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(54) Title: POLYPEPTIDE SPECIES USEFUL FOR THE TREATMENT OF NEUROLOGICAL DISORDERS

(57) Abstract: The invention discloses a new secreted polypeptide from Human Cerebro-Spinal Fluid which has therapeutic application for the treatment of neurological disorders. The invention also provides methods of using compositions including the polypeptides and polynucleotides encoding them from drug development.

POLYPEPTIDE SPECIES USEFUL FOR THE TREATMENT OF NEUROLOGICAL DISORDERS

FIELD OF THE INVENTION

The invention relates to polypeptide species useful for the treatment of neurological disorders. The polypeptides of the invention, of human origin, have shown activity in *in vitro* and *in vivo* neurological disease models, and as such are useful for the treatment of human neurological disorders. In addition the invention relates to isolated polynucleotides encoding such polypeptides, to polymorphic variants thereof, and to the use of said nucleic acids and polypeptides or compositions thereof for drug development.

BACKGROUND

Neurological disorders include, but are not limited to, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, stroke and ischemia associated with stroke, neural paropathy, other neural degenerative diseases, motor neuron diseases, sciatic crush, peripheral neuropathy, particularly neuropathy associated with diabetes, spinal cord injuries and facial nerve crush, as well as Traumatic Brain Injuries.

Multiple Sclerosis

Multiple Sclerosis (MS) is an acquired auto-immune disease which leads to destruction of the myelin sheath that surrounds the nerve axons and helps the conduction of electrical impulses. Factors triggering this auto-immune reaction are unknown. Evolution is chronic with occasional relapses and leads to a progressive disability in spite of therapeutic intervention.

The myelin destruction occurs in areas of the white matter of the central nervous system in random patches called plaques, starting by an inflammatory process (edema, inflammatory and immune cells migration) followed by a phase of scarification of the tissue, called sclerosis. Regeneration of damaged myelin by the oligodendrocytes then occurs, with a gliosis reaction (astrocytosis).

When myelin is damaged, neurological transmission of messages may be slowed or blocked completely, leading to diminished or lost function (Harrisson's : Principles of Internal medicine, 14th ed./editors, Anthony S: Fauci et al.).

Prevalence of MS is high in regard of the chronic disability status that it induces. It is

believed that 250,000 to 350,000 people in the United States are suffering from MS diagnosed by a physician. This estimate suggests that approximately 200 new cases are diagnosed each week. In France, around 50 000 people are suffering from MS. Global incidence varies according to a South-North gradient and may increase in some specific clustered populations. Despite intense studies of these clusters, no direct environmental factors (e.g., viral) have been identified. Symptom onset usually begins between ages 20 to 40. Women are affected at almost twice the rate of men.

Causes of this myelin destruction are due to i) an auto-immune process: it is thought that this process occurs in the periphery (outside the brain), where T-cell receptors acquire capability to recognize myelin epitopes. Experimental animal models confirm this hypothesis, injection of Myelin Basic Protein (and other myelin components) being able to induce Experimental Allergic Encephalitis (EAE), a disease that resembles MS, ii) the disruption of the Blood-Brain Barrier (BBB), likely due to an inflammatory process.
Then, after contacting myelin antigen, specific T-cells mediate toxic activity by usual immune mechanisms: production of pro-inflammatory cytokines (Il-1, Il-6, Rantes, TNF-alpha, TNF-beta), of INF-gamma, release of perforines/granzymes, activation of macrophages, activation of T-cells helper for auto-antibodies (IgM, IgG) production (Ristori G, *et al.*, J Neuroimmunol. 2000 Jul 24; 107(2):216-, Review).

Multiple triggering factors have been envisaged: viral, environmental, immunologic, genetic (linked to a particular MHC class II (HLA DR) that presents myelin epitopes to T-cells), but no study has shown yet that a single factor could, on its own, induce the pathology. These studies reinforce the theory that MS is the result of a combination of factors rather due to a single gene or other agent (Granieri E, *et al.*, Neurol Sci. 2001; Apr;22(2):179-85; Xiao BG, Link H, Cell Mol Life Sc.;1999 Oct 1;56(1-2):5-21; Shapiro S, *et al.*, J Neuroimmunol. 2003 Nov;144(1-2):116-24).

Clinical manifestations: MS displays several patterns of presentation and subsequent course. Most commonly, MS first manifests itself as a series of attacks followed by complete or partial remissions as symptoms diminish, before returning later after a period of stability (80% of cases). This is called Relapsing-Remitting (RR) MS (see profiles A and B in Figure 15). 50% of RR patients develop secondarily progressive disability, so-called Secondary-Progressive (SP) MS (see profiles C and D in Figure 15). Primary-Progressive (PP) MS is characterized by a gradual clinical decline with either no distinct remissions (profiles E and F,

10% of patients) or distinct relapses (profile G, 5% of patients). In addition, twenty percent of the MS population has a benign form of the disease in which symptoms show little or no progression after the initial attack; these patients remain fully functional. In general, MS is very rarely fatal and most people with MS have a fairly normal life expectancy.

MS assessment: the diagnosis of a patient whose symptoms, neurological examination, and medical history suggest MS, requires a variety of examinations to be completed, none of them being MS-specific (Fangerau T, *et al.*, Acta Neurol. Scand. 2004 Jun;109(6):385-9).

- Magnetic Resonance Imaging (MRI), often used in conjunction with a gadolinium contrast agent, helps distinguish new plaques from old ones (as the contrast agent does not go through a non-altered Blood Brain Barrier), and thus helps locate central nervous system lesions resulting from myelin loss.
- Evoked potential tests, a measure of the speed of the brain's response to visual, auditory, and sensory stimuli, can sometimes detect lesions that the MRI does not detect.
- The analysis of the patient's CerebroSpinal Fluid (CSF) for cellular and chemical abnormalities often associated with MS (*e.g.*, increased numbers of white blood cells, and above average amounts of proteins, especially myelin basic protein and IgG antibodies which may be distributed according to a characteristic pattern called oligoclonal bands), may reinforce the diagnosis.

Treatments of MS are not curative. They alleviate the symptoms and/or reduce the number of relapses. In the case of acute relapses, boluses of corticosteroid agents are given for 3 days. They have been shown to reduce the duration and the severity of relapses *(symptoms-modifying drugs)* and are likely to play a role in reducing the inflammatory response. There is no evidence to support the use of these drugs for treating progressive forms of MS. The treatment of RR MS relies on immunomodulator agents (Interferons Beta) and on agents mimicking myelin composition *(disease-modifying drugs)*:

- Interferons Beta (Avonex from Biogen, Rebif from Serono, or Betaseron from Schering) reduce the inflammatory response (increases expression of Mx protein 2'-5' oligoadenylate synthethase, Beta2-microglobuline, class I MHC, Neopterin) and inhibits IFN-gamma expression.
- Glatiramer (Copaxone from Teva / Aventis) simulates the Myelin Basic Protein and is supposed to blocks action of myelin damaging T-cells by acting as a decoy agent.

For rapidly progressive MS, Immunosuppressor agents (such as Mitoxantrone, a topo-isomerase II inhibitor, from Wyeth-Lederle) are proposed: they inhibit ADN replication, thus reducing the number of activated immune cells (Stevenson VL, Thompson AJ, Drugs Today (Barc). 1998 Mar;34(3):267-82).

Current Experimental Treatments focus on immuno- and vaccinotherapy, therapies that improve nerve impulse conduction, cytokines and remyelinisation.

Immunosuppressive agents can affect temporarily the course of MS, but their toxic side effects often preclude their widespread use. Examples are Cladribine (deoxyadenosine) and SC 12267 (dihydroorate deshydrogenase), both from Serono.

Monoclonal antibodies are also being tested, such as Campath®, an anti-CD52 antibody by Ilex Pharmaceuticals, to target CD52-presenting lymphocytes.

Vaccine/peptide therapy is exploiting the fact that an immune response against the myelin-damaging T cells can be initiated through the injection of antigens specific of these T-cells.

Improvement of nerve impulses transmission is attempted with drugs blocking or favoring ions channels involved in nerve conduction. A large range of cytokines and substances that block harmful cytokines are being tested: TGF, Il-4.

In addition, factors supporting the oligodendrocytes growth are envisaged to repair the damaged myelin, as it is known that new myelin can be formed by proliferating oligodendrocytes after an attack. A number of compounds are being tested for enhancing myelin repair, such as: Ciliary Neurotrophic Factor (CNTF), Glial Growth Factor (GGF2), Brain-Derived Neurotrophic Factor (BDNF), Myelin Associated Glycoprotein (MAG), Insulin-like Growth Factor-1, Leukemia Inhibitory Factor (LIF), Osteopontin, and MMP-12 inhibitor (Stangel M., Expert Opin Investig Drugs 2004 Apr; 13(4):331-47; Albrecht PJ, *et al.*, Exp Neurol. 2002; 173(1): 46-62; Cannella B, *et al.*, Proc Natl Acad Sci U S A. 1998;95(17):10100-5; Kerschensteiner M, *et al.*, J Exp Med. 1999 Mar 1;189(5):865-70; De Bellard ME, Filbin MT, J Neurosci Res. 1999 Apr 15;56(2):213-8; Mason JL, *et al.*, J Neurosci. 2003 Aug 20;23(20):7710-8; and Butzkueven H, *et al.*, Nat Med. 2002 Jun;8(6):613-9).

Alzheimer's Disease

Alzheimer's disease represents a major public health issue for health care providers and long term care facilities. It is the most common cause of dementia in persons 65 years of age and older and is the fourth leading cause of death among the elderly. It currently afflicts

an estimated four million people in the United States and at least 12 million people worldwide.

Alzheimer's disease is an incurable, progressive, terminal brain disease that afflicts mainly the elderly. It is marked by an irreversible decline in mental abilities, including memory, language and comprehension, and often accompanied by changes in behavior and personality. The course of the illness is typically from 8 to 12 years from the onset of symptoms to death. The definition of the disease is histologic, with 2 hallmarks being the shrinkage or disappearance of brain cells that are replaced by neuritic (amyloid) plaques, and the apparition of thread-like tangles within brain cells (neurofibrillary tangles). As a consequence, there is a loss of neurotransmitter Acetylcholine (AC) in the cerebral cortex. Risk factors of the disease include: age, as incidence increases with aging (from 1% at 65 years up to 25% at 85 years) – genetics, although the most common form of AD is sporadic (90 % in elderly *vs.* inherited form: 10 %, in adults 40 to 50 years old) - Down syndrome, as patients develop AD like illness if they are older than 35 years. The latter is linked to the fact that the amyloid precursor protein gene is on Ch 21.

Familial AD (autosomal dominant) can involve the Presenilin-1 gene (on Ch 14), which encodes the S182 protein (muted in FAD). In addition, the Presenilin-2 gene (on Ch 1) encodes the STM2 protein, which also causes FAD upon mutations.

The ApoE gene on Ch 19 had also been reported to be linked with AD. This protein is involved in cholesterol transport. The gene is composed of 3 alleles: ApoE2, ApoE3, ApoE4. ApoE, and the AD risk increases with phenotype ApoE4/ApoE4, whereas it decreases with phenotype ApoE2.

There is an urgent need for an effective treatment for the illness, caused in part by the rising health care, institutional and social costs for the treatment and care of Alzheimer's disease sufferers. The Surgeon General's Report on Mental Health released on December 13, 1999 put the direct health care costs for the illness in the United States at almost $18 billion for 1996. In a 1998 statement to the House Appropriations Subcommittee, the Director of the National Institute on Aging, Dr. Richard J. Hodes, estimated that the cost of care to family, caregivers and society in general was as much as $100 billion per year.

These costs are expected to rise sharply as the baby boom generation ages and more people become at risk for the disease. According to Dr. Hodes, the number of Americans aged 65 or over, now some 34 million, is expected to more than double by year 2030. Within this group, the population of persons over the age of 85 is the fastest growing segment. As people live longer, they become more at risk of developing Alzheimer's disease.

At present, there is no cure for Alzheimer's disease. Currently available treatments such as Aricept® (donepezil HCl), Exelon® (rivastigmine), Reminyl® (galantamine HBr) and Cognex® (tacrine) are cholinesterases inhibitors, but only provide a measure of symptomatic relief for patients with mild to moderate AD. Namenda® (Memantime) is an N-methyl-D-aspartate receptor (NMDA) antagonist which blocks glutamate activity.

The present invention therefore discloses protein compositions that are useful for the treatment of neurological disorders. In particular, the compositions of the invention have demonstrated efficacy in promoting neuronal survival (see Example 2 hereinbelow), and accelerating nerve regeneration in animals after a sciatic nerve crush (see Example 3 hereinbelow).

SUMMARY OF THE INVENTION

The present invention is directed to protein compositions that are useful for the treatment of neurological disorders. These polypeptide species and compositions are designated herein "Neurological Disorder Treatment Polypeptides", or NDTPs. Such Neurological Disorder Treatment Polypeptides comprise an amino acid sequence selected from the group consisting of SEQ ID NO:1. Compositions include NDTP precursors, and NDTP variants or enhanced forms, e.g. to provide a longer *in vivo* half life. Further included are methods of making and using these compositions.

In another aspect, the invention includes NDTPs comprising a sequence which is at least 75 percent identical to a sequence selected from the group consisting of SEQ ID NO:1. Preferably, the invention includes polypeptides comprising at least 80 percent, and more preferably at least 85 percent, and still more preferably at least 90 percent, identity with any one of the sequences selected from SEQ ID NO:1. Most preferably, the invention includes polypeptides comprising a sequence at least 95 percent identical to a sequence selected from the group consisting of SEQ ID NO:1.

In an additional aspect, the invention includes modified NDTPs. Such modifications include protecting/blocking groups, linkage to an antibody molecule or other cellular ligand, and detectable labels, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein. Chemical modifications may be carried out by known techniques, including but not limited to, specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH4, acetylation, formylation, oxidation, reduction, or metabolic synthesis in the presence of tunicamycin.

Also provided by the invention are chemically modified derivatives of the polypeptides of the invention which may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (e.g., water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol). The NDTPs are modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

In another aspect, the invention includes polynucleotides encoding an NDTP of the invention, polynucleotides encoding a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:1, and vectors for expressing NDTPs.

In another aspect, the invention provides a vector comprising DNA encoding an NDTP. The invention also includes host cells and transgenic nonhuman animals comprising such a vector. There is also provided a method of making an NDTP or NDTP precursor. One preferred method comprises the steps of (a) providing a host cell containing an expression vector as disclosed above; (b) culturing the host cell under conditions whereby the DNA segment is expressed; and (c) recovering the protein encoded by the DNA segment. Another preferred method comprises the steps of: (a) providing a host cell capable of expressing an NDTP; (b) culturing said host cell under conditions that allow expression of said NDTP; and (c) recovering said NDTP. Within one embodiment the expression vector further comprises a secretory signal sequence operably linked to the DNA segment, the cell secretes the protein into a culture medium, and the protein is recovered from the medium. An especially preferred method of making an NDTP includes chemical synthesis using standard peptide synthesis techniques, as described in the section titled "Chemical Manufacture of NDTP Compositions" and in Example 1.

Further aspects of the invention are also described in the specification and in the claims.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1 to 14 illustrate the results of the *in vitro* and *in vivo* assays conducted with the GPB003 polypeptide of the invention, as further detailed in Examples 2 and 3.

Figure 15 illustrates the clinical manifestations of Multiple Sclerosis, as detailed above in the "Background" section.

Figure 16 illustrates the protein sequence (SEQ ID NO:1) for the GPB003 protein of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention described in detail below provides compositions useful for the treatment of neurological disorders in a mammalian individual and for drug development. The invention also encompasses the administration of therapeutic compositions to a mammalian individual to treat or prevent neurological disorders. The mammalian individual may be a non-human mammal, but is preferably human, more preferably a human adult.

Definitions

Neurological disorders include, but are not limited to, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, stroke and ischemia associated with stroke, neural paropathy, other neural degenerative diseases, motor neuron diseases, sciatic crush, peripheral neuropathy, particularly neuropathy associated with diabetes, spinal cord injuries and facial nerve crush, as well as Traumatic Brain Injuries.

As used herein, the term "nucleic acids" and "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic 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. Throughout the present specification, the expression "nucleotide sequence" may be employed to designate indifferently a polynucleotide or a nucleic acid. More precisely, the expression "nucleotide sequence" encompasses the nucleic material itself and is thus not restricted to the sequence information (i.e. the succession of letters chosen among the four base letters) that biochemically characterizes a specific DNA or RNA molecule. Also, used interchangeably herein are terms "nucleic acids", "oligonucleotides", and "polynucleotides".

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated NDTP nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Using all or a portion of the

nucleic acid as a hybridization probe, NDTP 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, N.Y., 1989).

As used herein, the term "hybridizes to" is intended to describe conditions for moderate stringency or high stringency hybridization, preferably where the hybridization and washing conditions permit nucleotide sequences at least 60% homologous to each other to 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%, 90%, 95% or 98% homologous to each other typically remain hybridized to each other. Stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. In a preferred, non-limiting example, stringent hybridization conditions for nucleic acid interactions are as follows: the hybridization step is realized at 65°C in the presence of 6 x SSC buffer, 5 x Denhardt's solution, 0,5% SDS and 100µg/ml of salmon sperm DNA. The hybridization step is followed by four washing steps:

- two washings during 5 min, preferably at 65°C in a 2 x SSC and 0.1%SDS buffer;
- one washing during 30 min, preferably at 65°C in a 2 x SSC and 0.1% SDS buffer,
- one washing during 10 min, preferably at 65°C in a 0.1 x SSC and 0.1%SDS buffer,

these hybridization conditions being suitable for a nucleic acid molecule of about 20 nucleotides in length. It will be appreciated that the hybridization conditions described above are to be adapted according to the length of the desired nucleic acid, following techniques well known to the one skilled in the art, for example be adapted according to the teachings disclosed in Hames B.D. and Higgins S.J. (1985) Nucleic Acid Hybridization: A Practical Approach. Hames and Higgins Ed., IRL Press, Oxford; and Current Protocols in Molecular Biology.

"Percent homology" is used herein to refer to both nucleic acid sequences and amino acid sequences. Amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology". To determine the percent homology of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence and non-homologous sequences can be disregarded for comparison purposes). The length of a reference sequence aligned for 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%, 90% or 95%

of the length of the reference sequence. 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 homologous at that position. The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology=# of identical positions/total # of positions 100).

The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-68, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-77, the disclosures of which are incorporated herein by reference in their entireties. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to the sequences of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the polypeptide sequences of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Research 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov, the disclosures of which are incorporated herein by reference in their entireties. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989), the disclosures of which are incorporated herein by reference in their entireties. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

The term "polypeptide" refers to a polymer of amino acids without regard to the length of the polymer; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not specify or exclude post-translational modifications of polypeptides, for example, polypeptides which include the covalent attachment of glycosyl, acetyl, phosphate, amide, lipid, carboxyl, acyl, or carbohydrate

groups are expressly encompassed by the term polypeptide. Also included within the definition are polypeptides which contain one or more analogs of an amino acid (including, for example, non-naturally occurring amino acids, amino acids which only occur naturally in an unrelated biological system, modified amino acids from mammalian systems etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The term "protein" as used herein may be used synonymously with the term "polypeptide" or may refer to, in addition, a complex of two or more polypeptides which may be linked by bonds other than peptide bonds, for example, such polypeptides making up the protein may be linked by disulfide bonds. The term "protein" may also comprehend a family of polypeptides having identical amino acid sequences but different post-translational modifications, particularly as may be added when such proteins are expressed in eukaryotic hosts.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which it is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of a protein according to the invention in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of a protein according to the invention having less than about 30% (by dry weight) of protein other than the protein of the invention (also referred to herein as a "contaminating protein"), more preferably less than about 20% of protein other than the protein according to the invention, still more preferably less than about 10% of protein other than the protein according to the invention, and most preferably less than about 5% of protein other than the protein according to the invention. When the protein according to the invention or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of a protein of the invention in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of a protein of the invention having less than about 30% (by dry weight)

of chemical precursors or non-protein chemicals, more preferably less than about 20% chemical precursors or non-protein chemicals, still more preferably less than about 10% chemical precursors or non-protein chemicals, and most preferably less than about 5% chemical precursors or non-protein chemicals.

The term "recombinant polypeptide" is used herein to refer to polypeptides that have been artificially designed and which comprise at least two polypeptide sequences that are not found as contiguous polypeptide sequences in their initial natural environment, or to refer to polypeptides which have been expressed from a recombinant polynucleotide.

The term "Neurological Disorder Treatment Polypeptide" or "NDTP" refers to a polypeptide comprising the sequence described by the polypeptide sequence listed in SEQ ID NO: 1 . NDTPs may also contain other structural or chemical modifications such as disulfide linkages or amino acid side chain interactions such as hydrogen and amide bonds that result in complex secondary or tertiary structures. NDTPs also include mutant polypeptides, such as deletion, addition, swap, or truncation mutants, fusion polypeptides comprising such polypeptides, and polypeptide fragments of at least three, but preferably 8, 10, 12, 15, or 21 contiguous amino acids of the sequence of NDTPs. The invention embodies polypeptides encoded by the nucleic acid sequences of NDTP genes or NDTP mRNA species, preferably human NDTP genes and mRNA species.

The term "biological activity" as used herein refers to any single function carried out by an NDTP. These include but are not limited to: promoting neuronal survival (see Example 2 hereinbelow), and accelerating nerve regeneration in animals after a sciatic nerve crush (see Example 3 hereinbelow).

As used herein, a "label group" is any compound that, when attached to a polynucleotide or polypeptide (including antibodies), allows for detection or purification of said polynucleotide or polypeptide. Label groups may be detected or purified directly or indirectly by a secondary compound, including an antibody specific for said label group. Useful label groups include radioisotopes (e.g., ^{32}P, ^{35}S, ^{3}H, ^{125}I), fluorescent compounds (e.g., 5-bromodesoxyuridin, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, phycoerythrin acetylaminofluorene, digoxigenin), luminescent compounds (e.g., luminol, GFP, luciferin, aequorin), enzymes or enzyme co-factor detectable labels (e.g., peroxidase, luciferase, alkaline phosphatase, galactosidase, or acetylcholinesterase), or compounds that are recognized by a secondary factor such as strepavidin, GST, or biotin. Preferably, a label group is attached to a polynucleotide or polypeptide in such a way as to not interfere with the biological activity of the polynucleotide or polypeptide.

Radioisotopes may be detected by direct counting of radioemission, film exposure, or by scintillation counting, for example. Enzymatic labels may be detected by determination of conversion of an appropriate substrate to product, usually causing a fluorescent reaction. Fluorescent and luminescent compounds and reactions may be detected by, e.g., radioemission, fluorescent microscopy, fluorescent activated cell sorting, or a luminometer.

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 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 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.

As used herein, "effective amount" describes the amount of an agent, preferably an NDTP or NDTP modulator of the invention, sufficient to have a desired effect. For example, an anti-multiple sclerosis effective amount is the amount of an agent required to reduce a symptom of multiple sclerosis in an individual by at least 1, 2, 5, 10, 15, or preferably 25%. The term may also describe the amount of an agent required to ameliorate a multiple sclerosis-caused symptom in an individual. Symptoms of multiple sclerosis are diverse and many, but can be for example of the following types: visual, motor, sensory, coordination and balance, and cognitive. The effective amount for a particular patient may vary depending on such factors as the diagnostic method of the symptom being measured, the state of the condition being treated, the overall health of the patient, method of administration, and the severity of side-effects.

NDTPs of the invention

The NDTPs of the present invention are characterized by the polypeptide sequence of SEQ ID NO:1, as shown in Figure 16.

This polypeptide was identified in human Cerebro-Spinal Fluid and is CSF-specific, as it was never detected during proteomic studies of plasma or serum.

This polypeptide can be chemically synthesized, as described in Example 1, and display therapeutic efficacy in neurological assays, as detailed in Examples 2 and 3.

NDTP nucleic acids

One aspect of the invention pertains to purified or isolated nucleic acid molecules that encode NDTPs or biologically active portions thereof as further described herein, as well as nucleic acid fragments thereof. Said nucleic acids may be used for example in therapeutic (DNA vaccine) and in drug screening assays as further described herein.

An object of the invention is a purified, isolated, or recombinant nucleic acid coding for an NDTP, complementary sequences thereto, and fragments thereof. The invention also pertains to a purified or isolated nucleic acid comprising a polynucleotide having at least 95% nucleotide identity with a polynucleotide coding for an NDTP, advantageously 99 % nucleotide identity, preferably 99.5% nucleotide identity and most preferably 99.8% nucleotide identity with a polynucleotide coding for an NDTP, or a sequence complementary thereto or a biologically active fragment thereof. Another object of the invention relates to purified, isolated or recombinant nucleic acids comprising a polynucleotide that hybridizes, under the stringent hybridization conditions defined herein, with a polynucleotide coding for an NDTP, or a sequence complementary thereto or a variant thereof or a biologically active fragment thereof.

In another preferred aspect, the invention pertains to purified or isolated nucleic acid molecules that encode a portion or variant of an NDTP, wherein the portion or variant displays an NDTP biological activity. Preferably said portion or variant is a portion or variant of a naturally occurring NDTP or precursor thereof.

Another object of the invention is a purified, isolated, or recombinant nucleic acid encoding an NDTP comprising, consisting essentially of, or consisting of the amino acid sequence selected from the group consisting of the polypeptide sequence listed in SEQ ID NO: 1.

The nucleotide sequence determined from the cloning of the NDTP-encoding gene allows for the generation of probes and primers designed for use in identifying and/or cloning

other NDTPs (e.g. sharing the novel functional domains), as well as NDTP homologues from other species.

A nucleic acid fragment encoding a "biologically active portion of an NDTP" can be prepared by isolating a portion of a nucleotide sequence coding for an NDTP, which encodes a polypeptide having an NDTP biological activity, expressing the encoded portion of the NDTP (e.g., by recombinant expression *in vitro* or *in vivo*) and assessing the activity of the encoded portion of the NDTP.

The invention further encompasses nucleic acid molecules that differ from the NDTP nucleotide sequences of the invention due to degeneracy of the genetic code and encode the same NDTPs of the invention.

In addition to the NDTP nucleotide sequences described above, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of the NDTPs may exist within a population (e.g., the human population). Such genetic polymorphism may exist among individuals within a population due to natural allelic variation. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of an NDTP-encoding gene or nucleic acid sequence.

Nucleic acid molecules corresponding to natural allelic variants and homologues of the NDTP nucleic acids of the invention can be isolated based on their homology to the NDTP nucleic acids disclosed herein using the cDNAs disclosed herein, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions.

It will be appreciated that the invention comprises polypeptides having an amino acid sequence encoded by any of the polynucleotides of the invention.

Uses of NDTP nucleic acids

The invention includes primer pairs for carrying out a PCR to amplify a segment of a polynucleotide of the invention. Each primer of a pair is an oligonucleotide having a length of between 15 and 30 nucleotides such that i) one primer of the pair forms a perfectly matched duplex with one strand of a polynucleotide of the invention and the other primer of the pair form a perfectly match duplex with the complementary strand of the same polynucleotide, and ii) the primers of a pair form such perfectly matched duplexes at sites on the polynucleotide that separated by a distance of between 10 and 2500 nucleotides. Preferably, the annealing temperature of each primer of a pair to its respective complementary sequence is substantially the same.

Hybridization probes derived from polynucleotides of the invention can be used, for example, in performing in situ hybridization on tissue samples, such as fixed or frozen tissue sections prepared on microscopic slides or suspended cells. Briefly, a labeled DNA or RNA probe is allowed to bind its DNA or RNA target sample in the tissue section on a prepared microscopic, under controlled conditions. Generally, dsDNA probes consisting of the DNA of interest cloned into a plasmid or bacteriophage DNA vector are used for this purpose, although ssDNA or ssRNA probes may also be used. Probes are generally oligonucleotides between about 15 and 40 nucleotides in length. Alternatively, the probes can be polynucleotide probes generated by PCR random priming primer extension or in vitro transcription of RNA from plasmids (riboprobes). These latter probes are typically several hundred base pairs in length. The probes can be labeled by any of a number of label groups and the particular detection method will correspond to the type of label utilized on the probe (e.g., autoradiography, X-ray detection, fluorescent or visual microscopic analysis, as appropriate). The reaction can be further amplified in situ using immunocytochemical techniques directed against the label of the detector molecule used, such as an antibody directed to a fluorescein moiety present on a fluorescently labeled probe. Specific labeling and in situ detection methods can be found, for example, in Howard, G. C., Ed., *Methods in Nonradioactive Detection,* Appleton & Lange, Norwalk, Conn., (1993), herein incorporated by reference.

Hybridization probes and PCR primers may also be selected from the genomic sequences corresponding to the full-length proteins identified in accordance with the present invention, including promoter, enhancer elements and introns of the gene encoding the naturally occurring polypeptide.

Oligonucleotide Compounds

Oligonucleotides of the invention, including PCR primers, are synthesized by conventional means on a commercially available automated DNA synthesizer, e.g. an Applied Biosystems (Foster City, CA) model 380B, 392 or 394 DNA/RNA synthesizer, or like instrument. Preferably, phosphoramidite chemistry is employed, e.g. as disclosed in the following references: Beaucage and Iyer, Tetrahedron, 48: 2223-2311 (1992); Molko et al, U.S. patent 4,980,460; Koster et al, U.S. patent 4,725,677; Caruthers et al, U.S. patents 4,415,732; 4,458,066; and 4,973,679; and the like. For therapeutic use, nuclease resistant backbones are preferred. Many types of modified oligonucleotides are available that confer nuclease resistance, e.g. phosphorothioate, phosphorodithioate, phosphoramidate, or the like, described in many references, e.g. phosphorothioates: Stec et al, U.S. patent 5,151,510;

Hirschbein, U.S. patent 5,166,387; Bergot, U.S. patent 5,183,885; phosphoramidates: Froehler et al, International application PCT/US90/03138; and for a review of additional applicable chemistries: Uhlmann and Peyman (cited above).

Primers and probes

Primers and probes of the invention can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphodiester method of Narang SA et al (Methods Enzymol 1979;68:90-98), the phosphodiester method of Brown EL et al (Methods Enzymol 1979;68:109-151), the diethylphosphoramidite method of Beaucage et al (Tetrahedron Lett 1981, 22: 1859-1862) and the solid support method described in EP 0 707 592, the disclosures of which are incorporated herein by reference in their entireties.

Detection probes are generally nucleic acid sequences or uncharged nucleic acid analogs such as, for example peptide nucleic acids which are disclosed in International Patent Application WO 92/20702, morpholino analogs which are described in U.S. Patents Numbered 5,185,444; 5,034,506 and 5,142,047. If desired, the probe may be rendered "non-extendable" in that additional dNTPs cannot be added to the probe. In and of themselves analogs usually are non-extendable and nucleic acid probes can be rendered non-extendable by modifying the 3' end of the probe such that the hydroxyl group is no longer capable of participating in elongation. For example, the 3' end of the probe can be functionalized with the capture or detection label to thereby consume or otherwise block the hydroxyl group.

Any of the polynucleotides of the present invention can be labeled, if desired, by incorporating any label group known in the art to be detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. Additional examples include non-radioactive labeling of nucleic acid fragments as described in Urdea et al. (Nucleic Acids Research. 11:4937-4957, 1988) or Sanchez-Pescador et al. (J. Clin. Microbiol. 26(10):1934-1938, 1988). In addition, the probes according to the present invention may have structural characteristics such that they allow the signal amplification, such structural characteristics being, for example, branched DNA probes as those described by Urdea et al (Nucleic Acids Symp. Ser. 24:197-200, 1991) or in the European patent No. EP 0225807 (Chiron).

A label can also be used to capture the primer, so as to facilitate the immobilization of either the primer or a primer extension product, such as amplified DNA, on a solid support. A capture label is attached to the primers or probes and can be a specific binding member which forms a binding pair with the solid's phase reagent's specific binding member (e.g.

biotin and streptavidin). Therefore depending upon the type of label carried by a polynucleotide or a probe, it may be employed to capture or to detect the target DNA. Further, it will be understood that the polynucleotides, primers or probes provided herein, may, themselves, serve as the capture label. For example, in the case where a solid phase reagent's binding member is a nucleic acid sequence, it may be selected such that it binds a complementary portion of a primer or probe to thereby immobilize the primer or probe to the solid phase. In cases where a polynucleotide probe itself serves as the binding member, those skilled in the art will recognize that the probe will contain a sequence or "tail" that is not complementary to the target. In the case where a polynucleotide primer itself serves as the capture label, at least a portion of the primer will be free to hybridize with a nucleic acid on a solid phase. DNA labeling techniques are well known to the skilled technician.

The probes of the present invention are useful for a number of purposes. They can be notably used in Southern hybridization to genomic DNA. The probes can also be used to detect PCR amplification products. They may also be used to detect mismatches in NDTP-encoding genes or mRNA using other techniques.

Any of the nucleic acids, polynucleotides, primers and probes of the present invention can be conveniently immobilized on a solid support. Solid supports are known to those skilled in the art and include the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, sheep (or other animal) red blood cells, duracytes and others. The solid support is not critical and can be selected by one skilled in the art. Thus, latex particles, microparticles, magnetic or non-magnetic beads, membranes, plastic tubes, walls of microtiter wells, glass or silicon chips, sheep (or other suitable animal's) red blood cells and duracytes are all suitable examples. Suitable methods for immobilizing nucleic acids on solid phases include ionic, hydrophobic, covalent interactions and the like. A solid support, as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid support can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can include a charged substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member attached to the solid support and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid support material before the performance of the assay or during the performance of the assay. The solid phase thus can be

a plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon surface of a test tube, microtiter well, sheet, bead, microparticle, chip, sheep (or other suitable animal's) red blood cells, duracytes and other configurations known to those of ordinary skill in the art. The nucleic acids, polynucleotides, primers and probes of the invention can be attached to or immobilized on a solid support individually or in groups of at least 2, 5, 8, 10, 12, 15, 20, or 25 distinct polynucleotides of the invention to a single solid support. In addition, polynucleotides other than those of the invention may be attached to the same solid support as one or more polynucleotides of the invention.

Any polynucleotide provided herein may be attached in overlapping areas or at random locations on a solid support. Alternatively the polynucleotides of the invention may be attached in an ordered array wherein each polynucleotide is attached to a distinct region of the solid support which does not overlap with the attachment site of any other polynucleotide. Preferably, such an ordered array of polynucleotides is designed to be "addressable" where the distinct locations are recorded and can be accessed as part of an assay procedure. Addressable polynucleotide arrays typically comprise a plurality of different oligonucleotide probes that are coupled to a surface of a substrate in different known locations. The knowledge of the precise location of each polynucleotides location makes these "addressable" arrays particularly useful in hybridization assays. Any addressable array technology known in the art can be employed with the polynucleotides of the invention. One particular embodiment of these polynucleotide arrays is known as the Genechips, and has been generally described in US Patent 5,143,854; PCT publications WO 90/15070 and 92/10092, the disclosures of which are incorporated herein by reference in their entireties.

Methods for obtaining variant nucleic acids and polypeptides

In addition to naturally-occurring allelic variants of the NDTP sequences that may exist in the population, the skilled artisan will appreciate that changes can be introduced by mutation into the nucleotide sequences coding for NDTPs, thereby leading to changes in the amino acid sequence of the encoded NDTPs, with or without altering the functional ability of the NDTPs.

Several types of variants are contemplated including 1) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue and such substituted amino acid residue may or may not be one encoded by the genetic code, or 2) one in which one or more of the amino acid residues includes a substituent group, or 3) one in which the mutated NDTP is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or 4) one in which

the additional amino acids are fused to the NDTP, such as a leader, a signal or anchor sequence, a sequence which is employed for purification of the NDTP, or sequence from a precursor protein. Such variants are deemed to be within the scope of those skilled in the art.

For example, nucleotide substitutions leading to amino acid substitutions can be made in the sequences that do not substantially change the biological activity of the protein. An amino acid residue-can be altered from the wild-type sequence encoding an NDTP, or a biologically active fragment or homologue thereof without altering the biological activity. In general, amino acid residues that are shared among the NDTPs of the present invention are predicted to be less amenable to alteration.

In another aspect, the invention pertains to nucleic acid molecules encoding NDTPs that contain changes in amino acid residues that result in increased biological activity, or a modified biological activity. In another aspect, the invention pertains to nucleic acid molecules encoding NDTPs that contain changes in amino acid residues that are essential for an NDTP biological activity. Such NDTPs differ in amino acid sequence from NDTPs and display reduced activity, or essentially lack one or more NDTP biological activities.

Mutations, substitutions, additions, or deletions can be introduced into any of NDTPs, by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. For example, conservative amino acid substitutions may be 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 with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., 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, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in an NDTP, or a biologically active fragment or homologue thereof may be 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 an NDTP coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for NDTP biological activity to identify mutants that retain activity. Following mutagenesis of the nucleotide encoding one of NDTPs, the encoded protein can be expressed recombinantly and the activity of the protein can be determined in any suitable assay, for example, as provided herein.

The invention also provides NDTP chimeric or fusion proteins. As used herein, an NDTP "chimeric protein" or "fusion protein" comprises an NDTP of the invention or fragment thereof, operatively linked or fused in frame to a non-NDTP polypeptide sequence. In a preferred embodiment, an NDTP fusion protein comprises at least one biologically active portion of an NDTP. In another preferred embodiment, an NDTP fusion protein comprises at least two biologically active portions of an NDTP. For example, in one embodiment, the fusion protein is a GST-NDTP fusion protein in which NDTP domain sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant NDTPs. In another embodiment, the fusion protein is an NDTP containing a heterologous signal sequence at its N-terminus, for example, to allow for a desired cellular localization in a certain host cell. In yet another embodiment, the fusion is an NDTP biologically active fragment and an immunoglobulin molecule. Such fusion proteins are useful, for example, to increase the valency of NDTP binding sites. For example, a bivalent NDTP binding site may be formed by fusing biologically active NDTP fragments to an IgG Fc protein.

NDTP fusion proteins of the invention can be used as immunogens to produce anti-NDTP antibodies in a subject, to purify NDTP or NDTP ligands and in screening assays to identify NDTP modulators.

Furthermore, isolated fragments of NDTPs can also be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, an NDTP of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments with an NDTP biological activity, for example, by microinjection assays or in vitro protein binding assays. In an illustrative embodiment, peptidyl portions of an NDTP, such as an NDTP target binding region, can be tested for NDTP activity by expression as thioredoxin fusion proteins, each of which contains a discrete fragment of the NDTP (see, for example, U.S. Patents 5, 270,181 and 5,292,646; and PCT publication WO94/02502, the disclosures of which are incorporated herein by reference).

In addition, libraries of fragments of an NDTP coding sequence can be used to generate a variegated population of NDTP fragments for screening and subsequent selection of variants of an NDTP. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of NDTP 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 fragments of various sizes of the NDTP.

Modified NDTPs can be used for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo). Such modified peptides, when designed to retain at least one activity of the naturally occurring form of the protein, are considered functional equivalents of the NDTP described in more detail herein. Such modified peptide can be produced, for instance, by amino acid substitution, deletion, or addition.

Whether a change in the amino acid sequence of a peptide results in a functional NDTP homolog can be readily determined by assessing at least one NDTP biological activity of the variant peptide. Peptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method of generating sets of combinatorial mutants of the presently disclosed NDTPs, as well as truncation and fragmentation mutants, and is especially useful for identifying potential variant sequences which are functional in binding to an NDTP target protein but differ from a wild-type form of the protein by, for example, efficacy, potency and/or intracellular half-life. One purpose for screening such combinatorial libraries is, for example, to isolate novel NDTP homologs with altered biological activity, when compared with the wild-type protein, or alternatively, possessing novel activities all together. For example, mutagenesis can give rise to NDTP homologs which have intracellular half-lives dramatically different than the corresponding wild-type protein. The altered protein can be rendered either more stable or less stable to proteolytic degradation, or cellular processes which result in destruction of, or otherwise inactivation of, an NDTP. Such NDTP homologs, and the genes which encode them, can be utilized to alter the envelope of expression for a particular recombinant NDTP by modulating the half-life of the recombinant protein. For instance, a short half-life can give rise to more transient biological effects associated with a particular recombinant NDTP and, when part of an inducible expression system, can allow tighter control of recombinant protein levels within a cell and in circulating plasma. As above, such proteins, and particularly their recombinant nucleic acid constructs, can be used in gene therapy protocols.

In an illustrative embodiment of this method, the amino acid sequences for a population of NDTP homologs or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, NDTP homologs from one or more species, or NDTP homologs from the same species but which differ due to mutation. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. There are many ways by which the library of potential NDTP homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential NDTP sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example. Narang, SA (1983) Tetrahedron 393; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp. 273-289; 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). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Patents Nos: 5, 223,409, 5,198,346, and 5,096,815). The disclosures of the above references are incorporated herein by reference in their entireties.

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library, particularly where no other naturally occurring homologs have yet been sequenced. For example, NDTP homologs can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al. (1994) Biochemistry 33:1565-1572; Wang et al. (1994) J Biol. Chem. 269:3095-3099; Balint et al. (1993) Gene 137:109-118; Grodberg et al. (1993) Eur. J Biochem. 218:597-601; Nagashima et al. (1993) J Biol. Chem. 268:2888-2892; Lowman et al. (1991) Biochemistry 30:10832-10838; and Cunningham et al. (1989) Science 244:1081-1085), by linker scanning mutagenesis (Gustin et al. (1993) Virology 193:653-660; Brown et al. (1992) Mol. Cell Biol. 12:2644 2652; McKnight et al. (1982) Science 232:316); by saturation mutagenesis (Meyers et al. (1986) Science 232:613); by PCR mutagenesis (Leung et al. (1989) Method Cell Mol Biol 1: 1-19); or by random mutagenesis (Miller et al. (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener et al. (1994) Strategies in Mol Biol 7:32-34, the disclosures of which are incorporated herein by reference in their entireties).

A further method exploits automatic protein design to generate protein libraries for screening and optimization of the sequence of a protein of the invention. See, for example, U.S. Patent 6403312, disclosure of which is incorporated herein by reference. Briefly, a primary library is generated using computational processing based on the sequence and structural characteristics of the NDTP. Generally speaking, the goal of the computational processing is to determine a set of optimized protein sequences that result in the lowest energy conformation of any possible sequence. However, a plurality of sequences that are not the global minimum may have low energies and be useful. Thus, a primary library comprising a rank ordered list of sequences, generally in terms of theoretical quantitative stability, is generated. These sequences may be used to synthesize or express peptides displaying an extended half-life or stabilized interactions with NDTP binding compounds and proteins.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, as well as for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of NDTPs. The most widely used techniques for screening large gene libraries typically comprises 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 relatively easy isolation of the vector encoding the gene whose product was detected.

Each of the illustrative assays described below are amenable to high throughput analysis as necessary to screen large numbers of degenerate NDTP sequences created by combinatorial mutagenesis techniques. In one screening assay, the candidate gene products are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to bind an NDTP target molecule (for example a modified peptide substrate) via this gene product is detected in a "panning assay". For instance, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) BioTechnology 9:1370-1371, and Goward et al. (1992) TIBS 18:136 140). In a similar fashion, fluorescently labeled NDTP target can be used to score for potentially functional NDTP homologs. Cells can be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, separated by a fluorescence- activated cell sorter.

In an alternate embodiment, the gene library is expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide

sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phages can be applied to affinity matrices at very high concentrations, a large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical E. coli filamentous phages M13, fd, and fl are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) J Biol. Chem. 267:16007-16010; Griffiths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457 4461, the disclosures of which are incorporated herein by reference in their entireties). In an illustrative embodiment, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing NDTP combinatorial libraries, and the NDTP phage library can be panned on immobilized NDTP target molecule (glutathione immobilized NDTP target-GST fusion proteins or immobilized DNA). Successive rounds of phage amplification and panning can greatly enrich for NDTP homologs which retain an ability to bind an NDTP target and which can subsequently be screened further for biological activities in automated assays, in order to distinguish between agonists and antagonists.

The invention also provides for identification and reduction to functional minimal size of the NDTP functional domains, to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt binding of a polypeptide of the present invention with an NDTP target molecule. Thus, such mutagenic techniques as described above are also useful to map the determinants of NDTPs participating in protein-protein interactions involved in, for example, binding to an NDTP target protein. To illustrate, the critical residues of an NDTP involved in molecular recognition of the NDTP target can be determined and used to generate NDTP target-13P-derived peptidomimetics that competitively inhibit binding of the NDTP to the NDTP target. For instance, non hydrolysable peptide analogs of such residues can be generated using retro-inverse peptides (e.g., see U.S. Patents 5,116,947 and 5,219,089; and Pallai et al. (1983) Int J Pept Protein Res 21:84-92), benzodiazepine (e.g., see Freidinger et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in Peptides. Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM

Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) J Med Chem 29:295; and Ewenson et al. in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), P-turn dipeptide cores (Nagai et al. (1985) Tetrahedron Left 26:647; and Sato et al. (1986) J Chem Soc Perkin Trans 1: 123 1), and P-aminoalcohols (Gordon et al. (1985) Biochem Biophys Res Commun 126:419; and Dann et al. (1986) Biochem Biophys Res Commun 134:71, the disclosures of which are incorporated herein by reference in their entireties).

Chemical Manufacture of NDTP Compositions

Peptides of the invention are synthesized by standard techniques (e.g. Stewart and Young, Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Company, Rockford, IL, 1984). Preferably, a commercial peptide synthesizer is used, e.g. Applied Biosystems, Inc. (Foster City, CA) model 430A, and polypeptides of the invention may be assembled from multiple, separately synthesized and purified, peptide in a convergent synthesis approach, e.g. Kent et al, U.S. patent 6,184,344 and Dawson and Kent, Annu. Rev. Biochem., 69: 923-960 (2000). Peptides of the invention may be assembled by solid phase synthesis on a cross-linked polystyrene support starting from the carboxyl terminal residue and adding amino acids in a stepwise fashion until the entire peptide has been formed. The following references are guides to the chemistry employed during synthesis: Schnolzer et al, Int. J. Peptide Protein Res., 40: 180-193 (1992); Merrifield, J. Amer. Chem. Soc., Vol. 85, pg. 2149 (1963); Kent et al., pg 185, in Peptides 1984, Ragnarsson, Ed. (Almquist and Weksell, Stockholm, 1984); Kent et al., pg. 217 in Peptide Chemistry 84, Izumiya, Ed. (Protein Research Foundation, B.H. Osaka, 1985); Merrifield, Science, Vol. 232, pgs. 341-347 (1986); Kent, Ann. Rev. Biochem, Vol. 57, pgs. 957-989 (1988), and references cited in these latter two references.

Preferably, chemical synthesis of polypeptides of the invention is carried out by the assembly of peptide fragments by native chemical ligation, as described by Dawson et al, Science, 266: 776-779 (1994) and Kent el al, U.S. patent 6,184,344. Briefly, in the approach a first peptide fragment is provided with an N-terminal cysteine having an unoxidized sulfhydryl side chain, and a second peptide fragment is provided with a C-terminal thioester. The unoxidized sulfhydryl side chain of the N-terminal cysteine is then condensed with the C-terminal thioester to produce an intermediate peptide fragment which links the first and second peptide fragments with a β-aminothioester bond. The β-aminothioester bond of the intermediate peptide fragment then undergoes an intramolecular rearrangement to produce the peptide fragment product which links the first and second peptide fragments with an amide bond. Preferably, the N-terminal cysteine of the internal fragments is protected from

undesired cyclization and/or concatenation reactions by a cyclic thiazolidine protecting group as described below. Preferably, such cyclic thiazolidine protecting group is a thioprolinyl group.

Peptide fragments having a C-terminal thioester may be produced as described in the following references, which are incorporated by reference: Kent et al, U.S. patent 6,184,344; Tam et al, Proc. Natl. Acad. Sci., 92: 12485-12489 (1995); Blake, Int. J. Peptide Protein Res., 17: 273 (1981); Canne et al, Tetrahedron Letters, 36: 1217-1220 (1995); Hackeng et al, Proc. Natl. Acad. Sci., 94: 7845-7850 (1997); or Hackeng et al, Proc. Natl. Acad. Sci., 96: 10068-10073 (1999). Preferably, the method described by Hackeng et al (1999) is employed. Briefly, peptide fragments are synthesized on a solid phase support (described below) typically on a 0.25 mmol scale by using the in situ neutralization/HBTU activation procedure for Boc chemistry disclosed by Schnolzer et al, Int. J. Peptide Protein Res., 40: 180-193 (1992), which reference is incorporated herein by reference. (HBTU is 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and Boc is tert-butoxycarbonyl). Each synthetic cycle consists of N^{α}-Boc removal by a 1- to 2- minute treatment with neat TFA, a 1-minute DMF flow wash, a 10- to 20-minute coupling time with 1.0 mmol of preactivated Boc-amino acid in the presence of DIEA, and a second DMF flow wash. (TFA is trifluoroacetic acid, DMF is N,N-dimethylformamide, and DIEA is N,N-diisopropylethylamine). N^{α}-Boc-amino acids (1.1 mmol) are preactivated for 3 minutes with 1.0 mmol of HBTU (0.5 M in DMF) in the presence of excess DIEA (3 mmol). After each coupling step, yields are determined by measuring residual free amine with a conventional quantitative ninhydrin assay, e.g. as disclosed in Sarin et al, Anal. Biochem., 117: 147-157 (1981). After coupling of Gln residues, a DCM flow wash is used before and after deprotection by using TFA, to prevent possible high-temperature (TFA/DMF)-catalyzed pyrrolidone formation. After chain assembly is completed, the peptide fragments are deprotected and cleaved from the resin by treatment with anhydrous HF for 1 hour at 0°C with 4% *p*-cresol as a scavenger. The imidazole side-chain 2,4-dinitrophenyl (dnp) protecting groups remain on the His residues because the dnp-removal procedure is incompatible with C-terminal thioester groups. However, dnp is gradually removed by thiols during the ligation reaction. After cleavage, peptide fragments are precipitated with ice-cold diethylether, dissolved in aqueous acetonitrile, and lyophilized.

Thioester peptide fragments described above are preferably synthesized on a trityl-associated mercaptopropionic acid-leucine (TAMPAL) resin, made as disclosed by Hackeng et al (1999), or comparable protocol. Briefly, N^{α}-Boc-Leu (4 mmol) is activated with 3.6 mmol of HBTU in the presence of 6 mmol of DIEA and coupled for 16 minutes to 2 mmol of

p-methylbenzhydrylamine (MBHA) resin, or the equivalent. Next, 3 mmol of S-trityl mercaptopropionic acid is activated with 2.7 mmol of HBTU in the presence of 6 mmol of DIEA and coupled for 16 minutes to Leu-MBHA resin. The resulting TAMPAL resin can be used as a starting resin for polypeptide-chain assembly after removal of the trityl protecting group with two 1-minute treatments with 3.5% triisopropylsilane and 2.5% H_2O in TFA. The thioester bond can be formed with any desired amino acid by using standard in situ-neutralization peptide coupling protocols for 1 hour, as disclosed in Schnolzer et al (cited above). Treatment of the final peptide fragment with anhydrous HF yields the C-terminal activated mercaptopropionic acid-leucine (MPAL) thioester peptide fragments.

Preferably, thiazolidine-protected thioester peptide fragment intermediates are used in native chemical ligation under conditions as described by Hackeng et al (1999), or like conditions. Briefly, 0.1 M phosphate buffer (pH 8.5) containing 6 M guanidine, 4% (vol/vol) benzylmercaptan, and 4% (vol/vol) thiophenol is added to dry peptides to be ligated, to give a final peptide concentration of 1-3 mM at about pH 7, lowered because of the addition of thiols and TFA from the lyophilized peptide. Preferably, the ligation reaction is performed in a heating block at 37°C and is periodically vortexed to equilibrate the thiol additives. The reaction may be monitored for degree of completion by MALDI-MS or HPLC and electrospray ionization MS.

After a native chemical ligation reaction is completed or stopped, the N-terminal thiazolidine ring of the product is opened by treatment with a cysteine deprotecting agent, such as O-methylhydroxylamine (0.5 M) at pH 3.5-4.5 for 2 hours at 37° C, after which a 10-fold excess of Tris-(2-carboxyethyl)-phosphine is added to the reaction mixture to completely reduce any oxidizing reaction constituents prior to purification of the product by conventional preparative HPLC. Preferably, fractions containing the ligation product are identified by electrospray MS, are pooled, and lyophilized.

After the synthesis is completed and the final product purified, the final polypeptide product may be refolded by conventional techniques, e.g. Creighton, Meth. Enzymol., 107: 305-329 (1984); White, Meth. Enzymol., 11: 481-484 (1967); Wetlaufer, Meth. Enzymol., 107: 301-304 (1984); and the like. Preferably, a final product is refolded by air oxidation by the following, or like: The reduced lyophilized product is dissolved (at about 0.1 mg/mL) in 1 M guanidine hydrochloride (or like chaotropic agent) with 100 mM Tris, 10 mM methionine, at pH 8.6. After gentle overnight stirring, the re-folded product is isolated by reverse phase HPLC with conventional protocols.

Recombinant Expression Vectors and Host Cells

The polynucleotide sequences described herein can be used in recombinant DNA molecules that direct the expression of the corresponding polypeptides in appropriate host cells. Because of the degeneracy in the genetic code, other DNA sequences may encode the equivalent amino acid sequence, and may be used to clone and express the NDTPs. Codons preferred by a particular host cell may be selected and substituted into the naturally occurring nucleotide sequences, to increase the rate and/or efficiency of expression. The nucleic acid (e.g., cDNA or genomic DNA) encoding the desired NDTP may be inserted into a replicable vector for cloning (amplification of the DNA), or for expression. The polypeptide can be expressed recombinantly in any of a number of expression systems according to methods known in the art (Ausubel, et al., editors, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1990). Appropriate host cells include yeast, bacteria, archebacteria, fungi, and insect and animal cells, including mammalian cells, for example primary cells, including stem cells, including, but not limited to bone marrow stem cells. More specifically, these include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors, and yeast transformed with yeast expression vectors. Also included, are insect cells infected with a recombinant insect virus (such as baculovirus), and mammalian expression systems. The nucleic acid sequence to be expressed may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

The NDTPs of the present invention are produced by culturing a host cell transformed with an expression vector containing a nucleic acid encoding an NDTP, under the appropriate conditions to induce or cause expression of the protein. The conditions appropriate for NDTP expression will vary with the choice of the expression vector and the host cell, as ascertained by one skilled in the art. For example, the use of constitutive promoters in the expression vector may require routine optimization of host cell growth and proliferation, while the use of an inducible promoter requires the appropriate growth conditions for induction. In addition, in some embodiments, the timing of the harvest is important. For example, the baculoviral systems used in insect cell expression are lytic viruses, and thus harvest time selection can be crucial for product yield.

A host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the protein include, but are not limited to, glycosyl, acetyl, phosphate, amide, lipid, carboxyl, acyl, or carbohydrate groups. Post-translational processing, which cleaves a "prepro" form of the protein, may also be important for correct insertion, folding and/or function. By way of example, host cells such as CHO, HeLa, BHK, MDCK, 293, W138, etc. have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the introduced, foreign protein. Of particular interest are *Drosophila melanogaster* cells, *Saccharomyces cerevisiae* and other yeasts, *E. coli, Bacillus subtilis,* SF9 cells, Cl29 cells, 293 cells, Neurospora, BHK, CHO, COS, and HeLa cells, fibroblasts, Schwanoma cell lines, immortalized mammalian myeloid and lymphoid cell lines, Jurkat cells, human cells and other primary cells.

The nucleic acid encoding an NDTP must be "operably linked" by placing it into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" DNA sequences are contiguous, and, in the case of a secretory leader or other polypeptide sequence, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. Promoter sequences encode either constitutive or inducible promoters. The promoters may be either naturally occurring promoters or hybrid promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention. The expression vector may comprise additional elements, for example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a procaryotic host for cloning and amplification. Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2: plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for

cloning vectors in mammalian cells. Further, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably, two homologous sequences which flank the expression construct. The integrating vector may be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art. In an additional embodiment, a heterologous expression control element may be operably linked with the endogenous gene in the host cell by homologous recombination (described in US Patents 6410266 and 6361972, disclosures of which are hereby incorporated by reference in their entireties). This technique allows one to regulate expression to a desired level with a chosen control element while ensuring proper processing and modification of NDTP endogenously expressed by the host cell. Useful heterologous expression control elements include but are not limited to CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous Sarcoma Virus (RSV), and metallothionein promoters.

Preferably, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used. Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available for from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

Host cells transformed with a nucleotide sequence encoding an NDTP may be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The protein produced by a recombinant cell may be secreted, membrane-bound, or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides encoding the NDTP can be designed with signal sequences which direct secretion of the NDTP through a prokaryotic or eukaryotic cell membrane. The desired NDTP may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the NDTP-encoding DNA that is inserted into the vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp,

or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* a-factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published Apr. 4, 1990), or the signal described in WO 90113646 published Nov. 15, 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders. According to the expression system selected, the coding sequence is inserted into an appropriate vector, which in turn may require the presence of certain characteristic "control elements" or "regulatory sequences." Appropriate constructs are known generally in the art (Ausubel, et al., 1990) and, in many cases, are available from commercial suppliers such as Invitrogen (San Diego, Calif.), Stratagene (La Jolla, Calif.), Gibco BRL (Rockville, Md.) or Clontech (Palo Alto, Calif.).

Expression in Bacterial Systems

Transformation of bacterial cells may be achieved using an inducible promoter such as the hybrid lacZ promoter of the "BLUESCRIPT" Phagemid (Stratagene) or "pSPORT1" (Gibco BRL). In addition, a number of expression vectors may be selected for use in bacterial cells to produce cleavable fusion proteins that can be easily detected and/or purified, including, but not limited to "BLUESCRIPT" (a-galactosidase; Stratagene) or pGEX (glutathione S-transferase; Promega, Madison, Wis.). A suitable bacterial promoter is any nucleic acid sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of the coding sequence of the NDTP gene into mRNA. A bacterial promoter has a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region typically includes an RNA polymerase binding site and a transcription initiation site. Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose and maltose, and sequences derived from biosynthetic enzymes such as tryptophan. Promoters from bacteriophage may also be used and are known in the art. In addition, synthetic promoters and hybrid promoters are also useful; for example, the tat promoter is a hybrid of the trp and lac promoter sequences. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. An efficient ribosome-binding site is also desirable. The expression vector may also include a signal peptide sequence that provides for secretion

of the NDTP in bacteria. The signal sequence typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell, as is well known in the art. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). The bacterial expression vector may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed. Suitable selection genes include drug resistance genes such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways. When large quantities of NDTPs are needed, e.g., for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be desirable. Such vectors include, but are not limited to, multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene), in which the NDTP coding sequence may be ligated into the vector in-frame with sequences for the amino-terminal Met and the subsequent 7 residues of beta-galactosidase so that a hybrid protein is produced; PIN vectors (Van Heeke & Schuster *JBiol Chem 264:5503-5509* 1989)); PET vectors (Novagen, Madison Wis.); and the like. Expression vectors for bacteria include the various components set forth above, and are well known in the art. Examples include vectors for *Bacillus subtilis, E. coli, Streptococcus cremoris,* and *Streptococcus lividans,* among others. Bacterial expression vectors are transformed into bacterial host cells using techniques well known in the art, such as calcium chloride mediated transfection, electroporation, and others.

Expression in Yeast

Yeast expression systems are well known in the art, and include expression vectors for *Saccharomyces cerevisiae, Candida albicans* and *C. maltosa, Hansenula polymorpha, Kluyveromyces fragilis* and *K. lactis, Pichia guillermondii* and *P pastoris, Schizosaccharomyces pombe,* and *Yarrowia lipolytica.* Examples of suitable promoters for use in yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073 (1980)) or other glycolytic enzymes (Hess et al., J. *Adv. Enzyme Reg.* 7:149 (1968); Holland, *Biochemistry* 17:4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose- 6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, alpha factor, the ADH2IGAPDH promoter, glucokinase alcohol oxidase, and PGH. See, for example, Ausubel, et al., 1990; Grant et al., *Methods in Enzymology* 153:516-544, (1987). Other yeast promoters, which are

inducible have the additional advantage of transcription controlled by growth conditions, include the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast selectable markers include ADE2. HIS4. LEU2. TRP1. and ALG7, which confers resistance to tunicamycin; the neomycin phosphotransferase gene, which confers resistance to G418; and the CUP1 gene, which allows yeast to grow in the presence of copper ions. Yeast expression vectors can be constructed for intracellular production or secretion of an NDTP from the DNA encoding the NDTP of interest. For example, a selected signal peptide and the appropriate constitutive or inducible promoter may be inserted into suitable restriction sites in the selected plasmid for direct intracellular expression of the NDTP. For secretion of the NDTP, DNA encoding the NDTP can be cloned into the selected plasmid, together with DNA encoding the promoter, the yeast alpha-factor secretory signal/leader sequence, and linker sequences (as needed), for expression of the NDTP. Yeast cells, can then be transformed with the expression plasmids described above, and cultured in an appropriate fermentation media. The protein produced by such transformed yeast can then be concentrated by precipitation with 10% trichloroacetic acid and analyzed following separation by SDS-PAGE and staining of the gels with Coomassie Blue stain. The recombinant NDTP can subsequently be isolated and purified from the fermentation medium by techniques known to those of skill in the art.

Expression in Mammalian Systems

The NDTP may be expressed in mammalian cells. Mammalian expression systems are known in the art, and include retroviral vector mediated expression systems. Mammalian host cells may be transformed with any of a number of different viral-based expression systems, such as adenovirus, where the coding region can be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a nonessential El or E3 region of the viral genome results in a viable virus capable of expression of the polypeptide of interest in infected host cells. A preferred expression vector system is a retroviral vector system such as is generally described in PCT/US97/01019 and PCT/US97/101048. Suitable mammalian expression vectors contain a mammalian promoter which is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence for NDTP into mRNA. A promoter will have a transcription initiating region, which is usually placed

proximal to the 5' end of the coding sequence, and a TATA box, using a located 25-30 base pairs upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element (enhancer element), typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as mammalian promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211, 504 published Jul. 5,1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems. Transcription of DNA encoding an NDTP by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, a-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer is preferably located at a site 5' from the promoter. In general, the transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenylation signals include those derived from SV40. Long term, high-yield production of recombinant proteins can be effected in a stable expression system. Expression vectors which contain viral origins of replication or endogenous expression elements and a selectable marker gene may be used for this purpose. Appropriate vectors containing selectable markers for use in mammalian cells are readily available commercially and are known to persons skilled in the art. Examples of such selectable markers include, but are not limited to herpes simplex virus thymidine kinase and adenine phosphoribosyltransferase for use in tk- or hprt-cells, respectively. The methods of introducing exogenous nucleic acid into mammalian hosts, as well as other hosts, is well known in the art, and will vary with the host cell used. Techniques include dextran-mediated transfection, calcium phosphate precipitation,

polybrene mediated transfection, protoplast fusion, electroporation, viral infection, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

NDTPs can be purified from culture supernatants of mammalian cells transiently transfected or stably transformed by an expression vector carrying an NDTP-encoding sequence. Preferably, NDTP is purified from culture supernatants of COS 7 cells transiently transfected by the pcD expression vector. Transfection of COS 7 cells with pcD proceeds as follows: One day prior to transfection, approximately 10^6 COS 7 monkey cells are seeded onto individual 100 mm plates in Dulbecco's modified Eagle medium (DME) containing 10% fetal calf serum and 2 mM glutamine. To perform the transfection, the medium is aspirated from each plate and replaced with 4 ml of DME containing 50 mM Tris.HCl pH 7.4, 400 mg/ml DEAE-Dextran and 50 μg of plasmid DNA. The plates are incubated for four hours at 37°C, then the DNA-containing medium is removed, and the plates are washed twice with 5 ml of serum-free DME. DME is added back to the plates which are then incubated for an additional 3 hrs at 37°C. The plates are washed once with DME, after which DME containing 4% fetal calf serum, 2 mM glutamine, penicillin (100 U/L) and streptomycin (100 μg/L) at standard concentrations is added. The cells are then incubated for 72 hrs at 37°C, after which the growth medium is collected for purification of NDTP. Plasmid DNA for the transfections is obtained by growing pcD(SRα), or like expression vector, containing the NDTP-encoding cDNA insert in E. coli MC1061 (described by Casadaban and Cohen, J. Mol. Biol., Vol. 138, pgs. 179-207 (1980)), or like organism. The plasmid DNA is isolated from the cultures by standard techniques, e.g. Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, New York, 1989) or Ausubel et al (1990, cited above).

Expression in Insect Cells

NDTPs may also be produced in insect cells. Expression vectors for the transformation of insect cells, and in particular, baculovirus-based expression vectors, are well known in the art. In one such system, the NDTP-encoding DNA is fused upstream of an epitope tag contained within a baculovirus expression vector. *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* Sf9 cells or in Trichoplusia larvae. The NDTP-encoding sequence is cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of an NDTP-encoding sequence will render the

polyhedrin gene inactive and produce recombinant virus lacking coat protein coat. The recombinant viruses are then used to infect *S. frugiperda* cells or Trichoplusia larvae in which the NDTP is expressed (Smith et al., J. Wol. 46:584 (1994); Engelhard E K et al., *Proc. Nat. Acad. Sci.* 91:3224-3227 (1994)). Suitable epitope tags for fusion to the NDTP-encoding DNA include poly-his tags and immunoglobulin tags (like Fc regions of IgG). A variety of plasmids may be employed, including commercially available plasmids such as pVL1393 (Novagen). Briefly, the NDTP-encoding DNA or the desired portion of the NDTP-encoding DNA is amplified by PCR with primers complementary to the 5' and 3' regions. The 5' primer may incorporate flanking restriction sites. The PCR product is then digested with the selected restriction enzymes and subcloned into an expression vector. Recombinant baculovirus is generated by co-transfecting the above plasmid and BaculoGoldTM virus DNA (Pharmingen) into *Spodoptera frugiperda* ("Sf9") cells (ATCC CRL 1711) using lipofectin (commercially available from GIBCO-BRL), or other methods known to those of skill in the art. Virus is produced by day 4-5 of culture in Sf9 cells at 28°C, and used for further amplifications. Procedures are performed as further described in O'Reilley et al., *BACULOVIRUS EXPRESSION VECTORS: A LABORATORY MANUAL,* Oxford University Press (1994). Extracts may be prepared from recombinant virus-infected Sf9 cells as described in Rupert et al., *Nature* 362:175-179 (1993). Alternatively, expressed epitope-tagged NDTP can be purified by affinity chromatography, or for example, purification of an IgG tagged (or Fc tagged) NDTP can be performed using chromatography techniques, including Protein A or protein G column chromatography.

Evaluation of Gene Expression

Gene expression may be evaluated in a sample directly, for example, by standard techniques known to those of skill in the art, e.g., Northern blotting to determine the transcription of mRNA, dot blotting (DNA or RNA), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be used in assays for detection of polypeptides, nucleic acids, such as specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. Such antibodies may be labeled and the assay carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. Gene expression, alternatively, may be measured by immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to directly evaluate the expression of an NDTP polypeptide or polynucleotide. Antibodies useful for such immunological assays may be either monoclonal or polyclonal,

and may be prepared against a native sequence NDTP. Protein levels may also be detected by mass spectrometry. A further method of protein detection is with protein chips.

Purification of Expressed Protein

Expressed NDTP may be purified or isolated after expression, using any of a variety of methods known to those skilled in the art. The appropriate technique will vary depending upon what other components are present in the sample. Contaminant components that are removed by isolation or purification are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other solutes. The purification step(s) selected will depend, for example, on the nature of the production process used and the particular NDTP produced. As NDTPs are secreted, they may be recovered from culture medium. Alternatively, the NDTP may be recovered from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or by enzymatic cleavage. Alternatively, cells employed in expression of NDTP can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or by use of cell lysing agents. Exemplary purification methods include, but are not limited to, ion-exchange column chromatography; chromatography using silica gel or a cation-exchange resin such as DEAE; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; chromatography using metal chelating columns to bind epitope-tagged forms of the NDTP; ethanol precipitation; reverse phase HPLC; chromatofocusing; SDS-PAGE; and ammonium sulfate precipitation. Ordinarily, an isolated NDTP will be prepared by at least one purification step. For example, the NDTP may be purified using a standard anti-NDTP antibody column. Ultrafiltration and dialysis techniques, in conjunction with protein concentration, are also useful (see, for example, Scopes, R., *PROTEIN PURIFICATION,* Springer-Verlag, New York, N.Y., 1982). The degree of purification necessary will vary depending on the use of the NDTP. In some instances no purification will be necessary. Once expressed and purified as needed, the NDTPs and nucleic acids of the present invention are useful in a number of applications, as detailed herein.

Transgenic animals

The host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which NDTP-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous NDTP

sequences have been introduced into their genome or homologous recombinant animals in which endogenous NDTP sequences have been altered. Such animals are useful for studying the function and/or activity of an NDTP or fragment thereof and for identifying and/or evaluating modulators of NDTP biological activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal include a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing an NDTP-encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection or retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The NDTP cDNA sequence or a fragment thereof can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homologue of a human NDTP-encoding gene, such as from mouse or rat, can be used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to an NDTP transgene to direct expression of an NDTP to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al. and in Hogan, B., Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986, the disclosure of which is incorporated herein by reference in its entirety). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of an NDTP transgene in its genome and/or expression of NDTP mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding an NDTP can further be bred to other transgenic animals carrying other transgenes.

To create an animal in which a desired nucleic acid has been introduced into the genome via homologous recombination, a vector is prepared which contains at least a portion of an NDTP-encoding sequence into which a deletion, addition or substitution has been introduced to thereby alter, e.g., functionally disrupt, the NDTP-encoding sequence. The NDTP-encoding sequence can be a human gene, but more preferably, is a non-human homologue of a human NDTP-encoding sequence (e.g., a cDNA isolated by stringent hybridization with a nucleotide sequence coding for an NDTP). For example, a mouse NDTP-encoding sequence can be used to construct a homologous recombination vector suitable for altering an endogenous gene in the mouse genome. In a preferred embodiment, the vector is designed such that, upon homologous recombination, the endogenous NDTP-encoding sequence is functionally disrupted (i.e., no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous NDTP-encoding sequence is mutated or otherwise altered but still encodes functional protein (e.g., the upstream regulatory region can be altered to thereby alter the expression of the endogenous NDTP-encoding sequence). In the homologous recombination vector, the altered portion of the NDTP-encoding sequence is flanked at its 5' and 3' ends by additional nucleic acid sequence of the NDTP gene to allow for homologous recombination to occur between the exogenous sequence carried by the vector and an endogenous gene in an embryonic stem cell. The additional flanking nucleic acid sequence is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas, K. R. and Capecchi, M. R. (1987) Cell 51:503, the disclosure of which is incorporated herein by reference in its entirety, for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced NDTP-encoding sequence has homologously recombined with the endogenous gene are selected (see e.g., Li, E. et al. (1992) Cell 69:915, the disclosure of which is incorporated herein by reference in its entirety). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, A. in Teratocarcinomas and Embryonic Stem Cells. A Practical Approach, E. J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152, the disclosure of which is incorporated herein by reference in its entirety). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous

recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) Current Opinion in Biotechnology 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec et al.; WO 91/01140 by Smithies et al.; WO 92/0968 by Zijlstra et al.; and WO 93/04169 by Berns et al., the disclosures of which are incorporated herein by reference in their entireties.

In another embodiment, transgenic non-human animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the cre/loxP recombinase system of bacteriophage P1. For a description of the cre/loxP recombinase system, see, e.g., Lakso et al. (1992) PNAS 89:6232-6236, the disclosure of which is incorporated herein by reference in its entirety. Another example of a recombinase system is the FLP recombinase system of Saccharomyces cerevisiae (O'Gorman et al. (1991) Science 251:1351-1355, the disclosure of which is incorporated herein by reference in its entirety). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Assessing NDTP activity

It will be appreciated that the invention further provides methods of testing the activity of or obtaining functional fragments and variants of NDTPs and NDTP sequences. Such methods involve providing a variant or modified NDTP-encoding nucleic acid and assessing whether the encoded polypeptide displays an NDTP biological activity. Encompassed is thus a method of assessing the function of an NDTP comprising: (a) providing an NDTP, or a biologically active fragment or homologue thereof; and (b) testing said NDTP, or a biologically active fragment or homologue thereof for an NDTP biological activity under conditions suitable for NDTP activity. Cell free, cell-based and *in vivo* assays may be used to test NDTP activity. For example, said assay may comprise expressing an NDTP nucleic acid in a host cell, and observing NDTP activity in said cell and other affected cells. In another example, an NDTP, or a biologically active fragment or homologue thereof is contacted with a cell, and an NDTP biological activity is observed.

NDTP biological activities include, but are not limited to: promoting neuronal survival (see Example 2 hereinbelow), and accelerating nerve regeneration in animals after a sciatic nerve crush (see Example 3 hereinbelow).

NDTP biological activity can be assayed by any suitable method known in the art, and in particular by the methods described in details in the Examples herein.

Anti-NDTP Antibodies

The present invention provides antibodies and binding compositions specific for NDTPs. Such antibodies and binding compositions include polyclonal antibodies, monoclonal antibodies, Fab and single chain Fv fragments thereof, bispecific antibodies, heteroconjugates, and humanized antibodies. Such antibodies and binding compositions may be produced in a variety of ways, including hybridoma cultures, recombinant expression in bacteria or mammalian cell cultures, and recombinant expression in transgenic animals. There is abundant guidance in the literature for selecting a particular production methodology, e.g. Chadd and Chamow, Curr. Opin. Biotechnol., 12: 188-194 (2001).

The choice of manufacturing methodology depends on several factors including the antibody structure desired, the importance of carbohydrate moieties on the antibodies, ease of culturing and purification, and cost. Many different antibody structures may be generated using standard expression technology, including full-length antibodies, antibody fragments, such as Fab and Fv fragments, as well as chimeric antibodies comprising components from different species. Antibody fragments of small size, such as Fab and Fv fragments, having no effector functions and limited pharmokinetic activity may be generated in a bacterial expression system. Single chain Fv fragments are highly selective for in vivo tumors, show good tumor penetration and low immunogenicity, and are cleared rapidly from the blood, e.g. Freyre et al, J. Biotechnol., 76: 157-163 (2000). Thus, such molecules are desirable for radioimmunodetection and in situ radiotherapy. Whenever pharmacokinetic activity in the form of increased half-life is required for therapeutic purposes, then full-length antibodies are preferable. For example, the immunoglobulin G (IgG) molecule may be one of four subclasses: γ1, γ2, γ3, or γ4. If a full-length antibody with effector function is required, then IgG subclasses γ1 or γ3 are preferred, and IgG subclass γ1 is most preferred. The γ1 and γ3 subclasses exhibit potent effector function, complement activation, and promote antibody-dependent cell-mediated cytotoxicity through interaction with specific Fc receptors, e.g. Raju et al, Glycobiology, 10: 477-486 (2000); Lund et al, J. Immunol., 147: 2657-2662 (1991).

Polyclonal Antibodies

The anti-NDTP antibodies of the present invention may be polyclonal antibodies. Such polyclonal antibodies can be produced in a mammal, for example, following one or more injections of an immunizing agent, and preferably, an adjuvant. Typically, the

immunizing agent and/or adjuvant will be injected into the mammal by a series of subcutaneous or intraperitoneal injections. The immunizing agent may include NDTPs or a fusion protein thereof. It may be useful to conjugate the antigen to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include, but are not limited to, keyhole limpet hemocyanin (KLH), methylated bovine serum albumin (mBSA), bovine serum albumin (BSA), Hepatitis B surface antigen, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Adjuvants include, for example, Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicoryno-mycolate). The immunization protocol may be determined by one skilled in the art based on standard protocols or by routine experimentation.

Alternatively, a crude protein preparation which has been enriched for an NDTP or a portion thereof can be used to generate antibodies. Such proteins, fragments or preparations are introduced into the non-human mammal in the presence of an appropriate adjuvant. If the serum contains polyclonal antibodies to undesired epitopes, the polyclonal antibodies are purified by immunoaffinity chromatography.

Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. Also, host animals vary in response to site of inoculations and dose, with both inadequate and excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appear to be most reliable. Techniques for producing and processing polyclonal antisera are known in the art, see for example, Mayer and Walker (1987), the disclosure of which is incorporated herein by reference in its entirety. An effective immunization protocol for rabbits can be found in Vaitukaitis, J. et al. J. Clin. Endocrinol. Metab. 33:988-991(1971), the disclosure of which is incorporated by reference in its entirety. Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony, O. et al., Chap. 19 in: Handbook of Experimental Immunology D. Wier (ed) Blackwell (1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum. Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher, D., Chap. 42 in: Manual of Clinical Immunology, 2d Ed. (Rose and Friedman, Eds.) Amer. Soc. For Microbiol., Washington, D. C. (1980).

Monoclonal Antibodies

Alternatively, the anti-NDTP antibodies may be monoclonal antibodies. Monoclonal antibodies may be produced by hybridomas, wherein a mouse, hamster, or other appropriate host animal, is immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent, e.g. Kohler and Milstein, Nature 256:495 (1975). The immunizing agent will typically include the NDTP or a fusion protein thereof and optionally a carrier. Alternatively, the lymphocytes may be immunized in vitro. Generally, spleen cells or lymph node cells are used if non-human mammalian sources are desired, or peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired. The lymphocytes are fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to produce a hybridoma cell, e.g. Goding, *MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE,* Academic Press, pp. 59-103 (1986); Liddell and Cryer, A Practical Guide to Monoclonal Antibodies (John Wiley & Sons, New York, 1991); Malik and Lillenoj, Editors, Antibody Techniques (Academic Press, New York, 1994). In general, immortalized cell lines are transformed mammalian cells, for example, myeloma cells of rat, mouse, bovine or human origin. The hybridoma cells are cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT), substances which prevent the growth of HGPRT-deficient cells. Preferred immortalized cell lines are those that fuse efficiently, support stable high level production of antibody, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine or human myeloma lines, which can be obtained, for example, from the American Type Culture Collection (ATCC), Rockville, MD. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies, e.g. Kozbor, J. Immunol. 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, pp. 51-63 (1987).

The culture medium (supernatant) in which the hybridoma cells are cultured can be assayed for the presence of monoclonal antibodies directed against an NDTP. Preferably, the binding specificity of monoclonal antibodies present in the hybridoma supernatant is determined by immunoprecipitation or by an in vitro binding assay, such as radio-immunoassay (RIA) or Enzyme-Linked Immuno Sorbent Assay (ELISA). Appropriate techniques and assays are known in the art. The binding affinity of the monoclonal antibody

can, for example, be determined by the Scatchard analysis of Munson and Pollard, *Anal. Biochem.* 107:220 (1980). After the desired antibody-producing hybridoma cells are identified, the cells may be cloned by limiting dilution procedures and grown by standard methods (Goding, 1986, *supra*). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown in vivo as ascites in a mammal. The monoclonal antibodies secreted by selected clones may be isolated or purified from the culture medium or ascites fluid by immunoglobulin purification procedures routinely used by those of skill in the art such as, for example, protein A-Sepharose, hydroxyl-apatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be isolated from the NDTP-specific hybridoma cells and sequenced, e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies. Once isolated, the DNA may be inserted into an expression vector, which is then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for the murine heavy and light chain constant domains for the homologous human sequences (Morrison et al., *Proc. Nat. Acad. Sci.* 81:6851-6855 (1984); Neuberger et al., *Nature* 312:604-608 (1984); Takeda et al., *Nature* 314:452-454 (1985)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. The non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody. The antibodies may also be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, in vitro methods are suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

Antibodies and antibody fragments characteristic of hybridomas of the invention can also be produced by recombinant means by extracting messenger RNA, constructing a cDNA library, and selecting clones which encode segments of the antibody molecule. The following are exemplary references disclosing recombinant techniques for producing antibodies: Wall et al., Nucleic Acids Research, Vol. 5, pgs. 3113-3128 (1978); Zakut et al.,

Nucleic Acids Research, Vol. 8, pgs. 3591-3601 (1980); Cabilly et al., Proc. Natl. Acad. Sci., Vol. 81, pgs. 3273-3277 (1984); Boss et al., Nucleic Acids Research, Vol. 12, pgs. 3791-3806 (1984); Amster et al., Nucleic Acids Research, Vol. 8, pgs. 2055-2065 (1980); Moore et al., U.S. Patent 4,642,334; Skerra et al, Science, Vol. 240, pgs. 1038-1041(1988); Huse et al, Science, Vol. 246, pgs. 1275-1281 (1989); and U.S. patents 6,054,297; 5,530,101; 4,816,567; 5,750,105; and 5,648,237; which patents are incorporated by reference. In particular, such techniques can be used to produce interspecific monoclonal antibodies, wherein the binding region of one species is combined with non-binding region of the antibody of another species to reduce immunogenicity, e.g. Liu et al., Proc. Natl. Acad. Sci., Vol. 84, pgs. 3439-3443 (1987), and patents 6,054,297 and 5,530,101. Preferably, recombinantly produced Fab and Fv fragments are expressed in bacterial host systems. Preferably, full-length antibodies are produced by mammalian cell culture techniques. More preferably, full-length antibodies are expressed in Chinese Hamster Ovary (CHO) cells or NSO cells.

Both polyclonal and monoclonal antibodies can be screened by ELISA. As in other solid phase immunoassays, the test is based on the tendency of macromolecules to adsorb nonspecifically to plastic. The irreversibility of this reaction, without loss of immunological activity, allows the formation of antigen-antibody complexes with a simple separation of such complexes from unbound material. To titrate anti-peptide serum, peptide conjugated to a carrier different from that used in immunization is adsorbed to the wells of a 96-well microtiter plate. The adsorbed antigen is then allowed to react in the wells with dilutions of anti-peptide serum. Unbound antibody is washed away, and the remaining antigen-antibody complexes are allowed to react with an antibody specific for the IgG of the immunized animal. This second antibody is conjugated to an enzyme such as alkaline phosphatase. A visible colored reaction produced when the enzyme substrate is added indicates which wells have bound antipeptide antibodies. The use of spectrophotometer readings allows better quantification of the amount of peptide-specific antibody bound. High-titer antisera yield a linear titration curve between 10^{-3} and 10^{-5} dilutions.

NDTP peptide carriers

The invention includes immunogens derived from NDTPs, and immunogens comprising conjugates between carriers and peptides of the invention. The term immunogen as used herein refers to a substance which is capable of causing an immune response. The term carrier as used herein refers to any substance which when chemically conjugated to a peptide of the invention permits a host organism immunized with the resulting conjugate to generate antibodies specific for the conjugated peptide. Carriers include red blood cells,

bacteriophages, proteins, or synthetic particles such as agarose beads. Preferably, carriers are proteins, such as serum albumin, gamma-globulin, keyhole limpet hemocyanin (KLH), thyroglobulin, ovalbumin, or fibrinogen.

The general technique of linking synthetic peptides to a carrier is described in several references, e.g. Walter and Doolittle, "Antibodies Against Synthetic Peptides," in Setlow et al., eds., Genetic Engineering, Vol. 5, pgs. 61-91 (Plenum Press, N.Y., 1983); Green et al. Cell, Vol. 28, pgs. 477-487 (1982); Lerner et al., Proc. Natl. Acad. Sci., Vol. 78, pgs. 3403-3407 (1981); Shimizu et al., U.S. Patent 4,474,754; and Ganfield et al., U.S. Patent 4,311,639. Accordingly, these references are incorporated by reference. Also, techniques employed to link haptens to carriers are essentially the same as the above-referenced techniques, e.g. chapter 20 in Tijssen, Practice and Theory of Enzyme Immunoassays (Elsevier, New York, 1985). The four most commonly used schemes for attaching a peptide to a carrier are (1) glutaraldehyde for amino coupling, e.g. as disclosed by Kagan and Glick, in Jaffe and Behrman, eds. Methods of Hormone Radioimmunoassay, pgs. 328-329 (Academic Press, N.Y., 1979), and Walter et al. Proc. Natl. Acad. Sci., Vol. 77, pgs. 5197-5200 (1980); (2) water-soluble carbodiimides for carboxyl to amino coupling, e.g. as disclosed by Hoare et al., J. Biol. Chem., Vol. 242, pgs. 2447-2453 (1967); (3) bis-diazobenzidine (BDB) for tyrosine to tyrosine sidechain coupling, e.g. as disclosed by Bassiri et al., pgs. 46-47, in Jaffe and Behrman, eds. (cited above), and Walter et al. (cited above); and (4) maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) for coupling cysteine (or other sulfhydryls) to amino groups, e.g. as disclosed by Kitagawa et al., J. Biochem. (Tokyo), Vol. 79, pgs. 233-239 (1976), and Lerner et al. (cited above). A general rule for selecting an appropriate method for coupling a given peptide to a protein carrier can be stated as follows: the group involved in attachment should occur only once in the sequence, preferably at the appropriate end of the segment. For example, BDB should not be used if a tyrosine residue occurs in the main part of a sequence chosen for its potentially antigenic character. Similarly, centrally located lysines rule out the glutaraldehyde method, and the occurrences of aspartic and glutamic acids frequently exclude the carbodiimide approach. On the other hand, suitable residues can be positioned at either end of chosen sequence segment as attachment sites, whether or not they occur in the "native" protein sequence. Internal segments, unlike the amino and carboxy termini, will differ significantly at the "unattached end" from the same sequence as it is found in the native protein where the polypeptide backbone is continuous. The problem can be remedied, to a degree, by acetylating the α-amino group and then attaching the peptide by way of its carboxy terminus. The coupling efficiency to the carrier protein is conveniently measured by using a radioactively labeled peptide, prepared either by using a radioactive

amino acid for one step of the synthesis or by labeling the completed peptide by the iodination of a tyrosine residue. The presence of tyrosine in the peptide also allows one to set up a sensitive radioimmune assay, if desirable. Therefore, tyrosine can be introduced as a terminal residue if it is not part of the peptide sequence defined by the native polypeptide.

Preferred carriers are proteins, and preferred protein carriers include bovine serum albumin, myoglobulin, ovalbumin (OVA), keyhole limpet hemocyanin (KLH), or the like. Peptides can be linked to KLH through cysteines by MBS as disclosed by Liu et al., Biochemistry, Vol. 18, pgs. 690-697 (1979). The peptides are dissolved in phosphate-buffered saline (pH 7.5), 0.1 M sodium borate buffer (pH 9.0) or 1.0 M sodium acetate buffer (pH 4.0). The pH for the dissolution of the peptide is chosen to optimize peptide solubility. The content of free cysteine for soluble peptides is determined by Ellman's method, Ellman, Arch. Biochem. Biophys., Vol. 82, pg. 7077 (1959). For each peptide, 4 mg KLH in 0.25 ml of 10 mM sodium phosphate buffer (pH 7.2) is reacted with 0.7 mg MBS (dissolved in dimethyl formamide) and stirred for 30 min at room temperature. The MBS is added dropwise to ensure that the local concentration of formamide is not too high, as KLH is insoluble in >30% formamide. The reaction product, KLH-MBS, is then passed through Sephadex G-25 equilibrated with 50 mM sodium phosphate buffer (pH 6.0) to remove free MBS, KLH recovery from peak fractions of the column eluate (monitored by OD280) is estimated to be approximately 80%. KLH-MBS is then reacted with 5 mg peptide dissolved in 1 ml of the chosen buffer. The pH is adjusted to 7-7.5 and the reaction is stirred for 3 hr at room temperature. Coupling efficiency is monitored with radioactive peptide by dialysis of a sample of the conjugate against phosphate-buffered saline, and may range from 8% to 60%. Once the peptide-carrier conjugate is available, polyclonal or monoclonal antibodies are produced by standard techniques, e.g. as disclosed by Campbell, Monoclonal Antibody Technology (Elsevier, New York, 1984); Hurrell, ed. Monoclonal Hybridoma Antibodies: Techniques and Applications (CRC Press, Boca Raton, FL, 1982); Schreier et al. Hybridoma Techniques (Cold Spring Harbor Laboratory, New York, 1980); U.S. Patent 4,562,003; or the like. In particular, U.S. Patent 4,562,003 is incorporated by reference.

Uses of NDTP antibodies

NDTP antibodies are preferably specific for the NDTPs of the invention and as such, do not bind peptides derived from other proteins with high affinity. As used herein, the term "heavy chain variable region" means a polypeptide (1) which is from 110 to 125 amino acids in length, and (2) whose amino acid sequence corresponds to that of a heavy chain of an antibody of the invention, starting from the heavy chain's N-terminal amino acid. Likewise,

the term "light chain variable region" means a polypeptide (1) which is from 95 to 115 amino acids in length, and (2) whose amino acid sequence corresponds to that of a light chain of an antibody of the invention, starting from the light chain's N-terminal amino acid. As used herein the term "monoclonal antibody" refers to homogeneous populations of immunoglobulins which are capable of specifically binding to NDTPs.

NDTP antibodies may be used as functional modulators, most commonly as antagonists. Preferably, antibody modulators of the invention are derived from monoclonal antibodies specific for NDTPs. Monoclonal antibodies capable of blocking, or neutralizing, NDTPs are generally selected by their ability to inhibit an NDTP biological activity.

The use of antibody fragments is also well known, e.g. Fab fragments: Tijssen, Practice and Theory of Enzyme Immunoassays (Elsevier, Amsterdam, 1985); and Fv fragments: Hochman et al. Biochemistry, Vol. 12, pgs. 1130-1135 (1973), Sharon et al., Biochemistry, Vol. 15, pgs. 1591-1594 (1976) and Ehrlich et al., U.S. Patent 4,355,023; and antibody half molecules: Auditore- Hargreaves, U.S. Patent 4,470,925.

Preferably, monoclonal antibodies, Fv fragments, Fab fragments, or other binding compositions derived from monoclonal antibodies of the invention have a high affinity to NDTPs. The affinity of monoclonal antibodies and related molecules to NDTPs may be measured by conventional techniques including plasmon resonance, ELISA, or equilibrium dialysis. Affinity measurement by plasmon resonance techniques may be carried out, for example, using a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden) in accordance with the manufacturer's recommended protocol. Preferably, affinity is measured by ELISA, as described in U.S. patent 6,235,883, for example. Preferably, the dissociation constant between NDTPs and monoclonal antibodies of the invention is less than 10^{-5} molar. More preferably, such dissociation constant is less than 10^{-8} molar; still more preferably, such dissociation constant is less than 10^{-9} molar; and most preferably, such dissociation constant is in the range of 10^{-9} to 10^{-11} molar.

In addition, the antibodies of the present invention are useful for detecting NDTPs. The antibodies of the invention may be used in most assays involving antigen-antibody reactions. The assays may be homogeneous or heterogeneous. In a homogeneous assay approach, the sample can be a biological sample or fluid such as serum, urine, whole blood, lymphatic fluid, plasma, saliva, cells, tissue, and material secreted by cells or tissues cultured *in vitro*. The sample can be pretreated if necessary to remove unwanted materials. The immunological reaction usually involves the specific antibody, a labeled analyte, and the sample suspected of containing the antigen. The signal arising from the label is modified, directly or indirectly, upon the binding of the antibody to the labeled analyte. Both

immunological reaction and detection of the extent thereof are carried out in a homogeneous solution. Immunochemical labels which may be employed include free radicals, fluorescent dyes, enzymes, bacteriophages, coenzymes, and so forth.

In a heterogeneous assay approach, the reagents are usually the sample, the specific antibody, and means for producing a detectable signal. The specimen is generally placed on a support, such as a plate or a slide, and contacted with the antibody in a liquid phase. The support is then separated from the liquid phase and either the support phase or the liquid phase is examined for a detectable signal employing means for producing such signal or signal producing system. The signal is related to the presence of the antigen in the sample. Means for producing a detectable signal includes the use of radioactive labels, fluorescent compounds, enzymes, and so forth. Exemplary heterogeneous immunoassays are the radioimmunoassay, immunofluorescence methods, enzyme-linked immunoassays, and the like.

For a more detailed discussion of the above immunoassay techniques, see "Enzyme-Immunoassay," by Edward T. Maggio, CRC Press, Inc., Boca Raton, Fla., 1980. See also, for example, U.S. Pat. Nos. 3,690,834; 3,791,932; 3,817,837; 3,850,578; 3,853,987; 3,867,517; 3,901,654; 3,935,074; 3,984,533; 3,966,345; and 4,098,876, which listing is not intended to be exhaustive. Methods for conjugating labels to antibodies and antibody fragments are well known in the art. Such methods may be found in U.S. Pat. Nos. 4,220,450; 4,235,869; 3,935,974; and 3,966,345. Another example of a technique in which the antibodies of the invention may be employed is immunoperoxidase labeling. (Sternberger, Immunocytochemistry (1979) pp. 104-169). Alternatively, the antibodies may be bound to a radioactive material or to a drug to form a radiopharmaceutical or pharmaceutical, respectively. (Carrasquillo, et al., Cancer Treatment Reports (1984) 68:317-328).

One embodiment of an assay employing an antibody of the present invention involves the use of a surface to which the monoclonal antibody of the invention is attached. The underlying structure of the surface may take different forms, have different compositions and may be a mixture of compositions or laminates or combinations thereof. The surface may assume a variety of shapes and forms and may have varied dimensions, depending on the manner of use and measurement. Illustrative surfaces may be pads, beads, discs, or strips which may be flat, concave or convex. Thickness is not critical, generally being from about 0.1 to 2 mm thick and of any convenient diameter or other dimensions. The surface typically will be supported on a rod, tube, capillary, fiber, strip, disc, plate, cuvette and will typically be porous and polyfunctional or capable of being polyfunctionalized so as to permit covalent binding of an antibody and permit bonding of other compounds which form a part of a means

for producing a detectable signal. A wide variety of organic and inorganic polymers, both natural and synthetic, and combinations thereof, may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethracrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and latex. Other surfaces include paper, glasses, ceramics, metals, metaloids, semiconductor materials, cements, silicates or the like. Also included are substrates that form gels, gelatins, lipopolysaccharides, silicates, agarose and polyacrylamides or polymers which form several aqueous phases such as dextrans, polyalkylene glycols (alkylene of 2 to 3 carbon atoms) or surfactants such as phospholipids. The binding of the antibody to the surface may be accomplished by well known techniques, commonly available in the literature (see, for example, "Immobilized Enzymes," Ichiro Chibata, Press, New York (1978) and Cuatrecasas, J. Bio. Chem., 245: 3059 (1970)). In carrying out the assay in accordance with this aspect of the invention, the sample is mixed with aqueous medium and the medium is contacted with the surface having an antibody bound thereto. Labels may be included in the aqueous medium, either concurrently or added subsequently so as to provide a detectable signal associated with the surface. The means for producing the detectable signal can involve the incorporation of a labeled analyte or it may involve the use of a second monoclonal antibody having a label conjugated thereto. Separation and washing steps will be carried out as needed. The signal detected is related to the presence of NDTP in the sample. It is within the scope of the present invention to include a calibration on the same support. A particular embodiment of an assay in accordance with the present invention, by way of illustration and not limitation, involves the use of a support such as a slide or a well of a petri dish. The technique involves fixing the sample to be analyzed on the support with an appropriate fixing material and incubating the sample on the slide with a monoclonal antibody. After washing with an appropriate buffer such as, for example, phosphate buffered saline, the support is contacted with a labeled specific binding partner for the antibody. After incubation as desired, the slide is washed a second time with an aqueous buffer and the determination is made of the binding of the labeled monoclonal antibody to the antigen. If the label is fluorescent, the slide may be covered with a fluorescent antibody mounting fluid on a cover slip and then examined with a fluorescent microscope to determine the extent of binding. On the other hand, the label can be an enzyme conjugated to the monoclonal antibody and the extent of binding can be determined by examining the slide for the presence of enzyme activity, which may be indicated by the formation of a precipitate, color, etc. A particular example of an assay utilizing the present antibodies is a double determinant ELISA assay. A support such as, e.g.,

a glass or vinyl plate, is coated with an antibody specific for NDTP by conventional techniques. The support is contacted with the sample suspected of containing NDTP, usually in aqueous medium. After an incubation period from 30 seconds to 12 hours, the support is separated from the medium, washed to remove unbound NDTP with, for example, water or an aqueous buffered medium, and contacted with an antibody specific for NDTP, again usually in aqueous medium. The antibody is labeled with an enzyme directly or indirectly such as, e.g., horseradish peroxidase or alkaline phosphatase. After incubation, the support is separated from the medium, and washed as above. The enzyme activity of the support or the aqueous medium is determined. This enzyme activity is related to the amount of NDTP in the sample.

Further, an anti-NDTP antibody (e.g., monoclonal antibody) can be used to isolate NDTPs by standard techniques, such as affinity chromatography or immunoprecipitation. For example, an anti-NDTP antibody can facilitate the purification of natural NDTPs from cells and of recombinantly produced NDTP expressed in host cells. Moreover, an anti-NDTP antibody can be used to isolate NDTP to aid in detection of low concentrations of NDTP (e.g., in plasma, cellular lysate or cell supernatant) or in order to evaluate the abundance and pattern of expression of the NDTP. Anti-NDTP antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, e.g., to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (i.e., physically linking) the antibody to a label group.

Drug Screening Assays

The invention provides a method (also referred to herein as a "screening assay") for identifying candidate modulators (e.g., small molecules, peptides, antibodies, peptidomimetics or other drugs) which bind to NDTPs, have a modulatory effect on, for example, NDTP expression or preferably NDTP biological activity. In some embodiments small molecules can be generated using combinatorial chemistry or can be obtained from a natural products library. Assays may be cell based or non-cell based assays. Drug screening assays may be binding assays or more preferentially functional assays, as further described.

When the invention is used for drug development, e.g., to determine the ability of an NDTP polypeptide or candidate modulator to induce an anti-neurological disorder response, the body fluid analyzed for the level of at least one NDTP is preferably from a non-human mammal. The non-human mammal is preferably one in which the induction of an anti-neurological disorder response by endogenous and/or exogenous agents is predictive of the

induction of such a response in a human. Rodents (mice, rats, etc.) and primates are particularly suitable for use in this aspect of the invention.

Agents that are found, using screening assays as further described herein, to modulate NDTP activity by at least 5%, more preferably by at least 10%, still more preferably by at least 30%, still more preferably by at least 50%, still more preferably by at least 70%, even more preferably by at least 90 %, may be selected for further testing as a prophylactic and/or therapeutic anti-neurological disorder agent.

In another aspect, agents that are found, using screening assays as further described herein, to modulate NDTP expression by at least 5%, more preferably by at least 10%, still more preferably by at least 30%, still more preferably by at least 50%, still more preferably by at least 70%, even more preferably by at least 90 %, may be selected for further testing as a prophylactic and/or therapeutic anti-neurological disorder agent.

Agents that are found to modulate NDTP activity may be used, for example, to modulate treatment regimens for neurological disorders or to reduce the symptoms of a neurological disorder alone or in combination with other appropriate agents or treatments.

Protein array methods are useful for screening and drug discovery. For example, one member of a receptor/ ligand pair is docked to an adsorbent, and its ability to bind the binding partner is determined in the presence of the test substance. Because of the rapidity with which adsorption can be tested, combinatorial libraries of test substances can be easily screened for their ability to modulate the interaction. In preferred screening methods, NDTPs are docked to the adsorbent. Binding partners are preferably labeled, thus enabling detection of the interaction. Alternatively, in certain embodiments, a test substance is docked to the adsorbent. The polypeptides of the invention are exposed to the test substance and screened for binding.

In other embodiments, an assay is a cell-based assay in which a cell which expresses an NDTP or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate NDTP activity determined. Determining the ability of the test compound to modulate NDTP activity can be accomplished by monitoring the bioactivity of the NDTP or biologically active portion thereof. The cell, for example, can be of mammalian origin, insect origin, bacterial origin or a yeast cell.

In one embodiment, the invention provides assays for screening candidate or test compounds which are target molecules of an NDTP or biologically active portion thereof. In another embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of an NDTP or biologically active portion thereof. 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 used with 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, the disclosure of which is incorporated herein by reference in its entirety).

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. U.S.A. 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 and 2061; and in Gallop et al. (1994) J. Med. Chem. 37:1233, the disclosures of which are incorporated herein by reference in their entireties.

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 U.S. Pat. No. 5,223,409), spores (Ladner U.S. Pat. No. '409), plasmids (Cull et al. (1992) Proc Natl Acad Sci USA 89:1865-1869) or on phage (Scott and Smith (1990) Science 249:386-390); (Devin (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).

Determining the ability of the test compound to modulate NDTP activity can also be accomplished, for example, by coupling the NDTP or biologically active portion thereof with a label group such that binding of the NDTP or biologically active portion thereof to its cognate target molecule can be determined by detecting the labeled NDTP or biologically active portion thereof in a complex. For example, the extent of complex formation may be measured by immunoprecipitating the complex or by performing gel electrophoresis.

It is also within the scope of this invention to determine the ability of a compound to interact with its cognate target molecule without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a compound with its cognate target molecule without the labeling of either the compound or the target molecule. McConnell, H. M. et al. (1992) Science 257:1906-1912, the disclosure of which is incorporated by reference in its entirety. A microphysiometer such as a cytosensor is an analytical instrument that measures the rate at which a cell acidifies its environment using a Light-Addressable Potentiometric Sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between compound and receptor.

In a preferred embodiment, the assay comprises: contacting a cell which expresses an NDTP or biologically active portion thereof with a target molecule to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to modulate the activity of the NDTP or biologically active portion thereof. Determining the ability of the test compound to modulate the activity of the NDTP or biologically active portion thereof comprises: determining the ability of the test compound to modulate a biological activity of the NDTP expressing cell (e.g., interaction with an NDTP target molecule, as discussed above).

In another preferred embodiment, the assay comprises contacting a cell which is responsive to an NDTP or biologically active portion thereof with an NDTP or biologically active portion thereof, to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to modulate the activity of the NDTP or biologically active portion thereof. Determining the ability of the test compound to modulate the activity of the NDTP or biologically active portion thereof comprises determining the ability of the test compound to modulate a biological activity of the NDTP-responsive cell.

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing an NDTP target molecule (i.e. a molecule with which NDTPs interact) with a test compound and determining the ability of the test compound to modulate the activity of the NDTP target molecule. Determining the ability of the test compound to modulate the activity of an NDTP target molecule can be accomplished, for example, by assessing the activity of a target molecule, or by assessing the ability of the NDTP to bind to or interact with the NDTP target molecule.

Determining the ability of the NDTP to bind to or interact with an NDTP target molecule, for example, can be accomplished by one of the methods described above for directly or indirectly determining binding. In a preferred embodiment, the assay includes contacting the NDTP or biologically active portion thereof with a known compound which binds said NDTP (e.g., an NDTP antibody or target molecule) to form an assay mixture, contacting the NDTP with a test compound before or after said known compound, and determining the ability of the test compound to interact with the NDTP. Determining the ability of the test compound to interact with an NDTP comprises determining the ability of the test compound to preferentially bind to the NDTP or biologically active portion thereof as compared to the known compound. Determining the ability of the NDTP to bind to an NDTP target molecule 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 and Szabo et al. (1995) Curr. Opin. Struct. Biol. 5:699-705, the disclosures of which are incorporated herein by reference in their entireties. 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 another embodiment, the assay is a cell-free assay in which an NDTP or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate the activity of the NDTP or biologically active portion thereof is determined. In a preferred embodiment, determining the ability of the NDTP to modulate or interact with an NDTP target molecule can be accomplished by determining the activity of the target molecule. For example, the activity of the target molecule can be determined by contacting the target molecule with the NDTP or a fragment thereof and measuring induction of a cellular second messenger of the target (e.g., cAMP, STAT3, Akt, intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic/enzymatic activity of the target for an appropriate substrate, detecting the induction of a reporter gene (comprising a target-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, e.g., luciferase), or detecting a target-regulated cellular response, for example, signal transduction or protein:protein interactions.

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. NDTPs or biologically active portions thereof or molecules to which NDTPs targets bind). In the case of cell-free assays in which a membrane-bound form 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 TM X-100, Triton TM X-114, Thesit TM, Isotridecypoly(ethylene glycol ether)n,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, it may be desirable to immobilize either an NDTP or its 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 an NDTP, or interaction of an NDTP with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants and by any

immobilization protocol described herein. Alternatively, the complexes can be dissociated from the matrix, and the level of NDTP binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either an NDTP or an NDTP target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated NDTP or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with NDTP or target molecules but which do not interfere with binding of the NDTP to its target molecule can be derivatized to the wells of the plate, and unbound target or NDTP 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 with the NDTP or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the NDTP or target molecule.

In another embodiment, modulators of NDTP expression are identified in a method wherein a cell is contacted with a candidate compound and the expression of NDTP mRNA or protein in the cell is determined. The level of expression of NDTP mRNA or protein in the presence of the candidate compound is compared to the level of expression of NDTP mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of NDTP expression based on this comparison. For example, when expression of NDTP mRNA or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of NDTP mRNA or protein expression. Alternatively, when expression of NDTP mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of NDTP mRNA or protein expression. The level of NDTP mRNA or protein expression in the cells can be determined by methods described herein for detecting NDTP mRNA or protein.

In yet another aspect of the invention, the NDTP can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Pat. 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, the disclosures of which are incorporated herein by reference in their

entireties), to identify other proteins, which bind to or interact with NDTPs ("NDTP-binding proteins" or "NDTP-bp") and are involved in NDTP activity. Such NDTP-binding proteins are also likely to be involved in the propagation of signals by the NDTP or NDTP targets as, for example, downstream elements of an NDTP-mediated signaling pathway.

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 an NDTP or a fragment thereof 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 an NDTP -dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the NDTP.

This invention further pertains to novel agents identified by the above-described screening assays and to processes for producing such agents by use of these assays. Accordingly, in one embodiment, the present invention includes a compound or agent obtainable by a method comprising the steps of any one of the aforementioned screening assays (e.g., cell-based assays or cell-free assays).

Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent identified as described herein (e.g., an NDTP modulating agent, or an NDTP -binding partner) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

The present invention also pertains to uses of novel agents identified by the above-described screening assays for diagnoses, prognoses, prevention, and treatments as described herein. Accordingly, it is within the scope of the present invention to use such agents in the design, formulation, synthesis, manufacture, and/or production of a drug or pharmaceutical

composition for use in diagnosis, prognosis, or treatment, as described herein. For example, in one embodiment, the present invention includes a method of synthesizing or producing a drug or pharmaceutical composition by reference to the structure and/or properties of a compound obtainable by one of the above-described screening assays. For example, a drug or pharmaceutical composition can be synthesized based on the structure and/or properties of a compound obtained by a method in which a cell which expresses an NDTP target molecule is contacted with a test compound and the ability of the test compound to bind to, or modulate the activity of, the NDTP target molecule is determined. In another exemplary embodiment, the present invention includes a method of synthesizing or producing a drug or pharmaceutical composition based on the structure and/or properties of a compound obtainable by a method in which an NDTP or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to, or modulate, the activity of the NDTP or biologically active portion thereof is determined.

Pharmaceutical Compositions

When polypeptides of the present invention are expressed in soluble form, for example as a secreted product of transformed yeast or mammalian cells, they can be purified according to standard procedures of the art, including steps of ammonium sulfate precipitation, ion exchange chromatography, gel filtration, electrophoresis, affinity chromatography, according to, e.g., "Enzyme Purification and Related Techniques," Methods in Enzymology, 22:233-577 (1977), and Scopes, R., Protein Purification: Principles and Practice (Springer-Verlag, New York, 1982) provide guidance in such purifications. Likewise, when polypeptides of the invention are expressed in insoluble form, for example as aggregates or inclusion bodies, they can be purified by appropriate techniques, including separating the inclusion bodies from disrupted host cells by centrifugation, solubilizing the inclusion bodies with chaotropic and reducing agents, diluting the solubilized mixture, and lowering the concentration of chaotropic agent and reducing agent so that the polypeptide takes on a biologically active conformation. The latter procedures are disclosed in the following references, which are incorporated by reference: Winkler et al, Biochemistry, 25: 4041-4045 (1986); Winkler et al, Biotechnology, 3: 992-998 (1985); Koths et al, U.S. patent 4,569,790; and European patent applications 86306917.5 and 86306353.3.

Compounds capable of detecting or modulating an NDTP or an NDTP biological activity, including small molecules, peptides, and NDTP nucleic acid molecules, can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise 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 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; 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 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® (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability 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, glycerol, propylene glycol, and liquid polyethylene glycol, 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 microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, 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, 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.

Where the active compound is a protein, sterile injectable solutions can be prepared by incorporating the active compound 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 other required 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 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 used in the form of tablets, troches, or capsules. 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 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. Most preferably, active compound is delivered to a subject by intravenous injection.

In one embodiment, the active compounds 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. Pat. No. 4,522,811, the disclosure of which is incorporated herein by reference in its entirety.

In a further embodiment, the active compound may be coated on a microchip drug delivery device. Such devices are useful for controlled delivery of proteinaceous compositions into the bloodstream, cerebrospinal fluid, lymph, or tissue of an individual without subjecting such compositions to digestion or subjecting the individual to injection. Methods of using microchip drug delivery devices are described in US Patents 6123861, 5797898 and US Patent application 20020119176A1, disclosures of which are hereby incorporated in their entireties.

It is especially advantageous to formulate oral or preferably 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 active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds 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 it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds 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 compounds 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 compound used in the method 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 given circulating

concentration, subsequently used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

Neurological Disorders Therapy

The NDTP modulators and NDTP-related compositions of the invention can be used in the treatment or prevention of neurological disorders. Thus, in one aspect the invention relates to pharmaceutical compositions containing an NDTP, preferably containing a pharmaceutically acceptable carrier or diluent. The carrier or diluent is preferably adapted for oral, intravenous, intramuscular or subcutaneous administration. Pharmaceutical compositions may comprise or consist essentially of any of the NDTP-related compositions.

A number of agents are useful for the treatment and prevention of neurological disorders. Such agents may be used advantageously in combination with an NDTP-related composition.

For example, Interferons Beta (Avonex from Biogen, Rebif from Serono, or Betaseron from Schering), Glatiramer (Copaxone from Teva / Aventis), and Immunosuppressor agents (such as Mitoxantrone, a topo-isomerase II inhibitor, from Wyeth-Lederle) are being used to alleviate the symptoms of multiple sclerosis, and may be used advantageously in combination with an NDTP-related composition of the invention.

Additionally, for example, cholinesterases inhibitors such as Aricept® (donepezil HCl), Exelon® (rivastigmine), Reminyl® (galantamine HBr) and Cognex® (tacrine), or N-methyl-D-aspartate receptor (NMDA) antagonists such as Namenda® (Memantime) are being used to alleviate the symptoms of Alzheimer's disease, and may be used advantageously in combination with an ADP-related composition of the invention.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLES

Example 1: Synthesis of GPB003:

The GPB003 polypeptide was prepared by SPPS on a 0.2 mmolscale using machine-assisted protocols on a custom–modified Applied Biosystems model 433A peptide synthesizer, using in situ neutralization/HCTU activation procedure for Boc chemistry as described (Schnölzer et al., Int. J. Peptide Protein Res., 40: 180-193, 1992). The peptide was synthesized on the appropriate Boc-amino acyl –Pam preloaded resin.

After chain assembly was completed, the peptide was deprotected and cleaved from the resin by treatment with anhydrous HF for 1h at 0°C with 5% p-cresol as scavenger. The peptide was precipitated with diethyl ether, dissolved in aqueous acetonitrile, lyophilized and purified by preparative RP-HPLC on a Waters 600 HPLC module using a Vydac C8 5µm 300A, 22x250mm column. Peptide identity was confirmed by ESI-MS with a Bruker Esquire 3000 Ion Trap (Bruker Daltonics, Bremen, DE).

Analytical RP-HPLC of all the products was performed on a Waters 2690 HPLC module with a 214nm UV detection, using a Symmetry 300 C18 column , with a linear gradient of buffer B in buffer A over 30 min at 1mL/min. Buffer A= 0.1%TFA in water; buffer B= 0.1% TFA in ACN. Data were recorded and analysed using the software system Millennium 32.

79 mg pure material was obtained from the purification of 200 mg crude peptide.

The synthesized sequence was:

TVPGASDLFYWPR

Number of residues: 13

Theoretical relative molecular mass, reduced form: 1508.71

Analytical data:

Experimental relative molecular mass, reduced form: 1509.08

Protein purity: 98.75 % (by RP-HPLC analysis, column Waters Symmetry 300A, C18, 5µm, UV detection 214nm).

Material available as a TFA salt; estimated net protein content: 70-80%.

Readily soluble in water at 5 mg/ml.

Example 2: Improved Survival of Primary Cortical Neurons:

In vitro effect of GPB003 on cortical neurons survival

Primary cultures of rat cortical neurons

A female rat of 17 days gestation was killed by cervical dislocation; the fetuses were removed from the uterus. Their brains were removed and placed in ice-cold medium of Leibovitz (L15, Gibco, Invitrogen, Cergy-Pontoise, France). Cortex were dissected and meninges were carefully removed. The cortical neurons were dissociated by trypsinization for 30 min at 37°C (trypsin-EDTA Gibco) in presence of DNAse I (Roche, Meylan). The reaction was stopped by addition of medium of Eagle modified by Dulbecco (DMEM ; Gibco) with 10 % of fetal bovine serum (FBS ; Gibco). The suspension was triturated with a 10-ml pipette and using a needle syringe 21G and centrifuged at 350 x g for 10 min at room temperature. The pellets of dissociated cells were resuspended in culture medium containing Neurobasal medium (Gibco) with 2% of B27 supplement (Gibco) and 0.5 mM of glutamine (Gibco). Viable cells were counted in a Neubauer cytometer using the trypan blue exclusion test (Sigma ref T8154) and seeded on the basis of 4 000 cells per well in 96 well-plates (TPP) precoated with poly-L-lysine. Cells were allowed to adhere 2-3h and maintained in a humidified incubator at 37°C in 5 % CO_2-95 air atmosphere. Test compounds were then added to the medium.

Stock solution of GPB003 was prepared in distilled water (Gibco) at 3 mM.
BDNF (Tebu, Peprotech) was tested at 50 ng/ml as a reference compound.
All dilutions were made in culture medium. Final concentration of water was fixed at 1%.

Evaluation of survival

After 3 days of treatment, neuronal survival was assayed by measuring acid phosphatase activity according to a method known in the art. Briefly, after removal of the culture medium, cells were rinsed with 100 µl of PBS (Phosphatase Buffer Saline, Gibco), after 100 µl of buffer containing 0.1 M sodium acetate (pH 5.5), 0.1% Triton X100 and 1mg/ml p-nitrophenyl phosphate (Sigma) was added. Reaction was stopped by addition of 10 µl of 1N NaOH. Enzyme activity was measured at 405 nm in a microplate reader (Labsystems Multiskan Bichromatic).

Data analysis

A global analysis of the data was done using a one way analysis of variance (ANOVA). Where applicable, Fisher's PLSD test was used for multiple pairwise comparison. The level of significance was set at $p < 0.05$.

*** significant from intoxicated group ($p \leq 0.001$, Fisher's test)

** significant from intoxicated group ($p \leq 0.01$, Fisher's test)

* significant from intoxicated group ($p \leq 0.05$, Fisher's test)

Results

BDNF, used (at 50 ng/ml) as reference compound, significantly enhanced cortical neurons survival.

The experiment was run in triplicate, on 3 different primary cells cultures.

The results for GPB003 are shown in Figure 1. GPB003 displays a significant effect on cortical neurons survival.

Example 3: Therapeutic effect of GPB003 in nerve crush model

BACKGROUND

The primary goal of research in neurodegenerative disorders is ultimately to develop interventions which prevent neuronal death, maintain neuronal phenotype and repair neuronal damage. Many studies have been devoted to the unraveling of molecular and cellular mechanisms responsible for the complete regeneration of axotomized spinal motor neurons (Fawcett JW and Keynes RJ, Peripheral nerve regeneration. *Annu Rev Neurosci*, 1990. 13: p. 43-60; Funakoshi H, *et al.*, Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J Cell Biol*, 1993. 123(2): p. 455-65). Injury-induced expression of neurotrophic factors and corresponding receptors may play an important role in the ability of nerve regeneration. Previous studies have shown a significant improvement of nerve regeneration with various peptides and nonpeptides compounds like insulin-like growth factor (IGF-1), ACTH (Lewis ME, *et al.*, Insulin-like growth factor-I: potential for treatment of motor neuronal disorders. *Exp Neurol*, 1993. 124(1): p. 73-88; Strand FL and Kung TT. ACTH accelerates recovery of neuromuscular function following crushing of peripheral nerve. *Peptides*, 1980. 1(2): p. 135-8) testosterone (Jones KJ. Gonadal steroids as promoting factors in axonal regeneration. *Brain Res Bull*, 1993. 30(3-4): p. 491-8), SR57746A (Fournier J, *et al.*, Protective effects of SR 57746A in central and peripheral models of neurodegenerative disorders in rodents and primates. *Neuroscience*, 1993. 55(3): p. 629-41).

The sciatic nerve crush is a widely used animal model to study nerve regeneration, of which the extent and speed of regeneration is usually defined by the return of nerve function (De Koning P, Brakkee JH, and Gispen WH. Methods for producing a reproducible crush in the

sciatic and tibial nerve of the rat and rapid and precise testing of return of sensory function. Beneficial effects of melanocortins. *J Neurol Sci*, 1986. 74(2-3): p. 237-46; Azzouz M, *et al.*, Enhancement of mouse sciatic nerve regeneration by the long chain fatty alcohol, N-Hexacosanol. *Exp Neurol*, 1996. 138(2): p. 189-97).

OBJECTIVES

The aim of the present study was to evaluate the *in-vivo* effects of GPB003 with regard to the functional recovery of the sciatic nerve crush and its histomorphological correlates.
9 weeks were needed to achieve the entire study. All experiments were conducted at the laboratory of Neurofit, les Algorithmes, Immeuble le Platon rue Jean Sapidus, 67400 Illkirch, France.

MATERIALS AND METHODS

Animals and experimental groups
8 weeks-old females C57bl/6 RJ mice (Charles River, L'Arbresle, France) were used. They were divided into 12 groups (n = 10 per group) as following: (a) sham operated mice receiving vehicle treatment; (b) mice subjected to nerve crush injury receiving vehicle treatment; and (c) 10 groups for the 5 test compounds at 2 doses each. All test compounds were tested at the doses of 30 and 300 µg/kg.
They were group-housed (10 animals per cage) and maintained in a room with controlled temperature (21-22°C) and a reversed light-dark cycle (12h/12h) with food and water available *ad libitum*. All experiments were carried out in accordance with institutional guidelines.

Sciatic nerve crush and pharmacological treatment
The animals were anaesthetized with IP injection of 100 mg/kg ketamine chlorhydrate (Imalgène 500®, Rhône Mérieux, Lyon, France). The right sciatic nerve was surgically exposed at mid thigh level and crushed at 5 mm proximal to the trifurcation of the sciatic nerve. The nerve was crushed twice for 30 s with a haemostatic forceps (width 1.5 mm; Koenig ; Strasbourg ; France) with a 90 degree rotation between each crush.
Treatments started the day of the crush injury and continued on a daily basis until the end of the study. Test compounds or its corresponding vehicle (0.9% NaCl) were administrated subcutaneously.

Planning of experiments

Body weight and survival rate were recorded on daily basis.

Gait walking and toe spreading tests were performed once a week until week 4 post-lesioning.

EMG testings and behavioural monitorings were performed once a week until the end of the study (week 4 post-lesioning).

Sciatic nerves were harvested at week 4 post-lesioning for histomorphometric analysis (n = 5 per group).

Walking test

The apparatus consisted of a 15 mm diameter rod, 800 mm long mounted 400 mm above a table. The mouse was placed on the center of the rod and the time taken to cross 400 mm was recorded. Three consecutive trials were performed and the mean time recorded. The test was stopped after an arbritrary limit of 60 s.

Toe spreading

The spreading of the toes was checked when the mice were lifted. The presence of this reflex reflects the innervation of the small muscles of the foot and may disappear after axonal degeneration. The toe spreading was graded as: 1 : no spreading ; 2 : intermediate spreading ; 3 : normal spreading. In case of hesitation, an intermediate scoring could be attributed (0.5 ; 1.5 ; 2.5).

Electromyography

Electrophysiological recordings were performed using a Neuromatic 2000M electromyograph (EMG) (Dantec, Les Ulis, France). Mice were anaesthetized by IP injection of 100 mg/kg ketamine chlorhydrate (Imalgene 500®, Rhône Mérieux, Lyon, France). The normal body temperature was maintained at 30°C with a heating lamp and verified using a contact thermometer (Quick, Bioblock Scientific, Illkirch, France) placed on the tail surface. Compound muscle action potential (CMAP) was measured in the *gastrocnemius* muscle after a single 0.2 ms stimulation of the sciatic nerve at a supramaximal intensity (12.8 mA). The amplitude (mV), the latency (ms) and the duration (time needed between depolarization and repolarization session) of the action potential were measured. The amplitude is indicative of the number of active motor units, while the distal latency indirectly reflects motor nerve conduction and neuromuscular transmission velocities. The duration is indicative of the functionality of the nerve fibers.

Morphometric analysis

Morphometric analysis was performed at week 4 post-lesioning on 5 animals per group. The animals were anesthetized by IP injection of 100 mg/kg Imalgène 500®. A 5 mm-segment of sciatic nerve was excised, fixed overnight with 4 % glutaraldehyde (Sigma, L'Isle d'Abeau-Chesnes, France) in PBS (pH = 7.4) and maintained in 30% sucrose and stored at 4°C until further processing. At the time of use, each nerve sample was post-fixed in 1 % osmium tetroxide (Sigma, L'Isle d'Abeau-Chesnes, France) in PBS for 2 h, dehydrated in serial alcohol solution, and embedded in Epon. Embedded tissues were then placed at 70°C for 3 days to allow polymerization of the tissue wax. Cross sections of 1.5 µm thick were performed until the lesioned site is reached. Sections were stained with 1 % toluidine blue solution (Sigma, L'Isle d'Abeau-Chesnes, France) for 2 min, dehydrated and mounted on Eukitt. One nerve section in the mid-lesion region was examined for each sample using an optical microscope (Nikon, Tokyo, Japan). Analysis was performed on the entire surface of the nerve section using a semi-automated digital image analysis software (Biocom, France). Once extraneous objects had been eliminated, the software reported the total number of myelinated fibers. The number of degenerated fibers was then counted manually by an operator. Myelinated fiber was considered as degenerated when the myelin buckles and prolapses within the axoplasm, forming the so-called onion bulbs. The number of non-degenerated fibers was obtained by subtraction of the number of degenerated fibers. Morphological analysis was performed only on fibers considered as non-degenerated. For each fiber, the axonal and myelin sizes were reported in surface area (μm^2). These two parameters were used to calculate the equivalent area of g-ratio (axonal diameter/fiber diameter) of each fiber (i.e., $[A/(A+M)]^{0.5}$, A = axonal area, M = myelin area), indicative of the relative myelin sheath thickness (see Figure 2). This latter parameter varies between 0.36 and 0.95 in mammalian nerves (Sunderland S. Peripheral nerve fibres. In: Sunderland S, Editor., 1968. *Nerves and Nerves Injuries*. Livingstones Eyre and Spottiswoode;(1-2): p. 9-10). The proportion of fibers having a g-ratio ≥ 0.8 was evaluated, i.e., fibers showing relatively thin sheaths, and they were therefore considered as hypomyelinated fibers (McCreery DB, *et al.*, A quantitative computer-assisted morphometric analysis of stimulation-induced injury to myelinated fibers in a peripheral nerve. *J Neurosci Methods*, 1997. 73(2): p. 159-68). Fibers having g-ratio ≤ 0.6, i.e., fibers showing relatively thick sheaths, were considered as hypermyelinated fibers. The mean axonal size of fibers having g-ratio between 0.6 and 0.8 were calculated as this parameter constitutes a good indicator of axonal regeneration (Funakoshi H, *et al.*, Targeted expression of a multifunctional chimeric

neurotrophin in the lesioned sciatic nerve accelerates regeneration of sensory and motor axons. *Proc Natl Acad Sci USA*, 1998. 95(9): p. 5269-74).

Data analysis

Repeated measures ANOVA was used to compare the body weight between experimental groups. ANOVA followed by post-hoc analysis (Dunnett's test) was used to compare groups of electrophysiological and behavioural data. For the histology results, each experimental group was compared to the group of lesioned nerves treated with vehicle using student *t*-test.

RESULTS

Animal weight

The body weight of mice with lesioned nerves and treatment did not significantly differ from that of control mice (Figure 3).

Walking time

One week after the lesion, mice with lesioned nerves demonstrated a marked increase in the time taken to achieve the 400 mm distance on the rod (Figure 4). However, it appeared that mice treated with GPB003 demonstrated better performance than vehicle-treated animals. The difference reached the statistical significance for the group treated with 300 µg/kg GPB003. The dysfunction was still observed at week 2 post-lesioning although the effect of GPB003 was less pronounced as compared to that at week 1. At week 3 and 4 post-lesioning, the performance of all mice was no longer different from that of sham-operated mice, suggesting a functional recovery.

Toe spreading score

One week after the lesion, all mice including those of the control group showed a dramatic decrease in the toe spreading score (Figure 5). At week 2 post-lesioning, sham-operated mice had regained their initial score while mice with lesioned nerves still demonstrated a significant degree of toe spreading dysfunction. In addition, no significant difference was observed between GPB003-treated mice and vehicle-treated animals. At week 3 and 4 post-lesioning, the score of all mice with lesioned nerves returned to the normal spreading score.

Electrophysiological measurements

Amplitude of CMAP

Mice with nerve lesion showed a dramatic decrease (up to 80%) in the amplitude of CMAP followed by a progressive recovery from week 2 onwards (Figure 6). Mice treated with

GPB003 showed a faster recovery of CMAP than vehicle-treated specimens. Indeed, mice receiving GPB003 treatment showed significantly greater amplitude than the corresponding vehicle-treated mice. By week 4, all GPB003-treated mice demonstrated up to 78% CMAP recovery of sham-operated mice, whereas it was around 53% only for the vehicle-treated specimens (Figure 6).

Latency of CMAP

A marked increase in CMAP latency was observed after sciatic nerve crush (Figure 7). The impairment became pronounced with time. This impairment in the motor wave conduction was significantly less pronounced in GPB003-treated animals than in vehicle-treated specimens. Indeed, 2 weeks after the lesioning, the CMAP latencies of GPB003-treated mice were significantly extended by less than 17% as compared to that of control mice whereas the one of vehicle-treated group was increased by more than 35%. By week 3, whilst the CMAP latency of the vehicle-treated mice remained largely above the control value (about 50% above the control), those of GPB003-treated mice showed an increase less than 17% only.

Duration of CMAP

Following nerve injury, the duration of CMAP signal was markedly extended and reached a value superior to 9 ms 2 weeks after the lesion, whereas it was around 2 ms in control mice (Figure 8). At week 3 post-lesioning, spontaneous decrease was initiated in vehicle-treated mice, but still remained at a level about 50 % above the control by week 4. Treatment with GPB003 showed a slightly enhanced extension of the duration CMAP compared with vehicle-treated specimens at week 1 post-lesioning. From-week 2 onwards, mice treated with GPB003 showed shorter duration of CMAP than the corresponding vehicle-treated animals.

Morphometric analysis

g-ratio

At week 4 post-lesioning (Figure 9), GPB003 treatment was associated with a marked reduction of g-ratio.

Proportion of hypomyelinated fibers

In control mice, a small proportion (about 0.5%) of fibers showed a relatively thin myelin sheaths (g-ratio $\geq$ 0.8) at week 4 post-lesioning (Figure 10). This population of fibers was significantly greater (about 2%) in vehicle-treated mice. Marked reduction of the population of hypomyelinated fibers was observed in groups treated with GPB003 (significant effect at 300 µg/kg).

Proportion of hypermyelinated fibers

At week 4 post-lesioning, the proportion of hypermyelinated fibers, i.e., fibers with relatively thick myelin sheaths (g-ratio < 0.6), was markedly increased in mice treated with GPB003 (Figure 11).

Axon size

At week 4 post-lesioning, there was a dramatic decrease (about 50% as compared to the control value) in the overall axonal size in the vehicle-treated groups (Figure 12). The groups treated with GPB003 showed similar axonal size than vehicle-treated group.

Size distribution of fibers

As compared to the control nerves, the size distribution profile of fibers from lesioned nerves was markedly shifted towards smaller caliber fibers end (Figure 13). Indeed, the proportion of small caliber fibers (diameter < 5 μm) was increased by about a factor of 2. Conversely, the proportion of large caliber fibers (diameter > 9 μm) was decreased by about a factor of 20. The size distribution profile of fibers from GPB003-treated nerves was similar to that of vehicle-treated lesioned nerves.

Proportion of degenerated and non-degenerated fibers

At week 4 post-lesioning, the proportion of degenerated fibers was markedly increased (by about a factor of 7) in vehicle-treated lesioned nerves as compared to that of control nerves (Figure 14A). The proportion of degenerated fibers in lesioned nerves was not affected by GPB003 treatment. Conversely, the proportion of non-degenerated fibers was signifcantly decreased in vehicle-treated lesioned nerves as compared to that of control nerves (Figure 14B). GPB003 treatment did not improve the proportion of non-degenerated fibers.

CONCLUSION

The sciatic nerve crush model is the most commonly used to evaluate the regeneration of lesioned nerves. Various techniques such as electrophysiological measurements or histomorphometric analysis have been validated in this model to monitor the nerve regeneration. In this model indeed, dramatic loss of CMAP amplitude along with marked reduction of motor nerve conduction velocity (CMAP latency) is observed during the first two weeks following the lesioning. These changes are the consequence of fiber loss and/or a profound modification in the fiber morphology (Azzouz M, *et al.*, Enhancement of mouse sciatic nerve regeneration by the long chain fatty alcohol, N-Hexacosanol. *Exp Neurol*, 1996.

138(2): p. 189-97). The first sign of regeneration is not seen before week 2 post-lesioning and is demonstrated by a progressive improvement in the CMAP amplitude.

The above profile of pathology in the nerve crush paradigm was reproduced by the present study showing a dramatic loss of CMAP amplitude (up to 80%) along with significant alteration of characteristics of nerve conduction (CMAP latency and duration). These dysfunctions were correlated with an overall shrinkage of the axonal profile (up to 50%), marked loss of myelin reflected by the increase in the g-ratio and an increase in the population of degenerated fibers within 4 weeks after the lesioning. Spontaneous recovery indicative of nerve regeneration progressed from week 2 as demonstrated by the improvements in the nerve function (CMAP amplitude and duration).

In the present study, the potential regenerative action of GPB003 was investigated in the sciatic nerve crush model. The results showed that GPB003 accelerates nerve regeneration. This accelerated regeneration started at week 1 with an improved walking performance. At week 2 post-lesioning, this active regeneration was further supported by an enhanced recovery in the nerve function (CMAP measurement) in GPB003 groups as compared to that of vehicle-treated group. In addition, by week 4, fibers of lesioned nerves treated with GPB003 showed an overall increase in the size of myelin sheath as compared to that of the corresponding vehicle-treated specimens. Since the quality of the myelin sheath is an important component for optimal nerve conduction, increase in the size of myelin sheath may, *de facto*, in part explain the improvement in the CMAP parameters of lesioned nerves treated with GPB003. However, this idea does not exclude the possibility that GPB003 directly modifies the excitability of innervated muscles.

It was also noticed that a population of fibers with disproportional axon-myelin ratio appears in a significantly greater proportion at week 4 post-lesion in lesioned nerves treated with the vehicle. Treatments with GPB003 induced a significant reduction in the proportion of these abnormal fibers. These fibers were indeed identified as large caliber fibers (axon $\geq 20\ \mu m^2$ in size) with relatively thin myelin sheath (g-ratio > 0.8). However, it is not possible to clearly assert whether this phenomenon was due to myelin loss or a result of axonal swelling indicative of distal axonopathy. Whatever the case, it most likely appears that GPB003 contributes to preserve the axonal characteristics of the fibers.

Nerve crush lesion is also characterized by the presence of degenerated fibers. In the present study, this population of fibers was still observed at a significant proportion at week 4 post-lesioning. Treatments with GPB003 did not modify the proportion of degenerated fibers present in the lesioned nerves, suggesting that a direct neuroprotective action is unlikely to be involved in regenerative action of GPB003.

CLAIMS

1. A substantially purified polypeptide consisting of the amino acid sequence of SEQ ID NO:1.

2. A substantially purified polypeptide consisting of the amino acid sequence of SEQ ID NO:1, wherein said polypeptide is fused to a heterologous polypeptide sequence

3. A method of making the polypeptide of Claims 1 or 2, said method comprising
providing a population of host cells comprising a recombinant nucleic acid encoding said polypeptide of Claims 1 or 2; and
culturing said population of host cells under conditions conducive to the expression of said recombinant nucleic acid;
whereby said polypeptide is produced within said population of host cells.

4. The method of Claim 3, further comprising purifying said polypeptide from said population of cells.

5. A method of making the polypeptide of Claims 1or 2 by chemical synthesis.

6. The use of the polypeptide of Claims 1 or 2 in the preparation of a medicament for the prophylaxis and/or treatment of neurological disorders.

7. A method of treatment of neurological disorders comprising the step of administering to a subject an effective amount of a therapeutic composition comprising the polypeptide of Claims 1 or 2.

8. A method according to claim 7 wherein said neurological disorder is Multiple Sclerosis.

9. A method according to claim 7 wherein said neurological disorder is Alzheimer's Disease.

10. A method according to claim 7 wherein said therapeutic composition is administered subcutaneously.

Figure 1

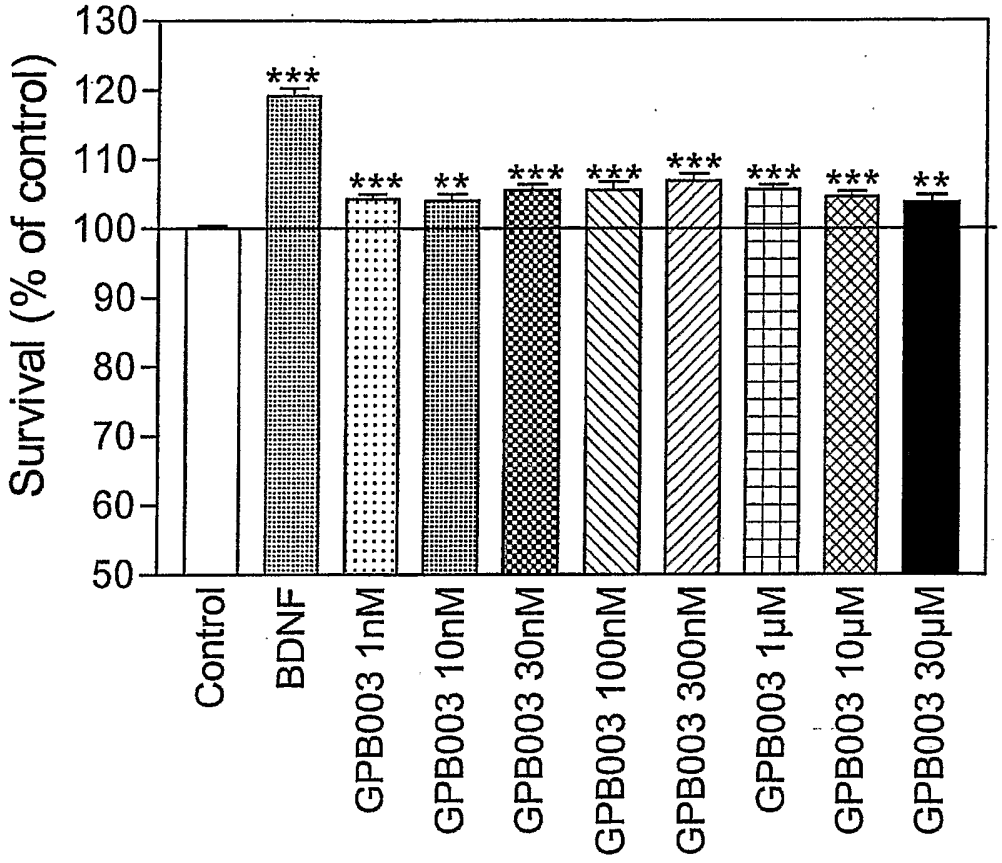

Survival (% of control)
130
120
110
100
90
80
70
60
50
***
***
**
***
***
***
***
***
**
Control
BDNF
GPB003 1nM
GPB003 10nM
GPB003 30nM
GPB003 100nM
GPB003 300nM
GPB003 1µM
GPB003 10µM
GPB003 30µM

Figure 2

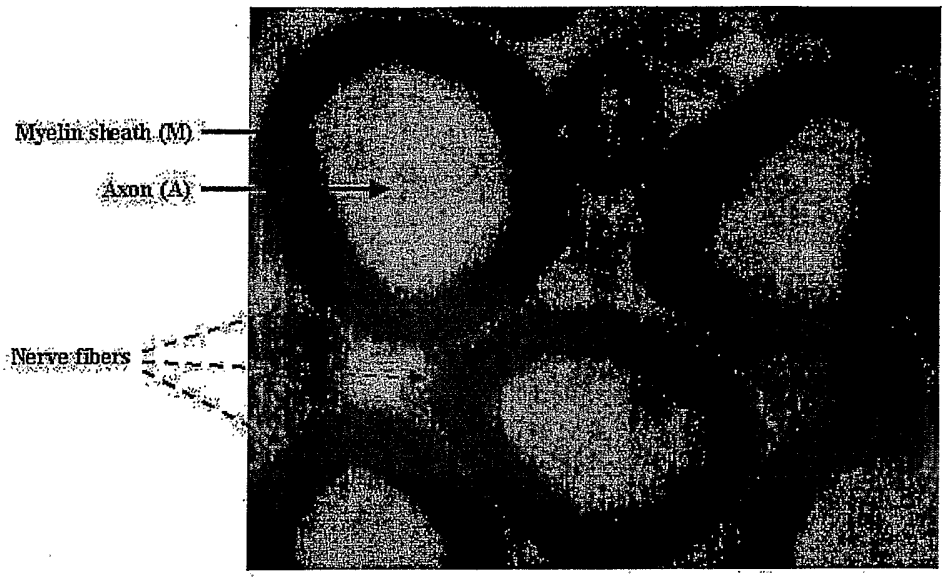
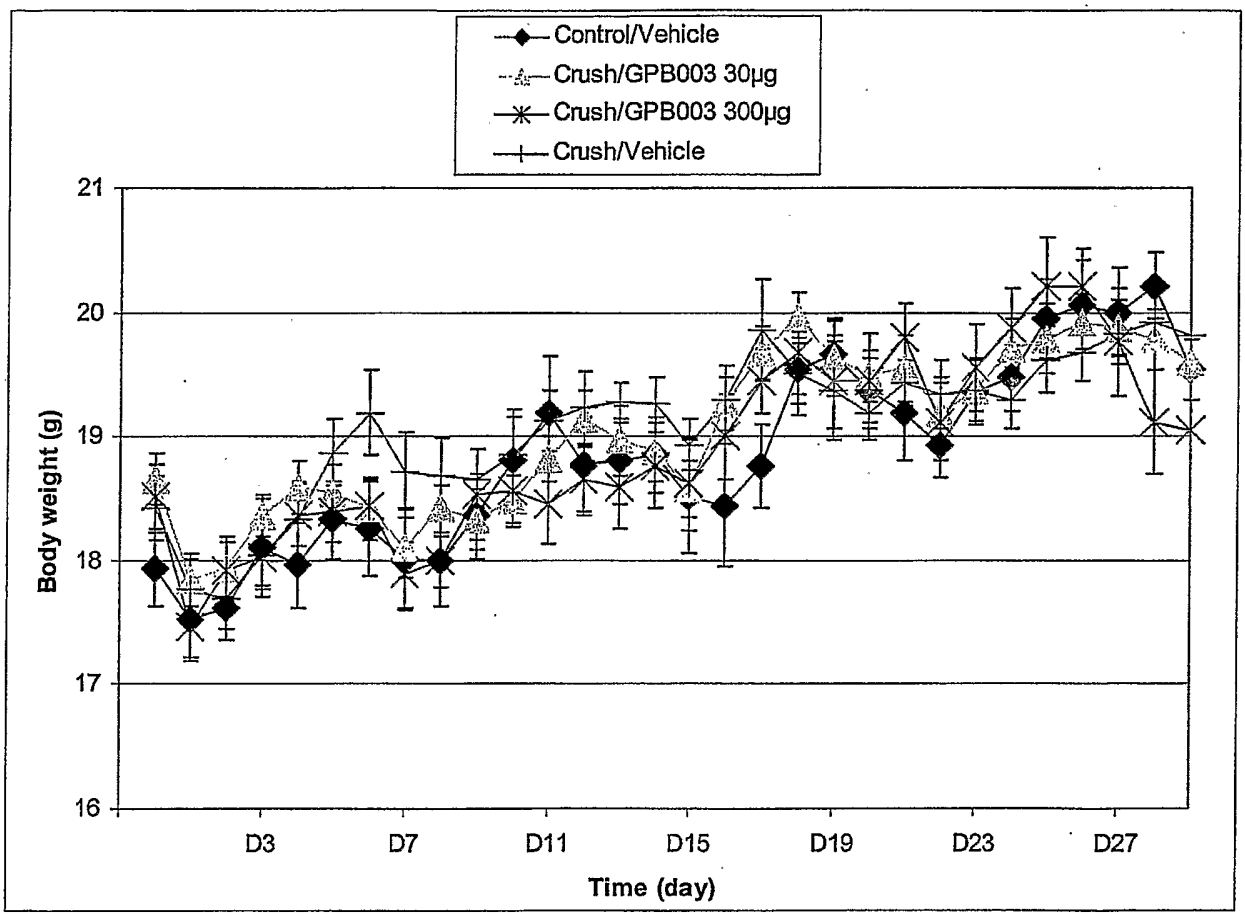


Figure 3

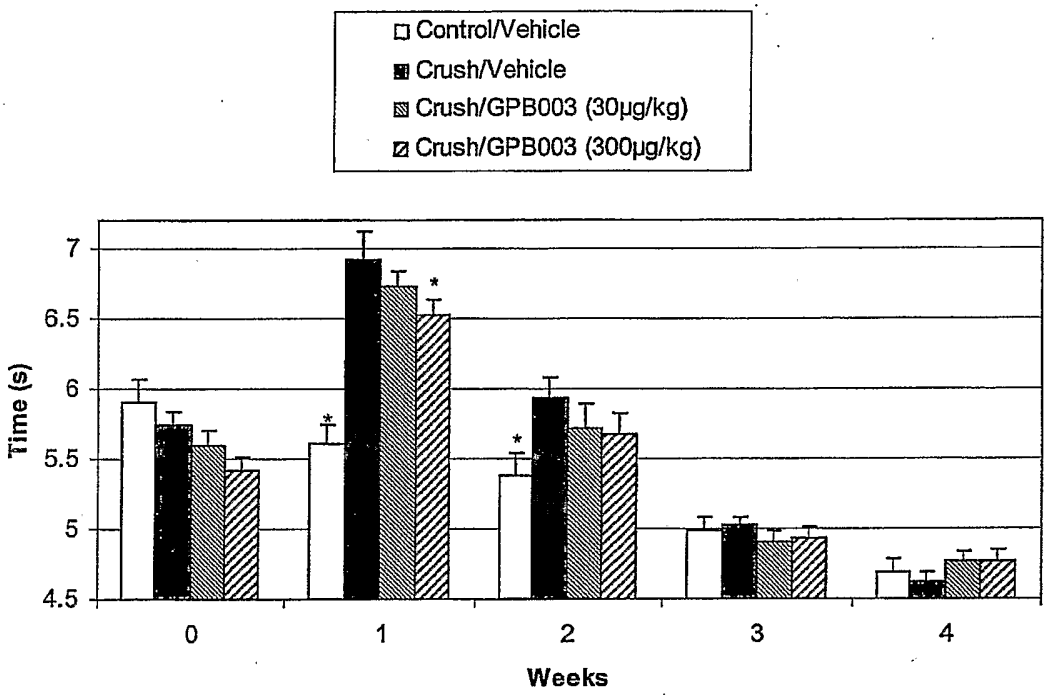
Body Weight



Mean ± s.e.m.

Figure 4

Walking Performance

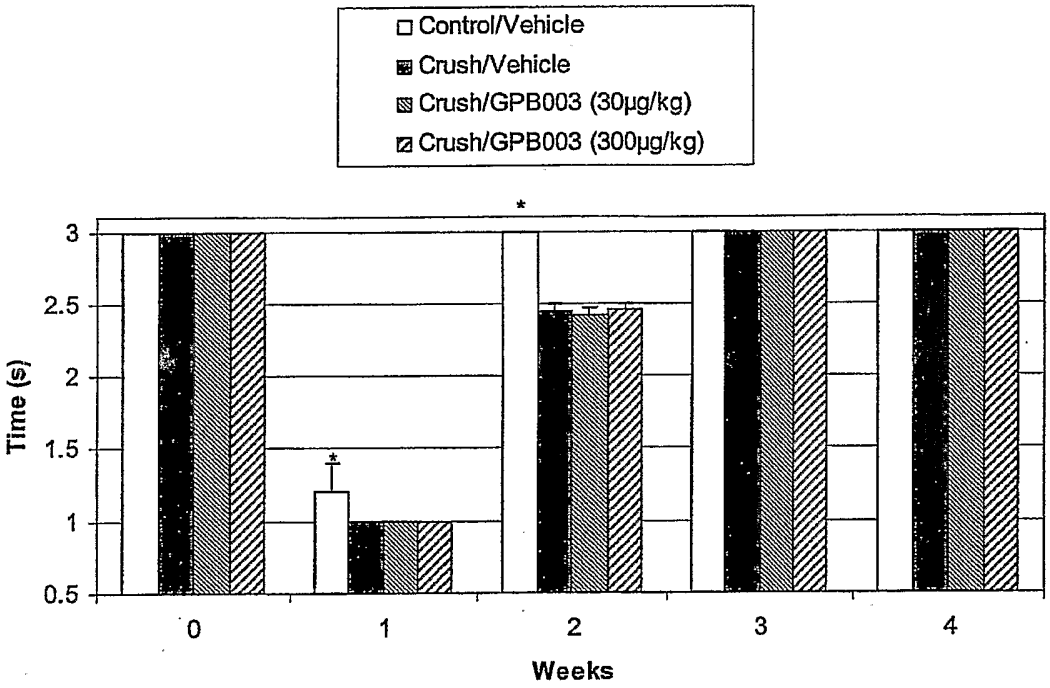


Mean ± s.e.m.

* $p \leq 0.05$, significantly different as compared to crush/Vehicle group.

Figure 5

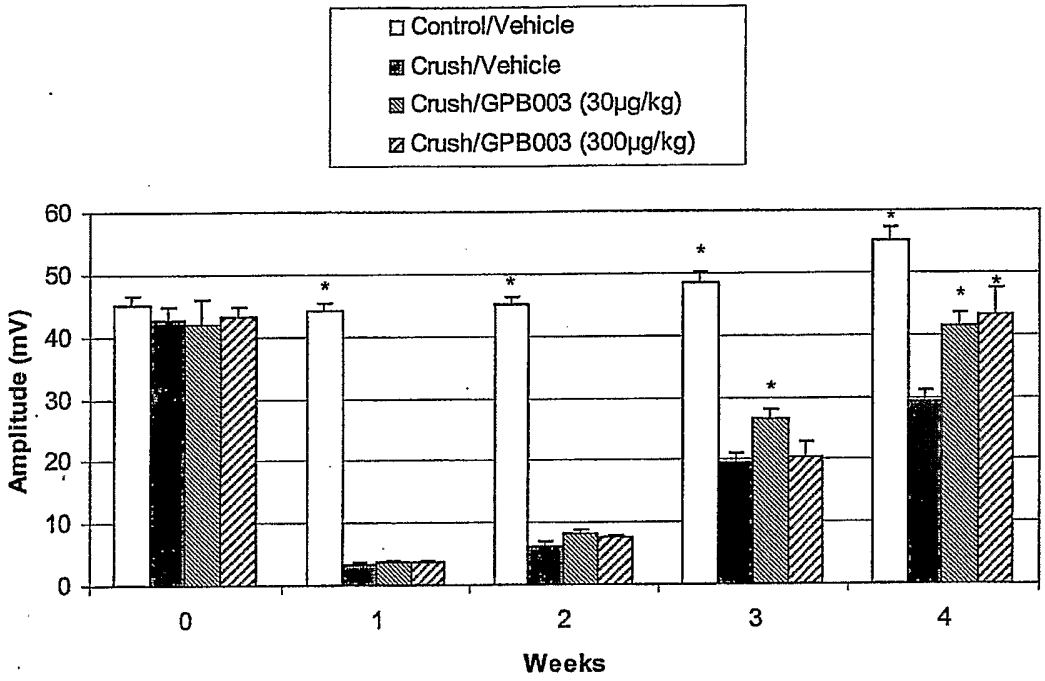
Toe Spreading



Mean ± s.e.m.
* $p \leq 0.05$, significantly different as compared to crush/Vehicle group.

Figure 6

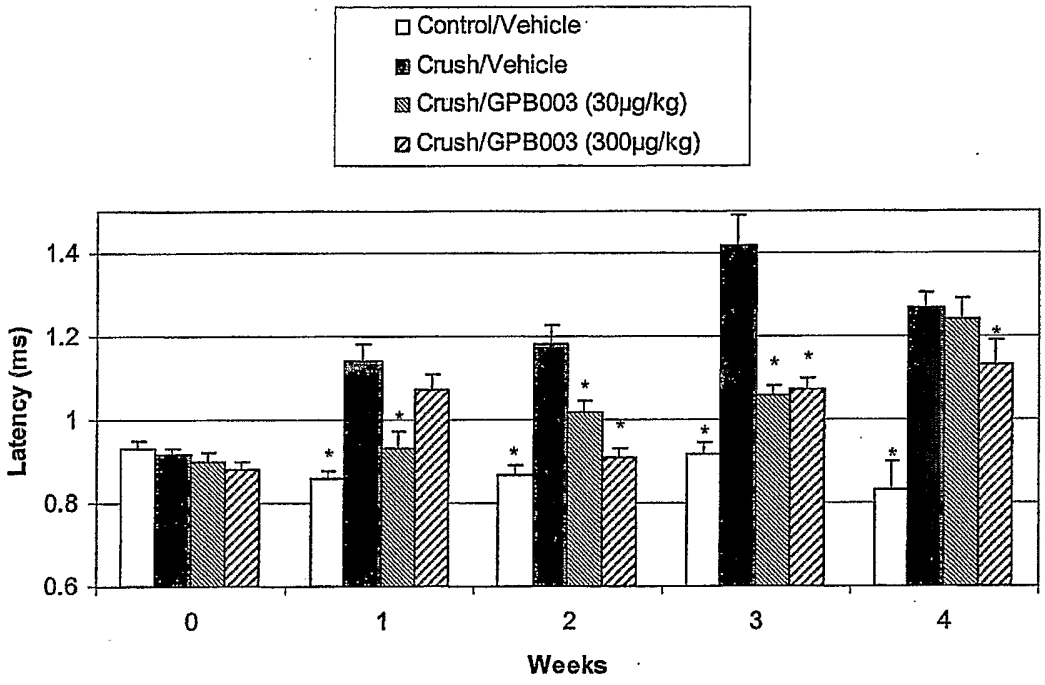
CMAP Amplitude



Mean ± s.e.m.
* $p \leq 0.05$, significantly different as compared to crush/Vehicle group.

Figure 7

CMAP Latency

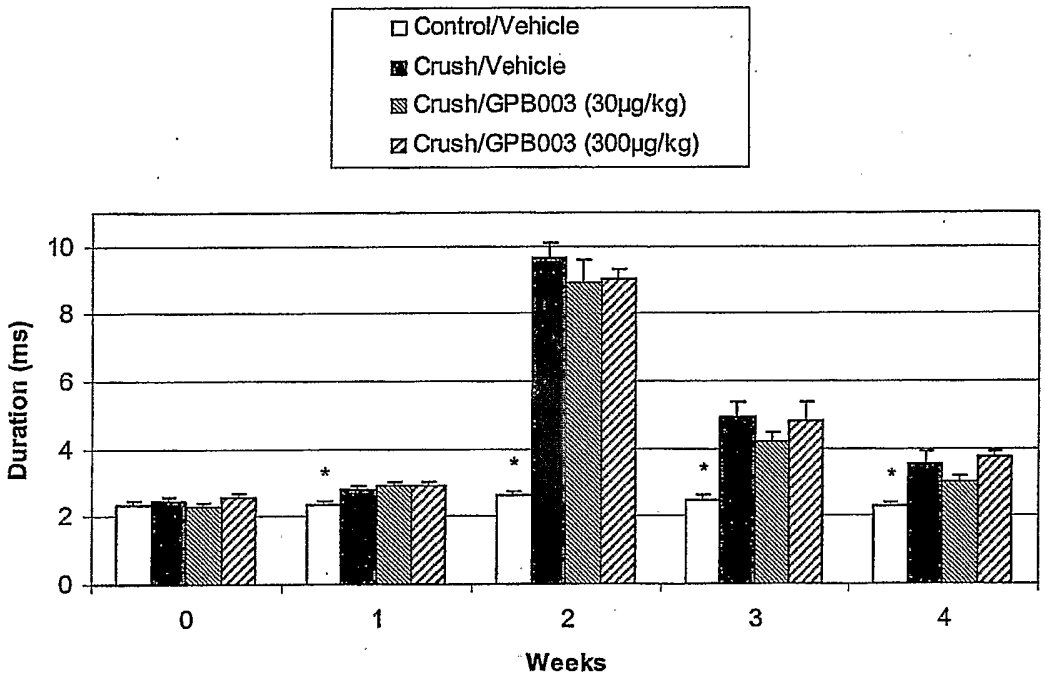


Mean ± s.e.m.

* $p \leq 0.05$, significantly different as compared to crush/Vehicle group.

Figure 8

CMAP Duration

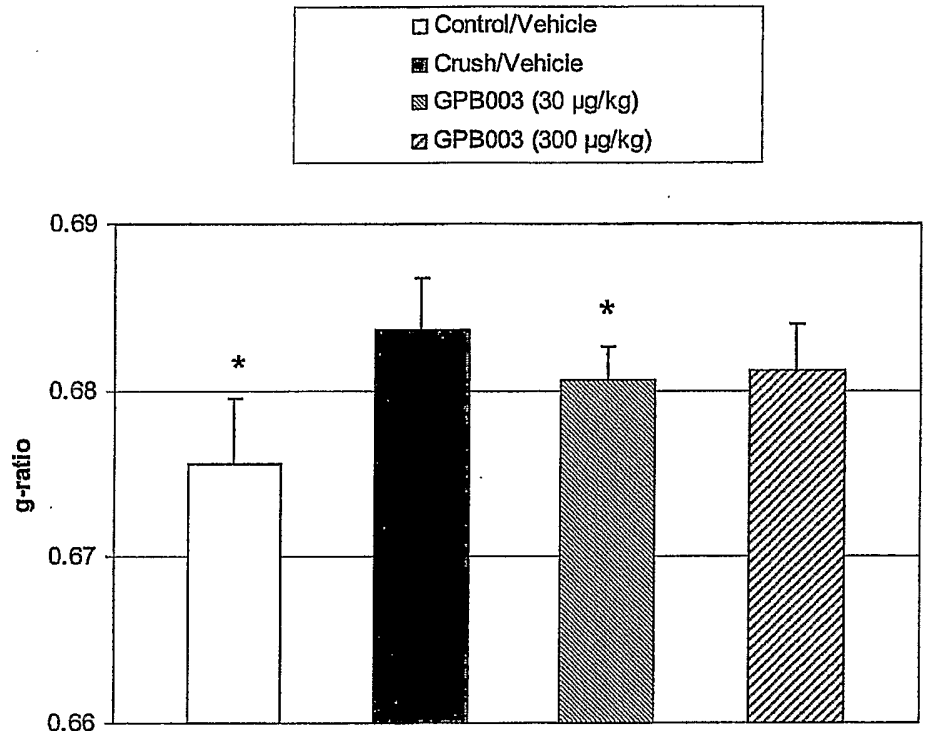


Mean ± s.e.m.

* $p \leq 0.05$, significantly different as compared to crush/Vehicle group.

Figure 9

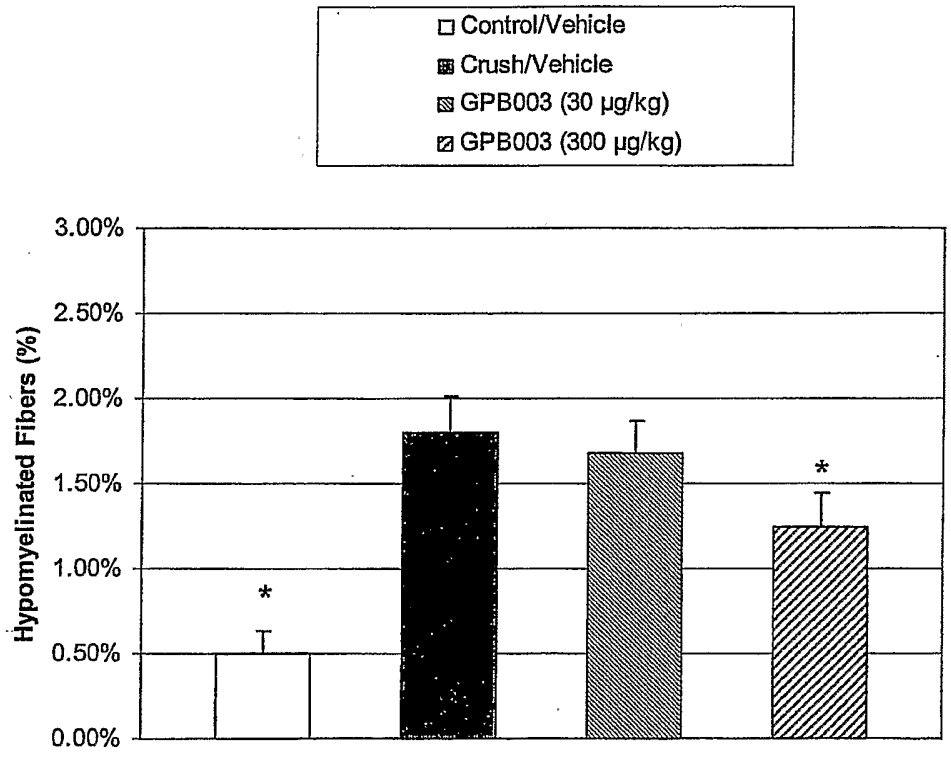
g-ratio



Mean ± s.e.m.
* $p \leq 0.05$, significantly different as compared to crush/Vehicle group.

Figure 10

Proportion of hypomyelinated fibers

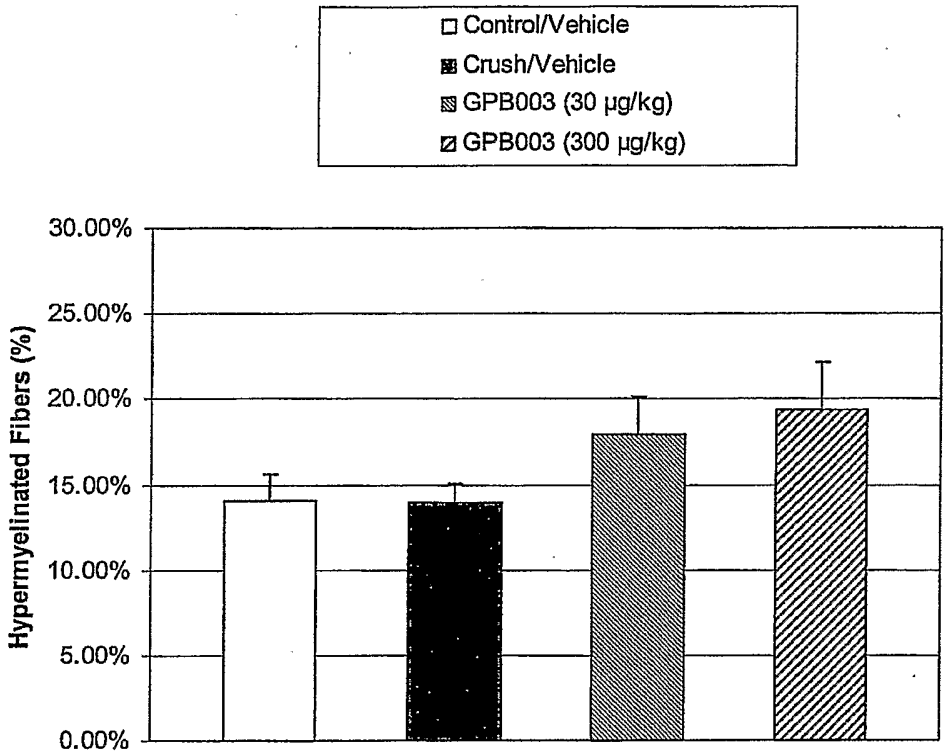


Mean ± s.e.m.

* $p \leq 0.05$, significantly different as compared to crush/Vehicle group.

Figure 11

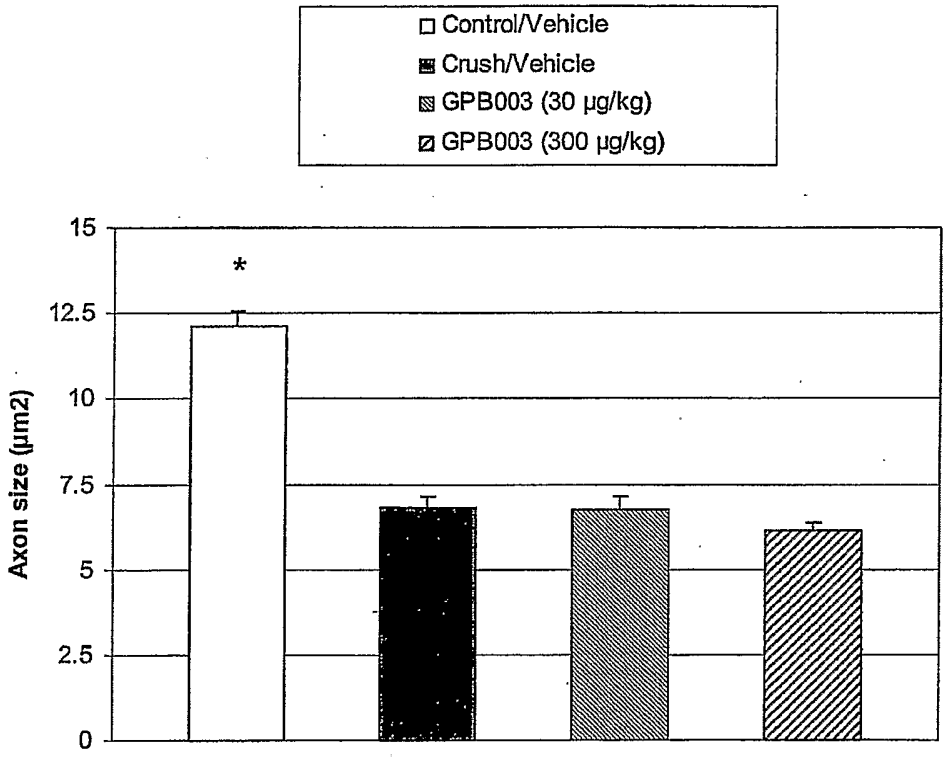
Proportion of hypermyelinated fibers



Mean ± s.e.m.
* $p \leq 0.05$, significantly different as compared to crush/Vehicle group.

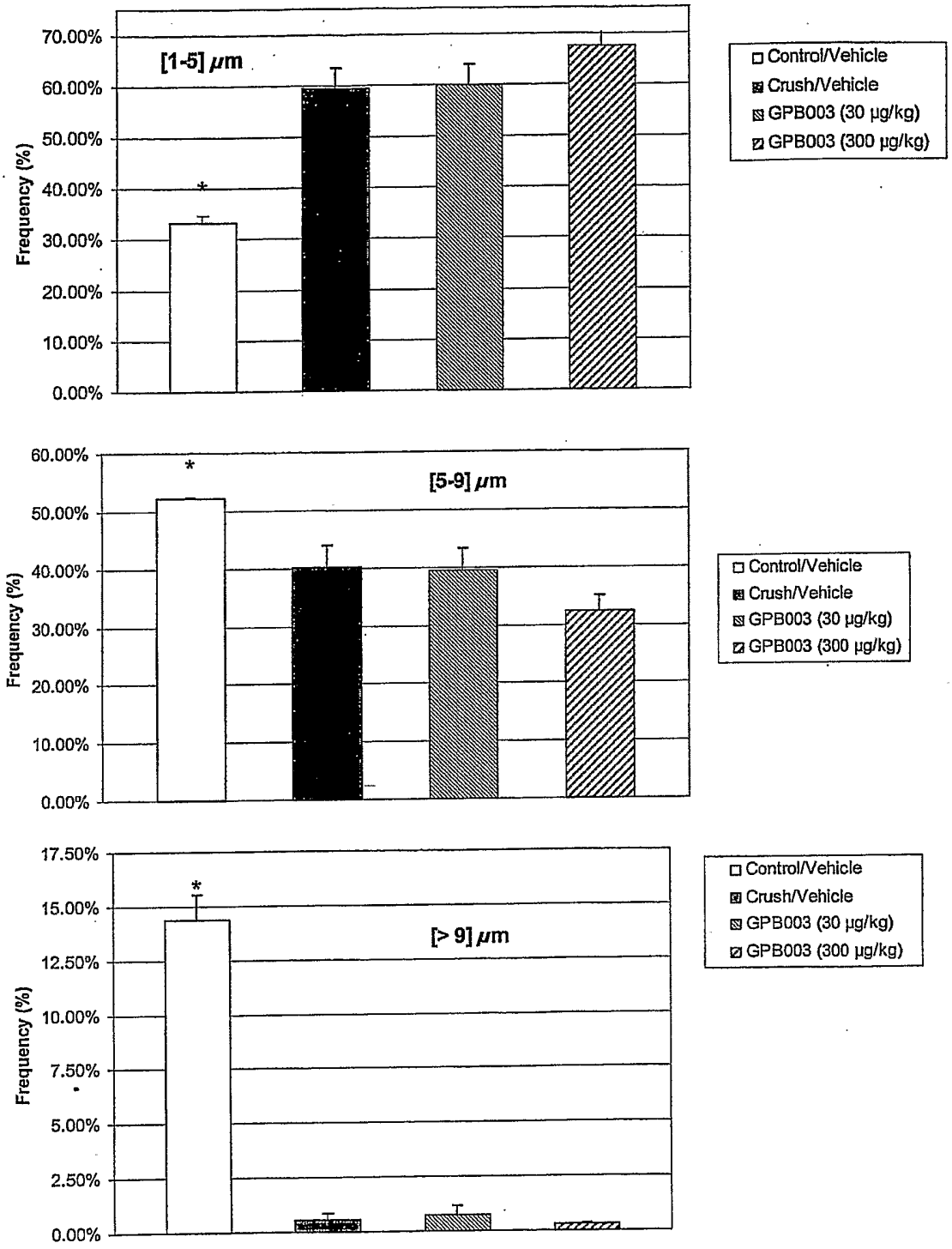
Figure 12

Axonal size



Mean ± s.e.m.
* $p \leq 0.05$, significantly different Crush/Vehicle group.

Size distribution **Figure 13**

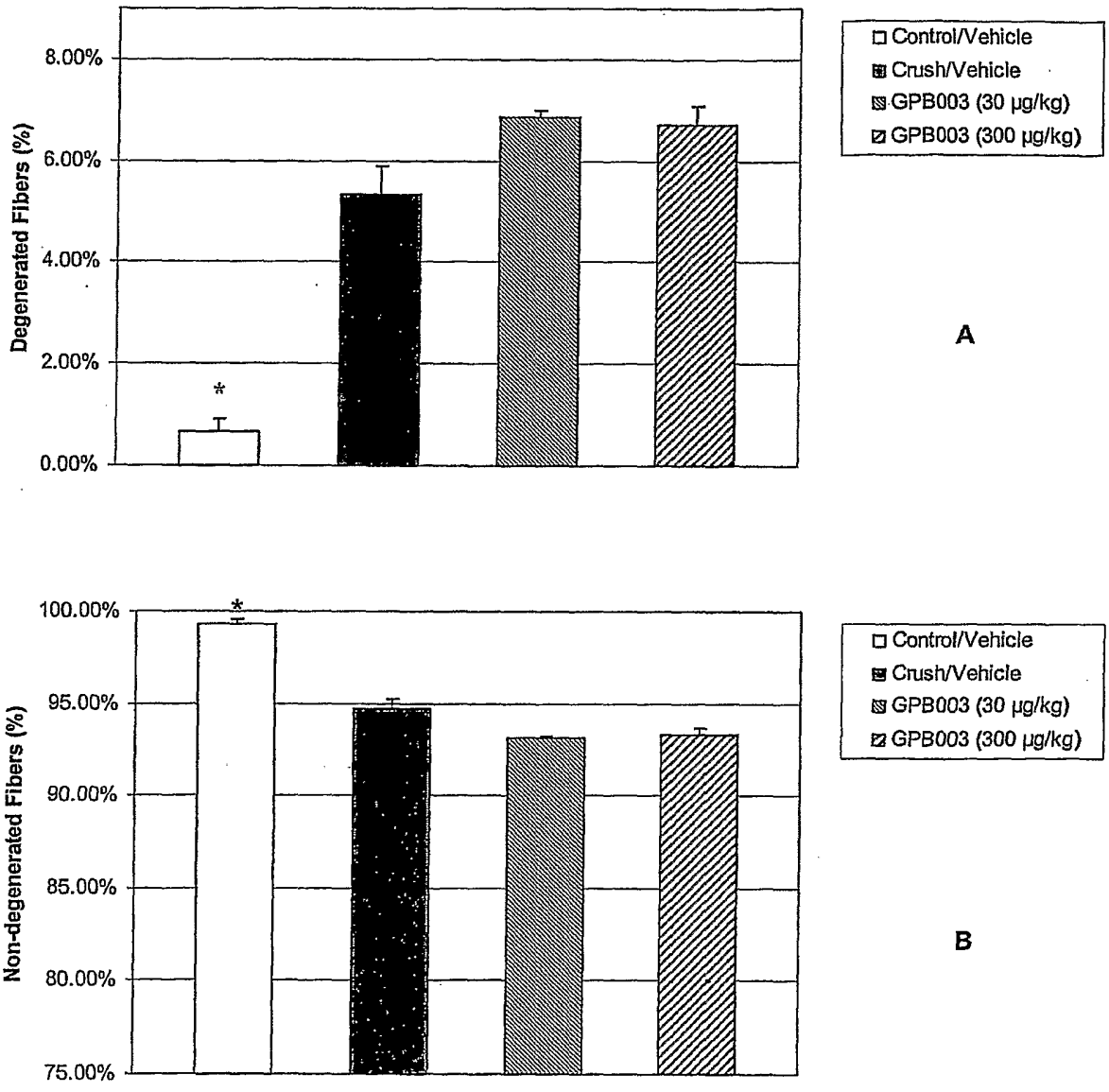


Mean ± s.e.m.

* $p \leq 0.05$, significantly different Crush/Vehicle group.

Figure 14

Proportion of degenerated and non-degenerated fibers



Mean ± s.e.m.

* $p \leq 0.05$, significantly different Crush/Vehicle group.

Figure 15: Clinical Features of Multiple Sclerosis

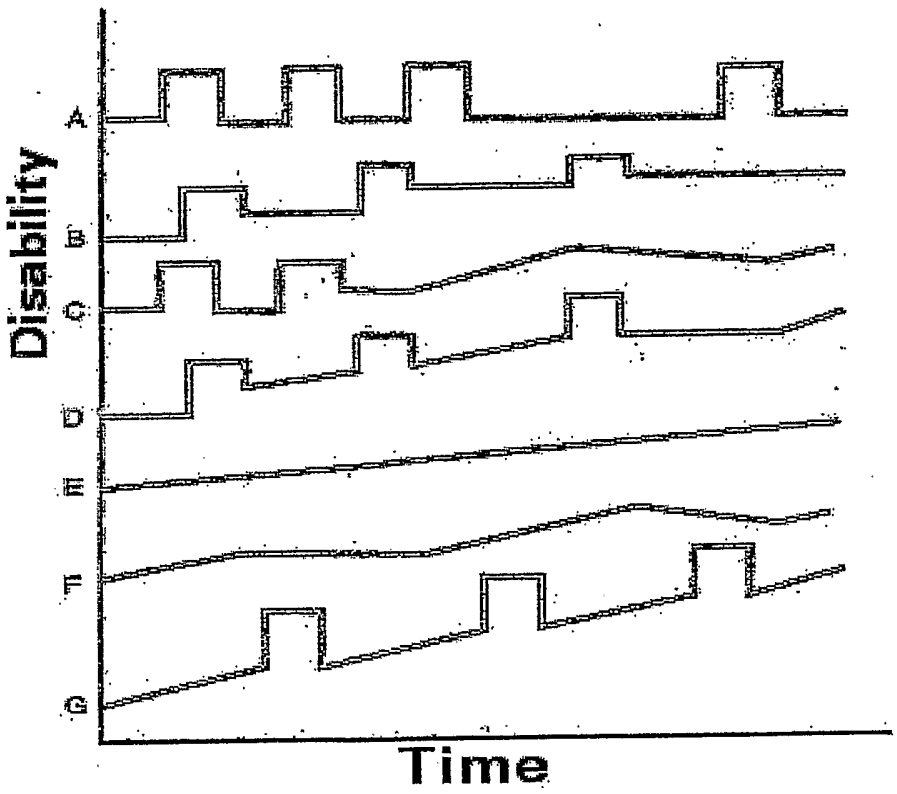


Figure 16

SEQ ID NO: 1

TVPGASDLFY WPR

SEQUENCE LISTING

<110> GeneProt, Inc.
Baussant, Thierry
Bougueleret, Lydie
Cusin, Isabelle
Mahe, Eve
Niknejad, Anne
Reffas, Samia

<120> POLYPEPTIDE SPECIES USEFUL FOR THE TREATMENT OF NEUROLOGICAL DISORDERS

<130> 5071-WO01

<150> US 60/587,641
<151> 2004-07-12

<150> US 60/663,882
<151> 2005-03-21

<160> 1

<170> PatentIn version 3.3

<210> 1
<211> 13
<212> PRT
<213> Homo sapiens

<400> 1

Thr Val Pro Gly Ala Ser Asp Leu Phe Tyr Trp Pro Arg
1 5 10

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/007566

A. CLASSIFICATION OF SUBJECT MATTER
C07K7/00 C07K14/705 C07K19/00 A61K38/08 A61P25/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 02/068600 A (ARENA PHARMACEUTICALS, INC; LIAW, CHEN, W; CHALMERS, DEREK, T; BEHAN,) 6 September 2002 (2002-09-06) page 4, lines 24,25 page 6, lines 4-7; figures 9,10; table B page 36, lines 1-19; table D examples 6,7; table F page 67, line 5 - page 68, line 18; sequence 9 ----- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
16 December 2005	02/01/2006

Name and mailing address of the ISA	Authorized officer
European Patent Office, P B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	MARAZZITI D ET AL: "Cloning of GPR37, a Gene Located on Chromosome 7 Encoding a Putative G-Protein-Coupled Peptide Receptor, from a Human Frontal Brain EST Library" GENOMICS, ACADEMIC PRESS, SAN DIEGO, US, vol. 45, no. 1, 1 October 1997 (1997-10-01), pages 68-77, XP004459139 ISSN: 0888-7543 abstract page 74, right-hand column, paragraph 1 - page 76, left-hand column, paragraph 1; figure 2	1-10
Y	MARAZZITI D ET AL: "MOLECULAR CLONING AND CHROMOSOMAL LOCALIZATION OF THE MOUSE GPR37 GENE ENCODING AN ORPHAN G-PROTEIN-COUPLED PEPTIDE RECEPTOR EXPRESSED IN BRAIN AND TESTIS" GENOMICS, ACADEMIC PRESS, SAN DIEGO, US, vol. 53, 1998, pages 315-324, XP004448985 ISSN: 0888-7543 abstract page 322, left-hand column, paragraph 2 - right-hand column, paragraph 1	1-10
Y	MARAZZITI DANIELA ET AL: "Altered dopamine signaling and MPTP resistance in mice lacking the Parkinson's disease-associated GPR37 / parkin-associated endothelin-like receptor" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 101, no. 27, 6 July 2004 (2004-07-06), pages 10189-10194, XP002359776 ISSN: 0027-8424 page 10189, left-hand column, paragraph 1 - right-hand column, paragraph 3 page 10192, right-hand column, last paragraph - page 10194, right-hand column, last paragraph	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2005/007566

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 7-10 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2005/007566

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02068600 A	06-09-2002	CA 2439383 A1	06-09-2002
		CN 1516706 A	28-07-2004
		EP 1412372 A2	28-04-2004
		JP 2004526441 T	02-09-2004