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(54) Title: PEPTIDE-BASED TREATMENT FOR NEURODEGENERATIVE DISEASES

(57) Abstract: Disclosed herein are peptide conjugates and methods for use of such for treating subjects with an axonal disorder. In an example, a conjugate includes a tetanus toxin peptide (Tet-1) with the amino acid sequence set forth by SEQ ID NO: 1 (HLNILSTLWKYR), an optional spacer moiety, an optional one or more amino acids, a tetrapeptide including at least two cysteine or two lysine residues. Methods of reducing or inhibiting one or more symptoms associated with an axonal disorder by administering a therapeutically effective amount of a pharmaceutical composition including a disclosed conjugate are also provided.



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PEPTIDE-BASED TREATMENT FOR NEURODEGENERATIVE DISEASES

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 61/264,076, filed on November 24, 2009, which is incorporated herein by reference in its entirety.

FIELD OF THE DISCLOSURE

This disclosure relates to the field of neurodegenerative diseases, specifically to Tet-1 fusion peptides and their use in the treatment of neurodegenerative diseases.

ACKNOWLEDGMENT OF GOVERNMENT SUPPORT

This invention was made with government support under Grant No. 5K01NS052183 awarded by the National Institutes of Health and Grant No. 1R21ES017225 awarded by the Fogarty International Center and the National Institutes of Environmental Health Sciences. The government has certain rights in the invention.

BACKGROUND

Neurodegeneration is often caused by misfolding of proteins such that they can no longer perform their cellular functions and instead trigger equivalent modifications in normal proteins, thus creating a cascade of damage that eventually results in significant neuronal death. Often neurodegeneration begins long before any symptoms are manifested. As such, diagnosis of a neurodegenerative disease tends to occur after the patient has already suffered the majority of the neural damages. Moreover, few therapies are available for the treatment of most neurodegenerative diseases even once the disease has been identified.

Amyotrophic lateral sclerosis (ALS) is one example of an adult-onset neurodegenerative disease characterized by the loss of specific motor neurons in the spinal cord, brainstem and cortex. ALS affects approximately 5 in 100,000 people

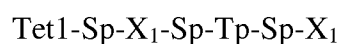
and has both familial and sporadic etiologies. As with many neurodegenerative diseases, currently available treatments have provided only temporary alleviations of the symptoms. Therefore, a therapeutic development that cures or inhibits the progression of this disorder as well as other neurodegenerative diseases is imperative.

SUMMARY OF THE DISCLOSURE

The treatment of neurodegenerative diseases has been disappointing for several reasons including, but not limited to: (1) lack of understanding of exact pathogenetic mechanisms; (2) possible involvement of multiple pathogenetic factors; (3) limitations in drug delivery to the central nervous system due to poor ability of certain compounds to cross the blood-brain barrier (BBB); and/or (4) intolerable side effects of drugs. Most drug candidates have been directed towards mitochondrial dysfunction, perturbation of energy metabolism, disruption of energy-dependent axonal transport, glutamate-mediated excitotoxicity, oxidative stress, and/or protein damage.

Herein are provided novel therapeutic agents that can be administered to axons/neurons by exploiting the physicochemical properties of Tet1 peptide (SEQ ID NO: 1: HLNILSTLWKYR), a tetanus toxin-derived peptide with no neurotoxic properties, that selectively translocates into neuronal networks.

As such, disclosed herein are Tet1 conjugates and methods of using such conjugates to prevent or inhibit an axonal disorder, such as a neurodegenerative disease. In one embodiment, a conjugate including the structure of:



is disclosed wherein Tet1 is a tetanus toxin peptide with the amino acid sequence set forth by SEQ ID NO: 1 (HLNILSTLWKYR), Sp is an optional spacer moiety, X₁ is an optional one or more amino acids, and Tp is a tetrapeptide including at least two cysteine or two lysine residues.

Also provided are pharmaceutical compositions including any of the disclosed conjugates and a pharmaceutically acceptable carrier. In one example, the pharmaceutical composition is for use in the manufacture of a medicament or for use as a medicament.

Methods of use of the disclosed conjugates are also provided, including methods of treating a neurodegenerative (axonal) disorder. In one example, a method of reducing or inhibiting one or more symptoms associated with an axonal disorder is disclosed. The method can include administering to the subject a therapeutically effective amount of one or more of the disclosed pharmaceutical compositions, thereby reducing or inhibiting one or more symptoms associated with the axonal disorder. Methods for modulating protein disulfide isomerase (PDI) and/or thioredoxin (THX) activity including contacting a cell, such as a neuronal cell (*e.g.*, a neuronal cell present in a mammal, such as a human) with a therapeutically effective amount of one or more agents including any one of the pharmaceutical compositions in which the pharmaceutical composition modulates the activity of PDI (such as membrane-associated PDI, mPDI) and/or THX in the treated cell relative to PDI and/or THX activity in an untreated cell, thereby reducing or inhibiting a 1,2-diacetylbenzene (1,2-DAB) mediated axonal disorder are also disclosed.

The foregoing and other features of the disclosure will become more apparent from the following detailed description of a several embodiments which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A provides the chemical structures of aliphatic and aromatic solvents (*left*), neurotoxic γ -diketone metabolites (*center*) and corresponding non-neurotoxic isomers (*right*).

FIG. 1B is a pair of schematics of motor neurons, the upper schematic showing a 2,5-HD-induced distal NF-filled swollen axon associated with axonal degeneration (d; m = muscle) and the lower image showing a 1,2-DAB-induced proximal axon swelling with nerve fiber atrophy distally along the length of the nerve fiber. Non-protein reactants 1,3-DAB and 2,3-HD did not cause axonopathy.

FIG. 2 is a chemical synthesis pathway showing amino acid (lysine) and protein reactivity of 2,5-HD and 1,2-DAB lead to similar high-molecular weight coloric adducts and/or polymers (numbered 1-9).

FIG. 3 is a schematic showing that unfolded protein response (UPR) signaling primarily involves interactions between the endoplasmic reticulum-associated degradation (ERAD)-master regulator polypeptide binding protein (BiP; also known as GRP-78) with three ER-membrane sensors namely inositol-requiring kinase 1 (IRE1), double-stranded RNA-activated protein-like ER kinase (PERK), and activating transcription factor 6 (ATF6). Under normal circumstances, BiP binds to IRE-1 and PERK. Under stress conditions including a variety of protein-PTM (*e.g.*, *S*-nitrosylation), BiP is released and permits the activation of IRE1, PERK, ATF6, and that of their downstream events, including the activation of repair (anti-oxidative) mechanisms, increased transcription/translation of chaperones (*e.g.*, PDI or of apoptotic executioners such as CHOP and caspases).

FIG. 4A is a diagram showing representative PDI-family members and conserved THX-like domains cysteine-glycine-histidine-cysteine (SEQ ID NO: 2) that make up their enzymatic active sites a and a' (b and b' have not yet well-understood functions). PDI = protein disulfide isomerase, ERp57 = ER protein 57, PDIP = PDI placenta, ERp72 = ER protein 72.

FIG. 4B is a schematic showing the mode of action of PDI involving sulfur exchange and isomerization reactions, and disulfide bond formation.

FIG. 5 is a graph showing the transport velocity of mitochondria over time for 1,2-DAB (solid line, filled circle), 1,3-DAB (dashed line, plus), or vehicle (dotted line, open circle). Although initial velocities (Time 0) are higher for 1,2-DAB and 1,3-DAB relative to vehicle, transport rate was decreased only by neurotoxic 1,2-DAB over time (11 % decrease every 10 minutes, $p < 0.001$). Transport of mitochondria remained steady with non-neurotoxic isomer 1,3-DAB or vehicle.

FIG. 6 is a series of diagrams including a Marquand diagram (right) showing the joint distribution of differentially expressed genes among the pairwise comparisons shown. Boxes in the Marquand diagram are indicated in the Venn diagram (left) for comparison. M_0 is the total number of genes analyzed. Numbers of genes modulated (up or down) are shown for the 1-hour and 1-week time points from the single-dose study and for four time points from the repeat-dose study (rep'd). Genes contained in boxes m_2 and m_3 are modulated by 1,3-DAB, which

does not induce motor neuron disease, while those in boxes m_4 and m_5 are modulated by 1,2-DAB, which does induce motor neuron disease. Those in m_5 represent the most conservative set of genes associated with the neurotoxic property of 1,2-DAB given their absence in comparisons with either vehicle (control) or 1,3-DAB as negative DAB control.

FIGS. 7A and 7B are digital images illustrating the relative abundance of PDI in rat spinal cord. Samples treated with vehicle (FIG. 7A) had 2.86 X higher amount of PDI relative to samples from animals treated with protein- reactive 1,2-DAB (FIG. 7B). Relative abundance of proteins was analyzed using the DeCyder software (GE Healthcare, NJ).

FIG. 8A is a series of protein immunoblots of cPDI (58kDa), mPDI (58kDa), and a *S*-nitrosylated protein band (approximately 60 kDa band, possibly PDI) in animals treated with vehicle or 1,2-DAB.

FIG. 8B is a bar graph illustrating 1,2-DAB lowered the abundance of cPDI (32.8%, $p < 0.001$, respectively, relative to vehicle).

FIG. 8C is a bar graph showing mPDI expression increased in 1,2-DAB-treated animals (18.0% relative to vehicle, $p < 0.01$).

FIG. 8D is a bar graph illustrating *S*-nitrosylation significantly occurred in 1,2-DAB- relative to vehicle-treated animals (2.7-fold, $p < 0.001$). Data analyzed by ANOVA followed by Tukey's post hoc analysis. Results are expressed as the mean $\pm$ standard error of the mean.

FIG. 9 is a protein immunoblot showing significant increased expression of ~ 150 KDa Spna2 -fragments in the lumbar spinal cord of animals treated systemically with vehicle versus 1,2-DAB, a pattern that suggests an activation of caspase/calpain proteolytic enzymes. Levels of native Spna2 appeared to be reduced in 1,2-DAB samples relative to vehicle-treated samples.

FIG. 10 is a flow chart illustrating an exemplary signal transduction pathway involved in axonal degeneration. First, 1,2-DAB or *S*-nitrosylation adducts free soluble (cytosolic) PDI/THX and reduces neuroprotein-folding capabilities which, in turn, leads to ER-associated degradation (ERAD) of proteins. ERAD mechanisms include transcription of ER chaperones, notably PDI, and increased synthesis of PDI that results in increase of membrane-bound PDI (mPDI), a well-known

transnitrosylating agent. Simultaneous increase in mPDI and decrease in the denitrosylating agent thioredoxin (THX) lead to increased *S*-nitrosylation of selected neuroproteins. Protein misfolding and *S*-nitrosylation trigger ERAD mechanisms including the induction/activation of CHOP, proteolytic enzymes, and increased cleavage of structural proteins *e.g.*, Spna 2.

FIGS. 11A-11D are schematic representations of the genomic structure of the wild-type murine *Spna2* sensitive allele (*Spna2*^S, FIG. 11A), the targeting vector (FIG. 11B), the targeted *Spna2* allele (FIG. 11C), and the *Spna2* resistant (*Spna2*^R) between exons 20 and 30 (FIG. 11D). Exons encoding the CSD domain are represented by white boxes. A floxed PGK-hygromycin cassette (PGK-hygro) (the arrow indicates the transcriptional orientation), was introduced into intron 24. The LoxP sites are represented by white arrowheads. After recombination with a Cre transgenic line, the resulting *Spna2*^R allele lacks the CSD domain and encodes the mutant *Spna2*.

FIG. 12 is a diagram showing the expected ordering of average response for protein expression, mRNA expression and sNO in animals treated with 1,2-DAB (left panel) and “therapeutic” agents (right panel).

FIGS. 13A -13D are mass spectra of Tet1 or Tet1-PLYS prior to (FIG. 13A and 13C, respectively) and following (FIG. 13B and 13D, respectively) 15 mM NaOCN treatment demonstrating carbamylation of Tet1 or Tet1-PLYS following such treatment.

FIGS. 14A and 14B are mass spectra showing intermolecular disulfide-bond formation was also detected following the reaction of Tet1-THO with hydrogen peroxide.

FIGS. 15A-15C are chromatographic MRM profiles of Tet1-PLYS (FIG. 15A), Tet1 (FIG. 15B) and Tet1-THO (FIG. 15C).

FIGS. 16A-16C are dose response curves of Tet1 and Tet1 derivatives.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid

sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NO: 1 is an amino acid sequence for a tetanus toxin peptide (Tet1);

SEQ ID NO: 2 is a consensus amino acid sequence for an exemplary tetrapeptide;

SEQ ID NO: 3 is an amino acid sequence for an exemplary conjugate;

SEQ ID NO: 4 is a consequence amino acid sequence for an exemplary tetrapeptide;

SEQ ID NO: 5 is an amino acid sequence for an exemplary tetrapeptide;

SEQ ID NO: 6 is an amino acid sequence for an exemplary conjugate; and

SEQ ID NOs: 7-18 are nucleic acid sequences for synthetic oligonucleotide primers.

The Sequence Listing is submitted as an ASCII text file in the form of the file named Sequence.txt, which was created on November 16, 2010, and is 4,314 bytes, which is incorporated by reference herein.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

I. Overview of Several Embodiments

Axonopathy-inducing neurotoxic chemicals bind to neuroproteins, but it is unclear which protein targets are critical for neurodegeneration. The inventor found that 1,2-DAB lowers the abundance of both soluble cysteine-dependent thioredoxin (THX) and protein disulfide isomerase (PDI), enzymes in controlling endoplasmic reticulum (ER)-mediated protein folding. The expression of (surface) membrane-associated PDI (mPDI) was increased and three protein-immunoblot bands are highly *S*-nitrosylated. Protein *S*-nitrosylation occurred without increased production of oxidative/nitrosative species suggesting that *S*-nitrosylation resulted from an increase in mPDI-mediated transnitrosylation and/or a reduction in THX-mediated denitrosylation. In addition, α 2-spectrin (Spna2), a protein that involved in maintaining axo-glial junctions and the integrity of the cytoskeleton, was cleaved producing two ~150 KDa Spna2 fragments, thus possibly *via* activation of caspase 3 and/or μ calpain. Dysregulation of the PDI/THX system and cleavage of Spna2

indicate that ER-mediated mechanisms of protein degradation (ERAD) are involved in 1,2-DAB axonopathy.

1,2-DAB and 2,5-hexanedione (HD), respectively induce proximal (1,2-DAB) and distal (2,5-HD) axonal swellings filled with 10nm-neurofilaments (NF) in elongated axons (FIGS. 1A and 1B). Atrophy and Wallerian-like degeneration occur distal to the swellings. These two compounds share a γ -spacing of their two carbonyl groups, a molecular arrangement that confers protein-reactive properties to the molecules. 1,2-DAB and 2,5-HD, but not their non-neurotoxic isomers (1,3-DAB and 2,3-HD, respectively), react with ϵ -amino groups or thiol-groups in lysine or cysteine moieties of proteins, respectively. This reaction leads to the formation of protein adducts (including polymers) as illustrated in FIG. 2. The non-protein-reactive isomers 1,3-DAB and 2,3-DAB do not cause axonopathy, indicating a link between the protein reactivity of the γ -diketone moiety of 1,2-DAB and 2,5-HD and their axonopathic property. Protein adduction by γ -diketone-like compounds, including 1,2-DAB and 2,5-HD, is accompanied by loss of function of the adducted proteins.

Survival of neurons relies on complex mechanisms of quality control to ensure that proteins function properly to maintain high levels of efficiency in cell signaling, maintenance, growth and differentiation, and death if needed. The ability of neuroproteins to carry out these activities depends on their ability to maintain proper structures and undergo conformational changes (*e.g.*, adopting proper folding) to adapt to constantly changing cellular environments. This capability may, however, be compromised by several factors, including genetic and/or aberrant posttranslational modifications (PTM). Aberrant PTM include oxidation, alkylation, and nitration, to name a few. *S*-nitrosylation regulates the activity of selected proteins in the nervous system.

Under normal circumstances, misfolded proteins are subjected to a clean-up process that is mediated through ER mechanisms. Typically, the first ER line of response to protein misfolding, termed “unfolded protein response (UPR)”, increases the transcription of chaperones, *e.g.*, PDI, to help repair misfolding (*vide infra*). This line of response may also lead to a reduction in levels of transcription and/or

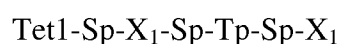
translation necessary for protein synthesis. The second line is aimed at “recycling” and degrading aberrantly folded proteins through ERAD. Upstream regulation of ERAD is mediated through the interactions between BiP (also known as GRP-78) and membrane sensors IRE1, PERK, and ATF6. The downstream effects of ERAD mechanisms include degradation of structural proteins and axonal (neuronal) death. Protein degradation is executed *via* induction/activation of pro-apoptotic CCAAT/enhancer-binding protein-homologous protein (CHOP), caspases, and that of other proteases including calpains that reportedly cross-talk with caspases (FIG. 3).

A central feature of the ER response to protein misfolding is the fine regulation (transcription/translation) of PDI, an enzyme that helps unfolded and/or misfolded proteins to fold correctly through sulfur exchange and isomerization reactions, and through formation of disulfide bonds (FIG. 4B). The activity of PDI is mediated through its two cysteine-dependent active domains a and a' (FIG. 4A).

PDI/THX family members are also involved in other functions including transferring of nitric oxide (NO) across biological membranes, participating in defense mechanisms against xenobiotics, and conferring mammalian resistance to drugs, just to name a few. Membrane-associated PDI (mPDI) acts as a transnitrosylating agent transferring NO across membranes, while THX acts as a denitrosylating agent. These two activities can be modulated through inhibition of PDI using bacitracin or administration of THX. The ability of 1,2-DAB to adduct neuroproteins, alter the functional properties of the PDI/THX system, and cause axonopathy makes it a probe to examine protein modifications and ER mechanisms associated with nerve fiber (axon) degeneration. Herein, the inventor exploits the neuroprotein-reactive properties of 1,2-DAB, the active neurotoxic metabolite of the solvent 1,2-diethylbenzene (1,2-DEB), to probe mechanisms of nerve fiber (axon) degeneration. 1,2-DAB induced proximal giant axonopathy featured by giant neurofilament-filled axonal swellings at proximal segments of axons. This pathological feature is seen in a host of neurodegenerative diseases notably amyotrophic lateral sclerosis. They may also be seen in Parkinson Disease, hereditary giant axonopathy, or dementia. The inventor tested the hypothesis that 1,2-DAB axonopathy was mediated by an imbalance in the protein disulfide

isomerase/thioredoxin (PDI/THX) system which, in turn, led to irreversible S-nitrosylation and activation of the ERAD of neuroproteins. A combination of functional assessment, molecular biology, and proteomic techniques were used to (1) determine time-course changes in the posttranslational modifications and expression/activity of PDI/THX in relation to S-nitrosylation and ERAD mechanisms in the Sprague-Dawley rat treated with axonopathy-inducing 1,2-DAB; (2) determine whether systemic treatment with bacitracin (PDI-inhibitor) or recombinant human THX (rhTHX, THX enhancer), or selective axon-delivery of Tet1PLYS or Tet1THO or Tet1-THX (putative custom-synthesized 1,2-DAB-scavengers), conferred protection against 1,2-DAB axonopathy; and (3) determine whether inhibition of calpains and/or a targeted mutation (deletion) of the caspase/calpain sensitive domain of α -2 spectrin (in the Spna2^{tm1.1Gnic} mutant) also protected against axonal pathology. The disclosed results are relevant to neurodegeneration and occupational/environmental health for they identify mechanisms by which protein posttranslational modifications, notably S-nitrosylation, led to nerve fiber (axon) degeneration, establish the ability of Tet1-mediated selective axon delivery of small molecules and targeted mutations (*e.g.*, Spna2^{tm1.1Gnic} mutation) in the treatment of axonal disease, and identify mechanisms of neurotoxicity associated with 1,2-DAB and hence, its parent solvent 1,2-DEB. Thus, this disclosure exploits the physicochemical properties of man-made and/or natural toxicants with high tropism for the nervous system to provide methods for treating and preventing nerve fiber (axon) damage.

As such, disclosed herein are conjugates and methods of using such conjugates to treat neurodegenerative diseases. In one embodiment, a conjugate including the structure of:



is disclosed wherein Tet1 is a tetanus toxin peptide with the amino acid sequence set forth by SEQ ID NO: 1 (HLNILSTLWKYR), Sp is an optional spacer moiety, X₁ is an optional one or more amino acids, and Tp is a tetrapeptide including at least two cysteine or two lysine residues. In one example, the tetrapeptide

includes at least two cysteine residues. In other examples, the tetrapeptide includes the amino acid sequence set forth by SEQ ID NO: 2 (CX₂X₃C) in which C is a cysteine and X₂ and X₃ are any amino acid, such as any polar or basic amino acid. In one particular example, X₂ and X₃ are glycine or histidine, such as in SEQ ID NO: 3 (HLNILSTLWKYRCGHC). In some examples, a tetrapeptide includes at least two lysine residues. For example, the tetrapeptide includes the amino acid sequence set forth by SEQ ID NO: 4 (KX₂X₃K) in which K is a lysine and X₂ and X₃ are any amino acid. In even further examples, the tetrapeptide includes at least three lysine residues. In one particular example, the tetrapeptide includes four lysines (SEQ ID NO: 5), such as the conjugate set forth by SEQ ID NO: 6 (HLNILSTLWKYRKKKK). In some examples, the disclosed conjugates include a label, such as a fluorescent label (*e.g.*, 5-carboxyfluorescein).

Also provided are pharmaceutical compositions including any of the disclosed conjugates and a pharmaceutically acceptable carrier. In one example, the pharmaceutical composition is for use in the manufacture of a medicament or for use as a medicament. In a specific example, the pharmaceutical composition is formulated for intracerebroventricular (ICV) administration.

Methods of use of the disclosed conjugates are also disclosed. In one example, a method of reducing or inhibiting one or more symptoms associated with an axonal disorder is disclosed. The method includes administering to the subject (for example via ICV administration) a therapeutically effective amount of one or more of the disclosed pharmaceutical compositions, thereby reducing or inhibiting one or more symptoms associated with the axonal disorder.

In certain examples, motor performance, PDI/THX expression and folding activity, *S*-nitrosylation, ERAD-specific response, or combinations thereof, are improved in the subject. Improvement can include one or more of an increase in motor performance, a decrease in membrane-bound PDI expression, an increase in soluble PDI expression, an increase in THX expression, or a decrease in *S*-nitrosylation as compared to motor performance, PDI/THX expression and folding activity, or *S*-nitrosylation prior to administration of the therapeutically effective amount of the conjugate-containing pharmaceutical composition.

Also disclosed is a method for modulating PDI and/or THX activity including contacting a cell, such as a neuronal cell (*e.g.*, a neuronal cell present in a mammal, such as a human) with a therapeutically effective concentration of one or more agents including any one of the pharmaceutical compositions in which the pharmaceutical composition modulates the activity of PDI (such as mPDI) and/or THX in the treated cell relative to PDI and/or THX activity in an untreated cell, thereby reducing or inhibiting a 1,2-DAB-mediated axonal disorder. In one example, modulating activity of PDI includes reducing and or inhibiting mPDI-mediated transnitrosylation. In other examples, modulating activity of THX includes increasing THX-mediated denitrosylation. In some examples, modulate the activity of PDI and/or THX includes decreasing *S*-nitrosylation of at least one target protein as compared to *S*-nitrosylation of the at least one target protein (such as mPDI) as in an untreated cell. In an example, contacting the cell with one or more agents comprises administering the one or more agents to the mammal.

In some examples, the axonal disorder is a disorder associated with proximal giant axonopathy. Exemplary axonal disorders, include, but are not limited to, a neuropathy associated with exposure to a neurotoxic solvent that form a gamma-diketone compound (such as n-hexane), solvent (1,2-diethylbenzene and/or n-hexane, or gamma-diketone) neuropathy, or neuropathies associated with production of protein adducts molecules (such as gamma-keto-aldehydes, oxidative metabolites of arachidonic acid), ALS (also known as Lou Gehrig's disease), Alzheimer's, diabetic neuropathy, uremic neuropathy (kidney failure), dementia, Corticobasal degeneration, Creutzfeldt-Jakob disease, familial fatal insomnia, frontotemporal lobar degeneration, Huntington's disease, HIV-associated dementia, Kennedy's disease, Krabbe's disease, Lewy body dementia disease, multiple sclerosis, konzo, tropical ataxic neuropathy, ALS/Parkinson's disease, Lathyrism, primary lateral sclerosis, spinal muscular atrophy or a combination thereof.

Kits for reducing or inhibiting one or more symptoms associated with an axonal disorder including one or more of the disclosed pharmaceutical compositions are also disclosed. Additionally, kits include additional compounds, such as protease and/or proteasome inhibitors. Pharmaceutical compositions can be used

alone or in association with other combinations such as protease and/or proteasome inhibitors.

II. Abbreviations and Terms

1,2-DAB:	1,2-diacetylbenzene
1,2-DEB:	1,2-diethylbenzene
2,5-HD:	2,5-hexanedione
ALD:	adrenoleukodystrophy
ALS:	amyotrophic lateral sclerosis
BBB:	blood brain barrier
BME:	β -mercaptoethanol
BSE:	bovine spongiform encephalopathy
CNS:	central nervous system
ER:	endoplasmic reticulum
ERAD:	ER-associated degradation
ICV:	intracerebroventricular
mPDI:	membrane-associated protein disulfide isomerase
MRI:	magnetic resonance imaging
MS:	multiple sclerosis
NAA:	n-acetyl aspartate
NF:	neurofilaments
NO:	nitric oxide
NOS:	nitric oxide synthase
PCR:	polymerase chain reaction
PD:	Parkinson's disease
PDI:	protein disulfide isomerase
PLP:	proteolipid protein
PMCA2:	ATPase calcium transporting plasma membrane 2
PTM:	posttranslational modification
SPNA2	α 2-spectrin
THX:	thioredoxin

The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. The singular forms “a,” “an,” and “the” refer to one or more than one, unless the context clearly dictates otherwise. For example, the term “comprising a nucleic acid molecule” includes single or plural nucleic acid molecules and is considered equivalent to the phrase “comprising at least one nucleic acid molecule.” The term “or” refers to a single element of stated alternative elements or a combination of two or more elements, unless the context clearly indicates otherwise. As used herein, “comprises” means “includes.” Thus, “comprising A or B,” means “including A, B, or A and B,” without excluding additional elements.

Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

Administration: To provide or give a subject an agent, such as a pharmaceutical composition including a disclosed conjugate, by any effective route. Exemplary routes of administration include, but are not limited to, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), oral, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes. A particular type of administration is intracerebroventricular (ICV) administration.

Alzheimer's disease (AD): A progressive brain disorder that occurs gradually and results in memory loss, behavioral and personality changes, and a decline in mental abilities. These losses are related to the death of brain cells and the breakdown of the connections between them. The course of this disease varies from person to person, as does the rate of decline. On average, AD patients live for 8 to 10 years after they are diagnosed, though the disease can last up to 20 years. AD advances by stages, from early, mild forgetfulness to a severe loss of mental function. At first, AD destroys neurons in parts of the brain that control memory,

especially in the hippocampus and related structures. As nerve cells in the hippocampus stop functioning properly, short-term memory fails. AD also attacks the cerebral cortex, particularly the areas responsible for language and reasoning.

Amyotrophic lateral sclerosis (ALS): A progressive, usually fatal, neurodegenerative disease caused by the degeneration of motor neurons. As a motor neuron disease, the disorder causes muscle weakness and atrophy throughout the body as both the upper and lower motor neurons degenerate, ceasing to send messages to muscles. Unable to function, the muscles gradually weaken, develop fasciculations (twitches) because of denervation, and eventually atrophy because of that denervation. The patient may ultimately lose the ability to initiate and control all voluntary movement except for the eyes. ALS is also known as Lou Gehrig's disease.

Axonal disorder: A disorder associated with axon damage. Axon damage includes axon degeneration and a reduction in axon density, for example in the white matter of the caudal spinal cord. White matter tissue damage includes axons undergoing Wallerian-like degeneration, reduced nerve fiber density, and demyelination. White matter tissue damage can be determined by histological examination of white matter, for example from the ventrolateral or dorsal thoracic spinal cord. White matter tissue damage may also be determined by MRI. Evidence of axonal damage can be inferred from presence of abnormal MRI signals, such as permanently decreased T₁ signals ("black holes"), decreased n-acetyl aspartate (NAA) and whole brain atrophy.

Axon damage also includes decreased neurofilament phosphorylation (NF-P) (see *e.g.* Trapp *et al.*, *N. Engl. J. Med.* 338:278-285, 1998). Neurofilaments in myelinated axons are normally heavily phosphorylated. NF-P can be determined by immunohistochemical staining. A reduction in NF-P reflects demyelination and axon damage.

Decreasing axon damage in a subject includes a reduction in white matter tissue damage as compared with an untreated subject, such as a reduction in the decrease in NF-P as compared with an untreated subject. Decreasing axon damage also encompasses preventing axon damage and repair of axon damage. Repair of axon damage in a subject includes a reduction in white matter tissue damage or a

reduction in the decrease in NF-P as compared with an earlier time point, for example prior to beginning treatment with other compounds used to treat an axonal disorder, including a neurodegenerative disease.

In one example, the axonal disorder is a disorder associated with proximal giant axonopathy. In some examples, an axonal disorder is a neuropathy associated with exposure to a neurotoxic solvent that form a gamma-diketone compound (such as n-hexane), solvent (1,2-diethylbenzene and/or n-hexane, or gamma-diketone) neuropathy, or neuropathies associated with production of protein adducts molecules (such as gamma-keto-aldehydes, oxidative metabolites of arachidonic acid), ALS (Lou Gehrig's), Alzheimer's, Corticobasal degeneration, Creutzfeldt-Jakob disease, familial fatal insomnia, frontotemporal lobar degeneration, Huntington's disease, HIV-associated dementia, Kennedy's disease, Krabbe's disease, Lewy body dementia disease, diabetic neuropathy, uremic neuropathy (kidney failure), dementia, multiple sclerosis, konzo, tropical ataxic neuropathy, Parkinson's disease (PD), ALS/PD, Lathyrism, primary lateral sclerosis, or spinal muscular atrophy or a combination thereof. In one example, an axonal disorder is a 1,2-diacetylbenzene (1,2-DAB) mediated axonal disorder.

Blood-brain barrier (BBB): The barrier formed by epithelial cells in the capillaries that supply the brain and central nervous system. This barrier selectively allows entry of substances such as water, oxygen, carbon dioxide, and nonionic solutes such as glucose, alcohol, and general anesthetics, while blocking entry of other substances. Some small molecules, such as amino acids, are taken across the barrier by specific transport mechanisms.

Conjugate: A molecule which does not occur in nature. In one example, a conjugate includes the structure of: Tet1-Sp-X1-Sp-Tp-Sp-X1 wherein Tet1 is a tetanus toxin peptide with the amino acid sequence set forth by SEQ ID NO: 1 (HLNILSTLWKYR), Sp is an optional spacer moiety, X1 is an optional one or more amino acids, and Tp is a tetrapeptide including at least two cysteine or two lysine residues.

Contacting: Placement in direct physical association; includes both in solid and liquid form. Contacting includes contact between one molecule and another

molecule. Contacting can occur *in vitro* with isolated cells or tissue or *in vivo* by administering to a subject.

Decrease: To reduce the quality, amount, or strength of something. In one example, a therapy decreases or reduces axonal degeneration. For example, a therapy decreases or inhibits the activity of THX and/or PDI (or one or more symptoms associated with an axonal disorder, such axonal degeneration), for example as compared to the response in the absence of the therapy (such as a therapy administered to affect axonal degeneration by, for example, modulating THX and/or PDI activity or expression). Such decreases can be measured using the methods disclosed herein as well as those known to one of ordinary skill in the art.

Differential expression: A difference, such as an increase or decrease, in the conversion of the information encoded in a gene (such as a gene that encodes THX or PDI) into messenger RNA, the conversion of mRNA to a protein, or both. In some examples, the difference is relative to a control or reference value, such as an amount of gene expression that is expected in a subject who does not have an axonal disorder or in a normal neuronal cell sample. Detecting differential expression can include measuring a change in gene expression.

Downregulated or inactivation: When used in reference to the expression of a nucleic acid molecule, such as a gene, refers to any process which results in a decrease in production of a gene product. A gene product can be RNA (such as mRNA, rRNA, tRNA, and structural RNA) or protein. Therefore, gene downregulation or deactivation includes processes that decrease transcription of a gene or translation of mRNA. Examples of genes whose expression is downregulated in association with axonal degeneration include those provided in Table 1 (such as myelin proteolipid protein (PLP), an abundant myelin structural protein, and ATPase calcium transporting plasma membrane 2 (PMCA2)).

Examples of processes that decrease transcription include those that facilitate degradation of a transcription initiation complex, those that decrease transcription initiation rate, those that decrease transcription elongation rate, those that decrease processivity of transcription and those that increase transcriptional repression. Gene downregulation can include reduction of expression above an existing level. Examples of processes that decrease translation include those that decrease

translational initiation, those that decrease translational elongation and those that decrease mRNA stability.

Gene downregulation includes any detectable decrease in the production of a gene product. In certain examples, production of a gene product decreases by at least 2-fold, for example at least 3-fold or at least 4-fold, as compared to a control (such an amount of gene expression in a normal neuronal cell). In one example, a control is a relative amount of gene expression or protein expression in a biological sample taken from a subject who does not have an axonal disorder.

Increase, upregulated or activation: When used in reference to the expression of a nucleic acid molecule, such as a gene, refers to any process which results in an increase in production of a gene product. A gene product can be RNA (such as mRNA, rRNA, tRNA, and structural RNA) or protein. Therefore, gene upregulation or activation includes processes that increase transcription of a gene or translation of mRNA. Specific examples of molecules that are upregulated in an axonal disorder include mPMI.

Examples of processes that increase transcription include those that facilitate formation of a transcription initiation complex, those that increase transcription initiation rate, those that increase transcription elongation rate, those that increase processivity of transcription and those that relieve transcriptional repression (for example by blocking the binding of a transcriptional repressor). Gene upregulation can include inhibition of repression as well as stimulation of expression above an existing level. Examples of processes that increase translation include those that increase translational initiation, those that increase translational elongation and those that increase mRNA stability.

Gene upregulation includes any detectable increase in the production of a gene product. In certain examples, production of a gene product increases by at least 2-fold, for example at least 3-fold or at least 4-fold, as compared to a control (such an amount of gene expression in a normal neuronal cell). In one example, a control is a relative amount of gene expression in a biological sample, such as in a sample obtained from a subject that does not have an axonal disorder.

Inhibiting or Treating a Disease: Inhibiting the full development of a disease or condition, for example, in a subject who is at risk for a disease such as

axonal disorder. “Treatment” refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. As used herein, the term “ameliorating,” with reference to a disease, pathological condition or symptom, refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, a reduction in the number of relapses of the disease, an improvement in the overall health or well-being of the subject, or by other parameters well known in the art that are specific to the particular disease, such as a particular axonal disorder.

Label: A detectable compound or composition that is conjugated directly or indirectly to another molecule to facilitate detection of that molecule. Specific, non-limiting examples of labels include fluorescent tags, enzymatic linkages, and radioactive isotopes. In some examples, a disclosed Tet1 conjugate is labeled.

Linker: A relatively short series of amino acids that separates elements or domains of a fusion protein.

Modulate or modulating: To adjust, alter, regulate an activity, a degree or rate of such. In one example, a pharmaceutical composition including one or more of the disclosed conjugates is administered to modulate, reduce or inhibit, one or more signs or symptoms of an axonal disorder (a neuronal disorder).

Multiple sclerosis (MS): A slowly progressive CNS disease characterized by disseminated patches of demyelination in the brain and spinal cord, resulting in multiple and varied neurological symptoms and signs, usually with remissions and exacerbation. An increased family incidence suggests genetic susceptibility, and women are somewhat more often affected than men. The symptoms of MS include weakness, lack of coordination, paresthesias, speech disturbances, and visual disturbances, most commonly double vision. More specific signs and symptoms depend on the location of the lesions and the severity and destructiveness of the inflammatory and sclerotic processes. Relapsing-remitting multiple sclerosis is a clinical course of MS that is characterized by clearly defined, acute attacks with full or partial recovery and no disease progression between attacks. Secondary-progressive multiple sclerosis is a clinical course of MS that initially is relapsing-

remitting, and then becomes progressive at a variable rate, possibly with an occasional relapse and minor remission. Primary progressive multiple sclerosis presents initially in the progressive form. A clinically isolated syndrome is the first neurologic episode, which is caused by inflammation/demyelination at one or more sites in the CNS.

Myelin: A lipid substance forming a sheath (known as the myelin sheath) around the axons of certain nerve fibers. Myelin is an electrical insulator that serves to speed the conduction of nerve impulses in nerve fibers. “**Myelination**” (also “myelinization”) refers to the development or formation of a myelin sheath around a nerve fiber. Similarly, “**remyelination**” (also, “remyelination”) refers to the repair or reformation of the myelin sheath, such as following injury, exposure to a toxic agent, or an inflammatory response, or during the course of a demyelinating disease.

Neurodegenerative disease: Refers to any type of disease that is characterized by the progressive deterioration of the nervous system.

Optional: A term used to indicate that the subsequently described event or circumstance can but need not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

Parenteral: Administered outside of the intestine, for example, not via the alimentary tract. Generally, parenteral formulations are those that will be administered through any possible mode except ingestion. This term especially refers to injections, whether administered intravenously, intrathecally, intramuscularly, intraperitoneally, or subcutaneously, and various surface applications including intranasal, intradermal, and topical application. In one example, parenteral refers to intracerebroventricular administration.

Parkinson's disease (PD): An idiopathic, slowly progressive, degenerative CNS disorder characterized by slow and decreased movement, muscular rigidity, resting tremor, and postural instability. The loss of substantia nigra neurons, which project to the caudate nucleus and putamen, results in the depletion of the neurotransmitter dopamine in these areas.

Peptide: Any compound composed of amino acids or amino acid analogs chemically bound together. Peptide as used herein includes oligomers of amino acids, amino acid analog, or small and large peptides, including polypeptides or

proteins. Any chain of amino acids, regardless of length or post-translational modification (such as glycosylation or phosphorylation). In one example, a peptide is two or more amino acids joined by a peptide bond. Typically, a peptide consists of fewer than fifty amino acids; for example, consisting of approximately 7 to approximately 40 amino acids, consisting of approximately 7 to approximately 30 amino acids, consisting of approximately 7 to approximately 20 amino acids. In one example, a peptide consists of 4 amino acids and is referred to as a tetrapeptide.

“Peptide” applies to amino acid polymers to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer as well as in which one or more amino acid residue is a non-natural amino acid, for example a artificial chemical mimetic of a corresponding naturally occurring amino acid.

A “**polypeptide**” is a polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used. The terms “polypeptide” or “protein” as used herein are intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. The term “polypeptide” is specifically intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically produced. The term “residue” or “amino acid residue” includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide.

The term "soluble" refers to a form of a polypeptide that is not inserted into a cell membrane.

Conservative amino acid substitutions are those substitutions that, when made, least interfere with the properties of the original protein, that is, the structure and especially the function of the protein is conserved and not significantly changed by such substitutions. Examples of conservative substitutions are shown below.

Original Residue	Conservative Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His

Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
His	Asn; Gln
Ile	Leu, Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Conservative substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

The substitutions which in general are expected to produce the greatest changes in protein properties will be non-conservative, for instance changes in which (a) a hydrophilic residue, for example, seryl or threonyl, is substituted for (or by) a hydrophobic residue, for example, leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, for example, lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, for example, glutamyl or aspartyl; or (d) a residue having a bulky side chain, for example, phenylalanine, is substituted for (or by) one not having a side chain, for example, glycine. In some examples, one or more conservative amino acid substitutions are made to Tet1 (SEQ ID NO: 1).

Pharmaceutical composition: A chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject. A pharmaceutical composition can include a therapeutic agent, a diagnostic agent or a pharmaceutical agent. A therapeutic or pharmaceutical agent is one that alone or together with an additional compound induces the desired response (such as inducing a therapeutic or prophylactic effect when administered to a subject). In a particular example, a pharmaceutical agent is an agent that significantly reduces one or more symptoms associated with an axonal disorder.

Pharmaceutically Acceptable Carriers or vehicles: The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional.

Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 19th Edition (1995), describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compounds or molecules, such as one or more Tet1 conjugates provided herein. In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. In a particular embodiment the carrier is one that allows the therapeutic compound to cross the blood-brain barrier. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Protease: An enzyme that catalyses the hydrolysis of peptide bonds, for example peptide bonds in a protein. Examples of proteolytic enzymes include endoproteases, such as trypsin, chymotrypsin, endoprotease ArgC, endoprotease aspN, endoprotease gluC, thermolysin, and endoprotease lysC. The specific bonds cleaved by an endoprotease or a chemical protein cleavage agents may be more

specifically referred to as “endoprotease cleavage sites” and “chemical protein cleavage agent sites,” respectively. Proteins typically contain one or more intrinsic protein cleavage agent sites recognized by one or more protein cleavage agents by virtue of the amino acid sequence of the protein. A protease inhibitor is an agent that inhibits the activity of a protease.

Protein disulfide isomerase (PDI): An enzyme in the endoplasmic reticulum in eukaryotes or periplasmic space of prokaryotes that catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold. This activity allows proteins to quickly find the correct arrangement of disulfide bonds in their fully-folded state, and therefore the enzyme acts to catalyze protein folding. In an example, the THX/PDI signaling pathway is involved in mediating an axonal disorder.

PDI sequences are publicly available. For example, GenBank Accession numbers CAA89996.1 and AAC50401.1 disclose human PDI mRNA and protein sequences, respectively, each of which is incorporated by reference as presented by GenBank on November 24, 2009. One skilled in the art will appreciate that PDI nucleic acid and protein molecules can vary from those

Additional human PDI sequences have been deposited with GenBank and are publically accessible. PDI sequences from other species also are publically available, such as rat PDI. GenBank Accession number AAH88305.1 discloses rat PDI protein sequence which is incorporated by reference as presented in GenBank on November 24, 2009. One skilled in the art will appreciate that PDI nucleic acid and protein molecules can vary from those publicly available, such as those having one or more substitutions, deletions, insertions, or combinations thereof, while still retaining PDI biological activity. In some embodiments, the PDI variants that retain biological activity are conservative variants of PDI. In other embodiments, the PDI variants are fragments of THX that retain biological activity.

Methods of determining whether a conjugate is capable of modulating PDI activity are known in the art, including commercially available PDI activity assay kits (such as an ScRNase assay or a Di-E-GSSG assay) and as described herein (*e.g.*, the Examples).

Purified: The term “purified” does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified polypeptide (such as a Tet1 polypeptide), protein or other active compound is one that is isolated in whole or in part from naturally associated proteins and other contaminants, in which the polypeptide or other active compound is purified to a measurable degree relative to its naturally occurring state, for example, relative to its purity within a cell extract or chemical synthesis checker.

In certain embodiments, the term “substantially purified” refers to a polypeptide, protein or other active compound that has been isolated from a cell, cell culture medium, or other crude preparation and subjected to fractionation to remove various components of the initial preparation, such as proteins, cellular debris, and other components. Such purified preparations can include materials in covalent association with the polypeptide, such as glycoside residues or materials admixed or conjugated with the polypeptide, which may be desired to yield a modified derivative or analog of the polypeptide or to produce a combinatorial therapeutic formulation, conjugate, fusion protein or the like. The term purified thus includes such desired products as peptide and protein analogs or mimetics or other biologically active compounds wherein additional compounds or moieties are bound to the polypeptide in order to allow for the attachment of other compounds and/or provide for formulations useful in therapeutic treatments.

Generally, substantially purified polypeptides, proteins or other active compounds include more than 80% of all macromolecular species present in a preparation prior to admixture or formulation of the respective compound with additional ingredients in a complete pharmaceutical formulation for therapeutic administration. Additional ingredients can include a pharmaceutical carrier, excipient, buffer, absorption enhancing agent, stabilizer, preservative, adjuvant or other like co-ingredients. More typically, the polypeptide or other active compound is purified to represent greater than 90%, often greater than 95% of all macromolecular species present in a purified preparation prior to admixture with other formulation ingredients. In other cases, the purified preparation can be essentially homogeneous, wherein other macromolecular species are less than 1%.

Recombinant: A recombinant nucleic acid or polypeptide is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis. One of ordinary skill in the art will appreciate that many different recombinant polynucleotides and recombinant polypeptides may be created by molecular engineering.

Subject: Living multi-cellular vertebrate organisms, a category that includes human and non-human mammals.

Substitution: The replacement of one thing with another. With reference to an amino acid in a polypeptide “substitution” means replacement of one amino acid with a different amino acid.

Symptom and sign: Any subjective evidence of disease or of a subject's condition, *e.g.*, such evidence as perceived by the subject; a noticeable change in a subject's condition indicative of some bodily or mental state. A “sign” is any abnormality indicative of disease, discoverable on examination or assessment of a subject. A sign is generally an objective indication of disease. Signs include, but are not limited to any measurable parameters such as tests for detecting an axonal disorder. In one example, reducing or inhibiting one or more symptoms or signs associated with an axonal disorder, includes reducing or inhibiting the activity of PDI and/or THX by a desired amount, for example by at least 20%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or even at least 100%, as compared to the activity and/or expression in the absence of the conjugate.

Tet-1 peptide: A tetanus toxin-derived peptide with the amino acid sequence as set forth by SEQ ID NO: 1 (HLNILSTLWKYR), has no neurotoxic properties, and selectively translocates into neuronal networks. This peptide is utilized herein to deliver small peptides to neuronal networks to inhibit or reduce one or more symptoms associated with an axonal disorder, thereby by treating the axonal disorder.

Therapeutically effective amount or concentration: An amount of a composition that alone, or together with an additional therapeutic agent(s) sufficient to achieve a desired effect in a subject, or in a cell, being treated with the agent. The

effective amount of the agent will be dependent on several factors, including, but not limited to the subject or cells being treated, and the manner of administration of the therapeutic composition. In one example, a therapeutically effective amount or concentration is one that is sufficient to prevent advancement, delay progression, or to cause regression of a disease, or which is capable of reducing symptoms caused by the disease, such as an axonal disorder.

In one example, a desired response is to reduce or inhibit one or more symptoms associated with the axonal disorder. The one or more symptoms do not have to be completely eliminated for the composition to be effective. For example, a composition can decrease the sign or symptom by a desired amount, for example by at least 20%, at least 50%, at least 80%, at least 90%, at least 95%, at least 98%, or even at least 100%, as compared to the sign or symptom in the absence of the conjugate. In one particular example, a desired response is to reduce or inhibit the activity of PDI and/or THX by a desired amount, for example by at least 20%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or even at least 100%, as compared to the activity and/or expression in the absence of the conjugate.

A therapeutically effective amount of a disclosed pharmaceutical composition can be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the therapeutically effective amount can depend on the subject being treated, the severity and type of the condition being treated, and the manner of administration. For example, a therapeutically effective amount of such agent can vary from about 100 µg -10 mg per kg body weight if administered intravenously.

Thioredoxin (THX): A protein that acts as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. THXs are found in nearly all known organisms and are essential for life in mammals.

THX is a 12-kD oxidoreductase enzyme containing a dithiol-disulfide active site. It is ubiquitous and found in many organisms from plants and bacteria to mammals. Multiple *in vitro* substrates for thioredoxin have been identified, including ribonuclease, choriogonadotropins, coagulation factors, glucocorticoid

receptor, and insulin. Reduction of insulin is classically used as a test of THX activity.

THXs are characterized at the level of their amino acid sequence by the presence of two vicinal cysteines in a CXXC (SEQ ID NO: 2) motif. These two cysteines are the key to the ability of thioredoxin to reduce other proteins. THX proteins also have a characteristic tertiary structure termed the THX fold.

THXs are kept in the reduced state by the flavoenzyme thioredoxin reductase, in a NADPH-dependent reaction. Thioredoxins act as electron donors to peroxidases and ribonucleotide reductase. The related glutaredoxins share many of the functions of thioredoxins, but are reduced by glutathione rather than a specific reductase.

THX sequences are publicly available. For example, GenBank Accession numbers NM_003329 and NP_003320 disclose human THX mRNA and protein sequences, respectively, each of which is incorporated by reference as presented by GenBank on November 24, 2009. Additional human THX sequences have been deposited with GenBank and are publically accessible. THX sequences from other species also are publically available, such as mouse THX. GenBank Accession numbers NM_011660 and NP_035790 disclose mouse THX mRNA and protein sequences, respectively, each of which is incorporated by reference as presented by GenBank on November 24, 2009. One skilled in the art will appreciate that THX nucleic acid and protein molecules can vary from those publicly available, such as those having one or more substitutions, deletions, insertions, or combinations thereof, while still retaining THX biological activity. In some embodiments, the THX variants that retain biological activity (such as THX-mediated denitrosylation) are conservative variants of THX. In other embodiments, the THX variants are fragments of THX that retain biological activity. Methods of determining whether a conjugate is capable of modulating a THX polypeptide activity are known in the art and are described in the Examples herein, including commercially available thioredoxin activity assay kits (such as from Redoxica, Little Rock Arkansas).

Toxic agent: Refers to any type of chemical or physical agent that can cause harmful effects to living organisms. Some types of toxic agents, such as

organophosphates, are capable of triggering demyelination or a demyelinating disease.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which a disclosed invention belongs. The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise. “Comprising” means “including.” Hence “comprising A or B” means “including A” or “including B” or “including A and B.”

Suitable methods and materials for the practice and/or testing of embodiments of a disclosed invention are described below. Such methods and materials are illustrative only and are not intended to be limiting. Other methods and materials similar or equivalent to those described herein can be used. For example, conventional methods well known in the art to which a disclosed invention pertains are described in various general and more specific references, including, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 3d ed., Cold Spring Harbor Press, 2001; Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates, 1992 (and Supplements to 2000); Ausubel *et al.*, *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, 4th ed., Wiley & Sons, 1999; Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1990; and Harlow and Lane, *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1999.

Additional terms commonly used in molecular genetics can be found in Benjamin Lewin, *Genes V* published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

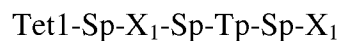
All sequences associated with the GenBank Accession Nos. mentioned herein are incorporated by reference in their entirety as were present on November 24, 2009, to the extent permissible by applicable rules and/or law.

III. Conjugates and mixtures thereof

Disclosed herein are conjugates, such as conjugates for use to treat neurodegenerative diseases. A disclosed conjugate includes at least one Tet1 peptide and at least one tetrapeptide having at least two cysteines or two lysine residues. In some examples, a conjugate includes a Tet1 peptide, a tetrapeptide and at least one spacer moiety and/or one or more additional amino acids. In additional examples, a conjugate includes a Tet1 peptide and multiple tetrapeptides.

The one or more tetrapeptides can be attached to the Tet1 peptide either directly or by a linker/spacer moiety by methods known to those of skill in the art (and as described in more detail below). For example, recombinant DNA technology can be used to add the tetrapeptide to Tet1 to produce conjugate. Details of suitable recombinant DNA technology can be found, for example, in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual* 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., 1989. In one example, the tetrapeptide is added to the N-terminus of Tet1. In another example, the tetrapeptide is added to the C-terminus of Tet1.

In one example, a conjugate including the structure of:



is disclosed wherein Tet1 is a tetanus toxin peptide with the amino acid sequence set forth by SEQ ID NO: 1 (HLNILSTLWKYR), Sp is an optional spacer moiety, X₁ is an optional one or more amino acids, and Tp is a tetrapeptide including at least two cysteine or two lysine residues.

In some examples, the disclosed conjugates include a label to assist with the detection of the conjugate. For example, a conjugate includes a Tet1 peptide, a tetrapeptide, at least one spacer moiety and/or additional amino acid and a label. Any label known to one of skill in the art can be employed that allows for conjugate detection without interfering with the delivery or activity of the conjugate. In one

example, the conjugate includes a fluorescent label. In a particular example, the fluorescent label is 5-carboxyfluorescein, rhodamine, Cy3, or Cy5.

In some embodiments, mixtures of two or more of the disclosed conjugates are provided. The various conjugates can be present in any ratio that is dictated by the specific properties, such as inhibiting or treating a particular axonal disorder, that are desired. For example, the ratio of a first conjugate to a second conjugate can be between about 0.01:99.99 to about 99.99:0.01, such as about 0.01:99.99, about 0.1:99.9, about 1:99, about 5:95, about 10:90, about 15:85, about 20:80, about 25:75, about 30:70, about 35:65, about 40:60, about 45:55, about 50:50, about 55:45, about 60:40, about 65:35, about 70:30, about 75:25, about 80:20, about 85:15, about 90:10, about 95:5, about 99:1, about 99.9:0.1, or about 99.99:0.01.

Tetrapeptide

The disclosed conjugate includes a tetrapeptide having at least two cysteine or lysine residues. In one example, the disclosed conjugate includes two cysteine residues. For example, the tetrapeptide can include the amino acid sequence set forth by SEQ ID NO: 2 (CX₂X₃C) in which C is a cysteine and X₂ and X₃ are any amino acid, such as any polar or basic amino acid. In one particular example, X₂ and X₃ are glycine or histidine, such as in SEQ ID NO: 3 (HLNILSTLWKYRCGHC). In some examples, a tetrapeptide comprises at least two lysine residues. For example, the tetrapeptide includes the amino acid sequence set forth by SEQ ID NO: 4 (KX₂X₃K) in which K is a lysine and X₂ and X₃ are any amino acid. In even further examples, the tetrapeptide includes at least three lysine residues. In one particular example, the tetrapeptide includes four lysines (SEQ ID NO: 5). For example, an exemplary conjugate includes the amino acid sequence set forth by SEQ ID NO: 6 (HLNILSTLWKYRKKKK).

Optional Spacer/Linker Moieties

According to the present disclosure, a disclosed tetrapeptide may be attached, such as covalently attached to a Tet1 peptide through an appropriate linker or spacer. For example, an optional spacer/linker moiety and/or additional amino acid may be added to any region of the conjugate, including, but not limited to, the

N- or C-terminus of the Tet1 peptide or tetrapeptide, provided that such moieties do not interfere with conjugate delivery and/or function.

In an example, the linker acts as a molecular bridge to link the Tet1 peptide to the tetrapeptide. The linker or spacer can serve, for example, simply as a convenient way to link the two entities, as a means to spatially separate the two entities, to provide an additional functionality to the conjugate, or a combination thereof. For example, it may be desirable to spatially separate Tet1 and the tetrapeptide to prevent Tet1 from interfering with the activity of the tetrapeptide and/or vice versa. The linker can also be used to provide a stability sequence, a molecular tag, a detectable label or various combinations thereof.

The selected linker can be bifunctional or polyfunctional, *e.g.*, contains at least a first reactive functionality at, or proximal to, a first end of the linker that is capable of bonding to, or being modified to bond to, the tetrapeptide and a second reactive functionality at, or proximal to, the opposite end of the linker that is capable of bonding to, or being modified to bond to Tet1. The two or more reactive functionalities can be the same (*i.e.*, the linker is homobifunctional) or they can be different (*i.e.*, the linker is heterobifunctional). A variety of bifunctional or polyfunctional cross-linking agents are known in the art that are suitable for use as linkers (for example, those commercially available from Pierce Chemical Co., Rockford, IL.). Alternatively, these reagents can be used to add the linker to tetrapeptide and/or Tet1.

The length and composition of the linker/spacer can be varied considerably provided that it can fulfill its purpose as a molecular bridge. The length and composition of the linker are generally selected taking into consideration the intended function of the linker, and optionally other factors such as ease of synthesis, stability, resistance to certain chemical and/or temperature parameters, and biocompatibility. For example, the linker or spacer should not significantly interfere with the targeting of the conjugate, such as the targeting of the conjugate to a neuronal cell, or with the activity of the conjugate relating to regulating one or more signs or symptoms of an axonal disorder.

Linkers suitable for use according to the present disclosure may be branched, unbranched, saturated, or unsaturated hydrocarbon chains, including peptides as

noted above. Furthermore, the linker can be attached to Tet1 and/or tetrapeptide using recombinant DNA technology. Such methods are well-known in the art and details of this technology can be found, for example, in Sambrook *et al.*, supra.

In one embodiment of the present disclosure, the linker is a branched or unbranched, saturated or unsaturated, hydrocarbon chain having from 1 to 100 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by -O- or -NR- (wherein R is H, or C1 to C6 alkyl), and wherein the chain is optionally substituted on carbon with one or more substituents selected from the group of (C1-C6) alkoxy, (C3-C6) cycloalkyl, (C1-C6) alkanoyl, (C1-C6) alkanoyloxy, (C1-C6) alkoxycarbonyl, (C1-C6) alkylthio, amide, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

Examples of suitable linkers include, but are not limited to, peptides having a chain length of 1 to 100 atoms, and linkers derived from groups such as ethanolamine, ethylene glycol, polyethylene with a chain length of 6 to 100 carbon atoms, polyethylene glycol with 3 to 30 repeating units, phenoxyethanol, propanolamide, butylene glycol, butyleneglycolamide, propyl phenyl, and ethyl, propyl, hexyl, steryl, cetyl, and palmitoyl alkyl chains.

In one example, the linker is a branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 50 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by -O- or -NR- (wherein R is as defined above), and wherein the chain is optionally substituted on carbon with one or more substituents selected from the group of (C1-C6) alkoxy, (C1-C6) alkanoyl, (C1-C6) alkanoyloxy, (C1-C6) alkoxycarbonyl, (C1-C6) alkylthio, amide, hydroxy, oxo (=O), carboxy, aryl and aryloxy.

In another example, the linker is an unbranched, saturated hydrocarbon chain having from 1 to 50 carbon atoms, wherein one or more of the carbon atoms is optionally replaced by -O- or -NR- (wherein R is as defined above), and wherein the chain is optionally substituted on carbon with one or more substituents selected from the group of (C1-C6) alkoxy, (C1-C6) alkanoyl, (C1-C6) alkanoyloxy, (C1-C6) alkoxycarbonyl, (C1-C6) alkylthio, amide, hydroxy, oxo (=O), carboxy, aryl and aryloxy.

In a specific example, the linker is a peptide having a chain length of 1 to 50 atoms. In another embodiment, the linker is a peptide having a chain length of 1 to 40 atoms. As known in the art, the attachment of a linker or spacer to a tetrapeptide or Tet1 need not be a particular mode of attachment or reaction. Various reactions providing a product of suitable stability and biological compatibility are acceptable.

Other modifications

The present disclosure contemplates further modifications of a Tet1 or tetrapeptide composition that do not affect the ability of the conjugate to selectively target neuronal cells and reduce or inhibit one or more symptoms associated with an axonal disorder. Such modifications include amino acid substitutions, insertions or deletions, and modifications, for example, to reduce antigenicity of the conjugate, to enhance the stability of the conjugate and/or to improve the pharmacokinetics of the conjugate. In one example, further modifications result in a polypeptide that differs by only a small number of amino acids. Such modifications include insertions (for example, of 1-3 or more residues), or substitutions that do not interfere with the ability of the conjugate to selectively target and modulate a neuronal cell.

Various modifications to reduce immunogenicity and/or improve the half-life of therapeutic proteins are known in the art. For example, the peptides can undergo glycosylation, isomerization, or deglycosylation according to standard methods known in the art. Similarly, the peptides can be modified by non-naturally occurring covalent modification for example by addition of polyethylene glycol moieties (pegylation) or lipidation. In one example, the compositions are conjugated to polyethylene glycol (PEGylated) to improve their pharmacokinetic profiles. Conjugation can be carried out by techniques known to those skilled in the art (see, for example, Deckert *et al.*, *Int. J. Cancer* 87: 382-390, 2000; Knight *et al.*, *Platelets* 15: 409-418, 2004; Leong *et al.*, *Cytokine* 16: 106-119, 2001; and Yang *et al.*, *Protein Eng.* 16: 761-770, 2003). In one example, antigenic epitopes can be identified and altered by mutagenesis. Methods of identifying antigenic epitopes are known in the art (see for example, Sette *et al.*, *Biologicals* 29:271-276, 2001), as are methods of mutating such antigenic epitopes. In one example, modifications are incorporated to decrease the toxicity of the conjugate. The general toxicity of the

conjugates according to the present disclosure can be tested according to methods known in the art. For example, the overall systemic toxicity of a disclosed conjugate can be tested by determining the dose that kills 100% of neuronal cells (*i.e.*, LD100) following a single treatment.

IV. Pharmaceutical Compositions

Provided herein are pharmaceutical compositions including any of the disclosed conjugates and a pharmaceutically acceptable carrier. The pharmaceutical composition may also include one or more agents or drugs as known to be therapeutically active in the treatment of a neurodegenerative disorder. In a further embodiment these agents may be selected from the group consisting of steroid, anti-inflammatory compound, immunosuppressive compound, and antioxidant compound. The pharmaceutical composition may be administered orally. Additional routes of administration may include sublingual, transdermal, transmucosal, or rectal (e.g., suppository or enema form). In a specific example, the pharmaceutical composition is formulated for intracerebroventricular (ICV) administration. In one example, the pharmaceutical composition is for use in the manufacture of a medicament or for use as a medicament.

Methods for preparing pharmaceutical compositions are known to those skilled in the art (see Remington's Pharmaceutical Science, 19th ed., Mack Publishing Company, Easton, Pa., 1995). Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. In

several embodiments, the composition includes a carrier which allows the conjugate to cross the blood-brain barrier.

Pharmaceutical compositions for oral use can be formulated, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion hard or soft capsules, or syrups or elixirs. Such compositions can be prepared according to standard methods known to the art for the manufacture of pharmaceutical compositions and may contain one or more agents selected from the group of sweetening agents, flavoring agents, coloring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with suitable non-toxic pharmaceutically acceptable excipients including, for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, such as corn starch, or alginic acid; binding agents, such as starch, gelatin or acacia, and lubricating agents, such as magnesium stearate, stearic acid or talc. The tablets can be uncoated, or they may be coated by known techniques in order to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. Pharmaceutical compositions for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium such as peanut oil, liquid paraffin or olive oil.

Pharmaceutical compositions can include pharmaceutically acceptable salts of the disclosed conjugates. Pharmaceutically acceptable salts of the presently disclosed compounds include those formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc, and from bases such as ammonia, ethylenediamine, N-methyl-glutamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chlorprocaine, diethanolamine, procaine, N-benzylphenethylamine, diethylamine, piperazine, tris(hydroxymethyl)aminomethane, and tetramethylammonium hydroxide. These salts may be prepared by standard procedures, for example by reacting the free acid

with a suitable organic or inorganic base. Any chemical compound recited in this specification may alternatively be administered as a pharmaceutically acceptable salt thereof. Pharmaceutically acceptable salts are also inclusive of the free acid, base, and zwitterionic forms. Description of suitable pharmaceutically acceptable salts can be found in Handbook of Pharmaceutical Salts, Properties, Selection and Use, Wiley VCH (2002).

In one example, the pharmaceutical compositions are administered in cerebrospinal fluid, such as in 2% acetone in artificial CSF.

The conjugates described herein can be administered to a subject for therapeutic treatment of an axonal disorder, such as a neurodegenerative disease. Thus, a therapeutically effective amount of a composition comprising one or more of the disclosed conjugates is administered to a subject already suffering from an axonal disorder, including a neurodegenerative disease (such as ALS), in an amount sufficient to improve a sign or a symptom of the disorder. Generally a suitable dose is about 1 milligram per kilogram (mg/kg) to about 50 mg/kg, such as a dose of about 1 mg/kg, about 2 mg/kg, about 5 mg/kg, or about 20 mg/kg administered parenterally. For example, a suitable dose is about 1 mg/kg to about 100 mg/kg, such as a dose of about 1 mg/kg, about 10 mg/kg, about 20 mg/kg, about 50 mg/kg, or about 100 mg/kg administered orally. Unit dosage forms are also possible, for example 50 mg, 100 mg, 150 mg or 200 mg, or up to 400 mg per dose. However, other higher or lower dosages also could be used, such as from about 0.001 mg/kg to about 1 g/kg, such as about 0.1 to about 500 mg/kg, including about 0.5 mg/kg to about 200 mg/kg.

Single or multiple administrations of the composition comprising one or more of the disclosed conjugates can be carried out with dose levels and pattern being selected by the treating physician. Generally, multiple doses are administered. In a particular example, the composition is administered parenterally once per day. However, the composition can be administered twice per day, three times per day, four times per day, six times per day, every other day, twice a week, weekly, or monthly. Treatment will typically continue for at least a month, more often for two or three months, sometimes for six months or a year, and may even continue indefinitely, *i.e.*, chronically. Repeat courses of treatment are also possible.

In one embodiment, the pharmaceutical composition is administered without concurrent administration of a second agent for the treatment of axonal disorder. In one specific, non-limiting example, one or more of the disclosed conjugates is administered without concurrent administration of other agents, such as without concurrent administration of an additional agent also known to target the axonal disorder. In other specific non-limiting examples, a therapeutically effective amount of a disclosed pharmaceutical composition is administered concurrently with an additional agent, including an additional axonal disorder therapy (such as, but not limited to, monoclonal antibodies, an anti-inflammatory agent, such as glatiramer acetate, an anti-oxidant, such as lipoic acid). For example, the disclosed compounds are administered in combination with protease and/or proteasome inhibitors, anti-oxidants, anti-inflammatory drugs or combinations thereof.

V. *Methods of Use*

It is shown herein that axonal damage is associated with increased THX/PDI activity. For example, the reducing or inhibiting THX/PDI activity has been demonstrated to reduced or inhibit axonal damage. It is also shown herein that the disclosed molecules are able to successfully cross the blood-brain barrier and be specifically translocated to neuronal/axonal circuitries. Studies demonstrate that this can be accomplished by intramuscular, continuous intrathecal infusion or ICV administration.

Based on these observations, methods of treatment to reduce or eliminate one or more symptoms or signs associated with an axonal disorder are disclosed. For example, a method includes administering to the subject a therapeutically effective amount of one or more of the disclosed pharmaceutical compositions, thereby reducing or inhibiting one or more symptoms associated with the axonal disorder.

In a particular example, the compound or a pharmaceutical composition comprising the conjugate readily penetrates the blood-brain barrier when peripherally administered. Compounds of this disclosure which cannot penetrate the blood-brain barrier can be effectively administered by an intraventricular route. In a further example, the pharmaceutical composition comprises a compound of the

disclosure and a pharmaceutically acceptable carrier that facilitates it to cross the blood-brain barrier.

In certain examples, motor performance, PDI/THX expression and folding activity, *S*-nitrosylation, ERAD-specific response is improved in the subject, wherein an improvement includes one or more of an increase in motor performance, a decrease in membrane-bound PDI expression, an increase in soluble PDI expression, an increase in THX expression, or a decrease in *S*-nitrosylation as compared to motor performance, PDI/THX expression and folding activity, or *S*-nitrosylation prior to administration of the therapeutically effective amount of the pharmaceutical composition.

Also disclosed is a method for modulating PDI and/or THX activity including contacting a cell, such as a neuronal cell (*e.g.*, a neuronal cell present in a mammal, such as a human) with a therapeutically effective amount of one or more of the disclosed conjugate-containing pharmaceutical compositions in which the composition modulates the activity of PDI (such as membrane-associated PDI, mPDI) and/or THX in the treated cell relative to PDI and/or THX activity in an untreated cell, thereby reducing or inhibiting a 1,2-diacetylbenzene (1,2-DAB) mediated axonal disorder. In one example, modulating activity of PDI includes reducing and or inhibiting mPDI-mediated transnitrosylation. In other examples, modulating activity of THX includes increasing THX-mediated denitrosylation. In some examples, modulate the activity of PDI and/or THX includes decreasing *S*-nitrosylation of at least one target protein as compared to *S*-nitrosylation of the at least one target protein (such mPDI) as in an untreated cell. In an example, contacting the cell with one or more agents comprises administering the one or more agents to the mammal, such as a human.

In some examples, the methods of use can include selecting a subject in need of treatment. For example, studies can be performed to identify a subject as being afflicted with an axonal disorder, including, but not limited to, any of the axonal disorders described herein. Methods of detecting an axonal disorder are known to those of skill in the art and can include methods of detecting PDI and/or THX activity or expression as described herein.

Therapeutically Effective Amount

In the methods disclosed herein, a therapeutically effective amount of a pharmaceutical composition including a conjugate described herein is administered to a subject in with an axonal disorder, such as a neurodegenerative disease. Assays to determine a therapeutically effective amount of a disclosed pharmaceutical composition for inhibiting or reducing one or more signs or symptoms associated with an axonal disorder are well known in the art.

In some examples, a therapeutic effective amount of a disclosed pharmaceutical composition is one in which one or more signs or symptoms associated with an axonal disorder is reduced or inhibited, such as by at least 10%, for example, about 15% to about 98%, about 30% to about 95%, about 40% to about 80%, about 50% to about 70%, including about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%, less than activity in the absence of the composition.

For example, a therapeutic effective amount of a disclosed pharmaceutical composition is one in which PDI/THX activity (such as folding activity) is reduced or inhibited, such as by at least 10%, for example, about 15% to about 98%, about 30% to about 95%, about 40% to about 80%, about 50% to about 70%, including about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%, as compared to such activity in the absence of the composition. Methods of assessing PDI and/or THX activity are known to one skilled in the art, including those described herein as well as commercially available PDI activity assay kits (such as an ScRNase assay or a Di-E-GSSG assay) or commercially available thioredoxin activity assay kits (such as from Redoxica, Little Rock Arkansas).

In other examples, a therapeutic effective amount of a disclosed pharmaceutical composition is one in which mPDI expression is reduced or inhibited, such as by at least 10%, for example, about 15% to about 98%, about 30% to about 95%, about 40% to about 80%, about 50% to about 70%, including about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%, relative to such activity in the absence of the composition. Methods of assessing mPDI are known to one skilled in the art,

including those described in the Examples below (*e.g.*, Western blot assay with commercially available antibodies).

In some examples, a therapeutically effective amount of a disclosed pharmaceutical composition is one in which motor performance is increased, such as by at least 10%, for example, about 15% to about 98%, about 30% to about 95%, about 40% to about 80%, about 50% to about 70%, including about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%, more than activity in the absence of the composition.

In other examples, a therapeutic effective amount of a disclosed pharmaceutical composition is one in which soluble PDI expression is increased, such as by at least 10%, for example, about 15% to about 98%, about 30% to about 95%, about 40% to about 80%, about 50% to about 70%, including about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%, relative to such activity in the absence of the composition. Methods of assessing soluble PDI are known to one skilled in the art, including those described in the Examples below (*e.g.*, Western blot assay with commercially available antibodies).

In other examples, a therapeutic effective amount of a disclosed pharmaceutical composition is one in which THX expression is increased, such as by at least 10%, for example, about 15% to about 98%, about 30% to about 95%, about 40% to about 80%, about 50% to about 70%, including about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%, relative to such activity in the absence of the composition. Methods of assessing THX expression are known to one skilled in the art, including those described in the Examples below (*e.g.*, Western blot assay with commercially available antibodies).

In further examples, a therapeutic effective amount of a disclosed pharmaceutical composition is one in which *S*-nitrosylation is reduced or inhibited, such as by at least 10%, for example, about 15% to about 98%, about 30% to about 95%, about 40% to about 80%, about 50% to about 70%, including about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%,

about 95%, about 98% or about 100%, relative to such activity in the absence of the composition. Methods of assessing THX expression are known to one skilled in the art, including those described in the Examples below.

Dosages, routes of administration of the disclosed pharmaceutical compositions for the methods of treatment are known to those of skill in the art and include, but are not limited to those described herein, including Section IV and the Examples.

Exemplary Axonal Disorders

Exemplary axonal disorders, include, but are not limited to, a neuropathy associated with exposure to a neurotoxic solvent that form a gamma-diketone compound (such as n-hexane), solvent (1,2-diethylbenzene and/or n-hexane, or gamma-diketone) neuropathy, or neuropathies associated with production of protein adducts molecules (such as gamma-keto-aldehydes, oxidative metabolites of arachidonic acid), ALS (Lou Gehrig's), diabetic neuropathy, uremic neuropathy (kidney failure), dementia, Alzheimer's, Corticobasal degeneration, Creutzfeldt-Jakob disease, familial fatal insomnia, frontotemporal lobar degeneration, Huntington's disease, HIV-associated dementia, Kennedy's disease, Krabbe's disease, Lewy body dementia disease, multiple sclerosis, konzo, tropical ataxic neuropathy, ALS/PDC, Lathyrism, primary lateral sclerosis, or spinal muscular atrophy. In some examples, the axonal disorder is ALS, Alzheimer's or PD. In one particular example, the axonal disorder is ALS. In some examples, the axonal disorder is a disorder associated with proximal giant axonopathy.

VI. Kits

Provided by this disclosure are kits that can be used to diagnose, prognose or treat an axonal disorder. For example, a kit is disclosed herein for preventing or inhibiting an axonal disorder, such as axonal degeneration, by reducing or inhibiting one or more symptoms associated with an axonal disorder in which the kit includes at least one of the disclosed pharmaceutical compositions. The disclosed kits can include instructional materials disclosing means of use of the compositions in the kit. The instructional materials can be written, in an electronic form (such as a

computer diskette or compact disk) or can be visual (such as video files). For example, instructions indicate to first perform a baseline measurement of a particular activity, such as measuring membrane-associated PDI, mPDI and/or THX activity. Then, administer a disclosed conjugate according to the teachings herein. Administration is followed by re-measuring the particular activity. The activity level prior to treatment is compared to activity observed following treatment. An alteration in activity of at least 10%, for example, about 15% to about 98%, about 30% to about 95%, about 40% to about 80%, about 50% to about 70%, including about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%, as compared to such activity in the absence of the composition indicates an effective treatment. In particular embodiments, a greater than 50% reduction indicates an effective treatment. An effective treatment can include, but are not limited to, an increase in patient survival, a slowing of the progression of the particular axonal disorder, a good prognosis, or a prevention of further axonal damage.

Kits are provided that can be used in the therapy assays disclosed herein. For example, kits can include one or more of the disclosed pharmaceutical compositions, agents (such as antibodies) capable of detecting one or more of the axonal disorder biomarkers (for example, measuring mPDI, cPDI, THX or *S*-nitrosylation), or combinations thereof. One skilled in the art will appreciate that the kits can include other agents to facilitate the particular application for which the kit is designed.

In one example, a kit is provided for treating an axonal disorder, such as ALS. For example, such kits can include one or more of the disclosed pharmaceutical compositions including one or more of the disclosed conjugates.

In some examples, a kit is provided for detecting one or more of the disclosed axonal disorder biomarkers in a biological sample. Kits for detecting axonal disorder-related molecules can include one or more probes that specifically bind to the molecules. In an example, a kit includes an array with one or more of the molecules provided in Table 1 and controls, such as positive and negative controls. In other examples, kits include antibodies that specifically bind to one of the axonal disorder biomarkers disclosed herein. In some examples, the antibody is labeled (for example, with a fluorescent, radioactive, or an enzymatic label). Such a diagnostic

kit can additionally contain means of detecting a label (such as enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a secondary antibody, or the like), as well as buffers and other reagents routinely used for the practice of a particular diagnostic method.

In certain examples, kits include additional compounds, such as protease and/or proteasome inhibitors. Pharmaceutical compositions can be used alone or in association with other combinations such as protease and/or proteasome inhibitors.

The present disclosure is illustrated by the following non-limiting Examples.

EXAMPLES

Example 1

Materials and Methods

This example provides the materials and methods utilized to perform the studies described herein.

Assessment of motor performance

A 4-animal rotorod apparatus suitable for rodents (AccuScan Instruments, Columbus, OH) was used to assess the neurological status of animals performing on a rotating bar. Once the rat was placed on the rotating rod, the rotation speed was increased in 5-RPM increments until the animal falls from the rod. Animals that fell in less than 10-15 seconds were given a second trial. If an animal was able to run for 120 seconds, the trial was stopped and the time recorded. Three trials per animal (for each rotation speed) were used to determine reliability of the measure. The rotation speed was increased for animals able to run for 120 seconds in each of the three trials. The mean time (seconds) when the animal falls from the rod rotating at a specific velocity (revolutions per minute) was used as a measure of neurologic status.

Neuropathology

Animals were anesthetized with isofluorane and perfused through the ascending aorta with 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH

7.4) for 10 sec followed by one liter of 2.5% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.4). Spinal cord tissues were removed, sampled and post-fixed with 2% osmium tetroxide, dehydrated in ascending concentrations of ethanol, and embedded in epoxy resin. Thick epoxy sections (1 micrometer) stained with 1% toluidine blue were examined by bright-field microscopy. Thin sections were treated with 2% uranyl acetate followed by 1% lead citrate, and examined by transmission electron microscope. *In vivo* uptake of fluorescein-conjugated Tet-1 peptides was assessed as previously described (Benn *et al.*, *J. Neurochem.* 2005;95(4):1118-31).

Genotype analysis

Genotype analysis was performed as described by Dr Gael Nicolas who generously donated the *Spna2* mutant (Meary *et al.*, *J. Biol. Chem.* 2007; 282(19):14226-14237). Wild-type and *Spna2* knock-out alleles were identified by polymerase chain reaction (PCR) using the following primers: 5'-tacatagagaatggccagctcttttgac-3' (forward; SEQ ID NO: 7) and 5'-gcacaactgggtaaggttcctattcc-3' (reverse; SEQ ID NO: 8) for the wild-type allele and 5'-gatctgaaagccaatgagtctcggc-3' (forward; SEQ ID NO: 9) and 5'-gcacaactgggtaaggttcctattcc-3' (reverse; SEQ ID NO: 10) for the resistant (mutant) allele. PCR was performed as follows: 35 cycles (each cycle consisting of 20 s at 94 °C, 20 s at 65 °C, and 30 s at 72 °C) with an initial denaturation at 94 °C for 4 min, and a final elongation at 72 °C for 5 min in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 0.2 mM each dNTP, 0.18 mM each primer 1–3, 0.2 mM each primer 4–5, 0.5 unit of Taq polymerase (Invitrogen, CA). The reaction was analyzed on 2% agarose gel containing SYBR Safe (Invitrogen, CA).

Western blots

Lumbar spinal cord tissues were homogenized in ice-cold buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.4) and a protease inhibitor mixture (Sigma Aldrich, MO). Cytosolic and membrane proteins were separated by ultracentrifugation. Proteins were resolved by SDS-polyacrylamide gels and transferred overnight to PVDF membranes (Bio-Rad Laboratories, CA). The filters were blocked for 1 hour in Tris-buffered saline containing 5% dry milk and 0.1% Tween 20, then incubated for 1 hour with either anti-PDI, or anti-BiP, or anti-PERK, or anti-IRE1, or anti-

ATF6 (1:600, SC), or anti-NOS isoforms, or anti-caspase-3, or anti-CHOP, or anti-caspase-3, or anti- μ calpain, such as those commercially available (*e.g.*, Sigma Aldrich, St. Louis, MO; Fischer Scientific, Hampton, NH; or ABCAM, Cambridge, UK). The blots were subsequently probed with a goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody (Perkin-Elmer, MA) diluted 1/10,000 in the same buffer. The peroxidase activity was detected by chemiluminescence using the ECL detection system (Amersham, IL).

Polymerase chain reaction

Total RNA was extracted from spinal cord tissues using TRIzol® (a registered trademark of Molecular Research Center, Inc.) reagent (Invitrogen, CA) according to the manufacturer's protocol. RNA samples were resuspended in diethylpyrocarbonate-treated water and kept at -70°C until use. Gene expression was investigated by reverse transcription-polymerase chain reaction. β -actin was used as an internal standard to monitor loading variations. Sequences of the primers used were: 5'-CCTCCCT GGAGAAGAGCTA-3' (SEQ ID NO: 11 and 5'-ACTCCTGCTTGCTGATCCAC-3' (SEQ ID NO: 12; β -actin, 384 bp), 5'-GCAAACTGAAGGCAGAAGG-3' (SEQ ID NO: 13) and 5'-GAGTCCACCAAGGACTCTGC-3' (SEQ ID NO: 14; PDI, 254 bp), 5'-AGTGGTGGCCACTAATGGAG-3' (SEQ ID NO: 15) and 5'-CTTCAAATTTG GCCCGAGTA-3' (SEQ ID NO: 16; BiP, 257 bp) and 5'-CGGAACCTGAGGAGAGAG-3' (SEQ ID NO: 17) and 5'-ATAGGTGCCCCCAATTTTCAT-3' (SEQ ID NO: 18; CHOP, 152 bp and 200 bp).

Assay for folding activity

The chaperone activity of PDI-family members were measured by analyzing the renaturation (refolding) of denatured rhodanese in the presence of equivalent amount of cytosolic vs. membrane fraction of spinal cord proteins. Rhodanese was denatured by guanidinium and then diluted in buffer (30 mM Tris-HCl, 50 mM KCl, pH 7.2) with or without the fraction of interest. The aggregation of denatured rhodanese was monitored by the increase in absorbance at 320 nm. The isomerase activity of was determined by measuring RNase A activity regenerated from scrambled RNase A as previously described (Uehara *et al.* Nature 2006;441(7092): 513-517).

Detection and quantification of S-nitrosylated proteins by LC-MS/MS*(i) Sample preparation*

Spinal cord tissues were homogenized in buffer containing 0.5% RapiGest SF (Waters, Billerica, MA), 100 mM Tris buffer (pH 8.5) and a BCA protein assay performed to measure protein content. The samples were reduced and alkylated using successive treatment with DTT and iodoacetamide, the RapiGest concentration reduced to 0.1% concentration, and sequencing grade modified trypsin (Trypsin Gold from Promega, Madison, WI) added at a ratio of 1:25 protease to substrate. Digestion was performed during an 8-hour incubation at 37°C with shaking. Following digestion, HCl was added to a final 250 mM concentration, the sample incubated for an additional 45 min, and hydrolyzed detergent removed by spinning the sample at 14,000 g for 10 minutes at 4°C. Peptides were solid phase-extracted using Sep-Pak Light cartridges (Millipore).

(ii)-DLC separations and MS/MS acquisition

The Sep-Pak-cleaned digests were injected onto a 100 x 2.1 mm polysulfoethyl A cation exchange column (The Nest Group, Inc., Southborough, MA) at a 200 µl/min flow rate. Mobile phase A contained 10 mM sodium phosphate (pH 3.0) and 25% acetonitrile, and mobile phase B was identical, except it contained 350 mM KCl. Following 5 minutes of loading and washing in mobile phase A, peptides were eluted using a linear gradient of 0-50% B over 45 minutes, followed by a linear gradient of 50-100% B over 20 min. One-minute fractions were collected, dried by vacuum centrifugation, and re-dissolved by shaking in 100 µl of 5% formic acid. Portions of each fraction (10 µl) were analyzed by LC/MS using an Agilent 1100 series capillary LC system and an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) using a standard electrospray source fitted with a 34 gauge metal needle (ThermoFinnigan, Cat. No. 97144-20040). Samples were applied at 20 µl/min to a trap cartridge and then switched onto a 0.5 X 250 mm Zorbax SB-C18 column (Agilent Technologies, Palo Alto, CA, USA) using a mobile phase A containing 0.1% formic acid. The gradient was 7-35% acetonitrile over 90 minutes at a 10 µl/min flow rate. Data-dependent collection of MS/MS spectra using the dynamic exclusion feature of the instrument's control software

(repeat count equal to 1, exclusion duration of 30 sec) was used to obtain MS/MS spectra of the three most abundant parent ions following each survey scan.

Estimation of changes in relative abundance of proteins and identification of modified peptides

Peptides were identified using the program Sequest (Version 27, rev. 12, ThermoFisher) which compares the observed MS/MS spectrum to theoretical fragmentation spectra of peptides generated from a database. Both rat and mouse sequences compiled from the Swiss-Prot database were used. DTA files were created with extract_msn software (ThermoFisher) with a molecular weight range of 400 to 3500, a minimum of 35 ions, and a TIC threshold of 500. In the Sequest searches, parent ion and fragment ion mass tolerances were used, 2.5 and 1.0 Da, respectively, monoisotopic masses selected, y- and b-ion series used in the scoring, and a static modification of +57 used due to alkylation of cysteine residues. A differential search for modified peptides was performed using mass shifts of +16 on Met and Trp (oxidation, possible under ER stress), and -28 on Cys (S-nitrosylation, possible under ER stress). The -28 mass shift for S-nitrosylation is the result of the differential mass following the static mass of +57 mass for alkylation and +29 for S-nitrosylation. List of identified proteins and their modifications were assembled using the program Scaffold (Proteome Software, Portland, OR) which assigns probability scores for identifications, tabulates list of modification sites, and estimates relative differences in protein abundance using spectral counting.

Analysis of S-nitrosylation of PDI and THX by mass spectrometry

Due to the labile nature of the S-nitrosyl group in proteins, it is difficult to directly detect this modification by mass spectrometry. To overcome this limitation, a recently developed His-Tag switch method is used to detect sites of S-nitrosylation (Camerini *et al. J Proteome Res* 2007;6(8): 3224-3231). The method utilizes a novel alkylating agent I-CH₂-CO-Gly-Arg-Ala-His₆ (His-Tag) that specifically reacts with S-nitrosyl groups in proteins. The simple synthesis of this His-Tag is accomplished by a single reaction between N-succinimidyl iodoacetate (Pierce, Richford, IL) and the synthetic peptide Gly-Arg-Ala-His₆. Sites of S-nitrosylation

are then simply but eloquently detected by specific reduction of *S*-nitrosyl groups by treatment with sodium ascorbate, followed by alkylation with the His-tag.

Previously *S*-nitrosylated proteins are then affinity-purified using a nickel column, digested with trypsin, and sites of *S*-nitrosylation detected by a mass shift occurring on specifically at previously nitrosylated cysteine residues.

Exemplary protocol

Membrane and cytosolic fractions of spinal cord proteins were obtained by ultracentrifugation. Free cysteines in all proteins were fully alkylated by treatment with 100 mM NEM for one hour at 37°C, followed by overnight dialysis to remove excess NEM. This treatment removed all free cysteines from the proteins so they were not available for alkylation with the His-tag peptide. Next, the proteins were treated with 10 mM sodium ascorbate in the presence of 0.2 mM His-tag peptide. This reaction converted all *S*-nitrosylated peptides to free cysteines so that they were alkylated with the His-tag, while leaving disulfide bonds in the proteins intact. The His-tagged proteins previously containing sites of *S*-nitrosylation were then purified by affinity chromatography using a nickel column (Qiagen) and eluted with imidazole. Purified proteins were separated by SDS-PAGE, stained with Imperial Coomassie Blue (Pierce), and PDI and THX proteins excised from the gels. These proteins were localized on the gel by comparison to Western blots using an antibody directed to PDI (Genway) or THX (Abcam). Excised PDI and THX were reduced with dithiothreitol (DTT), alkylated with NEM, and digested in-gel with trypsin. Peptides were analyzed by LC/MS using an Agilent 1100 series capillary LC system and an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA). Peptides were separated using a mobile phase A containing 0.1% formic acid and a 75 µm x 15 cm nanospray reverse phase column. The gradient was 7-35% acetonitrile over 90 minutes at a 300 nl/min flow rate. Data-dependent collection of MS/MS spectra using the dynamic exclusion feature of the instruments control software (repeat count equal to 1, exclusion duration of 30 sec) were used to obtain MS/MS spectra of the three most abundant parent ions following each survey scan. Peptides from PDI and THX were identified using Sequest software, which compared observed MS/MS spectra to predicted MS/MS spectra generated from peptide sequences in databases. Cysteines undergoing *S*-nitrosylation were

identified by differentially searching for mass shifts of either +271 or +125. The small mass shift of +271 is due to the cleavage of the His-tag by trypsin resulting from the presence of an Arg residue in the peptide. Other cysteine-containing peptides that either did not undergo reaction with NO, or were involved in disulfide bond formation, were detected from the resulting mass shift of +125 due to alkylation with NEM. The relative proportion of *S*-nitrosylation at each cysteine was calculated by integrating the extracted ion chromatograms for both forms of the peptides with +271 and +125 mass shifts. Reliable identification of peptides and their modifications was assured by filtering results of Sequest searches using Scaffold software (Proteome Software, Portland, OR) to estimate the false discovery rate for peptides.

Validation of Spna 2 cleavage

Since the preferred cleavage sites of spectrin have been determined for these proteases, which of these proteolytic systems that were activated can be examined by also examining sites of spectrin degradation occurring *in vivo*. The approximately 150 kDa fragments of spectrin are separated by SDS-PAGE, blotted onto PVDF membranes, stained with Coomassie Blue, the fragments cut from the blot, and the proteins subjected to Edman sequencing. The *N*-terminal sequence of the fragments is compared to the reported caspase and calpain cleavage sites to determine which protease was activated. As an alternate procedure, these 150 kDa spectrin fragments in gel slices will be digested using trypsin and other proteases to determine possible N- and C-terminal cleavage sites. This analysis is performed using mass spectrometry and examines the digest to identify peptides that are only partially tryptic and thus derived from either the shortened N- or C-terminus of the cleaved spectrin. These data are compared with the results using Edman degradation to more definitely identify sites of cleavage.

Statistical analysis

Analysis of individual protein and mRNA expression was conducted using linear regression to model trends over time for 1,2-DAB and vehicle and assess whether the trends differ. These same models can be used to estimate and test differences between the chemical treatments at each of the four time points. FIG. 12

illustrates the expected ordering of average response for protein expression, mRNA expression and sNO (*S*-nitrosylated proteins). The coefficient of variation (standard deviation divided by the mean) in a given response is expected to be approximately 15% for vehicle-treated animals. Comparisons made at each time point are conducted at a significance level of 0.012, which is the usual 0.05 level Bonferroni-corrected for four time points. Based on these assumptions, the use of $n=5$ animals per time point per group allows detection of a 36% change between 1,2-DAB and vehicle treatments with 80% power. The same sample size also gives 80% power to detect trends over time in which the overall increase or decrease is at least 6% the size of the average value (assuming the overall coefficient of variation in the response is at most 15%).

Example 2

1,2-DAB induces intraspinal giant axonopathy in low motor neurons of rodents

This example describes the 1,2-DAB inducement of intraspinal giant axonopathy in low motor neurons of rodents

The neuropathology associated with 1,2-DAB relative to that of its aliphatic γ -diketone cousin 2,5-hexanedione (2,5-HD) was characterized. 1,2-DAB induced giant intraspinal axonal swellings filled with 10-nm NF and organelles. This pathology is often observed in ALS. Further, 2,5-HD induced a dose-dependent combination of distal NF-axonal swelling, nerve fiber atrophy and Wallerian-like axon degeneration.

To determine whether accumulation of NF and organelles was due to deficits in mechanisms of axonal transport (*vide infra*), the following methods were used. First, the neurotoxicity of 1,2-DAB versus that of its non-neuroprotein reactive isomer 1,3-DAB in primary cultures of rat hippocampal neurons was evaluated. Electron micrograph studies demonstrated Wallerian-like degeneration in a neuron treated with 0.25 mM 1,2-DAB for 1 hour and a neuron treated with 1,3-DAB retained normal morphology.

Organelle trafficking in neuronal processes was examined by analyzing movements of mitochondria stained with 200 nM MitoTracker Green FM dye (Molecular Probes, Eugene) in cultures treated with 250 μ m DAB isomers for 60

min. Tracking of mitochondria was monitored *via* live-cell imaging on a heated stage of an inverted epifluorescence microscope coupled with a CCD camera and a computer system loaded with Metamorphe software (Molecular devices, Downingtown, PA). As illustrated in FIG. 5, movement of mitochondria (rate and velocity) initially increased following treatment with both 1,2-DAB or its non-neurotoxic isomer 1,3-DAB (negative control), but started declining approximately 30 minutes after treatment with 1,2-DAB.

The morphology observations and changes in axonal transport suggested that protein targets of 1,2-DAB reside inside the axons. The initial increase in the rate and velocity of moving mitochondria, which is an energy-mediated mechanism, possibly reflected cellular homeostatic response to treatment with 1,2-DAB or 1,3-DAB. However, reduction in axonal transport, seen only in 1,2-DAB could also have been caused by chemical modifications (adduction) of axonal proteins including motor proteins, or a mechanical blockade due to crosslinking of axonal proteins and accumulation of transported materials, or both.

Example 3

Isolation of the genomic profile of a γ -diketone axonopathy

This example describes the isolation of the genomic profile of a γ -diketone axonopathy.

To isolate the genomic profile of γ -diketone axonopathy, 11-week-old male C57BL/6 mice acclimated for 5 days were randomly selected to receive a single (50 mg/kg, i.p.) or multiple doses (35 mg/kg, 5 days/week) of 1,2-DAB or 1,3-DAB (negative control), or vehicle (saline containing 2% acetone). Animals were sacrificed 1, 6, 24, 48, 96 and 168 hours (after single dose) or 1 day, 1 week, 2 weeks and 3 weeks (multiple doses). Total RNA from right brain halves was extracted using Qiagen's RNeasy Mini Kit (Germantown, MD) and analyzed with an Agilent Bioanalyzer (Agilent Technologies, CA). Poly-A mRNA from each sample was amplified and labeled to produce Cy3 and Cy5 cRNA target molecules on Agilent's 60-mer Mouse Toxicology chip (~20,800 genes). Dye-flips were performed for all time-points. After washing and drying, microarrays were scanned

with an Agilent DNA Microarray “B” Scanner (Agilent Technologies, CA). Agilent Feature Extraction 7.1 software was used to collect and normalize microarray data prior to analysis with Rosetta Resolver (Rosetta inpharmatics, Kirkland, WA).

Identification of genes unique to 1,2-DAB followed the selection of genes that showed significant simultaneous differential expression comparing 1,2-DAB to vehicle and 1,2-DAB to 1,3-DAB, while excluding genes co-modulated by 1,3-DAB (box m₅ in FIG. 6). Analysis of modulation of 1,2-DAB-modulated genes revealed consistent (across a minimum of two time-points) downregulation of genes encoding for myelin proteolipid protein (PLP, GenBank Accession No. M154420), an abundant myelin structural protein, and ATPase calcium transporting plasma membrane 2 (PMCA2, GenBank Accession No. NM_009723), a cellular tool in the regulation and fine-tuning of intracellular calcium, among other genes that are reported in Table 1.

Table 1. List of six genes belonging to cell m₅ for at least two of the four comparison groups. The first arrow in each pair indicates the direction of the effect for 1,2-DAB relative to Vehicle, the second arrow gives similar information for the 1,2-DAB relative to 1,3-DAB comparison. Fold changes are at least 1.3 in the indicated direction coupled with a p-value ≤ 0.01 .

Doses	Single Dose		Repeated	
	1 hr	168 hr	1 wk	2 wks
Gene				
Proteolipid protein (myelin)	↑		↓↓	↓↓
Similar to hypothetical 139.5 kDa protein	↑↑			↓↓
Phospholipase C, β -1	↑↑			↓↓
RIKEN cDNA 9430072K23 gene	↑	↓↓	↓↓	
Mi-2 autoantigen 240 kDa protein (fragment)		↓	↓	↓
ATPase, Ca ⁺⁺ transporting plasma membrane 2	↑↑		↓↓	↓↓

Example 4

Proteomic modifications associated with 1,2-DAB axonopathy

This example describes proteomic modifications associated with 1,2-DAB axonopathy.

Two dimensional-DIGE and MALDI-TOF/MS-MS was used to analyze the lumbosacral spinal cord proteome of adult Sprague-Dawley rats treated systemically with 20 mg/kg/day 1,2-DAB, or equivalent dose of 1,3-DAB (negative control), or equivalent amount of vehicle (saline containing 2% acetone), 5 days a week, for up to three weeks.

As illustrated in FIGS. 7A and 7B, 1,2-DAB lowered the expression level of proteins involved in maintaining the physical integrity of axons (*e.g.*, Spna2) or assisting in folding mechanisms (*e.g.*, PDI, FIG. 7B). In contrast, most of the proteins involved in supporting the energy metabolism (*e.g.*, pyruvate kinase) were increased. The latter (somewhat consistent with the initial increase in axonal transport in the cell culture model) indicate that changes in energy metabolism represent a cellular homeostatic response to the pathology induced by 1,2-DAB

Example 5

Changes in the protein folding assistant protein disulfide isomerase (PDI), thioredoxin (THX), and structural protein Spna-2

This example describes the impact of 1,2-DAB on PDI family-members (*i.e.*, (surface) membrane-associated PDI (mPDI) versus soluble PDI (sPDI)) and THX was investigated and it was determined whether changes in PDI/THX were accompanied by changes in their transnitrosylation (mPDI) or denitrosylation (THX) activities. As shown in FIGS. 8A-8D, 1,2-DAB lowers the abundance of freely circulating sPDI and THX; however, the expression mPDI was increased and three immunoblot bands showed increased *S*-nitrosylation.

The expression levels of heme oxygenase-1 (HO-1) and isoforms of nitric oxide synthase (NOS-1, -2, or -3) remained unchanged indicating that (neuro)protein *S*-nitrosylation associated with 1,2-DAB axonopathy resulted from an increase in PDI-mediated transnitrosylation and/or reduced THX-mediated denitrosylation. Further, Spna2 was cleaved into two ~150 KDa Spna2-fragments in 1,2-DAB

axonopathy to an extent greatly in excess of that seen with vehicle treatment (FIG. 9).

These studies show that neuroprotein-reactant 1,2-DAB induces ALS-comparable proximal giant axonopathy. Further, the results indicate that changes in axonal transport may be secondary to 1,2-DAB-induced pathology. The genomic but mostly proteomic studies indicate a reduction in structural (axonal) proteins (*e.g.*, Spna 2 or PDI), possibly through activation of proteolytic mechanisms. Reduction in the abundance of PDI indicates that protein misfolding may occur in 1,2-DAB axonopathy. The decrease in THX and increase in mPDI/*S*-nitrosylation, coupled with NO increase in the production of oxidative/nitrosative species, indicates that *S*-nitrosylation occurs as a result of increased mPDI-transnitrosylation and decreased THX-denitrosylation. Moreover, down-regulation PMCA2 and modest increase in intracellular calcium, together with the cleavage pattern of Spna2, indicate that calcium-dependent proteases such as μ calpain may be activated and hence, involved in the degradation process of neuroproteins in 1,2-DAB axonopathy. Changes in the PDI/THX system indicate involvement of ER mechanisms. FIG. 10 provides a flow chart illustrating an exemplary signal transduction pathway involved in axonal degeneration.

Example 6

Elucidating the endoplasmic reticulum-time-course response to biochemical modifications induced by axonopathic 1,2-DAB

This example describes methods for elucidating pathological posttranslational modifications of PDI/THX and identifying which neuroproteins are aberrantly *S*-nitrosylated *via* ER or no-ER-mediated degradation of neuroproteins.

Young adult male Sprague-Dawely rats are treated intraperitoneally (IP) with 10 mg/kg/day 1,2-DAB or equivalent amount of vehicle-control (saline containing 2 % acetone), for up to 8 weeks ($n = 8$ animals per treatment group). Every other day, they are subjected to intracerebroventricular (ICV) infusion of 40 mg/kg bacitracin (PDI inhibitor), or 6 mg/kg of rhTHX (THX-enhancer), up to the study termination. Animals with ICV catheters are purchased from Charles Rivers Laboratories (CA).

At 14-day-intervals, animals are assessed for motor performance, PDI/THX expression/activity, S-nitrosylation, ER response (*vide infra*), and neuropathology.

Lumbar spinal cord proteins will be separated into cytosolic versus membrane fractions by ultracentrifugation and resolved on SDS-PAGE as previously described. Bands of PDI, THX and proteins that show increased S-nitrosylation (at least three including mPDI) are excised and subjected to analytical mass spectrometry. Other protein samples are subjected to molecular biology studies to capture the ER response (*e.g.*, detection of transcriptional and/or protein expression of PDI/THX, ERAD-master regulator BiP and ER-membrane transducers IRE1, PERK, and ATF6; ERAD-executioners CHOP, caspase-3, μ calpain; and cleavage of Spna2). In one example, prior to specific mass spectrometry analysis of PDI and THX posttranslational modifications, studies are performed *in vitro* by incubating test proteins, such as ovalbumin, with nitrosylated glutathione (GSH), a compound known to transfer S-nitrosyl groups to target proteins so that the procedure can be validated and optimized. Sequest searches are performed using sequence reversed protein entries to independently assess the false discovery rate for sites of S-nitrosylation. Pathological posttranslational modifications of PDI/THX are then identified.

Example 7

Effect of Caspase/Calpain Sensitive domain of Spna2 on γ -diketone axonopathy

This examples describes studies for determining whether a targeted mutation (deletion) of the caspase/calpain sensitive domain (CSD) of Spna2 (mutant Spna2tm1.1Gnic) confers protection against γ -diketone axonopathy.

Breeding pairs of mutant Spna2 mice are obtained (Institut National de la Santé et de la Recherche Médicale, the French National Institute of Health) and classical knockout molecular biological approaches known to those of skill in the art are employed to produce a mutant with the caspase-calpain-sensitive domain (CSD) of Spna2 (FIGS. 11A-11D) deleted.

Studies are performed to determine how the mutant reacts to chemically (1,2-DAB and/or S-nitrosylation) induced-stress and determine whether they display neuroprotective properties. It is hypothesized that the deletion of the Spna2-CSD

reduces the vulnerability of axons to death/proteolytic signals associated with 1,2-DAB axonopathy.

Young male homozygous (+/+) and heterozygous (+/-) mice, and their age- and sex-matched controls (wild-type littermates), bred on a C57Bl/6 background, will be treated with 1,2-DAB or vehicle-control as specified above. At 14-day-intervals, they will be assessed for motor performance, caspase 3 and m calpain activation, cleavage of Spna2, and neuropathology. The cleavage pattern of Spna2 will be assessed by Western blot and validated by analytical mass spectrometry and Edman protein sequencing as described herein. If Spna2 is cleaved by activated caspases and/or calpains in 1,2-DAB-axonopathy, then the Spna2 mutant that lacks the CSD domain can confer neuroprotection against 1,2-DAB. For example, the cleavage pattern of Spna2 in the Spna2 mutant will be altered in which a reduction of Spna2 cleavage will be observed, such as an at least 10% to 100% reduction, if the deletion of the Spna2 reduces 1,2-DAB axonopathy.

Example 8

Demonstration of *in vivo* uptake of Tet1-derivatives

This example demonstrates successful *in vivo* uptake of disclosed Tet1-derivatives.

Animals were injected intramuscularly, on alternate sides, in paravertebral lumbar muscles (latissimus dorsi at lumborum) with Tet1-derivatives (dissolved in 2% acetone in artificial CSF) at the dose of 1 μ l in /gm of body weight (n=3/each Tet1-derivatives with amino acid sequences corresponding to SEQ ID NOs: 1, 3, 6 and 7), once per day, for 3 days. On day 4, rats were deeply anesthetized with 4% isofluorane and systemically perfused with 0.1 M phosphate buffer, pH 7.4 (PB) followed by 4% paraformaldehyde in PB. The spinal cord was dissected out and put in 20% sucrose in PB overnight for cryoprotection. The lumbar segment of the spinal cord was cut under a dissecting stereomicroscope, embedded in OCT (optimal cutting temperature) compound, and frozen on dry ice. Forty μ m-thick transverse sections were cut at the cryostat and every eleventh section collected free-floating in PB.

Tet-1	HLNILSTLWKYR (SEQ ID NO: 1)	FLO	Amidation	95 %
Tet1PLYS	HLNILSTLWKYR KKKK (SEQ ID NO: 3)	FLO	Amidation	95 %
Tet1THO	HLNILSTLWKYR CGHC (SEQ ID NO: 6)	FLO	Amidation	95 %
Tet1LEU	HLNILSTLWKYR LLLL (SEQ ID NO: 5)	FLO	Amidation	95%

To determine whether Tet-1 derivatives targeted lower motor neurons, one series of sections from all rats was processed for double immunofluorescence with anti-GFP (which detects 5-FAM) and anti-choline acetyltransferase (ChAT) antibodies. Sections were preincubated in 3% bovine serum albumin (BSA) and 0.2% Triton X-100 in PB for 2 hours and then incubated in rabbit polyclonal anti-GFP antibodies (Invitrogen, Carlsbad, CA, USA; diluted 1:500) and goat polyclonal anti-ChAT antibodies (Millipore, Billerica, MA, USA; 1:80) in 3% BSA and 0.2% Triton X-100 in PB overnight. After repeated washes in PB, the sections were incubated with Cy^{TM2}-conjugated donkey anti-rabbit or Cy^{TM3}-conjugated mouse anti-goat (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; diluted 1:100), respectively, in 1% BSA and 0.2% Triton X-100 in PB for 2 hours. Control sections were processed as above omitting the primary antibodies as well as incubating at the same dilution of the primary antibodies with respective normal host sera. No immunopositivity was seen in these materials. After repeated washes, sections were mounted on slides, air dried, and covered with a coverslip with an anti-fading mounting medium (Dako, Glostrup, Denmark). Immunofluorescence was examined at a fluorescence microscope and images acquired with a Zeiss LSM 710 laser scanning confocal microscope, using the 488 nm and 533 nm excitation beams.

Examination of lumbar spinal cord sections from all Tet-1 injected rats under fluorescence microscope revealed immunopositivity for this peptide in the grey and white matter. In particular, immunopositive neuronal cell bodies were detected in

the ventral horn, while occasional and sparse immunosignal was observed in the dorsal horn. Uptake of Tet1-derivatives was also seen in axons.

Examination of sections from the rats injected with Tet-1 conjugated with the different amino acid sequences revealed no significant differences in the number of Tet-1-positive neurons in the ventral horn. Further study at the confocal microscope confirmed that most Tet1-positive neurons were motor neurons. These studies demonstrate *in vivo* uptake of Tet-1 conjugates.

Example 9

Preventing or Inhibiting Axonal Degradation *In Vivo*

This example describes methods of preventing or inhibiting axonal degradation by preventing PDI/THX from being adducted by 1,2-DAB to protect against 1,2-DAB-axonopathy.

Based upon the teachings herein, axonal degradation can be prevented or inhibited by modulating PDI/THX signal transduction pathway. As such, an axonal disorder can be treated by administering one or more of the disclosed pharmaceutical compositions that include one or more of the disclosed conjugates. Subjects are administered by ICV infusion a 3-mg/ml solution of one or more of the disclosed conjugates (such as Tet1PLYS conjugate or Tet1THO conjugate) every other day up to the study termination. At 14-day intervals, subjects will be assessed for axonal degradation as detailed herein, including Example 8. Neuroprotection is identified by detecting a decrease, such as a decrease in one or more activities of THX/PDI signal transduction pathway, including any one of the activities as described herein. An at least 10% decrease in one or more signs or symptoms will identify the treatment as useful for treating or preventing axonal degradation.

Additionally, preventing and inhibiting axonal degradation *in vivo* includes inhibiting proteolysis by either direct inhibition of calpain activity using E-64-d (cell-permeable calpain inhibitor) or indirect inhibition of calpain using anacardic acid, a putative inhibitor of SAE1-SUMO1 interaction. Neuroprotection is identified by detecting a decrease, such as a decrease in proteolysis. An at least 10% decrease in one or more signs or symptoms will identify the treatment as useful for treating or preventing axonal degradation.

Example 10

***Ex vivo* reactivity of tetrapeptides and *in vivo* delivery of such**

This example demonstrates the *ex vivo* reactivity of tetrapeptides and *in vivo* delivery of such peptides.

Ex vivo reactivity of tetrapeptides, with sequence homology to active sites of protein disulfide isomerase (PDI) family members or sequence of amino acids with chemical affinity for axonopathic cyanate, was assessed with oxidative species or sodium cyanate (NaOCN), respectively. Further, it was determined whether these tetrapeptides could be delivered *in vivo* to motor neurons using a synthetic analog of the non-virulent C fragment of tetanus toxin Tet1 (HLNILSTLWKYR; SEQ ID NO: 1) as vector.

Tetrapeptides made of lysine (KKKK; SEQ ID NO: 5) residues or a conserved active domain of PDI family members (CGHC; SEQ ID NO: 2) were conjugated with a GFP-tagged Tet1 to generate Tet1-PLYS or Tet1-THO, respectively. The *ex vivo* reactivity of Tet1-PLYS with the free amine carbamoylating agent NaOCN (1:2.5 to 1:37.5 molar ratios) or Tet1-THO with hydrogen peroxide (1:0.4 to 1:6.2 molar ratios) were assessed using mass spectrometry. Tet1-derivatives (3 mg/ml in artificial cerebrospinal fluid containing 2% acetone) were administered daily to rats by intra-muscular injection in the latissimus dorsi at lumborum at the dose of 1 µl/gm of body weight. The neuronal uptake of Tet1-derivatives in motor neurons was assessed after double immunolabeling for GFP and choline acetyltransferase (ChAT) in the lumbar spinal cord.

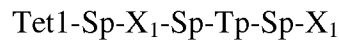
Mass analysis of end-products following reaction of Tet1-PLYS with NaOCN revealed carbamoylation of Tet1-PLYS lysine residues, which were concentration dependent (FIGS. 13A-13D). Intermolecular disulfide-bond formation was also detected by mass following the reaction of Tet1-THO with hydrogen peroxide (FIGS. 14A and 14B, respectively). Chromatographic MRM profiles of Tet1-derivatives are presented in FIGS. 15A-15C while dose response curves are provided in FIGS. 16A-16C. Confocal microscopy revealed immunopositivity for Tet1-derivatives in axons and motor neuron cell bodies,

prominently in the anterior horns of the lumbar spinal cord. These studies indicate that intramuscular delivery of Tet1-derivatives is a practical and efficient approach in target delivery of small molecules to the peripheral and central motor systems. These findings also suggest that short-sequence peptides with documented chemical affinity for neurotoxic species may display neuroprotective properties.

While this disclosure has been described with an emphasis upon particular embodiments, it will be obvious to those of ordinary skill in the art that variations of the particular embodiments may be used, and it is intended that the disclosure may be practiced otherwise than as specifically described herein. Features, characteristics, compounds, or examples described in conjunction with a particular aspect, embodiment, or example of the invention are to be understood to be applicable to any other aspect, embodiment, or example of the invention. Accordingly, this disclosure includes all modifications encompassed within the spirit and scope of the disclosure as defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A conjugate comprising the structure of:



wherein Tet1 is a tetanus toxin peptide with the amino acid sequence set forth by SEQ ID NO: 1 (HLNILSTLWKYR), Sp is an optional spacer moiety, X₁ is an optional one or more amino acids, and Tp is a tetrapeptide comprising at least two cysteine or two lysine residues.

2. The conjugate of claim 1, wherein the tetrapeptide comprises at least two cysteine residues.

3. The conjugate of claim 2, wherein the tetrapeptide comprises the amino acid sequence set forth by SEQ ID NO: 2 (CX₂X₃C) in which C is a cysteine and X₂ and X₃ are any amino acid.

4. The conjugate of claim 3, wherein X₂ and X₃ are any polar or basic amino acid.

5. The conjugate of claim 4, wherein X₂ and X₃ are glycine or histidine.

6. The conjugate of claim 5, wherein X₂ is a glycine and X₃ is a histidine.

7. The conjugate of claim 5, wherein the conjugate consists of the amino acid sequence set forth by SEQ ID NO: 3 (HLNILSTLWKYRCGHC).

8. The conjugate of claim 1, wherein the tetrapeptide comprises at least two lysine residues.

9. The conjugate of claim 8, wherein the tetrapeptide comprises the amino acid sequence set forth by SEQ ID NO: 4 (KX₂X₃K) in which K is a lysine and X₂ and X₃ are any amino acid.
10. The conjugate of claim 1, wherein the tetrapeptide comprises at least three lysine residues.
11. The conjugate of claim 10, wherein the tetrapeptide consists of the amino acid sequence set forth by SEQ ID NO: 5 (KKKK).
12. The conjugate of claim 11, wherein the conjugate consists of the amino acid sequence set forth by SEQ ID NO: 6 (HLNILSTLWKYRKKKK).
13. The conjugate of any one of claims 1-12, further comprising a fluorescent label.
14. The conjugate of claim 13, wherein the fluorescent label comprises 5-carboxyfluorescein.
15. A pharmaceutical composition comprising the conjugate of any one of claims 1-14 and a pharmaceutically acceptable carrier.
16. The pharmaceutical composition of claim 15, for use in the manufacture of a medicament.
17. The pharmaceutical composition of claim 15, for use as a medicament.
18. The pharmaceutical composition of any one of claims 15-17, wherein the pharmaceutical composition is formulated for intracerebroventricular (ICV) administration.

19. A kit for reducing or inhibiting one or more symptoms associated with an axonal disorder, comprising the conjugate of any one of claims 1-14, the pharmaceutical composition of any one of claims 15-18 and one or more protease or proteasome inhibitors.

20. A method of reducing or inhibiting one or more symptoms associated with an axonal disorder, comprising administering to a subject a therapeutically effective amount of the pharmaceutical composition according to any one of claims 15-18, thereby reducing or inhibiting one or more symptoms associated with the axonal disorder in the subject.

21. The method of claim 20, wherein the axonal disorder is a disorder associated with proximal giant axonopathy.

22. The method of any of claims 20-21, wherein motor performance, PDI/THX expression and folding activity, *S*-nitrosylation, ERAD-specific response, or combinations thereof are improved in the subject, wherein an improvement comprises one or more of an increase in motor performance, a decrease in membrane-bound PDI expression, an increase in soluble PDI expression, an increase in THX expression, or a decrease in *S*-nitrosylation as compared to motor performance, PDI/THX expression and folding activity, or *S*-nitrosylation prior to administration of the therapeutically effective amount of the pharmaceutical composition.

23. A method for modulating protein disulfide isomerase (PDI) and/or thioredoxin (THX) activity, comprising:

contacting a cell with a therapeutically effective amount of an agent comprising the pharmaceutical composition of any one of claims 15-18, wherein the pharmaceutical composition modulates the activity of PDI and/or THX in the treated cell relative to PDI and/or THX activity in an untreated cell, thereby reducing or inhibiting a 1,2-diacetylbenzene (1,2-DAB) mediated axonal disorder.

24. The method of claim 23, wherein the PDI is a membrane-associated PDI (mPDI).

25. The method of claim 24, wherein modulate the activity of PDI comprises reducing and or inhibiting mPDI-mediated transnitrosylation.

26. The method of any one of claims 23-25, wherein modulate the activity of THX comprises increasing THX-mediated denitrosylation.

27. The method of claim 23, wherein modulate the activity of PDI and/or THX comprises decreasing *S*-nitrosylation of at least one target protein as compared to *S*-nitrosylation of the at least one target protein in an untreated cell.

28. The method of claim 27, wherein the at least one target protein comprises a membrane-associated PDI (mPDI).

29. The method of any one of claims 23-28, wherein the cell is a neuronal cell.

30. The method of any one of claims 20-21 or 23-29, wherein the axonal disorder is a neuropathy associated a neuropathy associated with exposure to a neurotoxic solvent that form a gamma-diketone compound (such as n-hexane), solvent (1,2-diethylbenzene and/or n-hexane, or gamma-diketone) neuropathy, or neuropathies associated with production of protein adducts molecules (such as gamma-keto-aldehydes, oxidative metabolites of arachidonic acid), diabetic neuropathy, uremic neuropathy (kidney failure), dementia, amyotrophic lateral sclerosis (Lou Gehrig's), Alzheimer's, Corticobasal degeneration, Creutzfeldt-Jakob disease, familial fatal insomnia, frontotemporal lobar degeneration, Huntington's disease, HIV-associated dementia, Kennedy's disease, Krabbe's disease, Lewy body dementia disease, multiple sclerosis, konzo, tropical ataxic neuropathy, Parkinson's disease, Lathyrism, primary lateral sclerosis, spinal muscular atrophy or a combination thereof.

31. The method of claim 30, wherein the gamma-diketone compound is n-hexane.

32. The method of any one of claims 23-31, wherein the neuronal cell is present in a mammal, and wherein contacting the cell with an agent comprises administering the agent to the mammal.

FIG. 1B

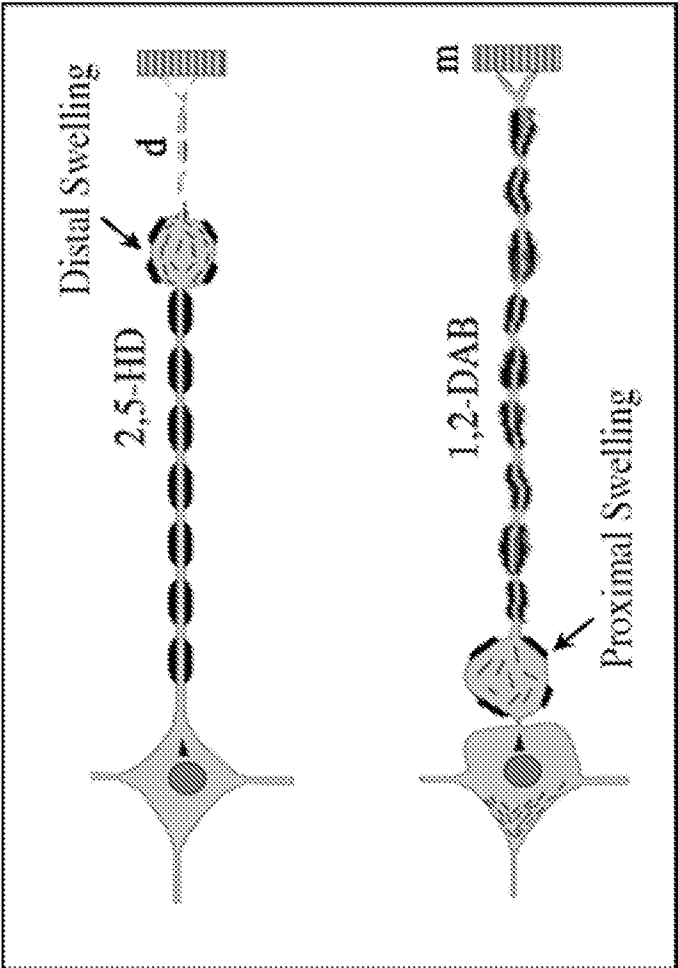


FIG. 1A

Solvent	<chem>CCCCC(=O)C</chem>	Non-Neurotoxic Isomer	<chem>CCCC(=O)C</chem>
Hexanone		γ -Diketone Metabolite	<chem>CCCC(=O)CC(=O)C</chem>
		2,5-HD	
		2,3-HD	
1,2-DEB	<chem>CC1=CC=CC=C1C2=CC=CC=C2C</chem>	1,2-DAB	<chem>CC(=O)C1=CC=CC=C1C(=O)C2=CC=CC=C2</chem>
		1,3-DAB	<chem>CC(=O)C1=CC=CC=C1C=CC2=CC=CC=C2</chem>

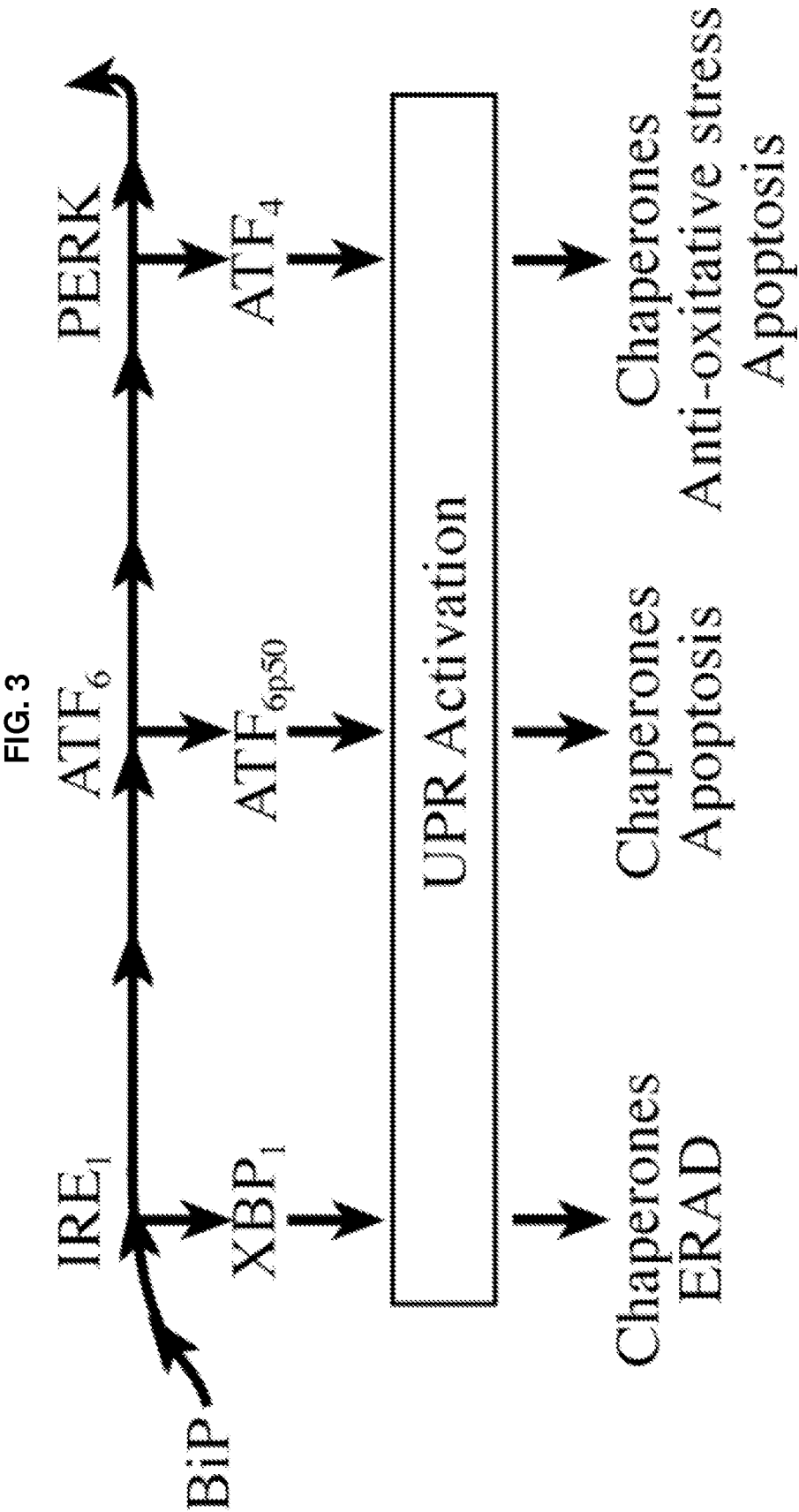


FIG. 4A

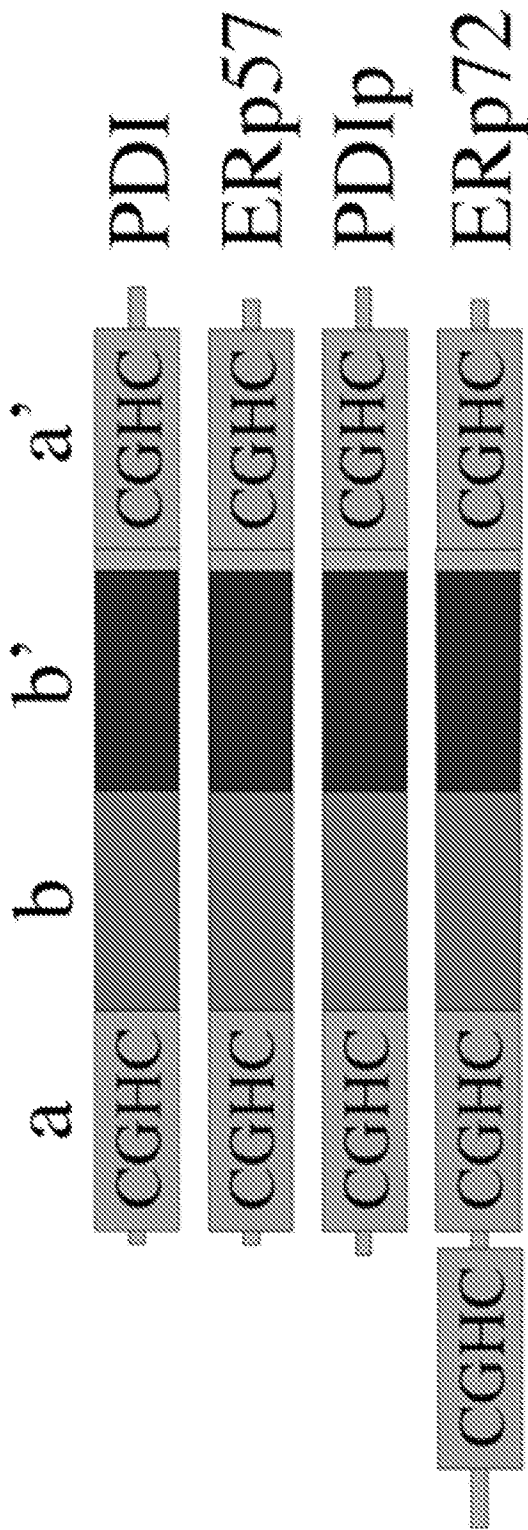


FIG. 4B

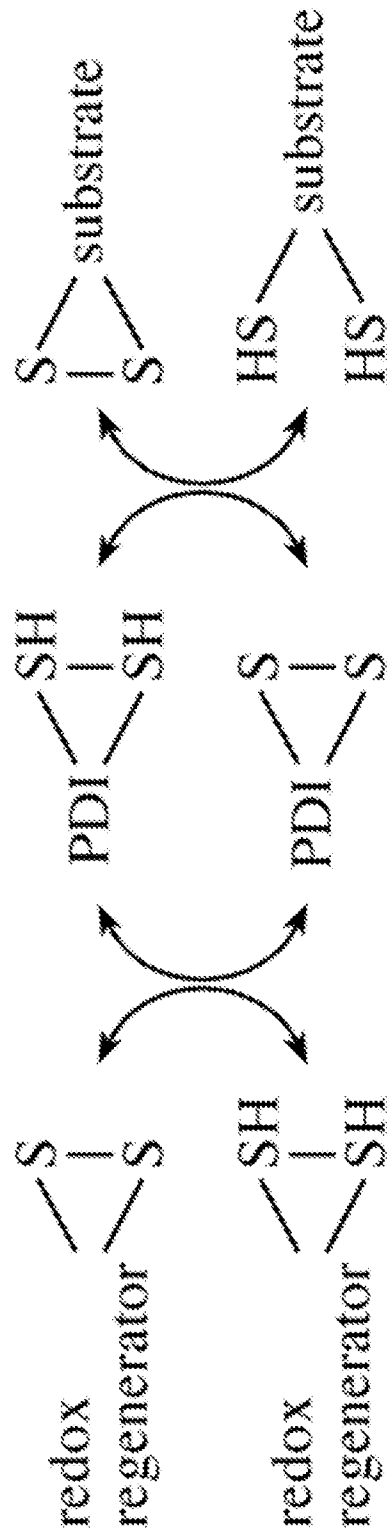


FIG. 5

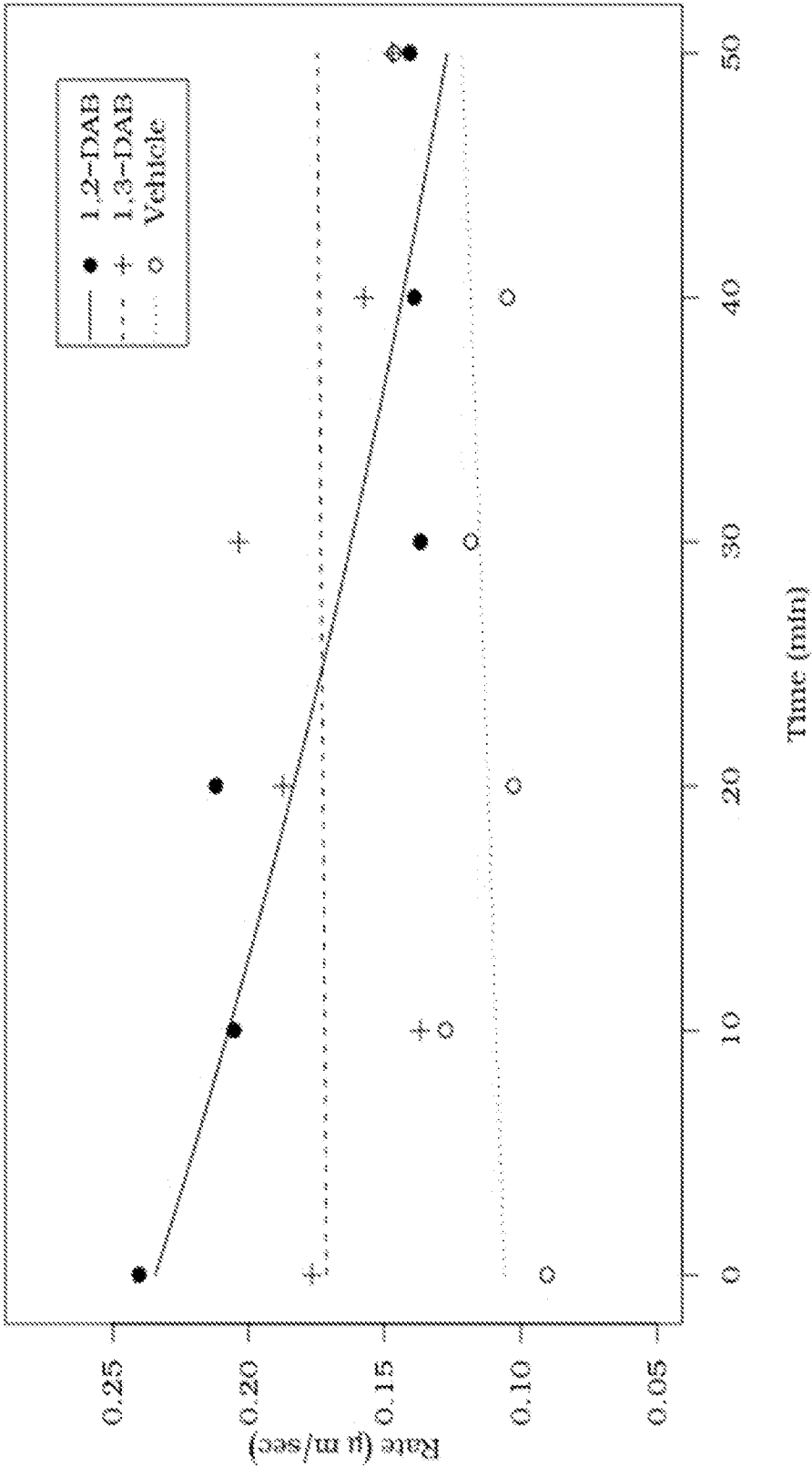


FIG. 7B

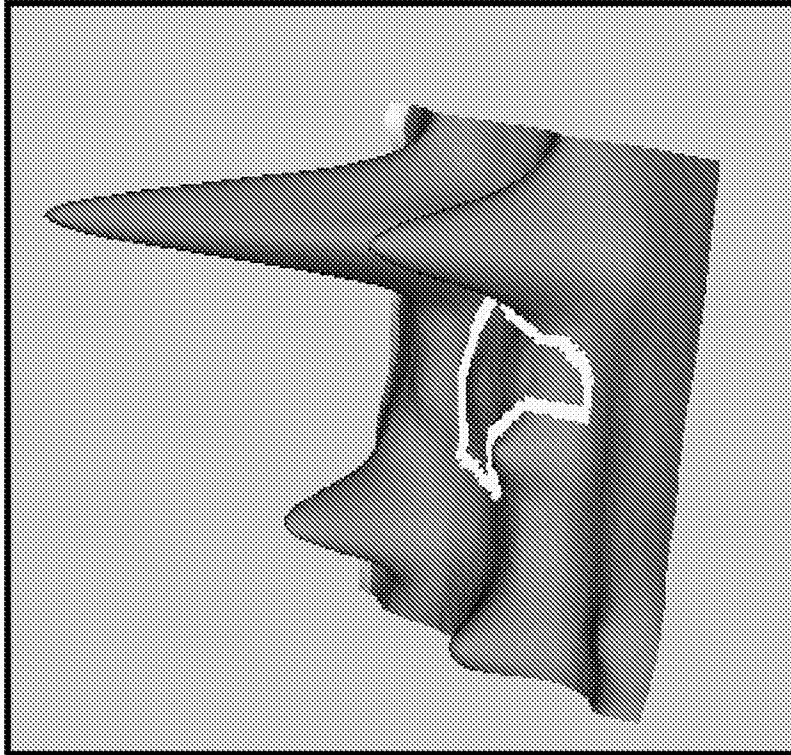
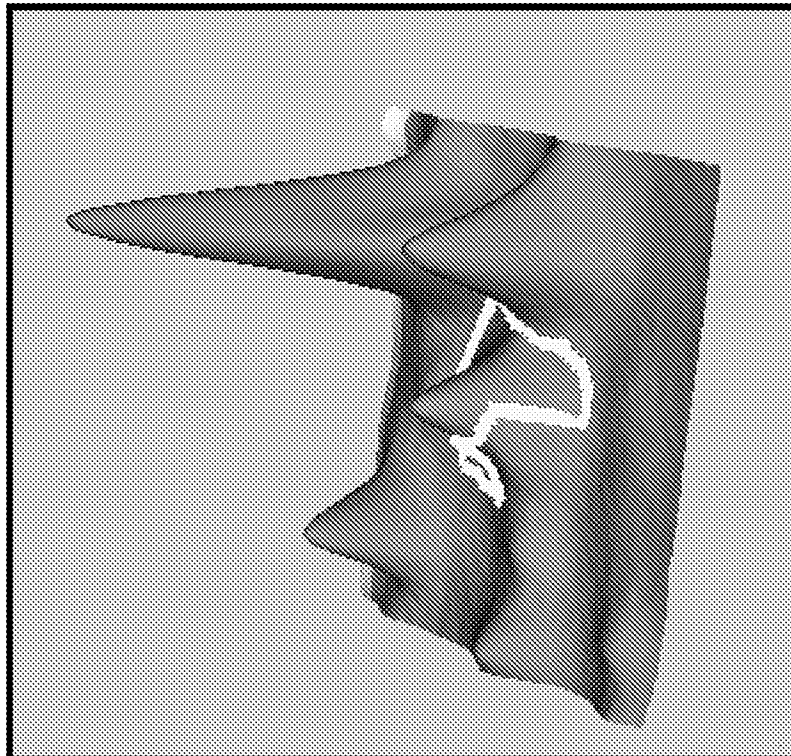


FIG. 7A



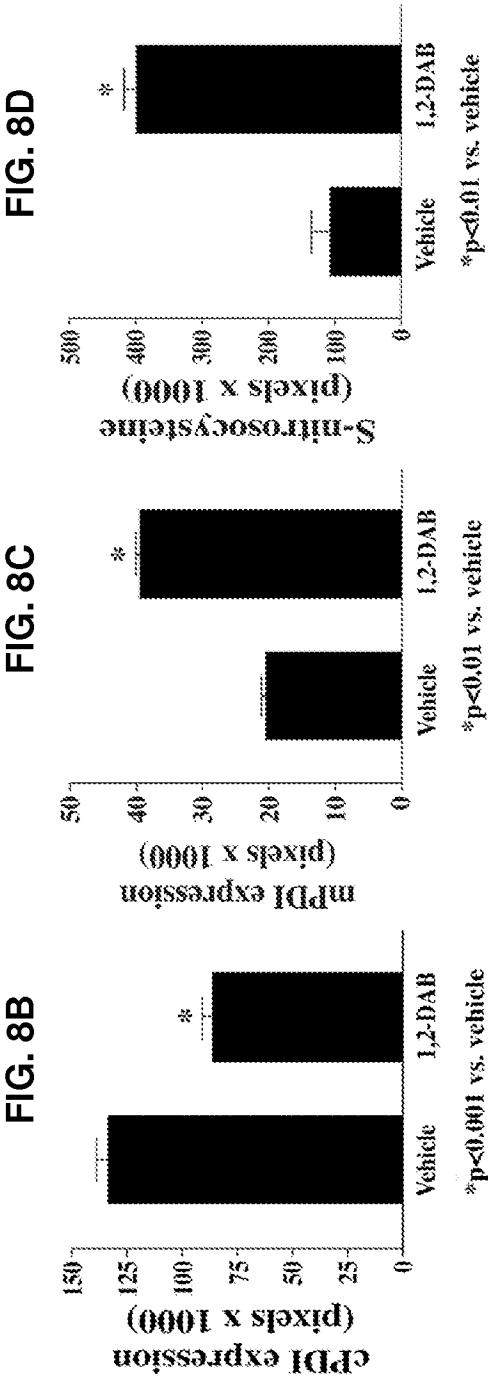
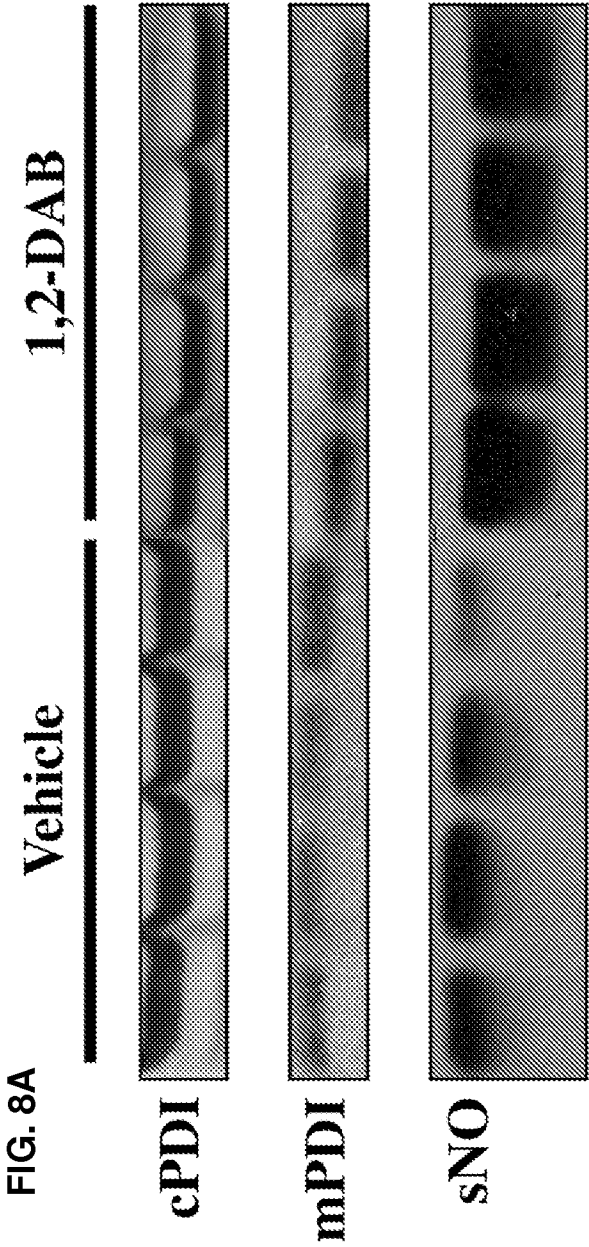


FIG. 9

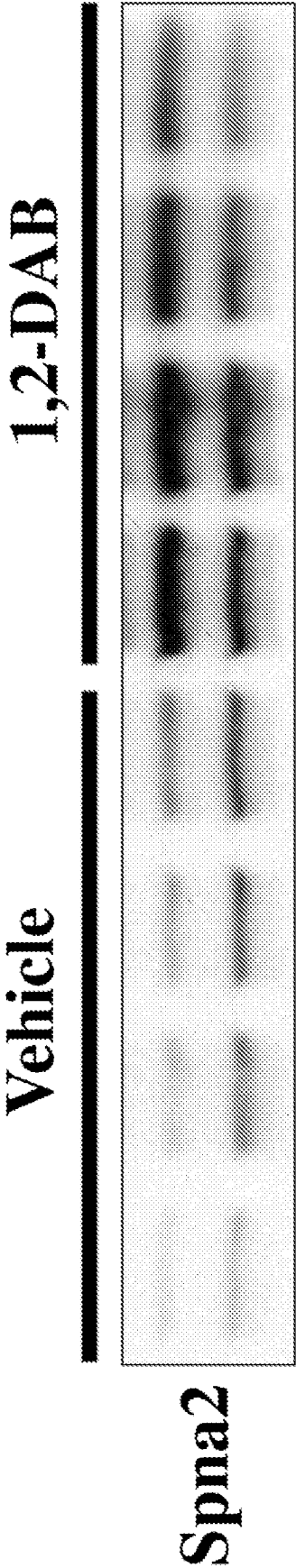
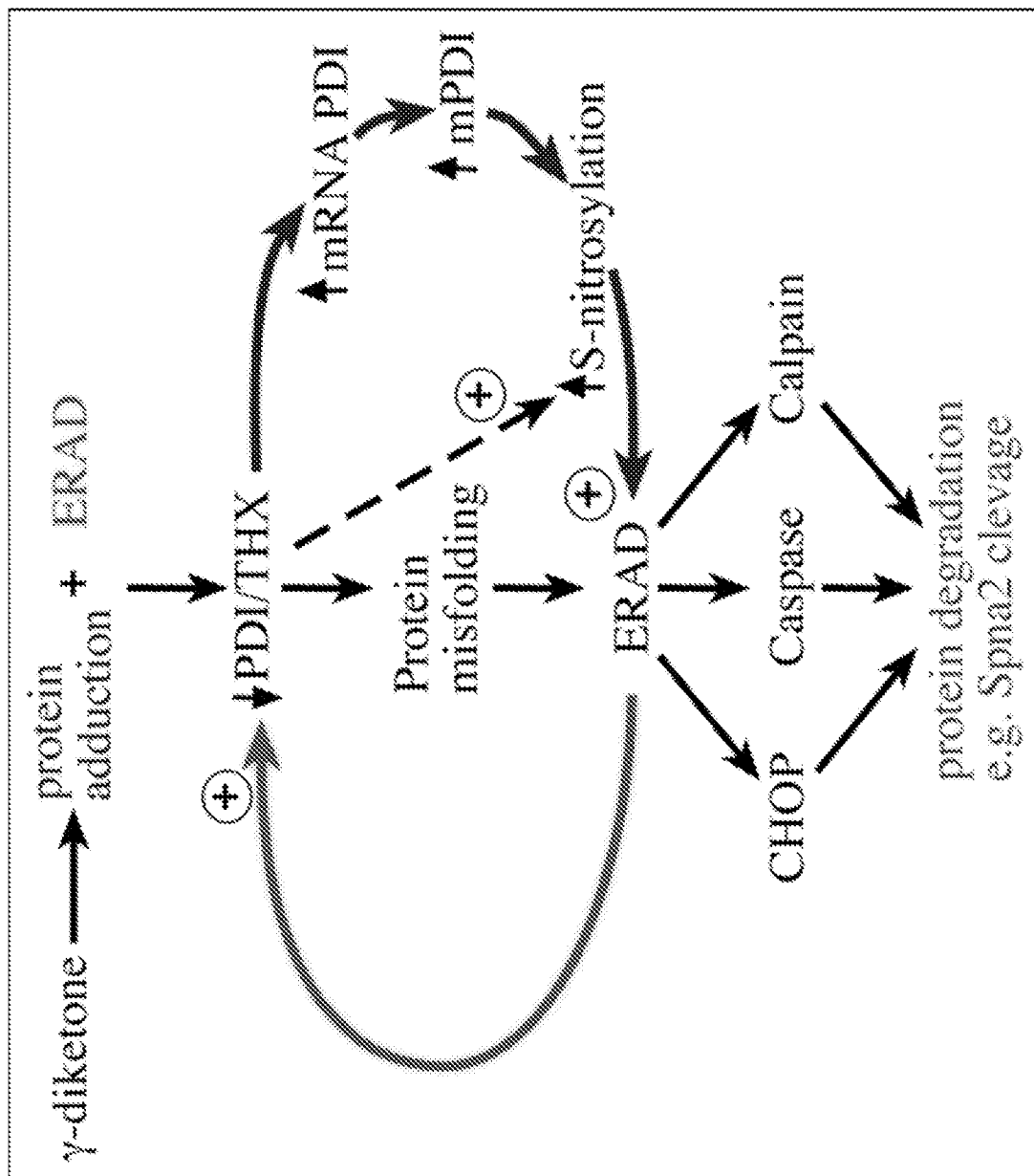


FIG. 10



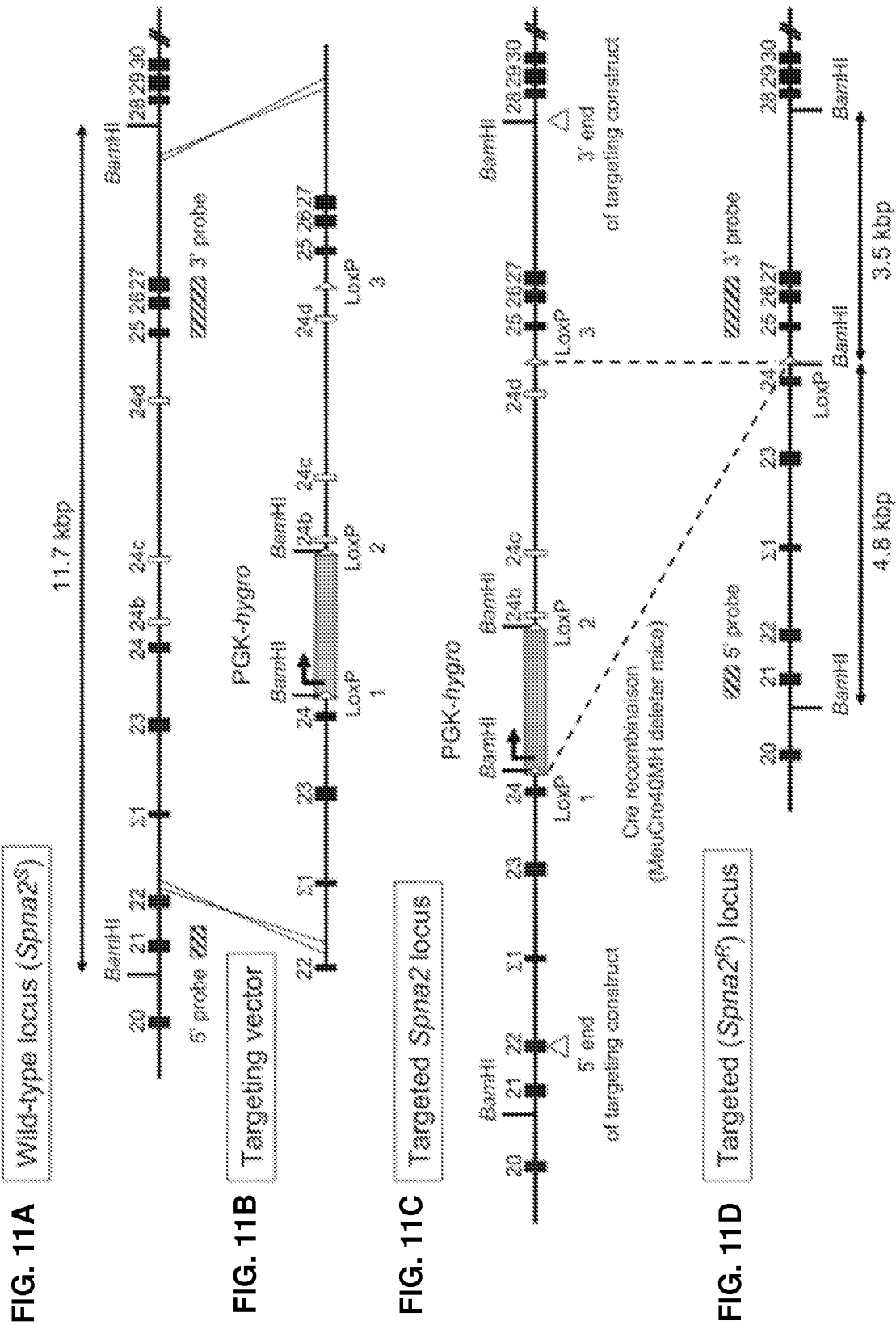
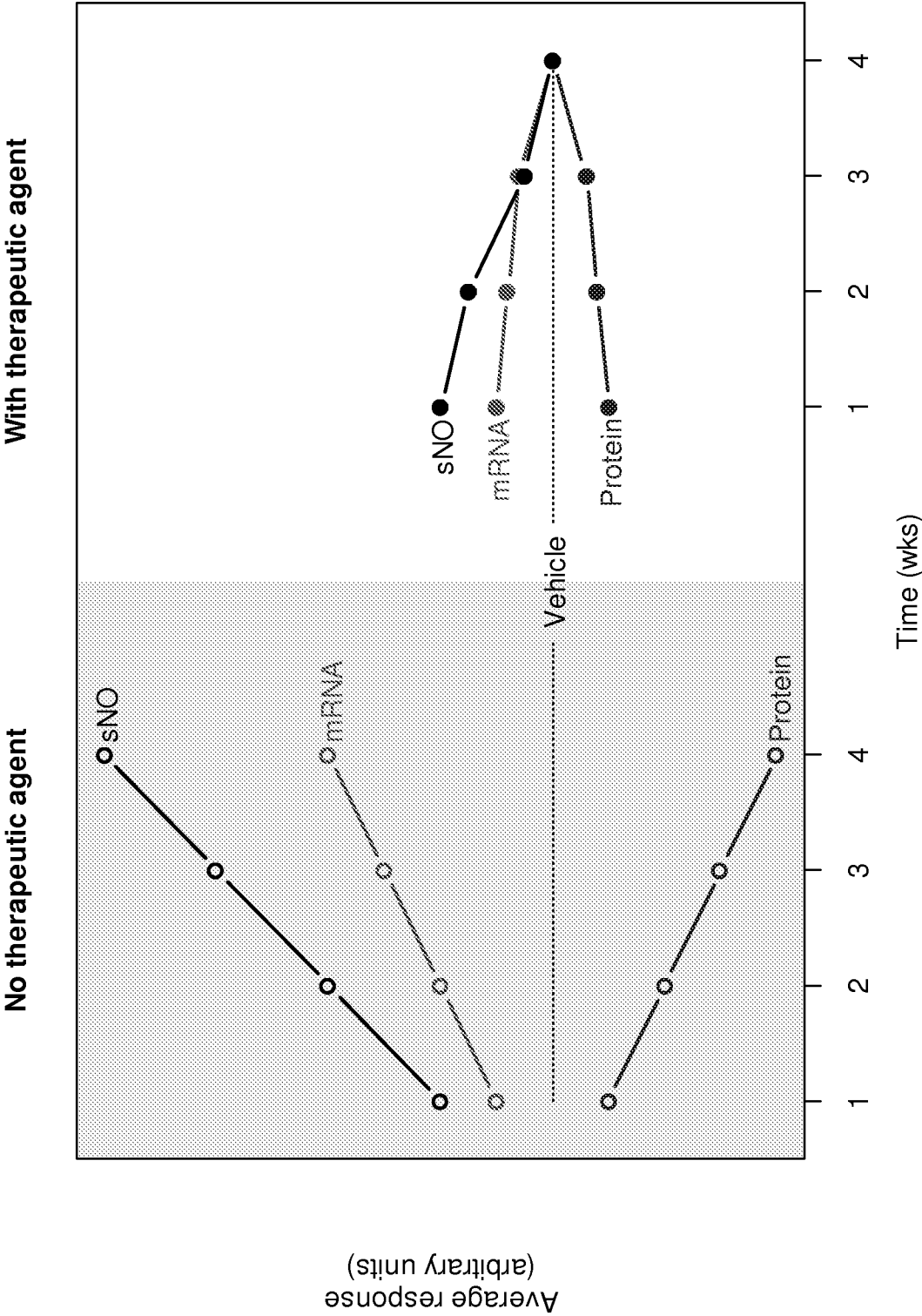
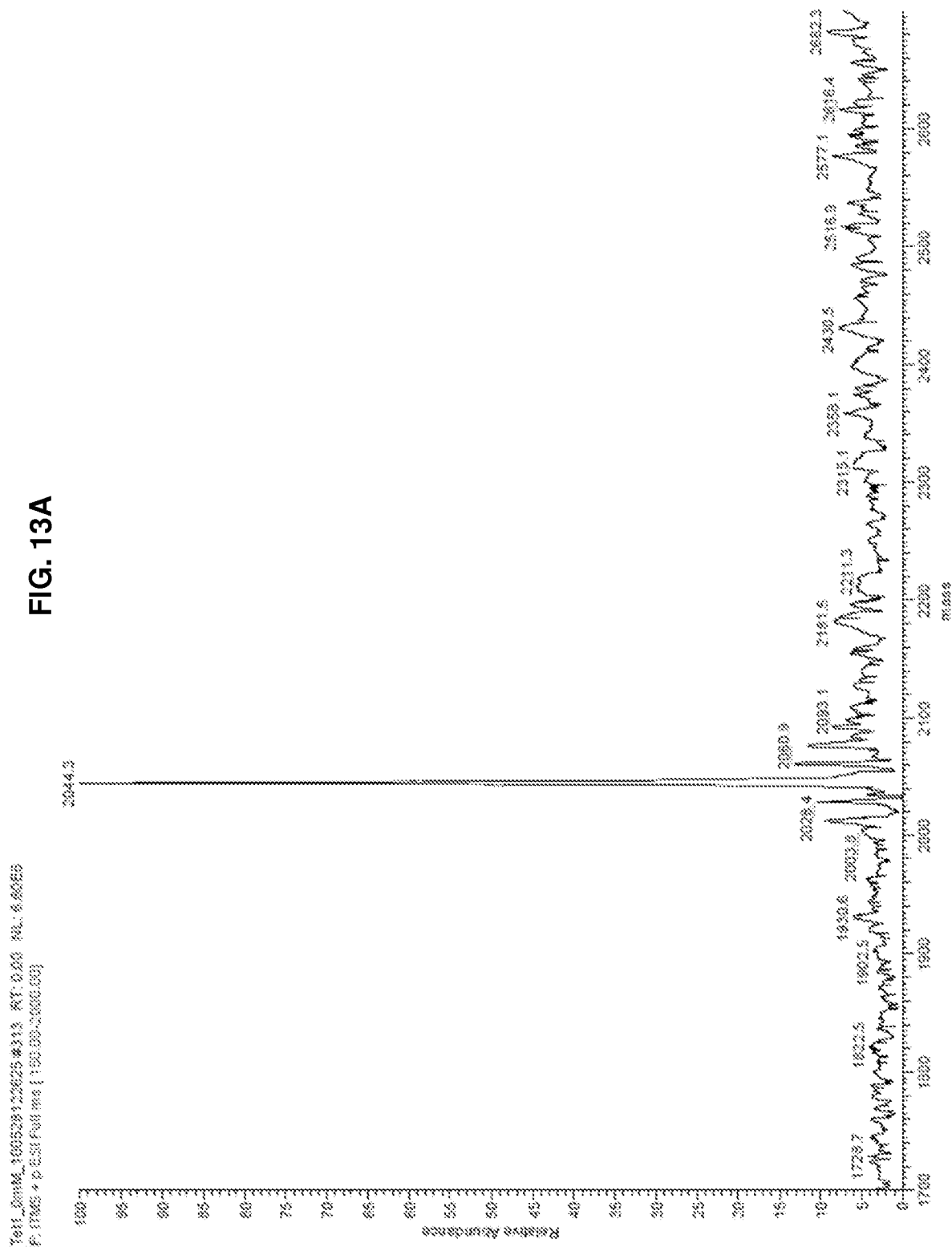
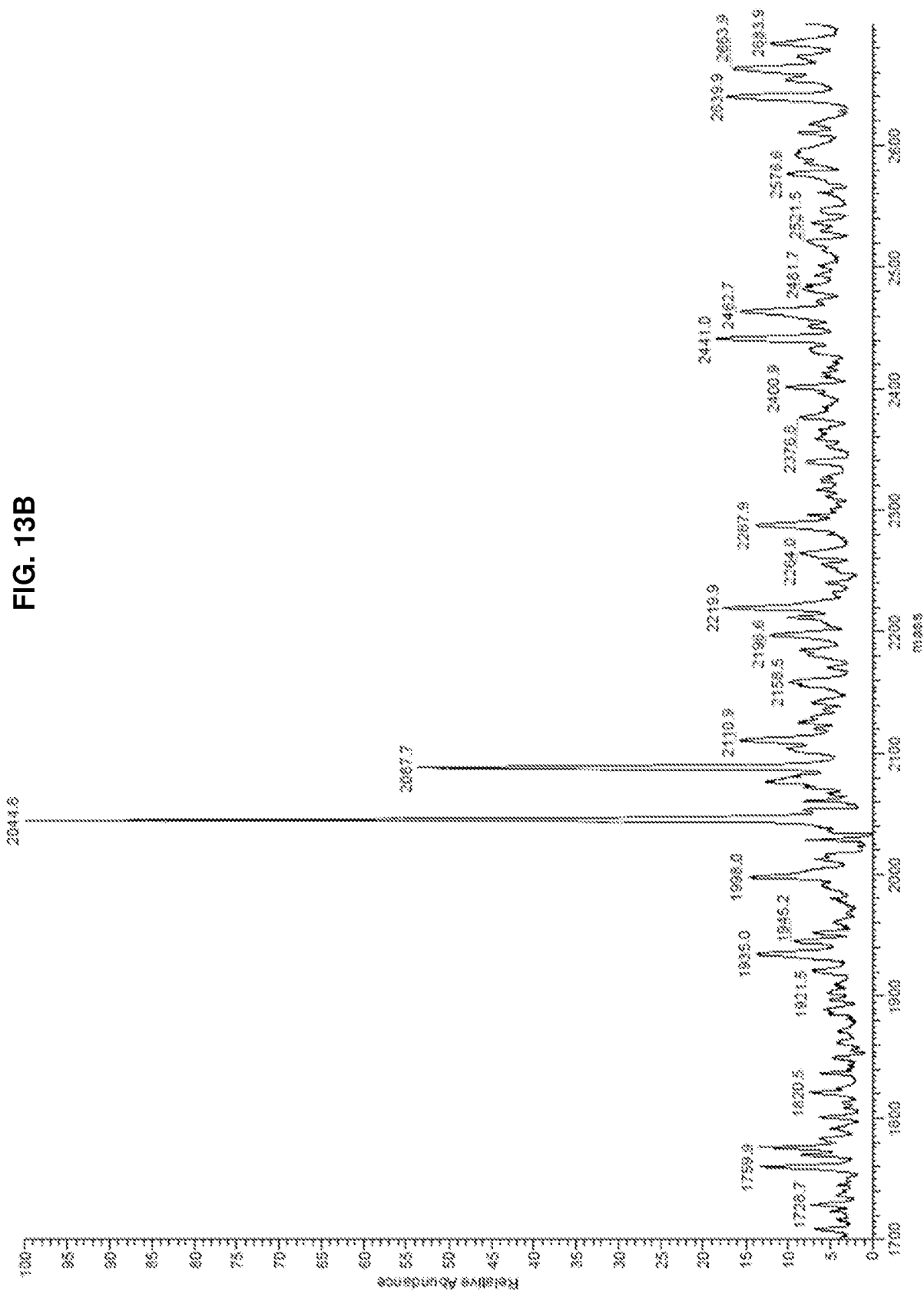


FIG. 12





Tert_15mM_10260111106 #313 RT: 0.00 ML: 7.04ES
F: (TMS + p ESI) ms (150.00-2000.00)



Test Log_DmM_100001111108 #312 RT: 0.80 NL: 1.18E9
 F: 17MS * 9 ESI Full ms [150.00-2000.00]

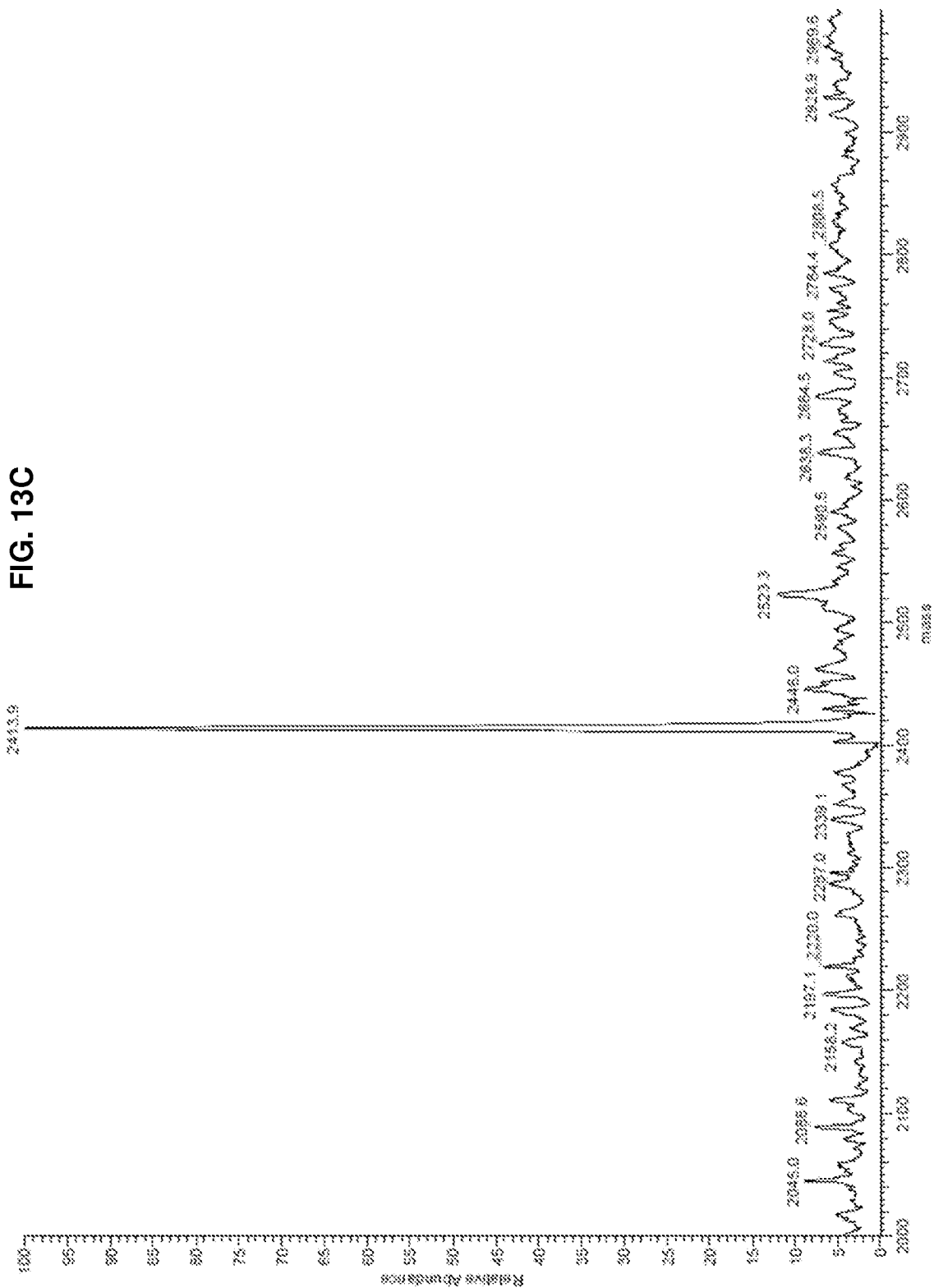


FIG. 13D

Test 1, 15mmM, 100601111188 #308 RT: 0.00 NL: 7.02E5
 F: 17000 * 0.251 Full ms { 150.00-2000.00 }

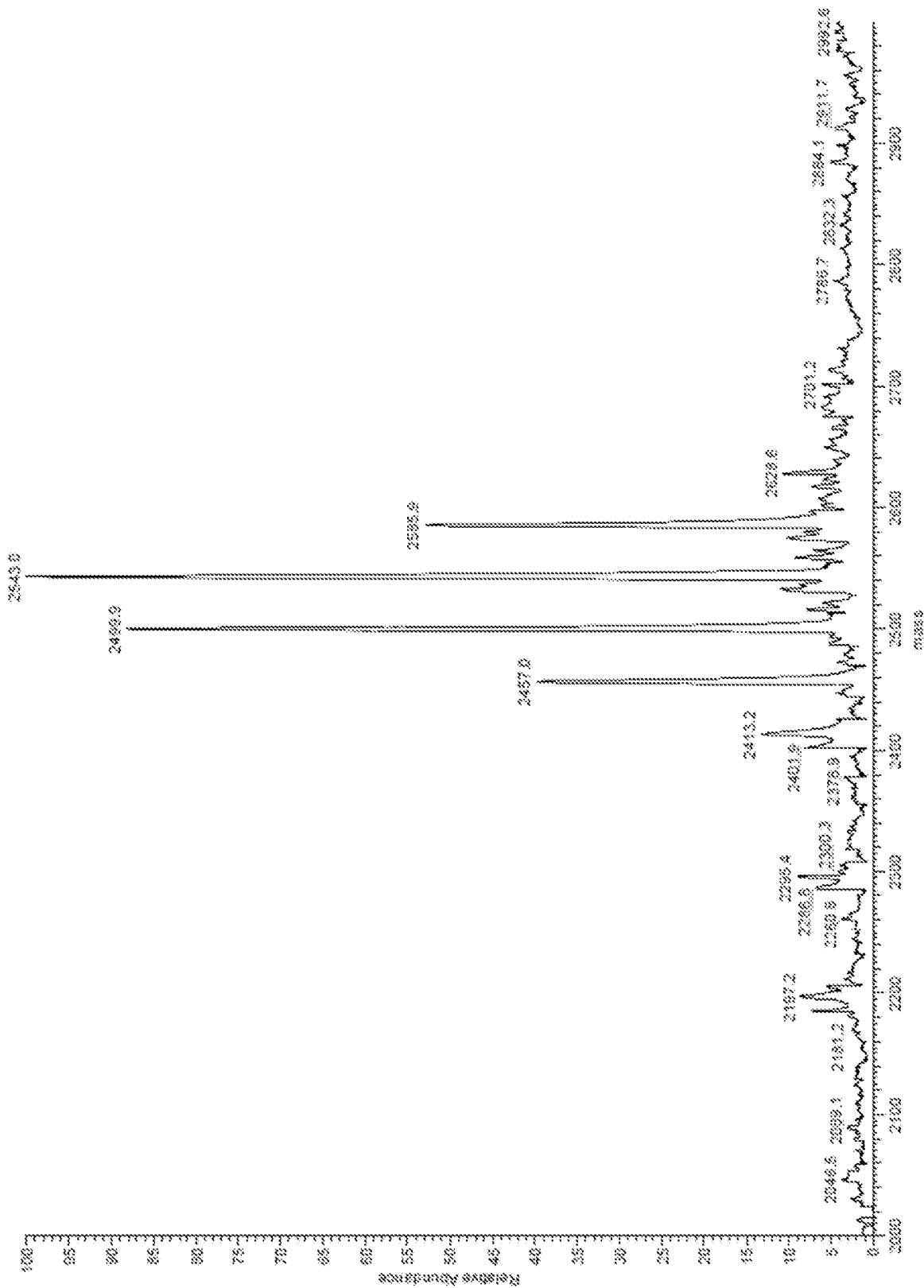


FIG. 14A

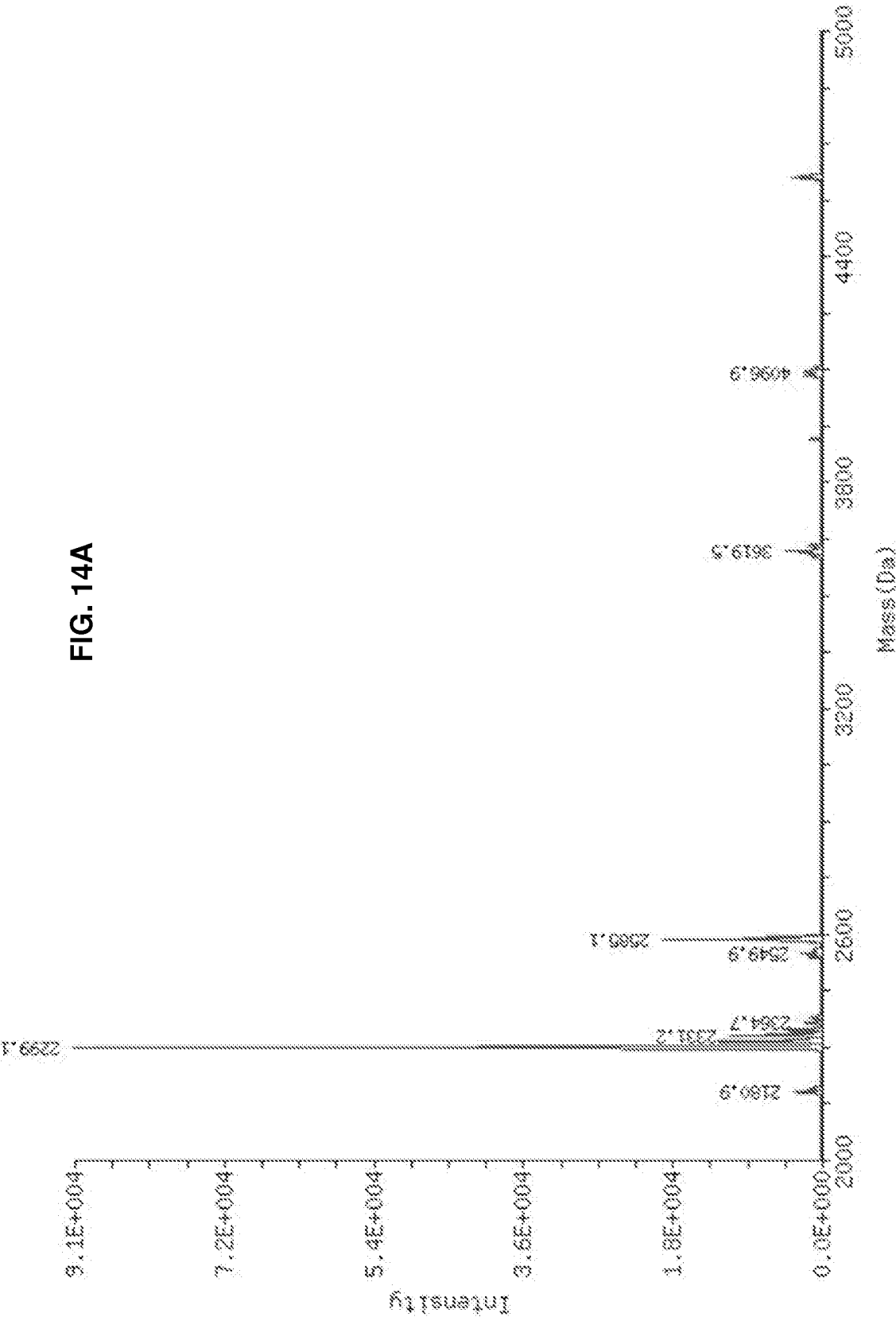


FIG. 14B

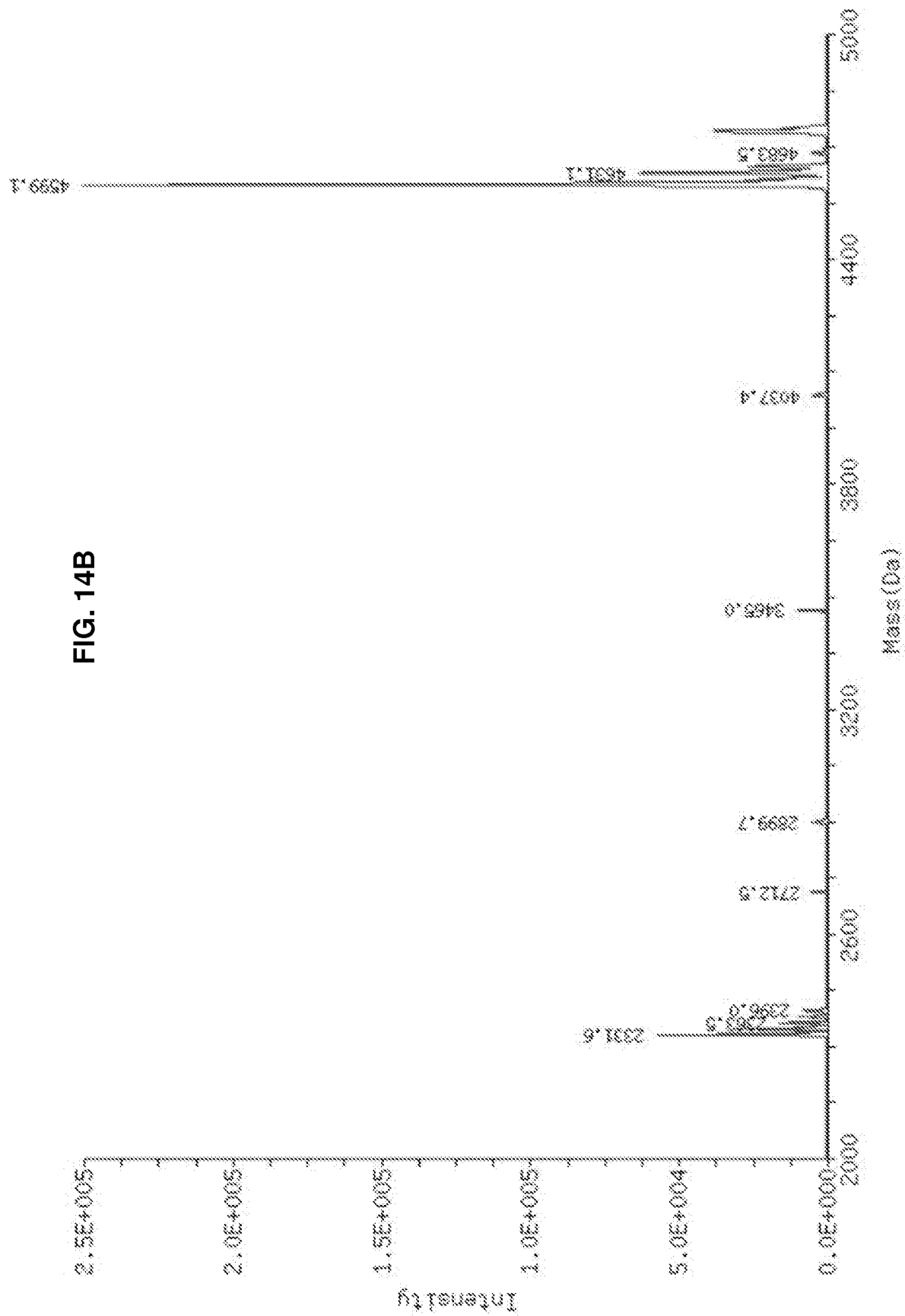


FIG. 15A

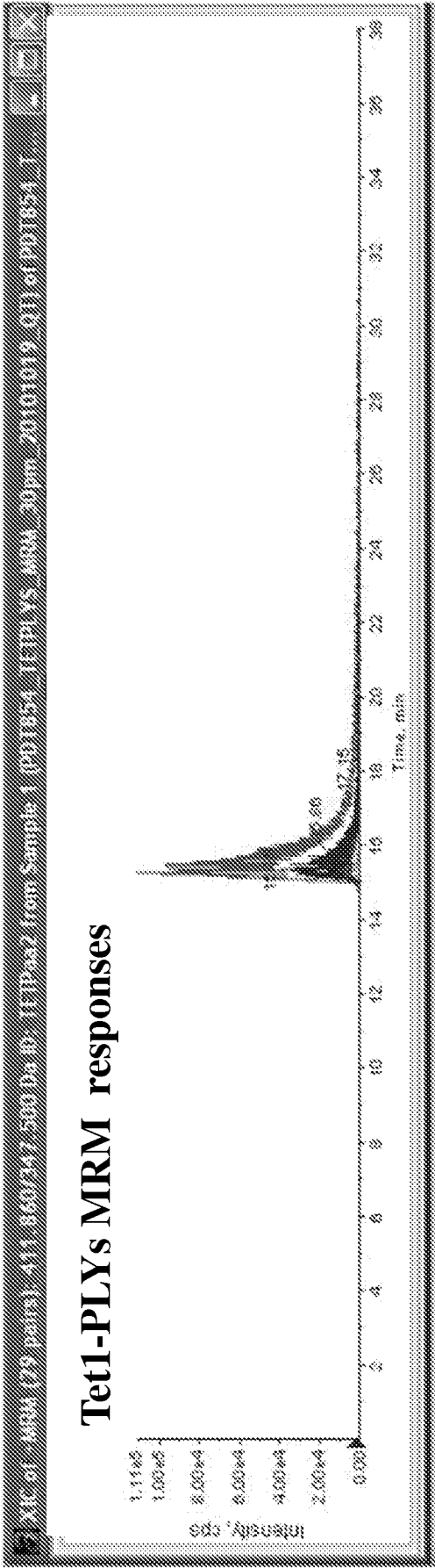


FIG. 15B

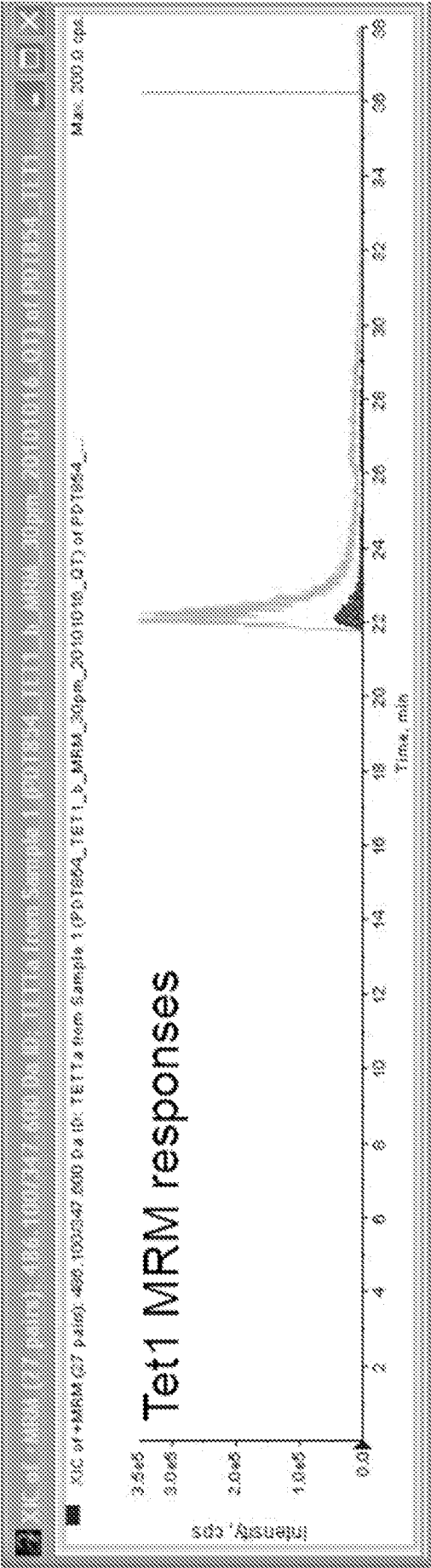


FIG. 15C

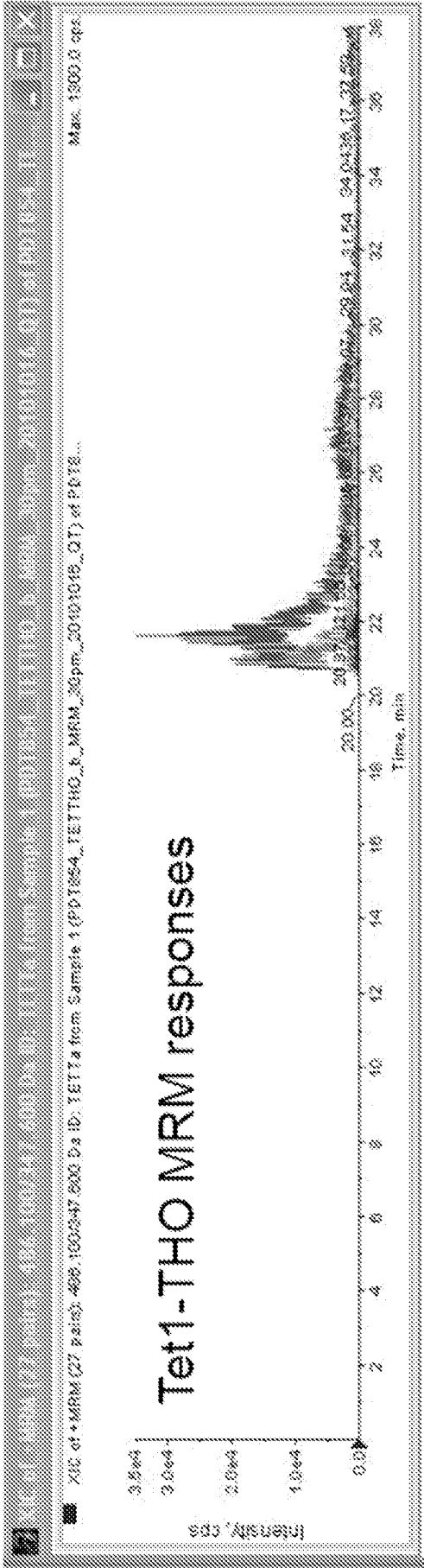


FIG. 16A

