according to that individual's drug response genotype.

25     Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to avoid treatment of patients who will experience toxic drug-related side effects.

A. Prophylactic Methods

30     In one aspect, the invention provides a method for preventing in a subject, a pain disorder by administering to the subject an agent which modulates 46566 expression or 46566 activity in a cell, *e.g.*, a neuron. Subjects at risk for developing a pain disorder can be identified by, for example, any or a combination of the diagnostic or prognostic assays

described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of aberrant 46566 expression or activity, such that a pain disorder is prevented or, alternatively, delayed in its progression. Depending on the type of 46566 aberrancy, for example, a 46566 molecule, 46566 agonist or 46566 antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

#### **B. Therapeutic Methods**

Another aspect of the invention pertains to methods for treating a subject suffering from a pain disorder. These methods involve administering to a subject an agent which modulates 46566 expression or activity (*e.g.*, an agent identified by a screening assay described herein), or a combination of such agents. In another embodiment, the method involves administering to a subject a 46566 protein or nucleic acid molecule as therapy to compensate for reduced, aberrant, or unwanted 46566 expression or activity.

Stimulation of 46566 activity is desirable in situations in which 46566 is abnormally downregulated and/or in which increased 46566 activity is likely to have a beneficial effect. Likewise, inhibition of 46566 activity is desirable in situations in which 46566 is abnormally upregulated and/or in which decreased 46566 activity is likely to have a beneficial effect.

The agents which modulate 46566 activity can be administered to a subject using pharmaceutical compositions suitable for such administration. Such compositions typically comprise the agent (*e.g.*, nucleic acid molecule, protein, or antibody) and a pharmaceutically acceptable carrier. As used herein the language “pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition used in the therapeutic methods of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral

(e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; 5 antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in 10 ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ .

15 (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, 20 glycerol, propylene glycol, and liquid polyethyleneglycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, 25 chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

30 Sterile injectable solutions can be prepared by incorporating the agent that modulates 46566 activity (e.g., a fragment of a 46566 protein or an anti-46566 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions

are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a 5 powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and 10 used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or 15 compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

20 For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For 25 transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

30 The agents that modulate 46566 activity can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the agents that modulate 46566 activity are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the agent that modulates 46566 activity and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an agent for the treatment of subjects.

Toxicity and therapeutic efficacy of such agents can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. Agents which exhibit large therapeutic indices are preferred. While agents that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such agents to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such 46566 modulating agents lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the

dosage form employed and the route of administration utilized. For any agent used in the therapeutic methods of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the 5 test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

As defined herein, a therapeutically effective amount of protein or polypeptide (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg pain, preferably about 0.01 to 25 mg/kg pain, more preferably about 0.1 to 20 mg/kg pain, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg pain. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous 15 treatments, the general health and/or age of the subject, and other diseases present.

Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments.

In a preferred example, a subject is treated with antibody, protein, or polypeptide in 20 the range of between about 0.1 to 20 mg/kg pain, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody, protein, or polypeptide used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and 25 become apparent from the results of diagnostic assays as described herein.

The present invention encompasses agents which modulate 46566 expression or activity. An agent may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, 30 organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams

per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or 5 researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention. Exemplary doses include milligram or microgram amounts 10 of the small molecule per kilogram of subject or sample weight (*e.g.*, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram). It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or 15 activity to be modulated. Such appropriate doses may be determined using the assays described herein. When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a 46566 polypeptide or nucleic acid molecule, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an 20 appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, pain, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

25 Further, an antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy 30 anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine),

alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics 5 (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response, the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired 10 biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, alpha-interferon, beta-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or biological response modifiers such as, for example, lymphokines, interleukin-1 (“IL-1”), interleukin-2 (“IL-2”), interleukin-6 (“IL-6”), 15 granulocyte macrophage colony stimulating factor (“GM-CSF”), granulocyte colony stimulating factor (“G-CSF”), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon *et al.*, “Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy”, in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld *et al.* (eds.), pp. 243-20 56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, “Antibodies For Drug Delivery”, in *Controlled Drug Delivery* (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, “Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review”, in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera *et al.* (eds.), pp. 475-506 (1985); “Analysis, Results, And Future Prospective Of The 25 Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy”, in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, “The Preparation And Cytotoxic Properties Of Antibody-Toxin 30 Conjugates”, *Immunol. Rev.*, 62:119-58 (1982). Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

The nucleic acid molecules used in the methods of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent

5,328,470) or by stereotactic injection (see, *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete 5 gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

### C. Pharmacogenomics

10 In conjunction with the therapeutic methods of the invention, pharmacogenomics (*i.e.*, the study of the relationship between a subject's genotype and that subject's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a 15 physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer an agent which modulates 46566 activity, as well as tailoring the dosage and/or therapeutic regimen of treatment with an agent which modulates 46566 activity.

Pharmacogenomics deals with clinically significant hereditary variations in the 20 response to drugs due to altered drug disposition and abnormal action in affected persons. See, for example, Eichelbaum, M. *et al.* (1996) *Clin. Exp. Pharmacol. Physiol.* 23(10-11): 983-985 and Linder, M.W. *et al.* (1997) *Clin. Chem.* 43(2):254-266. In general, two types 25 of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare genetic defects or as naturally-occurring polymorphisms. For example, glucose-6-phosphate aminopeptidase deficiency (G6PD) is a common inherited enzymopathy in which the main 30 clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

One pharmacogenomics approach to identifying genes that predict drug response, known as "a genome-wide association", relies primarily on a high-resolution map of the human genome consisting of already known gene-related markers (*e.g.*, a "bi-allelic" gene

marker map which consists of 60,000-100,000 polymorphic or variable sites on the human genome, each of which has two variants). Such a high-resolution genetic map can be compared to a map of the genome of each of a statistically significant number of patients taking part in a Phase II/III drug trial to identify markers associated with a particular observed drug response or side effect. Alternatively, such a high resolution map can be generated from a combination of some ten million known single nucleotide polymorphisms (SNPs) in the human genome. As used herein, a “SNP” is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every 1000 bases of DNA. A SNP may be involved in a disease process, however, the vast majority may not be disease-associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their individual genome. In such a manner, treatment regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals.

15        Alternatively, a method termed the “candidate gene approach” can be utilized to identify genes that predict drug response. According to this method, if a gene that encodes a drug target is known (*e.g.*, a 46566 protein of the present invention), all common variants of that gene can be fairly easily identified in the population and it can be determined if having one version of the gene versus another is associated with a particular drug response.

20        As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (*e.g.*, N-acetyltransferase 2 (NAT 2) and the cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed

metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Alternatively, a method termed the “gene expression profiling” can be utilized to 5 identify genes that predict drug response. For example, the gene expression of an animal dosed with a drug (*e.g.*, a 46566 molecule or 46566 modulator of the present invention) can give an indication whether gene pathways related to toxicity have been turned on.

Information generated from more than one of the above pharmacogenomics 10 approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment of a subject. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and, thus, enhance therapeutic or prophylactic efficiency when treating a subject suffering from a pain disorder with an agent which modulates 46566 activity.

15 IV. Recombinant Expression Vectors and Host Cells Used in the Methods of the Invention

The methods of the invention (*e.g.*, the screening assays described herein) include 20 the use of vectors, preferably expression vectors, containing a nucleic acid encoding a 46566 protein (or a portion thereof). As used herein, the term “vector” refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a “plasmid”, which refers to a circular double stranded DNA loop 25 into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the 30 genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as “expression vectors”. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, “plasmid” and “vector” can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression

vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors to be used in the methods of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, “operably linked” is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term “regulatory sequence” is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel (1990) *Methods Enzymol.* 185:3-7. Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, 46566 proteins, mutant forms of 46566 proteins, fusion proteins, and the like).

The recombinant expression vectors to be used in the methods of the invention can be designed for expression of 46566 proteins in prokaryotic or eukaryotic cells. For example, 46566 proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors), yeast cells, or mammalian cells. Suitable host cells are discussed further in Goeddel (1990) *supra*. Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein

encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion 5 expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and 10 Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Purified fusion proteins can be utilized in 46566 activity assays, (e.g., direct assays or competitive assays described in detail below), or to generate antibodies specific for 15 46566 proteins. In a preferred embodiment, a 46566 fusion protein expressed in a retroviral expression vector of the present invention can be utilized to infect bone marrow cells which are subsequently transplanted into irradiated recipients. The pathology of the subject recipient is then examined after sufficient time has passed (e.g., six weeks).

In another embodiment, a nucleic acid of the invention is expressed in mammalian 20 cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used 25 promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable 30 of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid).

The methods of the invention may further use a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an

antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to 46566 mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which 5 direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific, or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid, or attenuated virus in which antisense nucleic acids are produced under the control of a high 10 efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes, see Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to the use of host cells into which a 46566 15 nucleic acid molecule of the invention is introduced, *e.g.*, a 46566 nucleic acid molecule within a recombinant expression vector or a 46566 nucleic acid molecule containing sequences which allow it to homologously recombine into a specific site of the host cell's genome. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to 20 the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, a 46566 protein 25 can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via 30 conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or

transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

A host cell used in the methods of the invention, such as a prokaryotic or 5 eukaryotic host cell in culture, can be used to produce (*i.e.*, express) a 46566 protein. Accordingly, the invention further provides methods for producing a 46566 protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of the invention (into which a recombinant expression vector encoding a 46566 protein has been introduced) in a suitable medium such that a 46566 protein is produced. 10 In another embodiment, the method further comprises isolating a 46566 protein from the medium or the host cell.

#### **V. Isolated Nucleic Acid Molecules Used in the Methods of the Invention**

The cDNA sequence of the isolated human 46566 gene and the predicted amino 15 acid sequence of the human 46566 polypeptide are shown in Figures 1A-1B and in SEQ ID NOs:1 and 2, respectively. The coding region without the 5' or 3' untranslated regions of the human 2047 gene is shown in SEQ ID NO:3.

The methods of the invention include the use of isolated nucleic acid molecules that encode 46566 proteins or biologically active portions thereof, as well as nucleic acid 20 fragments sufficient for use as hybridization probes to identify 46566-encoding nucleic acid molecules (*e.g.*, 46566 mRNA) and fragments for use as PCR primers for the amplification or mutation of 46566 nucleic acid molecules. As used herein, the term “nucleic acid molecule” is intended to include DNA molecules (*e.g.*, cDNA or genomic 25 DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

A nucleic acid molecule used in the methods of the present invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, or a portion thereof, can be isolated using standard molecular biology techniques and the sequence 30 information provided herein. Using all or portion of the nucleic acid sequence of SEQ ID NO:1 as a hybridization probe, 46566 nucleic acid molecules can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring

*Harbor Laboratory*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1 can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide 5 primers designed based upon the sequence of SEQ ID NO:1.

A nucleic acid used in the methods of the invention can be amplified using cDNA, mRNA or, alternatively, genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. Furthermore, oligonucleotides corresponding to 46566 nucleotide sequences can be prepared by standard 10 synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In a preferred embodiment, the isolated nucleic acid molecules used in the methods of the invention comprise the nucleotide sequence shown in SEQ ID NO:1, a complement of the nucleotide sequence shown in SEQ ID NO:1, or a portion of any of these nucleotide sequences. A nucleic acid molecule which is complementary to the nucleotide sequence shown in SEQ ID NO:1, is one which is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:1 such that it can hybridize to the nucleotide sequence shown in SEQ ID NO:1 thereby forming a stable duplex. 15

In still another preferred embodiment, an isolated nucleic acid molecule used in the methods of the present invention comprises a nucleotide sequence which is at least about 20 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or more identical to the entire length of the nucleotide sequence shown in SEQ ID NO:1, or a portion of any of this nucleotide sequence.

Moreover, the nucleic acid molecules used in the methods of the invention can 25 comprise only a portion of the nucleic acid sequence of SEQ ID NO:1, for example, a fragment which can be used as a probe or primer or a fragment encoding a portion of a 46566 protein, *e.g.*, a biologically active portion of a 46566 protein. The probe/primer typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at 30 least about 12 or 15, preferably about 20 or 25, more preferably about 30, 35, 40, 45, 50, 55, 60, 65, or 75 consecutive nucleotides of a sense sequence of SEQ ID NO:1 or an anti-sense sequence of SEQ ID NO:1, or of a naturally occurring allelic variant or mutant of SEQ ID NO:1. In one embodiment, a nucleic acid molecule used in the methods of the

present invention comprises a nucleotide sequence which is greater than 50, 50-100, 100-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800, 800-900, 900-1000, 1000-1100 or more nucleotides in length and hybridizes under stringent hybridization conditions to a nucleic acid molecule of SEQ ID NO:1.

5 As used herein, the term “hybridizes under stringent conditions” is intended to describe conditions for hybridization and washing under which nucleotide sequences that are significantly identical or homologous to each other remain hybridized to each other. Preferably, the conditions are such that sequences at least about 70%, more preferably at least about 80%, even more preferably at least about 85% or 90% identical to each other 10 remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., John Wiley & Sons, Inc. (1995), sections 2, 4 and 6. Additional stringent conditions can be found in *Molecular Cloning: A Laboratory Manual*, Sambrook *et al.*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), chapters 7, 9 and 11. A preferred, non- 15 limiting example of stringent hybridization conditions includes hybridization in 4X or 6X sodium chloride/sodium citrate (SSC), at about 65-70°C (or hybridization in 4X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 1X SSC, at about 65-70°C. A further preferred, non-limiting example of stringent hybridization conditions includes hybridization at 6X SSC at 45°C, followed by one or more washes in 0.2X SSC, 20 0.1% SDS at 65°C. A preferred, non-limiting example of highly stringent hybridization conditions includes hybridization in 1X SSC, at about 65-70°C (or hybridization in 1X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 0.3X SSC, at about 65-70°C. A preferred, non-limiting example of reduced stringency 25 hybridization conditions includes hybridization in 4X or 6X SSC, at about 50-60°C (or alternatively hybridization in 6X SSC plus 50% formamide at about 40-45°C) followed by one or more washes in 2X SSC, at about 50-60°C. Ranges intermediate to the above-recited values, *e.g.*, at 65-70°C or at 42-50°C are also intended to be encompassed by the present invention. SSPE (1xSSPE is 0.15M NaCl, 10mM NaH<sub>2</sub>PO<sub>4</sub>, and 1.25mM EDTA, pH 7.4) can be substituted for SSC (1xSSC is 0.15M NaCl and 15mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes each after 30 hybridization is complete. The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature (T<sub>m</sub>) of the

hybrid, where  $T_m$  is determined according to the following equations. For hybrids less than 18 base pairs in length,  $T_m(^{\circ}\text{C}) = 2(\# \text{ of A} + \text{T bases}) + 4(\# \text{ of G} + \text{C bases})$ . For hybrids between 18 and 49 base pairs in length,  $T_m(^{\circ}\text{C}) = 81.5 + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G} + \text{C}) - (600/N)$ , where N is the number of bases in the hybrid, and  $[\text{Na}^+]$  is the concentration of 5 sodium ions in the hybridization buffer ( $[\text{Na}^+]$  for 1xSSC = 0.165 M). It will also be recognized by the skilled practitioner that additional reagents may be added to hybridization and/or wash buffers to decrease non-specific hybridization of nucleic acid molecules to membranes, for example, nitrocellulose or nylon membranes, including but not limited to blocking agents (e.g., BSA or salmon or herring sperm carrier DNA), 10 detergents (e.g., SDS), chelating agents (e.g., EDTA), Ficoll, PVP and the like. When using nylon membranes, in particular, an additional preferred, non-limiting example of stringent hybridization conditions is hybridization in 0.25-0.5M  $\text{NaH}_2\text{PO}_4$ , 7% SDS at about 65°C, followed by one or more washes at 0.02M  $\text{NaH}_2\text{PO}_4$ , 1% SDS at 65°C, see e.g., Church and Gilbert (1984) *Proc. Natl. Acad. Sci. USA* 81:1991-1995, (or alternatively 15 0.2X SSC, 1% SDS).

In preferred embodiments, the probe further comprises a label group attached thereto, e.g., the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress a 46566 protein, such as by measuring a level 20 of a 46566-encoding nucleic acid in a sample of cells from a subject e.g., detecting 46566 mRNA levels or determining whether a genomic 46566 gene has been mutated or deleted.

The methods of the invention further encompass the use of nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1 due to degeneracy of the genetic code and thus encode the same 46566 proteins as those encoded by the nucleotide 25 sequence shown in SEQ ID NO:1. In another embodiment, an isolated nucleic acid molecule included in the methods of the invention has a nucleotide sequence encoding a protein having an amino acid sequence shown in SEQ ID NO:2.

The methods of the invention further include the use of allelic variants of human 46566, e.g., functional and non-functional allelic variants. Functional allelic variants are 30 naturally occurring amino acid sequence variants of the human 46566 protein that maintain a 46566 activity. Functional allelic variants will typically contain only conservative substitution of one or more amino acids of SEQ ID NO:2, or substitution, deletion or insertion of non-critical residues in non-critical regions of the protein.

Non-functional allelic variants are naturally occurring amino acid sequence variants of the human 46566 protein that do not have a 46566 activity. Non-functional allelic variants will typically contain a non-conservative substitution, deletion, or insertion or premature truncation of the amino acid sequence of SEQ ID NO:2, or a substitution, insertion or 5 deletion in critical residues or critical regions of the protein.

The methods of the present invention may further use non-human orthologues of the human 46566 protein. Orthologues of the human 46566 protein are proteins that are isolated from non-human organisms and possess the same 46566 activity.

The methods of the present invention further include the use of nucleic acid 10 molecules comprising the nucleotide sequence of SEQ ID NO:1, or a portion thereof, in which a mutation has been introduced. The mutation may lead to amino acid substitutions at “non-essential” amino acid residues or at “essential” amino acid residues. A “non-essential” amino acid residue is a residue that can be altered from the wild-type sequence of 46566 (*e.g.*, the sequence of SEQ ID NO:2) without altering the biological activity, 15 whereas an “essential” amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the 46566 proteins of the present invention and other members of the short-chain dehydrogenase family are not likely to be amenable to alteration.

Mutations can be introduced into SEQ ID NO:1 by standard techniques, such as 20 site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids 25 with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, 30 tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a 46566 protein is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a 46566 coding sequence, such as by saturation mutagenesis, and the

resultant mutants can be screened for 46566 biological activity to identify mutants that retain activity. Following mutagenesis of SEQ ID NO:1, the encoded protein can be expressed recombinantly and the activity of the protein can be determined using an assay described herein.

5 Another aspect of the invention pertains to the use of isolated nucleic acid molecules which are antisense to the nucleotide sequence of SEQ ID NO:1. An “antisense” nucleic acid comprises a nucleotide sequence which is complementary to a “sense” nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, 10 an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire 46566 coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a “coding region” of the coding strand of a nucleotide sequence encoding a 46566. The term “coding region” refers to the region of the nucleotide sequence comprising codons which are translated into 15 amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a “noncoding region” of the coding strand of a nucleotide sequence encoding 46566. The term “noncoding region” refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (also referred to as 5' and 3' untranslated regions).

20 Given the coding strand sequences encoding 46566 disclosed herein, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of 46566 mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of 46566 mRNA. For 25 example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of 46566 mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic 30 acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine

substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, 5 dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiacytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules used in the methods of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a 46566 protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the

antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule used in the methods of the invention is an  $\alpha$ -anomeric nucleic acid molecule. An  $\alpha$ -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gaultier *et al.* (1987) *Nucleic Acids Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.* (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.* (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, an antisense nucleic acid used in the methods of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haseloff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave 46566 mRNA transcripts to thereby inhibit translation of 46566 mRNA. A ribozyme having specificity for a 46566-encoding nucleic acid can be designed based upon the nucleotide sequence of a 46566 cDNA disclosed herein (*i.e.*, SEQ ID NO:1). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a 46566-encoding mRNA. See, *e.g.*, Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, 46566 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel, D. and Szostak, J.W. (1993) *Science* 261:1411-1418.

Alternatively, 46566 gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the 46566 (*e.g.*, the 46566 promoter and/or enhancers) to form triple helical structures that prevent transcription of the 46566 gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6): 569-84; Helene, C. *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J. (1992) *Bioessays* 14(12):807-15.

In yet another embodiment, the 46566 nucleic acid molecules used in the methods of the present invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be

modified to generate peptide nucleic acids (see Hyrup, B. and Nielsen, P. E. (1996) *Bioorg. Med. Chem.* 4(1):5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup B. and Nielsen (1996) *supra* and Perry-O'Keefe *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:14670-675.

10 PNAs of 46566 nucleic acid molecules can be used in the therapeutic and diagnostic applications described herein. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication. PNAs of 46566 nucleic acid molecules can also be used in the analysis of single base pair mutations in a gene, (*e.g.*, by PNA-directed PCR clamping); as 'artificial restriction enzymes' when used in combination with other enzymes, (*e.g.*, S1 nucleases (Hyrup and Nielsen (1996) *supra*)); or as probes or primers for DNA sequencing or hybridization (Hyrup and Nielsen (1996) *supra*; Perry-O'Keefe *et al.* (1996) *supra*).

15 In another embodiment, PNAs of 46566 can be modified, (*e.g.*, to enhance their stability or cellular uptake), by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of 46566 nucleic acid molecules can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, (*e.g.*, RNase H and DNA polymerases), to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup and Nielsen (1996) *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup and Nielsen (1996) *supra* and Finn 20 P.J. *et al.* (1996) *Nucleic Acids Res.* 24 (17): 3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, *e.g.*, 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine 25 phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag, M. *et al.* 30

(1989) *Nucleic Acids Res.* 17: 5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn *et al.* (1996) *supra*). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser, K.H. *et al.* (1975) *Bioorganic Med. Chem. Lett.* 5: 1119-11124).

In other embodiments, the oligonucleotide used in the methods of the invention may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6553-6556; Lemaitre *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, *e.g.*, Krol *et al.* (1988) *Biotechniques* 6:958-976) or intercalating agents. (See, *e.g.*, Zon (1988) *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent).

## VI. Isolated 46566 Proteins and Anti-46566 Antibodies Used in the Methods of the Invention

The methods of the invention include the use of isolated 46566 proteins, and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogens to raise anti-46566 antibodies. In one embodiment, native 46566 proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, 46566 proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a 46566 protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

As used herein, a "biologically active portion" of a 46566 protein includes a fragment of a 46566 protein having a 46566 activity. Biologically active portions of a 46566 protein include peptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the 46566 protein, *e.g.*, the amino acid sequence shown in SEQ ID NO:2, which include fewer amino acids than the full length 46566 proteins, and exhibit at least one activity of a 46566 protein. Typically, biologically

active portions comprise a domain or motif with at least one activity of the 46566 protein. A biologically active portion of a 46566 protein can be a polypeptide which is, for example, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300 or more amino acids in length. Biologically active portions of a 46566 protein can be used as targets for developing agents 5 which modulate a 46566 activity.

In a preferred embodiment, the 46566 protein used in the methods of the invention has an amino acid sequence shown in SEQ ID NO:2. In other embodiments, the 46566 protein is substantially identical to SEQ ID NO:2, and retains the functional activity of the protein of SEQ ID NO:2, yet differs in amino acid sequence due to natural allelic variation 10 or mutagenesis, as described in detail in subsection V above. Accordingly, in another embodiment, the 46566 protein used in the methods of the invention is a protein which comprises an amino acid sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9% or more identical to SEQ ID NO:2.

15 To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for 20 comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (e.g., when aligning a second sequence to the 46566 amino acid sequence of SEQ ID NO:2 having 244 amino acid residues, at least 93, preferably at least 124, more preferably at least 156, even more preferably at least 187, and 25 even more preferably at least 200, 210, 215 or more amino acid residues are aligned). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or 30 nucleic acid “identity” is equivalent to amino acid or nucleic acid “homology”). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* 48:444-453 (1970)) algorithm which has been 5 incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blosum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at 10 <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.* 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0 or 2.0U), using a PAM120 weight 15 residue table, a gap length penalty of 12 and a gap penalty of 4.

The methods of the invention may also use 46566 chimeric or fusion proteins. As used herein, a 46566 "chimeric protein" or "fusion protein" comprises a 46566 polypeptide operatively linked to a non-46566 polypeptide. A "46566 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a 46566 molecule, whereas a 20 "non-46566 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the 46566 protein, e.g., a protein which is different from the 46566 protein and which is derived from the same or a different organism. Within a 46566 fusion protein the 46566 polypeptide can correspond to all or a portion of a 46566 protein. In a preferred embodiment, a 46566 25 fusion protein comprises at least one biologically active portion of a 46566 protein. In another preferred embodiment, a 46566 fusion protein comprises at least two biologically active portions of a 46566 protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the 46566 polypeptide and the non-46566 polypeptide are fused in-frame to each other. The non-46566 polypeptide can be fused to the N-terminus or C- 30 terminus of the 46566 polypeptide.

For example, in one embodiment, the fusion protein is a GST-46566 fusion protein in which the 46566 sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant 46566. In another

embodiment, this fusion protein is a 46566 protein containing a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of 46566 can be increased through use of a heterologous signal sequence.

The 46566 fusion proteins used in the methods of the invention can be incorporated 5 into pharmaceutical compositions and administered to a subject *in vivo*. The 46566 fusion proteins can be used to affect the bioavailability of a 46566 substrate. Use of 46566 fusion proteins may be useful therapeutically for the treatment of disorders caused by, for example, (i) aberrant modification or mutation of a gene encoding a 46566 protein; (ii) mis-regulation of the 46566 gene; and (iii) aberrant post-translational modification of a 10 46566 protein.

Moreover, the 46566-fusion proteins used in the methods of the invention can be used as immunogens to produce anti-46566 antibodies in a subject, to purify 46566 ligands and in screening assays to identify molecules which inhibit the interaction of 46566 with a 46566 substrate.

15 Preferably, a 46566 chimeric or fusion protein used in the methods of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling- 20 in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor 25 primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A 46566-encoding nucleic acid can be cloned into such an expression vector such that the fusion 30 moiety is linked in-frame to the 46566 protein.

The present invention also pertains to the use of variants of the 46566 proteins which function as either 46566 agonists (mimetics) or as 46566 antagonists. Variants of the 46566 proteins can be generated by mutagenesis, *e.g.*, discrete point mutation or

truncation of a 46566 protein. An agonist of the 46566 proteins can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of a 46566 protein. An antagonist of a 46566 protein can inhibit one or more of the activities of the naturally occurring form of the 46566 protein by, for example, competitively modulating a 5 46566-mediated activity of a 46566 protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function. In one embodiment, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the 46566 protein.

10 In one embodiment, variants of a 46566 protein which function as either 46566 agonists (mimetics) or as 46566 antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of a 46566 protein for 46566 protein agonist or antagonist activity. In one embodiment, a variegated library of 46566 variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a 15 variegated gene library. A variegated library of 46566 variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential 46566 sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of 46566 sequences therein. There are a variety of methods 20 which can be used to produce libraries of potential 46566 variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential 46566 sequences. 25 Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477).

30 In addition, libraries of fragments of a 46566 protein coding sequence can be used to generate a variegated population of 46566 fragments for screening and subsequent selection of variants of a 46566 protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a 46566 coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double

stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal

5 fragments of various sizes of the 46566 protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of 46566 proteins. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify 46566 variants (Arkin and Youvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delagrange *et al.* (1993) *Prot. Eng.* 6(3):327-331).

The methods of the present invention further include the use of anti-46566 antibodies. An isolated 46566 protein, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind 46566 using standard techniques for polyclonal and monoclonal antibody preparation. A full-length 46566 protein can be used or, alternatively, antigenic peptide fragments of 46566 can be used as immunogens. The antigenic peptide of 46566 comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an epitope of 46566 such that an antibody raised against the peptide forms a specific immune complex with the 46566 protein. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

30 Preferred epitopes encompassed by the antigenic peptide are regions of 46566 that are located on the surface of the protein, *e.g.*, hydrophilic regions, as well as regions with high antigenicity.

A 46566 immunogen is typically used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse, or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed 46566 protein or a chemically synthesized 46566 polypeptide. The preparation can further 5 include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic 46566 preparation induces a polyclonal anti-46566 antibody response.

The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain 10 an antigen binding site which specifically binds (immunoreacts with) an antigen, such as a 46566. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')<sub>2</sub> fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind 46566 molecules. The term "monoclonal antibody" or "monoclonal antibody 15 composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of 46566. A monoclonal antibody composition thus typically displays a single binding affinity for a particular 46566 protein with which it immunoreacts.

Polyclonal anti-46566 antibodies can be prepared as described above by 20 immunizing a suitable subject with a 46566 immunogen. The anti-46566 antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized 46566. If desired, the antibody molecules directed against 46566 can be isolated from the mammal (e.g., from the blood) and further purified by well known techniques, such as protein A 25 chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the anti-46566 antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also, Brown *et al.* (1981) *J. Immunol.* 127:539-46; 30 Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci. USA* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol. Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985) *Monoclonal Antibodies and Cancer Therapy*,

Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally Kenneth, R. H. in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); Lerner, E. A. (1981) *Yale J. Biol. Med.* 54:387-402; 5 Gefter, M. L. *et al.* (1977) *Somat. Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a 46566 immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds 46566.

10 Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-46566 monoclonal antibody (see, *e.g.*, G. Galfre *et al.* (1977) *Nature* 266:55052; Gefter *et al.* (1977) *supra*; Lerner (1981) *supra*; and Kenneth (1980) *supra*). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also 15 would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium 20 containing hypoxanthine, aminopterin and thymidine (“HAT medium”). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells 25 are fused to mouse splenocytes using polyethylene glycol (“PEG”). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind 46566, *e.g.*, using a standard ELISA assay.

30 Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-46566 antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with 46566 to thereby isolate immunoglobulin library members that bind 46566. Kits for generating

and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT International Publication No. WO 92/18619; Dower *et al.* PCT International Publication No. WO 91/17271; Winter *et al.* PCT International Publication WO 92/20791; Markland *et al.* PCT International Publication No. WO 92/15679; Breitling *et al.* PCT International Publication WO 93/01288; McCafferty *et al.* PCT International Publication No. WO 92/01047; Garrard *et al.* PCT International Publication No. WO 92/09690; Ladner *et al.* PCT International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J.* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrard *et al.* (1991) *Biotechnology (NY)* 9:1373-1377; Hoogenboom *et al.* (1991) *Nucleic Acids Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al.* (1990) *Nature* 348:552-554.

Additionally, recombinant anti-46566 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the methods of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Application No. PCT/US86/02269; Akira, *et al.* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT International Publication No. WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura *et al.* (1987) *Cancer Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986)

*Nature* 321:552-525; Verhoeven *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988)

*J. Immunol.* 141:4053-4060.

An anti-46566 antibody can be used to detect 46566 protein (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the 46566 protein. Anti-46566 antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or  $^{3}\text{H}$ .

## VII. Electronic Apparatus Readable Media and Arrays

Electronic apparatus readable media comprising a 46566 modulator of the present invention is also provided. As used herein, “electronic apparatus readable media” refers to any suitable medium for storing, holding or containing data or information that can be read and accessed directly by an electronic apparatus. Such media can include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as compact disc; electronic storage media such as RAM, ROM, EPROM, EEPROM and the like; general hard disks and hybrids of these categories such as magnetic/optical storage media. The medium is adapted or configured for having recorded thereon a marker of the present invention.

As used herein, the term “electronic apparatus” is intended to include any suitable computing or processing apparatus or other device configured or adapted for storing data or information. Examples of electronic apparatus suitable for use with the present invention include stand-alone computing apparatus; networks, including a local area

network (LAN), a wide area network (WAN) Internet, Intranet, and Extranet; electronic appliances such as a personal digital assistants (PDAs), cellular phone, pager and the like; and local and distributed processing systems.

As used herein, "recorded" refers to a process for storing or encoding information 5 on the electronic apparatus readable medium. Those skilled in the art can readily adopt any of the presently known methods for recording information on known media to generate manufactures comprising the 46566 modulators of the present invention.

A variety of software programs and formats can be used to store the marker 10 information of the present invention on the electronic apparatus readable medium. For example, the nucleic acid sequence corresponding to the 46566 modulators can be 15 represented in a word processing text file, formatted in commercially-available software such as WordPerfect and MicroSoft Word, or represented in the form of an ASCII file, stored in a database application, such as DB2, Sybase, Oracle, or the like, as well as in other forms. Any number of dataprocessor structuring formats (e.g., text file or database) 20 may be employed in order to obtain or create a medium having recorded thereon the 46566 modulators of the present invention.

By providing the 46566 modulators of the invention in readable form, one can 25 routinely access the marker sequence information for a variety of purposes. For example, one skilled in the art can use the nucleotide or amino acid sequences of the present invention in readable form to compare a target sequence or target structural motif with the sequence information stored within the data storage means. Search means are used to identify fragments or regions of the sequences of the invention which match a particular target sequence or target motif.

The present invention therefore provides a medium for holding instructions for 30 performing a method for determining whether a subject has a pain disorder or a pre-disposition to a pain disorder, wherein the method comprises the steps of determining the presence or absence of a 46566 modulator and based on the presence or absence of the 46566 modulator, determining whether the subject has a pain disorder or a pre-disposition to a pain disorder and/or recommending a particular treatment for the pain disorder or pre-pain disorder condition.

The present invention further provides in an electronic system and/or in a network, a method for determining whether a subject has a pain disorder or a pre-disposition to a pain disorder associated with a 46566 modulator wherein the method comprises the steps

of determining the presence or absence of the 46566 modulator, and based on the presence or absence of the 46566 modulator, determining whether the subject has a pain disorder or a pre-disposition to a pain disorder, and/or recommending a particular treatment for the pain disorder or pre-pain disorder condition. The method may further comprise the step of 5 receiving phenotypic information associated with the subject and/or acquiring from a network phenotypic information associated with the subject.

The present invention also provides in a network, a method for determining whether a subject has a pain disorder or a pre-disposition to a pain disorder associated with a 46566 modulator, said method comprising the steps of receiving information associated 10 with the 46566 modulator receiving phenotypic information associated with the subject, acquiring information from the network corresponding to the 46566 modulator and/or pain disorder, and based on one or more of the phenotypic information, the 46566 modulator, and the acquired information, determining whether the subject has a pain disorder or a pre-disposition to a pain disorder. The method may further comprise the step of 15 recommending a particular treatment for the pain disorder or pre- pain disorder condition.

The present invention also provides a business method for determining whether a subject has a pain disorder or a pre-disposition to a pain disorder, said method comprising the steps of receiving information associated with the 46566 modulator, receiving phenotypic information associated with the subject, acquiring information from the 20 network corresponding to the 46566 modulator and/or pain disorder, and based on one or more of the phenotypic information, the 46566 modulator, and the acquired information, determining whether the subject has a pain disorder or a pre-disposition to a pain disorder. The method may further comprise the step of recommending a particular treatment for the pain disorder or pre-pain disorder condition.

25 The invention also includes an array comprising a 46566 modulator of the present invention. The array can be used to assay expression of one or more genes in the array. In one embodiment, the array can be used to assay gene expression in a tissue to ascertain tissue specificity of genes in the array. In this manner, up to about 7600 genes can be simultaneously assayed for expression. This allows a profile to be developed showing a 30 battery of genes specifically expressed in one or more tissues.

In addition to such qualitative determination, the invention allows the quantitation of gene expression. Thus, not only tissue specificity, but also the level of expression of a battery of genes in the tissue is ascertainable. Thus, genes can be grouped on the basis of

their tissue expression *per se* and level of expression in that tissue. This is useful, for example, in ascertaining the relationship of gene expression between or among tissues. Thus, one tissue can be perturbed and the effect on gene expression in a second tissue can be determined. In this context, the effect of one cell type on another cell type in response to a biological stimulus can be determined. Such a determination is useful, for example, to know the effect of cell-cell interaction at the level of gene expression. If an agent is administered therapeutically to treat one cell type but has an undesirable effect on another cell type, the invention provides an assay to determine the molecular basis of the undesirable effect and thus provides the opportunity to co-administer a counteracting agent or otherwise treat the undesired effect. Similarly, even within a single cell type, undesirable biological effects can be determined at the molecular level. Thus, the effects of an agent on expression of other than the target gene can be ascertained and counteracted.

10 In another embodiment, the array can be used to monitor the time course of expression of one or more genes in the array. This can occur in various biological contexts, as disclosed herein, for example development of pain disorder, progression of pain disorder, and processes associated with a pain disorder.

15 The array is also useful for ascertaining the effect of the expression of a gene on the expression of other genes in the same cell or in different cells. This provides, for example, for a selection of alternate molecular targets for therapeutic intervention if the ultimate or downstream target cannot be regulated.

20 The array is also useful for ascertaining differential expression patterns of one or more genes in normal and abnormal cells. This provides a battery of genes that could serve as a molecular target for diagnosis or therapeutic intervention.

25 This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference.

## EXAMPLES

### EXAMPLE 1: 46566 EXPRESSION IN HUMAN TISSUES

#### 5 Materials and Methods

For analysis of human 46566 expression, the following methods were used.

Tissues were collected from various human tissues. Total RNA was prepared using the trizol method and treated with DNase to remove contaminating genomic DNA. cDNA was synthesized using standard techniques. Mock cDNA synthesis in the absence of 10 reverse transcriptase resulted in samples with no detectable PCR amplification of the control 18S RNA gene confirming efficient removal of genomic DNA contamination. 46566 expression was measured by TaqMan® quantitative PCR analysis, performed according to the manufacturer's directions (Perkin Elmer Applied Biosystems, Foster City, CA).

15 PCR probes were designed by PrimerExpress software (PE Biosystems) based on the sequence of human 46566 (SEQ ID NO:1).

To standardize the results between different tissues, two probes, distinguished by different fluorescent labels, were added to each sample. The differential labeling of the probe for the 46566 gene and the probe for 18S RNA as an internal control thus enabled 20 their simultaneous measurement in the same well. Forward and reverse primers and the probes for both 18S RNA and human or murine 46566 were added to the TaqMan Universal PCR Master Mix (PE Applied Biosystems). Although the final concentration of primer and probe could vary, each was internally consistent within a given experiment. A typical experiment contained 200 nM of forward and reverse primers, plus 100 nM of the 25 probe for the 18S RNA, and 4500 nM of each of the forward and reverse primers, plus 150 nM of the probe for murine NCE-SLC24A. TaqMan matrix experiments were carried out using an ABI PRISM 770 Sequence Detection System (PE Applied Biosystems). The thermal cycler conditions were as follows: hold for 2 minutes at 50°C and 10 minutes at 95°C, followed by two-step PCR for 40 cycles of 95°C for 15 seconds, followed by 60°C 30 for 1 minute.

The following method was used to quantitatively calculate human 46566 gene expression in the tissue samples, relative to the 18S RNA expression in the same tissue. The threshold values at which the PCR amplification started were determined using the

manufacturer's software. PCR cycle number at threshold value was designated as CT.

Relative expression was calculated as:

$$2^{-((CT_{\text{test}}-CT_{18S})_{\text{tissue of interest}} - (CT_{\text{test}}-CT_{18S})_{\text{lowest expressing tissue in panel}})}$$

5

Samples were run in duplicate and the averages of 2 relative expression determinations are shown. All probes were tested on serial dilutions of RNA from a tissue with high expression levels and only probes which gave relative expression levels that were linear to the amount of template cDNA with a slope similar to the slope for the 10 internal control 18S were used.

### Results

46566 was most highly expressed in nervous tissues. The highest levels of 46566 expression were found in the brain, followed by the spinal cord and dorsal root ganglia (DRG). *In situ* hybridization using a human probe confirmed the foregoing TaqMan data.

The results described above demonstrate that 46566 is a relatively brain specific gene.

## **EXAMPLE 2: 46566 EXPRESSION IN RAT TISSUES DERIVED FROM 20 ANIMAL MODELS FOR PAIN**

### Materials and Methods

For analysis of rat 46566 expression, the methods described in Example 1 were used.

25

### Results

TaqMan analysis using various rat tissues demonstrated that, 46566, like the human counterpart, is expressed in nervous tissues and showed the same pattern of expression as in the human panel (described in Example 1).

30 Expression of 46566 in animal models for pain/inflammation was also determined. One of the models used was the nerve ligation model (CCI), wherein chronic loose constriction of the sciatic nerve of the animal induces neuropathic pain. Nerve damage

results in sensory hypersensitivity or prolonged lowering of the primary afferent nociceptor threshold (hyperalgesia). Furthermore, after axonal injury, mechanical and thermal allodynia develop. Another animal-based paradigm used was the complete Freund's adjuvant (CFA) model, wherein CFA injection in the rodent paw or monkey knee joint 5 induces an inflammatory response with the development of altered pain responses manifested as reduced threshold to noxious stimuli (hyperalgesia) and lowered thresholds to innocuous stimuli (allodynia).

The results from these experiments indicate that there was no regulation of 46566 in the spinal cord of CCI animals and some down-regulation in the CFA induced model of 10 inflammatory pain. A more pronounced down-regulation of 46566 was observed in animals after axotomy.

**Equivalents** Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the 15 invention described herein. Such equivalents are intended to be encompassed by the following claims.

## What is claimed:

1. A method for identifying a compound capable of treating a pain disorder, comprising assaying the ability of the compound to modulate 46566 nucleic acid expression or 46566 polypeptide activity, thereby identifying a compound capable of treating a pain disorder.

2. A method for identifying a compound capable of modulating a pain signaling mechanism comprising:

10 a) contacting a cell which expresses 46566 with a test compound; and  
b) assaying the ability of the test compound to modulate the expression of a 46566 nucleic acid or the activity of a 46566 polypeptide, thereby identifying a compound capable of modulating pain signalling.

15 3. A method for modulating a pain signaling mechanism in a cell comprising contacting a cell with a 46566 modulator, thereby modulating a pain signaling mechanism in the cell.

4. The method of claim 2, wherein the cell is a brain cell, neuron, or  
20 cell derived from spinal cord or dorsal root ganglion.

5. The method of claim 3, wherein the 46566 modulator is a small organic molecule, peptide, antibody or antisense nucleic acid molecule.

25 6. The method of claim 3, wherein the 46566 modulator is capable of modulating 46566 polypeptide activity.

7. The method of claim 6, wherein the 46566 modulator is a small organic molecule, peptide, antibody or antisense nucleic acid molecule.

30 8. The method of claim 6, wherein the 46566 modulator is capable of modulating 46566 nucleic acid expression.

9. A method for treating a subject having a pain disorder characterized by aberrant 46566 polypeptide activity or aberrant 46566 nucleic acid expression comprising administering to the subject a 46566 modulator, thereby treating said subject having a pain disorder.

5

10. The method of claim 9, wherein said pain disorder includes inflammatory pain, chronic pain, neuropathic pain, causalgia, fibromyalgia, cancer pain, migraine/headache pain and tissue pain.

10

11. The method of claim 9, wherein said 46566 modulator is administered in a pharmaceutically acceptable formulation.

12. The method of claim 9, wherein the 46566 modulator is a small organic molecule, peptide, antibody or antisense nucleic acid molecule.

15

13. The method of claim 9, wherein the 46566 modulator is capable of modulating 46566 polypeptide activity.

> Fbh46566pub - Import - vector trimmed

CTCTCTCTTTGGCGGAGTGGCCACCACTGCCAGCCCAGCGCTGGGGGACCTGCT  
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 GCGTCCCCGGTCTCCCTGCCGTGACCCCAACTCCAGGCCATCCTGTTGGTGC  
 TATATCCCTGGGAAATTCCACCCAGTCCCCTCCCAAGGGAACCAACCCCCAGTAACCA

## FIGURE 1A

TCCTGGGGAGTTAAGGTCTCTCCTGGTACCCAGCCTGGCTTGCCCCAAAGTCT  
 CCCTCCCCTAGTGACCCCCCCCCACTTCACCCATGTCCCAGAGCCTCAGAACCCACCC  
 TCCCTGGGGACCCTCGAAGGAGGCTGTCAGAGGCCGTCAGCTCCAGCCCTCCCC  
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 TAAAACCTTGAAATTCTGCCTG

**FIGURE 1A (Continued)**

> 46566 protein  
 MAPLALVGVTLLAAPPCSGAATPTPSLPPPPANDSDTSTGGCQGSYRCQPGVLLPVWEPPDPSLGDKAARAVVYFVA  
 MVYMFGLVSIADRMAIEVITSKEKEITITKANGETSVGTVRIWNETVSNLTLMALGSSAPEILLSVIEVCGHNFQ  
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 REVIQILKDLKQKHPDKDLEQLVGIANYALLHQQKSRAFYRIQATRLMTGAGNVLRRHAADASRRAAPAEGAGEDED  
 DGASRIFFEPSLYHCLENCGSVLLSVCQGGEGNSTFYVDYRTEDGSAKAGSDYEYSEGTIVFKPGETQKELRIGIID  
 DDIFEEDEHFFVRLNLNRVGDQAQGMFEPDGGRPKRLVAPLLATVTILDDDHAGIFSQDRLLHVSECMGTVDRV  
 RSSGARGTVRLPYRTVDGTARGGGVHYEDACGELEFGDDETMTKLQVKIVDDEEYEKDNFFIELGQPQWLKRGISAL  
 LLNQGDGDRKLTAEEEEARRIAEMGPVLGENCRLEVIIIESYDFKNTVDKLKTKTNLALVIGTHSWREQFLEAITVS  
 AGDEEEEDGSREERLPSCDFYVMHFLTVFWKVLFACVPTEYCHGWACFGVSILVIGLLTALIGDLASHFGCTVGLK  
 DSVAVVFVALGTSIPDTFASKVAALQDQCADASIGNVTGSNAVNVFLGLGVAWSVAAVYWAQGRPFEVRTGTLAFS  
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**FIGURE 1B**

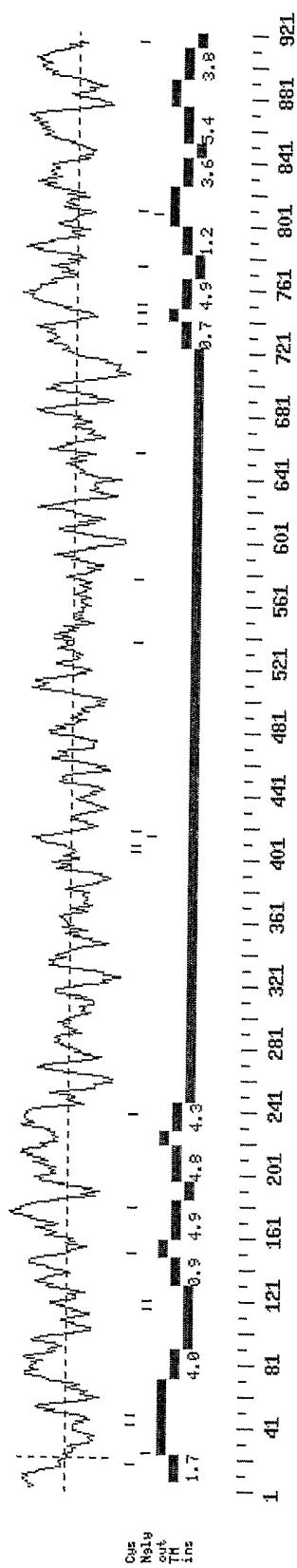


FIGURE 2

## SEQUENCE LISTING

<110> Silos-Santiago, Inmaculada

<120> Methods and compositions for the treatment and diagnosis of pain disorders using 46566

<130> MPI01-272

<150> 60/335,078

<151> 2001-10-31

<160> 3

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<211> 4282

<212> DNA

<213> Homo sapien

<400> 1

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acactccctcc tggcggtctcc cccatgctcc ggggcagccca ccccaacccc ctccctgccc 180  
cctccccccgg ccaatgacag cgacaccagc acagggggct gccagggtc ctaccgctgc 240  
cagccgggggg tgctgctgcc cgtgtggag cccgacgacc cgtcgctggg tgacaaggcg 300  
gcacgggcag tggtgtactt tgtggccatg gtctacatgt ttctggaggt gtccatcatc 360  
gccgaccgtt tcatggcgcc catcgaggtc atcacgtcaa aagagaagga gatcaccatc 420  
accaaggcca acggtgagac cagcgtggc accgttcgca tctggaatga gacggtgtcc 480  
aacctcacgc tcatggccct gggctccctcc gcacctgaga tcctgctgtc agtcatcgaa 540  
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 Tyr Met Phe Leu Gly Val Ser Ile Ile Ala Asp Arg Phe Met Ala Ala  
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 Ile Glu Val Ile Thr Ser Lys Glu Lys Glu Ile Thr Ile Thr Lys Ala  
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 Asn Gly Glu Thr Ser Val Gly Thr Val Arg Ile Trp Asn Glu Thr Val  
 115 120 125  
 Ser Asn Leu Thr Leu Met Ala Leu Gly Ser Ser Ala Pro Glu Ile Leu  
 130 135 140  
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Gly Pro Gly Thr Ile Val Gly Ser Ala Ala Phe Asn Met Phe Val Val  
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 Ile Ala Val Cys Ile Tyr Val Ile Pro Ala Gly Glu Ser Arg Lys Ile  
                  180                 185                 190  
 Lys His Leu Arg Val Phe Phe Val Thr Ala Ser Trp Ser Ile Phe Ala  
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 Tyr Val Trp Leu Tyr Leu Ile Leu Ala Val Phe Ser Pro Gly Val Val  
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 Gln Val Trp Glu Ala Leu Leu Thr Leu Val Phe Phe Pro Val Cys Val  
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 Val Phe Ala Trp Met Ala Asp Lys Arg Leu Leu Phe Tyr Lys Tyr Val  
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 Tyr Lys Arg Tyr Arg Thr Asp Pro Arg Ser Gly Ile Ile Gly Ala  
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 Glu Gly Asp Pro Pro Lys Ser Ile Glu Leu Asp Gly Thr Phe Val Gly  
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 Ala Glu Ala Pro Gly Glu Leu Gly Gly Leu Gly Pro Gly Pro Ala Glu  
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 Ala Arg Glu Leu Asp Ala Ser Arg Arg Glu Val Ile Gln Ile Leu Lys  
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 Ile Ala Asn Tyr Tyr Ala Leu Leu His Gln Gln Lys Ser Arg Ala Phe  
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 Ser Leu Tyr His Cys Leu Glu Asn Cys Gly Ser Val Leu Leu Ser Val  
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 Gly Ile Ile Asp Asp Asp Ile Phe Glu Glu Asp Glu His Phe Phe Val  
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                  580                 585                 590  
 Val Lys Ile Val Asp Asp Glu Glu Tyr Glu Lys Lys Asp Asn Phe Phe  
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Asp Glu Glu Glu Glu Asp Gly Ser Arg Glu Glu Arg Leu Pro Ser		
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Cys Phe Asp Tyr Val Met His Phe Leu Thr Val Phe Trp Lys Val Leu		
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Phe Ala Cys Val Pro Pro Thr Glu Tyr Cys His Gly Trp Ala Cys Phe		
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Gly Val Ser Ile Leu Val Ile Gly Leu Leu Thr Ala Leu Ile Gly Asp		
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Leu Ala Ser His Phe Gly Cys Thr Val Gly Leu Lys Asp Ser Val Asn		
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Ala Val Val Phe Val Ala Leu Gly Thr Ser Ile Pro Asp Thr Phe Ala		
785	790	795
Ser Lys Val Ala Ala Leu Gln Asp Gln Cys Ala Asp Ala Ser Ile Gly		
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Asn Val Thr Gly Ser Asn Ala Val Asn Val Phe Leu Gly Leu Gly Val		
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Ala Trp Ser Val Ala Ala Val Tyr Trp Ala Val Gln Gly Arg Pro Phe		
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Glu Val Arg Thr Gly Thr Leu Ala Phe Ser Val Thr Leu Phe Thr Val		
850	855	860
Phe Ala Phe Val Gly Ile Ala Val Leu Leu Tyr Arg Arg Arg Pro His		
865	870	875
Ile Gly Gly Glu Leu Gly Gly Pro Arg Gly Pro Lys Leu Ala Thr Thr		
885	890	895
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cca acc ccc tcc ctg ccg cct ccc ccg gcc aat gac agc gac acc agc 210  
Pro Thr Pro Ser Leu Pro Pro Pro Ala Asn Asp Ser Asp Thr Ser  
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Thr	Gly	Gly	Cys	Gln	Gly	Ser	Tyr	Arg	Cys	Gln	Pro	Gly	Val	Leu	Leu	
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ccc gtg tgg gag ccc gac gac ccg tcg ctg ggt gac aag gcg gca cgg															306	
Pro	Val	Trp	Glu	Pro	Asp	Asp	Pro	Ser	Leu	Gly	Asp	Lys	Ala	Ala	Arg	
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gca gtg gtg tac ttt gtg gcc atg gtc tac atg ttt ctg gga gtg tcc															354	
Ala	Val	Val	Tyr	Phe	Val	Ala	Met	Val	Tyr	Met	Phe	Leu	Gly	Val	Ser	
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atc atc gcc gac cgt ttc atg gcg gcc atc gag gtc atc acg tca aaa															402	
Ile	Ile	Ala	Asp	Arg	Phe	Met	Ala	Ala	Ile	Glu	Val	Ile	Thr	Ser	Lys	
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gag aag gag atc acc atc acc aag gcc aac ggt gag acc agc gtg ggc															450	
Glu	Lys	Glu	Ile	Thr	Ile	Thr	Lys	Ala	Asn	Gly	Glu	Thr	Ser	Val	Gly	
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acc gtt cgc atc tgg aat gag acg gtg tcc aac ctc acg ctc atg gcc															498	
Thr	Val	Arg	Ile	Trp	Asn	Glu	Thr	Val	Ser	Asn	Leu	Thr	Leu	Met	Ala	
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ctg ggc tcc tcc gca cct gag atc ctg ctg tca gtc atc gaa gtc tgc															546	
Leu	Gly	Ser	Ser	Ala	Pro	Glu	Ile	Leu	Leu	Ser	Val	Ile	Glu	Val	Cys	
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ggc cac aac ttc cag gcg ggt gag ctg ggc cca ggc acc atc gtg ggc															594	
Gly	His	Asn	Phe	Gln	Ala	Gly	Glu	Leu	Gly	Pro	Gly	Thr	Ile	Val	Gly	
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agc gct gcc ttc aac atg ttt gtg gtc atc gcc gtg tgc atc tac gtc															642	
Ser	Ala	Ala	Phe	Asn	Met	Phe	Val	Val	Ile	Ala	Val	Cys	Ile	Tyr	Val	
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Leu	Ala	Val	Phe	Ser	Pro	Gly	Val	Val	Gln	Val	Trp	Glu	Ala	Leu	Leu	
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acc ctg gtc ttc ttc ccg gtg tgc gtg gta ttc gcc tgg atg gcc gac															834	
Thr	Leu	Val	Phe	Phe	Pro	Val	Cys	Val	Val	Phe	Ala	Trp	Met	Ala	Asp	
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Lys	Arg	Leu	Leu	Phe	Tyr	Lys	Tyr	Val	Tyr	Lys	Arg	Tyr	Arg	Thr	Asp	
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Pro	Arg	Ser	Gly	Ile	Ile	Gly	Ala	Glu	Gly	Asp	Pro	Pro	Lys	Ser		
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Glu Cys Met Gly Thr Val Asp Val Arg Val Val Arg Ser Ser Gly Ala				
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Arg Gly Thr Val Arg Leu Pro Tyr Arg Thr Val Asp Gly Thr Ala Arg				
ggc ggc ggc gtg cac tac gag gac gcg tgc gga gag ctg gag ttt ggc	570	575	580	1842
Gly Gly Gly Val His Tyr Glu Asp Ala Cys Gly Glu Leu Glu Phe Gly				
gac gac gag acc atg aaa act ctt cag gtg aag ata gtt gat gac gag	585	590	595	1890
Asp Asp Glu Thr Met Lys Thr Leu Gln Val Lys Ile Val Asp Asp Glu				
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Glu Tyr Glu Lys Lys Asp Asn Phe Phe Ile Glu Leu Gly Gln Pro Gln				
tgg ctt aag cga ggg att tca gct ctg cta ctc aat caa ggg gat ggg	620	625	630	1986
Trp Leu Lys Arg Gly Ile Ser Ala Leu Leu Asn Gln Gly Asp Gly				
gac agg aag cta aca gcc gag gag gag gct cgg agg ata gca gag	635	640	645	2034
Asp Arg Lys Leu Thr Ala Glu Glu Glu Ala Arg Arg Ile Ala Glu				
atg ggc aag cca gtt ctt ggg gag aac tgc cgg ctg gag gtc atc atc	650	655	660	2082
Met Gly Lys Pro Val Leu Gly Glu Asn Cys Arg Leu Glu Val Ile Ile				
gag gag tca tat gat ttt aag aac acg gtg gat aaa ctc atc aag aaa	665	670	675	2130
Glu Glu Ser Tyr Asp Phe Lys Asn Thr Val Asp Lys Leu Ile Lys Lys				
acg aac ttg gcc ttg gta att ggg acc cat tca tgg agg gag cag ttt	680	685	690	2178
Thr Asn Leu Ala Leu Val Ile Gly Thr His Ser Trp Arg Glu Gln Phe				
tta gag gca att acg gtg agc gca ggg gac gag gag gag gag gag	700	705	710	2226
Leu Glu Ala Ile Thr Val Ser Ala Gly Asp Glu Glu Glu Glu Asp				
ggg tcc cgg gag gag cgg ctg ccg tcg tgc ttt gac tac gtg atg cac	715	720	725	2274
Gly Ser Arg Glu Glu Arg Leu Pro Ser Cys Phe Asp Tyr Val Met His				
ttc ctg acg gtg ttc tgg aag gtg ctc ttc gcc tgt gtg ccc ccc acc	730	735	740	2322
Phe Leu Thr Val Phe Trp Lys Val Leu Phe Ala Cys Val Pro Pro Thr				
gag tac tgc cac ggc tgg gcc tgc ttt ggt gtc tcc atc ctg gtc atc	745	750	755	2370
Glu Tyr Cys His Gly Trp Ala Cys Phe Gly Val Ser Ile Leu Val Ile				
ggc ctg ctc acc gcc ctc att ggg gac ctc gcc tcc cac ttc ggc tgc	760	765	770	2418
Gly Leu Leu Thr Ala Leu Ile Gly Asp Leu Ala Ser His Phe Gly Cys				

acc gtt ggc ctc aag gac tct gtc aat gct gtt gtc ttc gtt gcc ctg 2466  
 Thr Val Gly Leu Lys Asp Ser Val Asn Ala Val Val Phe Val Ala Leu  
 780 785 790  
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 Tyr Trp Ala Val Gln Gly Arg Pro Phe Glu Val Arg Thr Gly Thr Leu  
 840 845 850 855  
 gcc ttc tcc gtc acg ctc ttc acc gtc ttc gcc ttc gtg ggc att gcc 2706  
 Ala Phe Ser Val Thr Leu Phe Thr Val Phe Ala Phe Val Gly Ile Ala  
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 Val Leu Leu Tyr Arg Arg Pro His Ile Gly Gly Glu Leu Gly Gly  
 875 880 885  
 ccg cgc gga ccc aag ctc gcc acc acc gcg ctc ttc ctg ggc ctc tgg 2802  
 Pro Arg Gly Pro Lys Leu Ala Thr Thr Ala Leu Phe Leu Gly Leu Trp  
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 Leu Leu Tyr Ile Leu Phe Ala Ser Leu Glu Ala Tyr Cys His Ile Arg  
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 Gly Phe \*  
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ctg 4282