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(72) Inventeur/Inventor:  
GARMAN, JONATHAN DAVID, CA  
(73) Propriétaire/Owner:  
NONO INC., CA  
(74) Agent: SMART & BIGGAR LLP

(54) Titre : FORMULATION LYOPHILISEE DE TAT-NR2B9C  
(54) Title: LYOPHILIZED FORMULATION OF TAT-NR2B9C

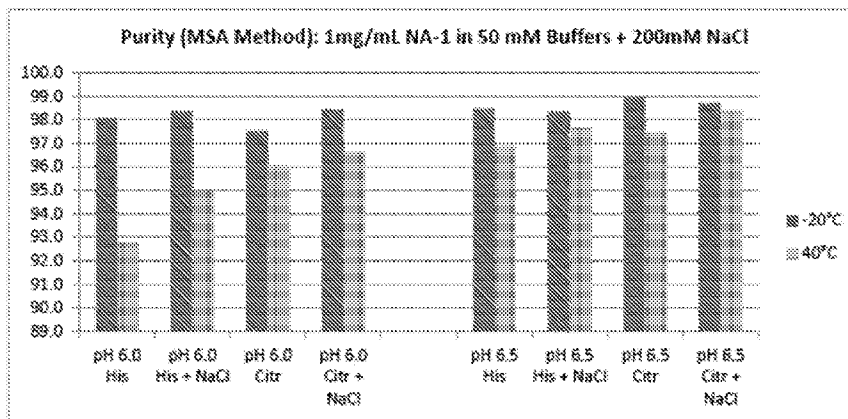


Figure B. Plot of Purity Results, MSA Method (Ordered by pH)

(57) **Abrégé/Abstract:**

The present invention provides lyophilized formulations of active agents, particularly of TAT-NR2B9c. TAT-NR2B9c has shown promise for treating stroke, aneurysm, subarachnoid hemorrhage and other neurological or neurotraumatic conditions. Such formulations are stable at room temperature thus facilitating maintenance of supplies of such a formulation in ambulances for administration at the scene of illness or accident or in transit to a hospital.

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- (71) Applicant: **NONO INC.** [CA/CA]; 88 Strath Avenue,  
Toronto, Ontario M8X 1R5 (CA).
- (72) Inventor; and  
(71) Applicant: **GARMAN, Jonathan David** [US/CA]; 148  
Lawrie Road, Thornhill, Ontario L4J 8N1 (CA).
- (74) Agents: **LIEBESCHUETZ, Joe** et al.; Alston & Bird  
LLP, Bank of America Plaza, 101 South Tryon Street,  
Suite 4000, Charlotte, North Carolina 28280-4000 (US).
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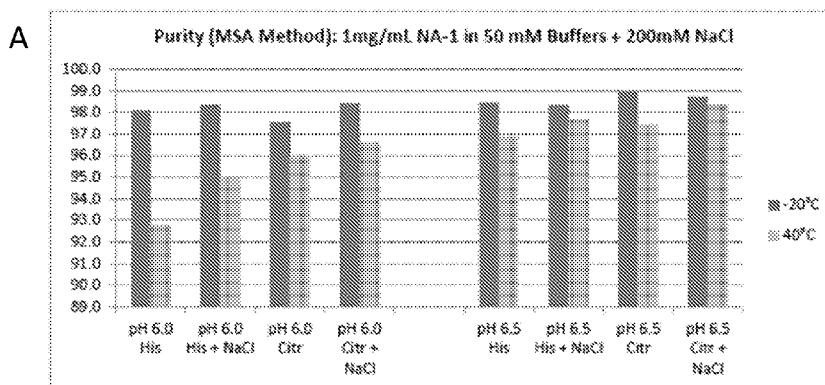


Figure 8. Plot of Purity Results, MSA Method (Ordered by pH)

FIG. 2A

(57) Abstract: The present invention provides lyophilized formulations of active agents, particularly of TAT-NR2B9c. TAT-NR2B9c has shown promise for treating stroke, aneurysm, subarachnoid hemorrhage and other neurological or neurotraumatic conditions. Such formulations are stable at room temperature thus facilitating maintenance of supplies of such a formulation in ambulances for administration at the scene of illness or accident or in transit to a hospital.

## LYOPHILIZED FORMULATION OF TAT-NR2B9C

### CROSS-REFERENCE TO RELATED APPLICATION

**[001]** The present application claims priority to US 61/730,952; filed November 28, 2012.

### SEQUENCE LISTING

**[002]** This description contains a sequence listing in electronic form in ASCII text format. A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

### BACKGROUND

**[003]** Tat-NR2B9c (NA-1) is an agent that inhibits PSD-95, thus disrupting binding to N-methyl-D-aspartate receptors (NMDARs) and neuronal nitric oxide synthases (nNOS) and reducing excitotoxicity induced by cerebral ischemia. Treatment reduces infarction size and functional deficits. TAT-NR2B9c (NA-1) has undergone a successful phase II trial (see WO 2010144721 and Aarts et al., Science 298, 846-850 (2002), Hill et al., The Lancet Neurology, 11:942 – 950 (2012)).

**[004]** Because TAT-NR2B9c is free of serious side effects, it can be administered when stroke or other ischemic conditions or hemorrhagic conditions are suspected without a diagnosis according to art-recognized criteria having been made to confirm that no hemorrhage is present. For example, TAT-NR2B9c can be administered at the location where the stroke or neurotrauma has occurred (e.g., in the patients' home) or in an ambulance transporting a subject to a hospital.

### SUMMARY OF THE CLAIMED INVENTION

**[005]** The invention provides a prelyophilized formulation comprising TAT-NR2B9c (SEQ ID NO:6), histidine and trehalose at a pH of 6-7. Optionally, the TAT-NR2B9c is at a concentration of 70-120 mg/ml, the histidine is at a concentration of 15-100 mM, and the trehalose is at a concentration of 80-160 mM. Optionally, the TAT-NR2B9c is at a concentration of 70-120 mg/ml, the histidine is at a concentration of 20-100 mM, and the trehalose is at a concentration of 100-140 mM.

**[006]** Optionally, the Tat-NR2B9c is at a concentration of 70-120 mg/ml, the concentration of histidine 20-50 mM, and the concentration of trehalose is 100-140 mM, and the pH is 6-7.

Optionally, the concentration of histidine is 20 mM and the concentration of trehalose is 100-200 mM, preferably 120 mM and the concentration of TAT-NR2B9c is 90 mg/ml.

**[007]** The invention further provides a lyophilized formulation prepared by lyophilizing any of the above described prelyophilized formulations.

**[008]** The invention further provides a reconstituted formulation prepared by combining a lyophilized formulation as described above with an aqueous solution. Optionally, the aqueous solution is water or normal saline. Optionally, the volume of the reconstituted formulation is 3-6 times the volume of the prelyophilized formulation.

**[009]** The invention further provides a reconstituted formulation comprising TAT-NR2B9c at concentration of 15-25 mg/ml, histidine at a concentration of 4-20 mM and trehalose at a concentration of 20-30 mM at pH 6-7.

**[0010]** The invention further provides a method of preparing a formulation, comprising storing a lyophilized formulation sample as described herein for at least a week at room temperature; and reconstituting the lyophilized formulation. The method can further include administering the reconstituted formulation, optionally after further dilution in normal saline, to a patient. Optionally, the patient has stroke or traumatic injury to the CNS. Optionally, the lyophilized sample is stored in an ambulance. Optionally, the patient has a subarachnoid hemorrhage. Optionally, the patient is undergoing endovascular repair for an aneurysm.

**[0011]** Various embodiments of the claimed invention relate to a prelyophilized formulation comprising TAT-NR2B9c (SEQ ID NO:6), histidine and trehalose at a pH of 6-7, wherein the TAT-NR2B9c is at a concentration of 70-120 mg/ml, the histidine is at a concentration of 15-100 mM, and the trehalose is at a concentration of 80-160 mM.

**[0012]** Various embodiments of the claimed invention relate to a reconstituted formulation comprising TAT-NR2B9c at concentration of 15-25 mg/ml, histidine at a concentration of 4-20 mM and trehalose at a concentration of 20-30 mM at pH 6-7.

**[0013]** Various embodiments of the claimed invention relate to a prelyophilized formulation comprising a peptidomimetic of TAT-NR2B9c (SEQ ID NO:6), which differs from SEQ ID NO:6 in that one or more amino acids are D-amino acids, histidine and trehalose at a pH of 6-7, wherein the peptidomimetic of TAT-NR2B9c is at a concentration of 70-120 mg/ml, the

histidine is at a concentration of 15-100 mM, and the trehalose is at a concentration of 80-160 mM.

#### BRIEF DESCRIPTIONS OF THE FIGURES

**[0014]** Figure 1: Graph shows the infarct area of the rat brain after 3PVO stroke following treatment with different formulations of NA-1.

**[0015]** Figures 2A, B: A) Bar graph demonstrating the stability of different NA-1 formulations at -20°C and 40°C. Y axis represents purity of the NA-1 after 1 week at the storage temperature as measured by % total area using RP-HPLC. B) same data as A, but sorted by buffer and pH.

**[0016]** Figure 3: Bar graph demonstrating the stability (by HPLC) of 20 mg/ml NA-1 in Histidine buffer, pH 6.5, in the presence of different bulking agents and salt at -20oC and 40oC.

**[0017]** Figures 4A, B: Differential scanning calorimetry graphs of 20 mg/ml NA-1 in histidine buffer pH 6.5 in the presence of Mannitol (A) or Mannitol and NaCl (B).

**[0018]** Figures 5A, B: Differential scanning calorimetry graphs of 20 mg/ml NA-1 in histidine buffer pH 6.5 in the presence of Trehalose (A) or Trehalose and NaCl (B).

**[0019]** Figures 6A, B: Differential scanning calorimetry graph of 20 mg/ml NA-1 in histidine buffer pH 6.5 in the presence of Dextran-40 (A) or Dextran-40 and NaCl (B).

**[0020]** Figures 7A, B: A) Cake appearance following lyophilization of 3 mL of 90 mg/ml NA-1 in 100 mM Histidine pH 6.5 with 120 mM Trehalose. B). Cake appearance of alternative NA-1 formulations with different amounts of histidine and trehalose.

## DEFINITIONS

**[0021]** As well as active ingredients, lyophilized formulations can include one or more of the following classes of components. The classes are not mutually exclusive; in other words the same agent can component can fall within multiple classes.

**[0022]** A "bulking agent" provides structure to a freeze-dried peptide. Bulking agents include, mannitol, trehalose, dextran-40, glycine, lactose, sorbitol, and sucrose among others. In addition to providing a pharmaceutically elegant cake, bulking agents may also impart useful qualities in regard to modifying the collapse temperature, providing freeze-thaw protection, glass transition temperature and enhancing the protein stability over long-term storage. These agents can also serve as tonicity modifiers.

**[0023]** A buffer is an agent that maintains the solution pH in an acceptable range prior to lyophilization. A preferred buffer is histidine. Other buffers include succinate (sodium or potassium), histidine, citrate (sodium), gluconate, acetate, phosphate, Tris and the like. Preferred buffers are effective in a pH range from about 5.5 to about 7 or about 6 to about 7.7; preferably a pH of about 6.5. Examples of buffers that control the pH in this range include succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers.

**[0024]** A "cryoprotectant" provides stability to a peptide against freezing-induced stresses, presumably by being preferentially excluded from the protein surface. It may also offer protection during primary and secondary drying, and long-term product storage. Examples are polymers such as dextran and polyethylene glycol; sugars such as sucrose, glucose, trehalose,

and lactose; and surfactants such as polysorbates; and amino acids such as glycine, arginine, and serine.

**[0025]** A lyoprotectant provides stability to the peptide during the drying or `dehydration` process (primary and secondary drying cycles), presumably by providing an amorphous glassy matrix and by binding with the protein through hydrogen bonding, replacing the water molecules that are removed during the drying process. This helps to maintain the peptide conformation, minimize peptide degradation during the lyophilization cycle and improve the long-term product stability. Examples include polyols or sugars such as sucrose and trehalose.

**[0026]** To the extent not already mentioned, other stabilizers or inhibitors of degradations can be included deamidation inhibitors, surfactants, some common ones are fatty acid esters of sorbitan polyethoxylates (e.g., polysorbate 20 or polysorbate 80), poloxamer 188, and detergents.

**[0027]** The terms "lyophilization," "lyophilized," and "freeze-dried" refer to a process by which the material to be dried is first frozen and then the ice or frozen solvent is removed by sublimation in a vacuum environment.

**[0028]** A "pharmaceutical formulation" or composition is a preparation that permits an active agent to be effective, and lacks additional components which are toxic to the subjects to which the formulation would be administered.

**[0029]** "Reconstitution time" is the time that is required to rehydrate a lyophilized formulation with a solution to solution which is free of particles to the naked eye.

**[0030]** A "stable" lyophilized peptide formulation is one with no significant changes observed at 20° C for at least one week, month, or more preferably at least three months, at least six months or a year. Changes are considered insignificant if no more than 10%, preferably 5%, of peptide is degraded as measured by SEC-HPLC. The rehydrated solution is colorless, or clear to slightly opalescent by visual analysis. The concentration, pH and osmolality of the formulation have no more than +/-10% change after storage. Potency is within 70-130%, preferably 80-120% or sometimes 80-100% of a freshly prepared control sample. No more than 10%, preferably 5% of clipping is observed. No more than 10%, preferably 5% of aggregation is formed. . Stability can be measured by various methods reviewed in Peptide and Protein Drug Delivery, 247-301,

Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10:29-90 (1993).

**[0031]** The term "isotonic" means that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 270-328 mOsm. Slightly hypotonic pressure is 250-269 and slightly hypertonic pressure is 328-350 mOsm. Osmotic pressure can be measured, for example, using a vapor pressure or ice-freezing type osmometer.

**[0032]** Tonicity Modifiers: Salts (NaCl, KCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>) can be used as tonicity modifiers to control osmotic pressure. In addition, cryoprotectants/lyoprotectants and/or bulking agents such as sucrose, mannitol, or glycine can serve as tonicity modifiers.

**[0033]** Numeric values such as concentrations or pH's are given within a tolerance reflecting the accuracy with which the value can be measured. Unless the context requires otherwise, fractional values are rounded to the nearest integer. Unless the context requires otherwise, recitation of a range of values means that any integer or subrange within the range can be used.

## DETAILED DESCRIPTION

### I. General

**[0034]** The present invention provides lyophilized formulations of active agents, particularly of TAT-NR2B9c. Such formulations are stable at room temperature thus facilitating maintenance of supplies of such a formulation in ambulances or the like or with emergency personnel for administration at the scene of illness or accident or between such scene and a medical facility.

**[0035]** Lyophilized formulations are prepared from a prelyophilized formulation comprising an active agent, a buffer, a bulking agent and water. Other components, such as cryo or lyopreservatives, a tonicity agent pharmaceutically acceptable carriers and the like may or may be present. A preferred active agent is TAT-NR2B9c. A preferred buffer is histidine. A preferred bulking agent is trehalose. Trehalose also serves as a cryo and lyo-preservative. An exemplary prelyophilized formulation comprises the active agent (e.g., TAT-NR2B9c), histidine (10-100 mM, 15-100 mM 15-80 mM, 40-60 mM or 15-60 mM, for example, 20 mM or optionally 50 mM, or 20-50mM)) and trehalose (50-200 mM, preferably 80-160 mM, 100-140 mM, more preferably 120 mM). The pH is 5.5 to 7.5, more preferably, 6-7, more preferably 6.5.



The concentration of active agent (e.g., TAT-NR2B9c) is 20-200 mg/ml, preferably 50-150 mg/ml, more preferably 70-120 mg/ml or 90 mg/ml. Thus, an exemplary prelyophilized formulation is 20 mM histidine, 120 mM trehalose, and 90 mg/ml TAT-NR2B9c.

**[0036]** After lyophilization, lyophilized formulations have a low-water content, preferably from about 0%-5% water, more preferably below 2.5% water by weight. Lyophilized formulations can be stored in a freezer (e.g., -20 or -70° C), in a refrigerator (0-4° C) or at room temperature (20-25° C).

**[0037]** Active agents are reconstituted in an aqueous solution, preferably water for injection or optionally normal saline (0.8-1.0% saline and preferably 0.9% saline). Reconstitution can be to the same or a smaller or larger volume than the prelyophilized formulation. Preferably, the volume is larger post-reconstitution than before (e.g., 3-6 times larger). For example, a prelyophilization volume of 3-5 ml can be reconstituted as a volume of 13.5 ml. After reconstitution, the concentration of histidine is preferably 2-20 mM, e.g., 2-7 mM or 4.5 mM; the concentration of trehalose is preferably 15-45 mM or 20-30 mM or 25-27 mM. The active agent is preferably at a concentration of 10-30 mg/ml, for example 15-25, 18-20 or 20 mg/ml of active agent (e.g., TAT-NR2B9c). An exemplary formulation after reconstitution has 4-5 mM histidine, 26-27 mM trehalose and 20 mg/ml TAT-NR2B9c (with concentrations rounded to the nearest integer). The reconstituted formulation can be further diluted before administration such as by adding into a fluid bag containing normal saline for intravenous infusion.

**[0038]** Methods of freeze drying are set forth, for example, in *Methods in Enzymology*, Vol. 22, Pages 33-39, Academic Press, New York (1971); and in *Freeze-Drying*, E. W. Flosdorf, Rheinhold, New York (1949). TAT-NR2B9c is preferably lyophilized in the same vial as that in which it will be reconstituted for use. An aqueous solution of TAT-NR2B9c is added to the vial optionally after filtering through a sterilizing filtration system, such as a 0.22 micron filter standardly used for peptides. Formulations can be lyophilized in a controlled cycle, such as described in the Examples. A prelyophilized formulation can be placed in a vial, and lyophilized at reduced temperature and pressure. After lyophilization, vials can be sealed. For use, the lyophilizate is reconstituted with water for injection, normal saline or other pharmaceutically acceptable carrier or diluent.

**[0039]** A variety of containers are suitable for lyophilization. A container should be able to withstand the outside pressure when the container is sealed and stored under partial vacuum. The container should be made of a material that allows a reasonable transfer of heat from outside to inside. The size of the container should be such that the solution to be lyophilized occupies not more than 20% of the useful volume or may be overfilled with an excess, in accord with then-prevailing USP recommendations for the volume in a container. For example, a 0.5 ml solution may be filled in a 3 ml vial. The vials may be made of glass e.g. borosilicate, or plastic, e.g. polypropylene.

**[0040]** Glass bottles commonly used for lyophilizing biological materials can be used. Another suitable container is a two-compartment syringe wherein one compartment contains the lyophilized TAT-NR2B9c peptide cake and the other compartment contains the aqueous diluent. After lyophilization is complete, the vacuum within the vials or ampules may be released by filling the system with an inert gas, stoppered in place using standard equipment and then crimp sealed. Such a method will ensure a sterile final product. Other two-part solutions such as a bag with a breakable seal between the lyophilized drug compartment and the diluent can be used as well.

## II. Active Agents

**[0041]** Active agents inhibit interaction between PSD-95 and one or more NMDARs (e.g., 2A, 2B, 2C or 2D) or nNOS (e.g., Swiss-Prot P29475) by binding to PSD-95. Such agents are useful for reducing one or more damaging effects of stroke and other neurological conditions mediated at least in part by NMDAR excitotoxicity. Such agents include peptides having an amino acid sequence including or based on the PL motif of a NMDA Receptor or PDZ domain of PSD-95. Such peptides can also inhibit interactions between PSD-95 and nNOS and other glutamate receptors (e.g., kainite receptors or AMPA receptors), such as KV1-4 and GluR6. Preferred peptides inhibit interaction between PDZ domains 1 and 2 of postsynaptic density-95 protein (PSD-95)(human amino acid sequence provided by Stathakism, Genomics 44(1):71-82 (1997)) and the C-terminal PL sequence of one or more NMDA Receptor 2 subunits including the NR2B subunit of the neuronal N-methyl-D-aspartate receptor (Mandich et al., Genomics 22, 216-8 (1994)). NMDAR2B has GenBank ID 4099612, a C-terminal 20 amino acids FNGSSNGHVYEKLSSIESDV (SEQ ID NO:11) and a PL motif ESDV (SEQ ID NO:12). Preferred peptides inhibit the human forms of PSD-95 and human NMDAR receptors. However,

inhibition can also be shown from species variants of the proteins. A list of NMDA and glutamate receptors that can be used appears below:

#### NMDA Receptors With PL Sequences

Name	GI or Acc#	C-terminal 20mer sequence	C-terminal 4mer sequence	PL?	internal PL ID
NMDAR1	307302	HPTDITGPLNLSDPSVST VV (SEQ ID NO:13)	STVV (SEQ ID NO:27)	X	AA216
NMDAR1-1	292282	HPTDITGPLNLSDPSVST VV (SEQ ID NO:13)	STVV (SEQ ID NO:27)	X	AA216
NMDAR1-4	472845	HPTDITGPLNLSDPSVST VV (SEQ ID NO:13)	STVV (SEQ ID NO:27)	X	AA216
NMDAR1-3b	2343286	HPTDITGPLNLSDPSVST VV (SEQ ID NO:13)	STVV (SEQ ID NO:27)	X	AA216
NMDAR1-4b	2343288	HPTDITGPLNLSDPSVST VV (SEQ ID NO:13)	STVV (SEQ ID NO:27)	X	AA216
NMDAR1-2	11038634	RRAIEREEGQLQLCSRH RES (SEQ ID NO:14)	HRES (SEQ ID NO:28)		
NMDAR1-3	11038636	RRAIEREEGQLQLCSRH RES (SEQ ID NO:14)	HRES (SEQ ID NO:28)		
NMDAR2C	6006004	TQGFPGPCTWRRISLES EV (SEQ ID NO:15)	ESEV (SEQ ID NO:29)	X	AA180
NMDAR3	560546	FNGSSNGHVYEKLSSIES DV (SEQ ID NO:11)	ESDV (SEQ ID NO:12)	X	AA34.1
NMDAR3A	17530176	AVSRKTELEEYQRTSRT CES (SEQ ID NO:16)	TCES (SEQ ID NO:30)		
NMDAR2B	4099612	FNGSSNGHVYEKLSSIES DV (SEQ ID NO:11)	ESDV (SEQ ID NO:12)	X	
NMDAR2A	558748	LNCSNRRVYKKMPSIE SDV (SEQ ID NO:17)	ESDV (SEQ ID NO:12)	X	AA34.2
NMDAR2D	4504130	GGDLGTRRGS AHFSSLE SEV (SEQ ID NO:18)	ESEV (SEQ ID NO:18)	X	

Name	GI or Acc#	C-terminal 20mer sequence	C-terminal 4mer sequence	PL?	internal PL ID
			NO:29)		
Glutamate receptor delta 2	AF009014	QPTPTLGLNLGNDPDRG TSI (SEQ ID NO:19)	GTSI (SEQ ID NO:31)	X	
Glutamate receptor 1	I28953	MQSIPCMSHSSGMPLGA TGL (SEQ ID NO:20)	ATGL (SEQ ID NO:32)	X	
Glutamate receptor 2	L20814	QNFATYKEGYNVYGIES VKI (SEQ ID NO:21)	SVKI (SEQ ID NO:33)	X	
Glutamate receptor 3	AF167332	QNYATYREGYNVYGTE SVKI (SEQ ID NO:22)	SVKI (SEQ ID NO:33)	X	
Glutamate receptor 4	U16129	HTGTAIRQSSGLAVIASD LP (SEQ ID NO:23)	SDLP (SEQ ID NO:34)		
Glutamate receptor 5	U16125	SFTSILTCHQRRRTQRKET VA (SEQ ID NO:24)	ETVA (SEQ ID NO:35)	X	
Glutamate receptor 6	U16126	EVINMHTFNDRLPGKE TMA (SEQ ID NO:25)	ETMA (SEQ ID NO:36)	X	

**[0042]** Peptides can include or be based on a PL motif from the C-terminus of any of the above subunits and have an amino acid sequence comprising [S/T]-X-[V/L]. This sequence preferably occurs at the C-terminus of the peptides of the invention. Preferred peptides have an amino acid sequence comprising [E/D/N/Q]-[S/T]-[D/E/Q/N]-[V/L] (SEQ ID NO:38) at their C-terminus. Exemplary peptides comprise: ESDV (SEQ ID NO:12), ESEV (SEQ ID NO:29), ETDV (SEQ ID NO:39), ETEV (SEQ ID NO:40), DTDV (SEQ ID NO:41), and DTEV (SEQ ID NO:42) as the C-terminal amino acids. Two particularly preferred peptides are KLSSIESDV (SEQ ID NO:5), and KLSSIETDV (SEQ ID NO:43). Such peptides usually have 3-25 amino acids (without an internalization peptide), peptide lengths of 5-10 amino acids, and particularly 9 amino acids (also without an internalization peptide) are preferred. In some such peptides, all amino acids are from the C-terminus of an NMDA receptor (not including amino acids from an internalization peptide).

**[0043]** Peptides and peptidomimetics of the invention can contain modified amino acid residues for example, residues that are N-alkylated. N-terminal alkyl modifications can include e.g., N-

Methyl, N-Ethyl, N-Propyl, N-Butyl, N-Cyclohexylmethyl, N-Cyclohexylethyl, N-Benzyl, N-Phenylethyl, N-phenylpropyl, N-(3, 4-Dichlorophenyl)propyl, N-(3,4-Difluorophenyl)propyl, and N-(Naphthalene-2-yl)ethyl).

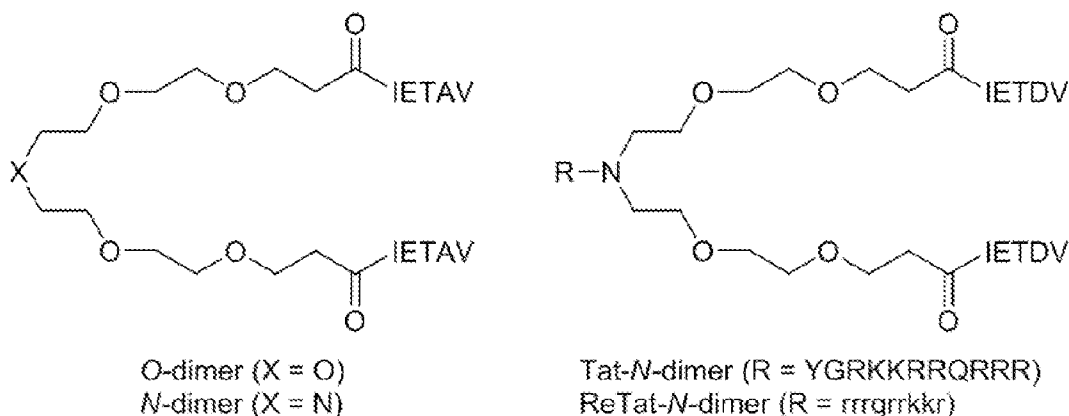
**[0044]** Bach, J. Med. Chem. 51, 6450-6459 (2008) and WO 2010/004003 have described a series of analogs of NR2B9c (SEQ ID NO:6). PDZ-binding activity is exhibited by peptides having only three C-terminal amino acids (SDV). Bach also reports analogs having an amino acid sequence comprising or consisting of X<sub>1</sub>tSX<sub>2</sub>V (SEQ ID NO:68), wherein t and S are alternative amino acids, X<sub>1</sub> is selected from among E, Q, and A, or an analogue thereof, X<sub>2</sub> is selected from among A, Q, D, N, N-Me-A, N-Me-Q, N-Me-D, and N-Me-N or an analog thereof. Optionally the peptide is N-alkylated in the P3 position (third amino acid from C-terminus, i.e. position occupied by tS). The peptide can be N-alkylated with a cyclohexane or aromatic substituent, and further comprises a spacer group between the substituent and the terminal amino group of the peptide or peptide analogue, wherein the spacer is an alkyl group, preferably selected from among methylene, ethylene, propylene and butylene. The aromatic substituent can be a naphthalen-2-yl moiety or an aromatic ring substituted with one or two halogen and/or alkyl group.

**[0045]** Other modifications can also be incorporated without adversely affecting the activity and these include substitution of one or more of the amino acids in the natural L-isomeric form with amino acids in the D-isomeric form. Thus, any amino acid naturally occurring in the L-configuration (which can also be referred to as the R or S, depending upon the structure of the chemical entity) can be replaced with the amino acid of the same chemical structural type or a peptidomimetic, but of the opposite chirality, generally referred to as the D-amino acid, but which can additionally be referred to as the R- or S-form. Thus, a peptidomimetic may include 1, 2, 3, 4, 5, at least 50%, or all D-amino acid residues. A peptidomimetic containing some or all D residues is sometimes referred to an “inverso” peptide.

**[0046]** Peptidomimetics also include retro peptides. A retro peptide has a reverse amino acid sequence. Peptidomimetics also include retro inverso peptides in which the order of amino acids is reversed from so the originally C-terminal amino acid appears at the N-terminus and D-amino acids are used in place of L-amino acids. WO 2008/014917 describes a retro-inverso analog of Tat-NR2B9c having the amino acid sequence vdseisslk-rrrqrkkrgyin (SEQ ID NO:69) (lower

case letters indicating D amino acids), and reports it to be effective inhibiting cerebral ischemia. Another effective peptide described herein is Rv-Tat-NR2B9c (RRRQRRKKRGYKLSSIESDV; SEQ ID NO:70).

[0047] A linker, e.g., a polyethylene glycol linker, can be used to dimerize the active moiety of the peptide or the peptidomimetic to enhance its affinity and selectivity towards proteins containing tandem PDZ domains. See e.g., Bach et al., (2009) *Angew. Chem. Int. Ed.* 48:9685-9689 and WO 2010/004003. A PL motif-containing peptide is preferably dimerized via joining the N-termini of two such molecules, leaving the C-termini free. Bach further reports that a pentamer peptide IESDV (SEQ ID NO:71) from the C-terminus of NMDAR 2B was effective in inhibiting binding of NMDAR 2B to PSD-95. IETDV (SEQ ID NO:73) can also be used instead of IESDV. Optionally, about 2-10 copies of a PEG can be joined in tandem as a linker. Optionally, the linker can also be attached to an internalization peptide or lipidated to enhance cellular uptake. Examples of illustrative dimeric inhibitors are shown below (see Bach et al., *PNAS* 109 (2012) 3317-3322). Any of the PSD-95 inhibitors disclosed herein can be used instead of IETDV, and any internalization peptide or lipidating moiety can be used instead of tat. Other linkers to that shown can also be used.



[0048] IETAV is assigned SEQ ID NO:26, YGRKKRRQRRR SEQ ID NO:2, and rrrqrrkkr, SEQ ID NO:10, lower case letters indicated D-amino acids.

[0049] Appropriate pharmacological activity of peptides, peptidomimetics or other agent can be confirmed if desired, using previously described rat models of stroke before testing in the

primate and clinical trials described in the present application. Peptides or peptidomimetics can also be screened for capacity to inhibit interactions between PSD-95 and NMDAR 2B using assays described in e.g., US 20050059597. Useful peptides typically have IC<sub>50</sub> values of less than 50  $\mu$ M, 25  $\mu$ M, 10  $\mu$ M, 0.1  $\mu$ M or 0.01  $\mu$ M in such an assay. Preferred peptides typically have an IC<sub>50</sub> value of between 0.001-1  $\mu$ M, and more preferably 0.001-0.05, 0.05-0.5 or 0.05 to 0.1  $\mu$ M. When a peptide or other agent is characterized as inhibiting binding of one interaction, e.g., PSD-95 interaction to NMDAR2B, such description does not exclude that the peptide or agent also inhibits another interaction, for example, inhibition of PSD-95 binding to nNOS.

**[0050]** Peptides such as those just described can optionally be derivatized (e.g., acetylated, phosphorylated, myristoylated, geranylated, pegylated and/or glycosylated) to improve the binding affinity of the inhibitor, to improve the ability of the inhibitor to be transported across a cell membrane or to improve stability. As a specific example, for inhibitors in which the third residue from the C-terminus is S or T, this residue can be phosphorylated before use of the peptide.

**[0051]** A pharmacological agent can be linked to an internalization peptide to facilitate uptake into cells and/or across the blood brain barrier. Internalization peptides are a well-known class of relatively short peptides that allow many cellular or viral proteins to traverse membranes. Internalization peptides, also known as cell membrane transduction peptides or cell penetrating peptides can have e.g., 5-30 amino acids. Such peptides typically have a cationic charge from an above normal representation (relative to proteins in general) of arginine and/or lysine residues that is believed to facilitate their passage across membranes. Some such peptides have at least 5, 6, 7 or 8 arginine and/or lysine residues. Examples include the antennapedia protein (Bonfanti, Cancer Res. 57, 1442-6 (1997)) (and variants thereof), the tat protein of human immunodeficiency virus, the protein VP22, the product of the UL49 gene of herpes simplex virus type 1, Penetratin, SynB1 and 3, Transportan, Amphipathic, gp41NLS, polyArg, and several plant and bacterial protein toxins, such as ricin, abrin, modeccin, diphtheria toxin, cholera toxin, anthrax toxin, heat labile toxins, and Pseudomonas aeruginosa exotoxin A (ETA). Other examples are described in the following references (Temsamani, Drug Discovery Today, 9(23):1012-1019, 2004; De Coupade, Biochem J., 390:407-418, 2005; Saalik Bioconjugate Chem. 15: 1246-1253, 2004; Zhao, Medicinal Research Reviews 24(1):1-12, 2004; Deshayes,

Cellular and Molecular Life Sciences 62:1839-49, 2005); Gao, ACS Chem. Biol. 2011, 6, 484–491, SG3 (RLSGMNEVL SFRWL (SEQ ID NO:9)), Stalmans PLoS ONE 2013, 8(8) e71752, 1-11 and supplement.

**[0052]** A preferred internalization peptide is tat from the HIV virus. A tat peptide reported in previous work comprises or consists of the standard amino acid sequence YGRKKRRQRRR (SEQ ID NO:2) found in HIV Tat protein. If additional residues flanking such a tat motif are present (beside the pharmacological agent) the residues can be for example natural amino acids flanking this segment from a tat protein, spacer or linker amino acids of a kind typically used to join two peptide domains, e.g., gly (ser)<sub>4</sub> (SEQ ID NO:44), TGEKP (SEQ ID NO:45), GGRRGGGS (SEQ ID NO:46), or LRQRDGERP (SEQ ID NO:47) (see, e.g., Tang et al. (1996), J. Biol. Chem. 271, 15682-15686; Hennecke et al. (1998), Protein Eng. 11, 405-410)), or can be any other amino acids that do not significantly reduce capacity to confer uptake of the variant without the flanking residues. Preferably, the number of flanking amino acids other than an active peptide does not exceed ten on either side of YGRKKRRQRRR (SEQ ID NO:2). One suitable tat peptide comprising additional amino acid residues flanking the C-terminus of YGRKKRRQRRR (SEQ ID NO:2) is YGRKKRRQRRRPQ (SEQ ID NO:48). However, preferably, no flanking amino acids are present. Other tat peptides that can be used include GRKKRRQRRRPQ (SEQ ID NO:4) and GRKKRRQRRRP (SEQ ID NO:72).

**[0053]** Variants of the above tat peptide having reduced capacity to bind to N-type calcium channels are described by WO/2008/109010. Such variants can comprise or consist of an amino acid sequence XGRKKRRQRRR (SEQ ID NO:49), in which X is an amino acid other than Y or nothing (in which case G is a free N-terminal residue). A preferred tat peptide has the N-terminal Y residue substituted with F. Thus, a tat peptide comprising or consisting of FGRKKRRQRRR (SEQ ID NO:3) is preferred. Another preferred variant tat peptide consists of GRKKRRQRRR (SEQ ID NO:1). Another preferred tat peptide comprises or consists of RRRQRRKKRG or RRRQRRKKRGY (amino acids 1-10 or 1-11 of SEQ ID NO:70). Other tat derived peptides that facilitate uptake of a pharmacological agent without inhibiting N-type calcium channels include those presented below.

X-FGRKKRRQRRR (F-Tat) (SEQ ID NO:8)
X-GKKKKKQKKK (SEQ ID NO:50)
X-RKKRRQRRR (SEQ ID NO:51)



X-GAKKRRQRRR (SEQ ID NO:52)
X-AKKRRQRRR (SEQ ID NO:53)
X-GRKARRQRRR (SEQ ID NO:54)
X-RKARRQRRR (SEQ ID NO:55)
X-GRKKARQRRR (SEQ ID NO:56)
X-RKKARQRRR (SEQ ID NO:57)
X-GRKKRRQARR (SEQ ID NO:58)
X-RKKRRQARR (SEQ ID NO:59)
X-GRKKRRQRAR (SEQ ID NO:60)
X-RKKRRQRAR (SEQ ID NO:61)
X-RRPRRRRRPRR (SEQ ID NO:62)
X-RRARRARRARR (SEQ ID NO:63)
X-RRRARRRARR (SEQ ID NO:64)
X-RRRPRRRPRR (SEQ ID NO:65)
X-RRPRRRPRR (SEQ ID NO:66)
X-RRARRARR (SEQ ID NO:67)

**[0054]** X can represent a free amino terminus, one or more amino acids, or a conjugated moiety. Internalization peptides can be used in inverso or retro or inverso retro form with or without the linked peptide or peptidomimetic being in such form. For example, a preferred chimeric peptide has an amino acid sequence comprising or consisting of YGRKKRRQRRR-KLSSIESDV (SEQ ID NO:6, also known as NA-1 or Tat-NR2B9c), or YGRKKRRQRRR-KLSSIETDV (SEQ ID NO:7). Other preferred peptides include RRRQRRKKRGY-KLSSIESDV (SEQ ID NO:70, also known as RvTat-NR2B9c or having an amino acid sequence comprising or consisting of RRRQRRKKRGY-KLSSIETDV (SEQ ID NO:37).

**[0055]** Internalization peptides can be attached to pharmacological agents by conventional methods. For example, the agents can be joined to internalization peptides by chemical linkage, for instance via a coupling or conjugating agent. Numerous such agents are commercially available and are reviewed by S. S. Wong, Chemistry of Protein Conjugation and Cross-Linking, CRC Press (1991). Some examples of cross-linking reagents include J-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) or N,N'-(1,3-phenylene) bismaleimide; N,N'-ethylene-bis-(iodoacetamide) or other such reagent having 6 to 11 carbon methylene bridges (which relatively specific for sulfhydryl groups); and 1,5-difluoro-2,4-dinitrobenzene (which forms irreversible linkages with amino and tyrosine groups). Other cross-linking reagents include p,p'-difluoro-m, m'-dinitrodiphenylsulfone (which forms irreversible cross-linkages with amino and phenolic

groups); dimethyl adipimidate (which is specific for amino groups); phenol-1,4-disulfonylchloride (which reacts principally with amino groups); hexamethylenediisocyanate or diisothiocyanate, or azophenyl-p-diisocyanate (which reacts principally with amino groups); glutaraldehyde (which reacts with several different side chains) and disdiazobenzidine (which reacts primarily with tyrosine and histidine).

**[0056]** For pharmacological agents that are peptides attachment to an internalization peptide can be achieved by generating a fusion protein comprising the peptide sequence fused, preferably at its N-terminus, to an internalization peptide.

**[0057]** Instead of or as well as linking a peptide (or other agent) inhibiting PSD-95 to an internalization peptide, such a peptide can be linked to a lipid (lipidation) to increase hydrophobicity of the conjugate relative to the peptide alone and thereby facilitate passage of the linked peptide across cell membranes and/or across the brain barrier. Lipidation is preferably performed on the N-terminal amino acid but can also be performed on internal amino acids, provided the ability of the peptide to inhibit interaction between PSD-95 and NMDAR 2B is not reduced by more than 50%. Preferably, lipidation is performed on an amino acid other than one of the four most C-terminal amino acids. Lipids are organic molecules more soluble in ether than water and include fatty acids, glycerides and sterols. Suitable forms of lipidation include myristoylation, palmitoylation or attachment of other fatty acids preferably with a chain length of 10-20 carbons, such as lauric acid and stearic acid, as well as geranylation, geranylgeranylation, and isoprenylation. Lipidations of a type occurring in posttranslational modification of natural proteins are preferred. Lipidation with a fatty acid via formation of an amide bond to the alpha-amino group of the N-terminal amino acid of the peptide is also preferred. Lipidation can be by peptide synthesis including a prelipidated amino acid, be performed enzymatically *in vitro* or by recombinant expression, by chemical crosslinking or chemical derivatization of the peptide. Amino acids modified by myristoylation and other lipid modifications are commercially available.

**[0058]** Lipidation preferably facilitates passage of a linked peptide (e.g., KLSSIESDV (SEQ ID NO:5), or KLSSIETDV (SEQ ID NO:43)) across a cell membrane and/or the blood brain barrier without causing a transient reduction of blood pressure as has been found when a standard tat

peptide is administered at high dosage (*e.g.*, at or greater than 3 mg/kg), or at least with smaller reduction than the same peptide linked to a standard tat peptide.

**[0059]** Pharmacologic peptides, optionally fused to tat peptides, can be synthesized by solid phase synthesis or recombinant methods. Peptidomimetics can be synthesized using a variety of procedures and methodologies described in the scientific and patent literature, *e.g.*, Organic Syntheses Collective Volumes, Gilman et al. (Eds) John Wiley & Sons, Inc., NY, al-Obeidi (1998) Mol. Biotechnol. 9:205-223; Hruby (1997) Curr. Opin. Chem. Biol. 1:114-119; Ostergaard (1997) Mol. Divers. 3:17-27; Ostresh (1996) Methods Enzymol. 267:220-234.

### III. Diseases

**[0060]** The lyophilized formulations are useful in treating a variety of diseases, particularly neurological diseases, and especially diseases mediated in part by excitotoxicity. Such diseases and conditions include stroke, epilepsy, hypoxia, subarachnoid hemorrhage, traumatic injury to the CNS not associated with stroke such as traumatic brain injury and spinal cord injury, other cerebral ischemia, Alzheimer's disease and Parkinson's disease. Other neurological diseases treatable by agents of the invention not known to be associated with excitotoxicity include anxiety and pain.

**[0061]** A stroke is a condition resulting from impaired blood flow in the CNS regardless of cause. Potential causes include embolism, hemorrhage and thrombosis. Some neuronal cells die immediately as a result of impaired blood flow. These cells release their component molecules including glutamate, which in turn activates NMDA receptors, which raise intracellular calcium levels, and intracellular enzyme levels leading to further neuronal cell death (the excitotoxicity cascade). The death of CNS tissue is referred to as infarction. Infarction Volume (*i.e.*, the volume of dead neuronal cells resulting from stroke in the brain) can be used as an indicator of the extent of pathological damage resulting from stroke. The symptomatic effect depends both on the volume of an infarction and where in the brain it is located. Disability index can be used as a measure of symptomatic damage, such as the Rankin Stroke Outcome Scale (Rankin, Scott Med J;2:200-15 (1957)) and the Barthel Index. The Rankin Scale is based on assessing directly the global conditions of a patient as follows.

0: No symptoms at all

1: No significant disability despite symptoms; able to carry out all usual duties and activities.

2: Slight disability; unable to carry out all previous activities but able to look after own affairs without assistance.

3: Moderate disability requiring some help, but able to walk without assistance

4: Moderate to severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance.

5: Severe disability; bedridden, incontinent, and requiring constant nursing care and attention.

**[0062]** The Barthel Index is based on a series of questions about the patient's ability to carry out 10 basic activities of daily living resulting in a score between 0 and 100, a lower score indicating more disability (Mahoney et al, Maryland State Medical Journal 14:56-61 (1965)).

**[0063]** Alternatively stroke severity/outcomes can be measured using the NIH stroke scale, available at world wide web [ninds.nih.gov/doctors/NIH Stroke ScaleJBooklet.pdf](http://ninds.nih.gov/doctors/NIH_Stroke_ScaleJBooklet.pdf).

**[0064]** The scale is based on the ability of a patient to carry out 11 groups of functions that include assessments of the patient's level of consciousness, motor, sensory and language functions.

**[0065]** An ischemic stroke refers more specifically to a type of stroke that caused by blockage of blood flow to the brain. The underlying condition for this type of blockage is most commonly the development of fatty deposits lining the vessel walls. This condition is called atherosclerosis. These fatty deposits can cause two types of obstruction. Cerebral thrombosis refers to a thrombus (blood clot) that develops at the clogged part of the vessel "Cerebral embolism" refers generally to a blood clot that forms at another location in the circulatory system, usually the heart and large arteries of the upper chest and neck. A portion of the blood clot then breaks loose, enters the bloodstream and travels through the brain's blood vessels until it reaches vessels too small to let it pass. A second important cause of embolism is an irregular heartbeat, known as arterial fibrillation. It creates conditions in which clots can form in the heart, dislodge and travel to the brain. Additional potential causes of ischemic stroke are hemorrhage, thrombosis, dissection of an artery or vein, a cardiac arrest, shock of any cause including hemorrhage, and iatrogenic

causes such as direct surgical injury to brain blood vessels or vessels leading to the brain or cardiac surgery. Ischemic stroke accounts for about 83 percent of all cases of stroke.

**[0066]** Transient ischemic attacks (TIAs) are minor or warning strokes. In a TIA, conditions indicative of an ischemic stroke are present and the typical stroke warning signs develop. However, the obstruction (blood clot) occurs for a short time and tends to resolve itself through normal mechanisms. Patients undergoing heart surgery are at particular risk of transient cerebral ischemic attack.

**[0067]** Hemorrhagic stroke accounts for about 17 percent of stroke cases. It results from a weakened vessel that ruptures and bleeds into the surrounding brain. The blood accumulates and compresses the surrounding brain tissue. The two general types of hemorrhagic strokes are intracerebral hemorrhage and subarachnoid hemorrhage. Hemorrhagic stroke result from rupture of a weakened blood vessel ruptures. Potential causes of rupture from a weakened blood vessel include a hypertensive hemorrhage, in which high blood pressure causes a rupture of a blood vessel, or another underlying cause of weakened blood vessels such as a ruptured brain vascular malformation including a brain aneurysm, arteriovenous malformation (AVM) or cavernous malformation. Hemorrhagic strokes can also arise from a hemorrhagic transformation of an ischemic stroke which weakens the blood vessels in the infarct, or a hemorrhage from primary or metastatic tumors in the CNS which contain abnormally weak blood vessels. Hemorrhagic stroke can also arise from iatrogenic causes such as direct surgical injury to a brain blood vessel. An aneurysm is a ballooning of a weakened region of a blood vessel. If left untreated, the aneurysm continues to weaken until it ruptures and bleeds into the brain. An arteriovenous malformation (AVM) is a cluster of abnormally formed blood vessels. A cavernous malformation is a venous abnormality that can cause a hemorrhage from weakened venous structures. Any one of these vessels can rupture, also causing bleeding into the brain. Hemorrhagic stroke can also result from physical trauma. Hemorrhagic stroke in one part of the brain can lead to ischemic stroke in another through shortage of blood lost in the hemorrhagic stroke.

**[0068]** One patient class amenable to treatments are patients undergoing a surgical procedure that involves or may involve a blood vessel supplying the brain, or otherwise on the brain or CNS. Some examples are patients undergoing cardiopulmonary bypass, carotid stenting, diagnostic angiography of the brain or coronary arteries of the aortic arch, vascular surgical

procedures and neurosurgical procedures. Additional examples of such patients are discussed in section IV above. Patients with a brain aneurysm are particularly suitable. Such patients can be treated by a variety of surgical procedures including clipping the aneurysm to shut off blood, or performing endovascular surgery to block the aneurysm with small coils or introduce a stent into a blood vessel from which an aneurysm emerges, or inserting a microcatheter. Endovascular procedures are less invasive than clipping an aneurysm and are associated with a better patient outcome but the outcome still includes a high incidence of small infarctions. Such patients can be treated with an inhibitor of PSD95 interaction with NMDAR 2B and particularly the agents described above including the peptide YGRKKRRQRRRLSSIESDV (SEQ ID NO:6, also known as Tat-NR2B9c). The timing of administration relative to performing surgery can be as described above for the clinical trial.

**[0069]** Another class of patients amenable to treatment are patients having a subarachnoid hemorrhage with or without an aneurysm (see US 61/570,264).

#### IV. Effective Regimes of Administration

**[0070]** After reconstitution, a lyophilized formulation, is administered such that the active agent (e.g., NR2B9c) is administered in an amount, frequency and route of administration effective to cure, reduce or inhibit further deterioration of at least one sign or symptom of a disease in a patient having the disease being treated. A therapeutically effective amount means an amount of active agent sufficient significantly to cure, reduce or inhibit further deterioration of at least one sign or symptom of the disease or condition to be treated in a population of patients (or animal models) suffering from the disease treated with an agent of the invention relative to the damage in a control population of patients (or animal models) suffering from that disease or condition who are not treated with the agent. The amount is also considered therapeutically effective if an individual treated patient achieves an outcome more favorable than the mean outcome in a control population of comparable patients not treated by methods of the invention. A therapeutically effective regime involves the administration of a therapeutically effective dose at a frequency and route of administration needed to achieve the intended purpose.

**[0071]** For a patient suffering from stroke or other ischemic condition, the active agent is administered in a regime comprising an amount frequency and route of administration effective

to reduce the damaging effects of stroke or other ischemic condition. When the condition requiring treatment is stroke, the outcome can be determined by infarction volume or disability index, and a dosage is considered therapeutically effective if an individual treated patient shows a disability of two or less on the Rankin scale and 75 or more on the Barthel scale, or if a population of treated patients shows a significantly improved (i.e., less disability) distribution of scores on a disability scale than a comparable untreated population, see Lees et al, N Engl J Med 2006;354:588-600. A single dose of agent is usually sufficient for treatment of stroke.

**[0072]** The invention also provides methods and formulations for the prophylaxis of a disorder in a subject at risk of that disorder. Usually such a subject has an increased likelihood of developing the disorder (e.g., a condition, illness, disorder or disease) relative to a control population. The control population for instance can comprise one or more individuals selected at random from the general population (e.g., matched by age, gender, race and/or ethnicity) who have not been diagnosed or have a family history of the disorder. A subject can be considered at risk for a disorder if a "risk factor" associated with that disorder is found to be associated with that subject. A risk factor can include any activity, trait, event or property associated with a given disorder, for example, through statistical or epidemiological studies on a population of subjects. A subject can thus be classified as being at risk for a disorder even if studies identifying the underlying risk factors did not include the subject specifically. For example, a subject undergoing heart surgery is at risk of transient cerebral ischemic attack because the frequency of transient cerebral ischemic attack is increased in a population of subjects who have undergone heart surgery as compared to a population of subjects who have not.

**[0073]** Other common risk factors for stroke include age, family history, gender, prior incidence of stroke, transient ischemic attack or heart attack, high blood pressure, smoking, diabetes, carotid or other artery disease, atrial fibrillation, other heart diseases such as heart disease, heart failure, dilated cardiomyopathy, heart valve disease and/or congenital heart defects; high blood cholesterol, and diets high in saturated fat, trans fat or cholesterol.

**[0074]** In prophylaxis, a lyophilized formulation after reconstitution is administered to a patient at risk of a disease but not yet having the disease in an amount, frequency and route sufficient to prevent, delay or inhibit development of at least one sign or symptom of the disease. A prophylactically effective amount means an amount of agent sufficient significantly to prevent,

inhibit or delay at least one sign or symptom of the disease in a population of patients (or animal models) at risk of the disease relative treated with the agent compared to a control population of patients (or animal models) at risk of the disease not treated with a chimeric agent of the invention. The amount is also considered prophylactically effective if an individual treated patient achieves an outcome more favorable than the mean outcome in a control population of comparable patients not treated by methods of the invention. A prophylactically effective regime involves the administration of a prophylactically effective dose at a frequency and route of administration needed to achieve the intended purpose. For prophylaxis of stroke in a patient at imminent risk of stroke (e.g., a patient undergoing heart surgery), a single dose of agent is usually sufficient.

**[0075]** Depending on the agent, administration can be parenteral, intravenous, nasal, oral, subcutaneous, intra-arterial, intracranial, intrathecal, intraperitoneal, topical, intranasal or intramuscular. Intravenous administration is preferred for peptide agents.

**[0076]** For administration to humans, a preferred dose of active agent (e.g., Tat-NR2B9c) is 2-3mg/kg and more preferably 2.6 mg/kg. Indicated dosages should be understood as including the margin of error inherent in the accuracy with which dosages can be measured in a typical hospital setting. Such amounts are for single dose administration, i.e., one dose per episode of disease.

**[0077]** Active agents, such as Tat-NR2B9c are preferably delivered by infusion into a blood vessel, more preferably by intravenous infusion. The time of the infusion can affect both side effects (due e.g., to mast cell degranulation and histamine release) and efficacy. In general, for a given dosage level, a shorter infusion time is more likely to lead to histamine release. However, a shorter infusion time also may result in improved efficacy. Although practice of the invention is not dependent on an understanding of mechanism, the latter result can be explained both because of the delay being significant relative to development of pathology in the patient and because of the delay being significant relative to the plasma half-life of the chimeric agent, as a result of which the chimeric agent does not reach an optimal therapeutic level. For the chimeric agent Tat-NR2B9c, a preferred infusion time providing a balance between these considerations is 5-15 minutes and more preferably 10 min. Indicated times should be understood as including a marking of error of +/- 10%. Infusion times do not include any extra time for a wash out



diffusion to wash out any remaining droplets from an initial diffusion that has otherwise proceeded to completion. The infusion times for Tat-NR2B9c can also serve as a guide for other active agents.

[0078] Although the invention has been described in detail for purposes of clarity of understanding, certain modifications may be practiced within the scope of the appended claims. To the extent more than one sequence is associated with an accession number at different times, the sequences associated with the accession number as of the effective filing date of this application is meant. The effective filing date is the date of the earliest priority application disclosing the accession number in question. Unless otherwise apparent from the context any element, embodiment, step, feature or aspect of the invention can be performed in combination with any other.

#### EXAMPLES

**Example 1: Demonstration that Standard buffers and excipients do not interfere with the efficacy of NA-1 in vivo.**

[0079] Five liquid toxicology formulations were compounded targeted at a 20 mg/mL concentration of NA-1. Table 1 includes the vehicle composition, Lot Number, and the potency, purity and pH at the time of compounding. Approximately 5 mL of each formulation was vialled for testing. Vials were frozen at -20 to simulate transport or liquid storage conditions.

Table 1: composition of NA-1 formulations for efficacy testing in vivo

Formulation #	Vehicle Composition	PTek Lot #	Potency <sup>1</sup> (mg/mL)	Purity <sup>2</sup> (% Area of NA-1 peak)	pH <sup>3</sup>
1	50 mM sodium phosphate, 76.9 mM NaCl, pH 7.0	1205-1-17-1	20.5	97.87	6.7
2	50 mM sodium phosphate, 154 mM Mannitol, pH 7.0	1205-1-17-2	20.0	96.34 <sup>3</sup>	6.5
3	50 mM histidine, 154 mM Mannitol, pH 6.5	1205-1-17-3	19.9	98.38	6.4
4	50 mM histidine, 154 mM Trehalose, pH 6.5	1205-1-17-4	20.8	99.16	6.4
5	50 mM histidine, 5% Dextran-40, pH 6.5	1205-1-18-1	19.4	98.81	6.4

<sup>1</sup>Potency and purity were evaluated by RP-HPLC analysis using a TFA method

<sup>2</sup>The purity of formulation #2 is notably lower than the other formulations.

<sup>3</sup>The pH of the phosphate buffered formulations noticeably deviated from the initial buffer pH of 7.0.

[0080] It was noted that phosphate buffered formulation did not maintain pH as well as the histidine buffers did between formulation and testing, indicating that histidine may be a superior buffer for formulation.

[0081] Formulations 1-5 were tested in the 3-PIAL Vessel Occlusions (3PVO) model of stroke in rats. Rats subjected to stroke were given one of the formulations by intravenous administration into the femoral vein, and then the animals were sacrificed 24 hours after the stroke. Brains were harvested, fixed and stained with triphenyltetrazolium chloride (TTC) to visualize the ischemic portions of the brain. All of the formulations tested were able to provide significant neuroprotection in animals relative to the saline-only control (Fig. 1).

## Methods

### *Three pial vessel occlusion model of ischemia*

[0082] Experiments were performed on rats. For permanent three pial vessels occlusion (3PVO) was performed as described previously [Angiogenic protection from focal ischemia with angiotensin II type 1 receptor blockade in the rat. Forder et al., Am J Physiol Heart Circ Physiol. 2005 Apr;288(4):H1989-96]. In brief, 250 g to 350 g rats were anesthetized with a 0.5 ml/kg intramuscular injection of ketamine (100 mg/kg), acepromazine (2 mg/kg), and xylazine (5

mg/kg), supplemented with one-third of the initial dose as required. An anal temperature probe was inserted, and the animal was placed on a heating pad maintained at about 37°C. The skull was exposed via a midline incision and scraped free of tissue. Using a dissecting microscope and a pneumatic dental drill, a 6- to 8-mm cranial window was made over the right somatosensory cortex (2 mm caudal and 5 mm lateral to bregma) by drilling a rectangle through the skull and lifting off the piece of skull while keeping the dura intact. The 3 pial arteriolar middle cerebral artery branches were cauterized around the barrel cortex were selected and electrically cauterized through the dura. After the cauterizations, the scalp was sutured. Each rat was returned to its individual cage under a heating lamp to maintain body temperature until the rat fully recovered. Food and water was supplied. One hour after 3PVO ischemia the rats were injected with NA1 formulations at 3 nmol/g in ~0.45 mL saline based upon rat weight. Doses were administered over 5 minutes.

**[0083]** Twenty-four hours after surgery, the brain was quickly harvested. Coronal slices (2 mm) were taken through the brain and incubated in 2% triphenyltetrazolium chloride (TTC) (*Sigma-Aldrich* St. Louis MO) for 15 min at 37°C. Images were scanned (CanoScan 4200F Canon) and quantitated.

## **Example 2: Determination of NA-1 stability in different buffers and at different pH values**

### Screening of buffers

**[0084]** Ten buffers were compounded at 1 mg/mL NA-1 for excipient screening. Samples were stored at 25°C/60% relative humidity (RH) and 40°C/75%RH. Samples were tested for stability (purity) at t=0 and t=1 week for purity by RP-HPLC (TFA and MSA methods), and the results are shown in Tables 2 and 3.

**[0085]** Results indicate improved stability for NA-1 in liquid media buffered between pH 6.0 and pH 6.5. Degradation appears to increase outside of this range in either direction. Data generated with the MSA method showed clear degradation patterns that were both pH and buffering species dependent, and provided valuable insight into future formulation development. Results for main peak purity by % HPLC Area using the MSA method are provided in Table 2, while results for main peak purity by %HPLC Area using the TFA method are provided in Table 3.

**Table 2. % Area of the Main Peak by MSA Method, NA-1**

Sample	t=0	t=1 week 25°C	t=1 week 40°C
His, 6.0	98.5	98.5	98.0
His, 6.5	98.5	98.6	97.3
His, 7.0	98.5	98.4	97.0
Phos, 6.0	98.5	98.2	97.0
Phos, 6.5	98.5	97.9	97.3
Phos, 7.0	98.5	97.9	96.0
Phos, 7.5	98.5	97.6	95.2
Citr, 5.5	98.5	98.3	94.4
Citr, 6.0	98.5	98.4	97.4
Citr, 6.5	98.5	98.7	97.7

**Table 3. % Area of the Main Peak by TFA Method, NA-1**

Sample	t=0	t=1 week 25°C	t=1 week 40°C
His, pH 6.0	99.5	98.8	98.2
His, pH 6.5	99.5	98.4	99.4
His, pH 7.0	99.5	98.3	96.7
Phos, 6.0	98.5	99.8	97.9
Phos, 6.5	99.5	99.5	98.6
Phos, 7.0	98.5	98.6	98.3
Phos, 7.5	98.5	98.0	93.2
Citr, 5.5	99.5	98.0	95.1
Citr, 6.0	98.5	99.0	98.2
Citr, 6.5	99.5	98.5	99.1

**[0086]** Results indicate that NA-1 solution stability is best maintained in pH 6.0 to 6.5 buffered media and the vehicle is still well tolerated for administration by IV. In general, histidine and citrate buffering systems were able to maintain NA-1 in an intact form even when kept at accelerated stability conditions of 25°C or 40°C for 1 week.

**[0087]** There are several factors to consider when selecting a buffering species: the specific degradation patterns that occur in each media, and any data on identified related substances or toxicology concerns may streamline the decision process if specified related substances should be avoided. For the period tested, histidine and citrate buffers between pH 6 and 6.5 showed few degradation products. The histidine buffer itself used in this study contained a contaminant that was present in the histidine buffer in the absence of added NA-1. Therefore, identification of a

supplier of histidine without such a contaminant would make analysis simpler. Table 4 provides a summary of the buffer species from the standpoint of NA-1 stability.

**Table 4. Buffering Species Selection**

Species	pH	Pro	Con
Histidine	6.0	Excellent stability, historic use in lyophilization applications, well within buffering range	Chromatographic interference, but chromatography could possibly be improved by new histidine vendor
Citrate	6.0	Improved stability, historic use in lyophilization applications, well within buffering range	
Histidine	6.5	Improved stability, historic use in lyophilization applications, well within buffering range	Chromatographic interference, but chromatography could possibly be improved by new histidine vendor
Citrate	6.5	Excellent stability, historic use in lyophilization applications, well within buffering range	Target pH of 6.5 may be on the edge of the ideal buffering range for the citrate species
Phosphate	6.5	Improved stability, historic use of phosphate species in NA-1 formulations	Phosphate species has been historically avoided for lyophilization formulations

**Example 3: Determination of NA-1 stability in histidine and citrate buffers and at different pH values with varying amounts of sodium chloride.**

[0088] The goal of this study was to demonstrate the effects of sodium chloride (NaCl) on pH and NA-1 stability in liquid formulations. Buffer formulations with 1 mg/mL NA-1 are listed in Table 5, and results for pH are provided in Table 6. The data show fairly consistent results for the duration of the study. However, notable shifts occurred in citrate with the addition of NaCl, where the buffering capacity was impacted and the pH dropped by ~0.2 units. Selected pH's of 6.0 and 6.5 are on the outside edge of citrate's ideal buffering range (pH 2.5-5.6), so this may cause difficulties with various additives during the compounding process and should be considered when evaluating formulation robustness.

Table 5: Buffer formulations for examining the effect of salt on pH

Vehicle #	Buffer	Target pH	NaCl
1	50 mM Citrate	6.0	NA
2	50 mM Citrate	6.0	200 mM
3	50 mM Citrate	6.5	NA
4	50 mM Citrate	6.5	200 mM
5	50 mM Histidine	6.0	NA
6	50 mM Histidine	6.0	200 mM
7	50 mM Histidine	6.5	NA
8	50 mM Histidine	6.5	200 mM

Table 6: pH Stability of NA-1 formulations in under frozen and accelerated temperature conditions

Vehicle #	Buffer	Target pH	Measured pH t=0 Vehicle	Measured pH t=0 Vehicle + NA-1	Measured pH t=1 week Vehicle + NA-1	
					-20°C	40°C/75%RH
1	Citr	6.0	6.1	6.0	6.1	6.0
2		6.5	6.6	6.5	6.6	6.6
3	His	6.0	6.0	5.9	6.2	6.0
4		6.5	6.5	6.5	6.6	6.7
5	Citr + NaCl	6.0	5.8	5.8	5.9	5.8
6		6.5	6.3	6.2	6.3	6.3
7	His + NaCl	6.0	6.1	6.0	6.2	6.0
8		6.5	6.6	6.6	6.7	6.7

[0089] The results indicate that the addition of 200 mM NaCl to the histidine and citrate buffered NA-1 solutions does not significantly affect the pH of the solutions whether stored for a week frozen or at the accelerated temperature of 40°C.

[0090] Next, we examined the stability of NA-1 in these formulations when stored 1 week at frozen and accelerated temperatures. Table 7 shows the results of the testing using the RP-HPLC method with an MSA gradient. The data is also presented in Figures 2A and 2B. Figure 2A presents the accelerated stability of formulations sorted from left to right (low to high stability). Figure 2B shows the relative accelerated stability by buffering agent.

**Table 7. Purity (MSA Method), NA-1**

Vehicle	pH 6.0		pH 6.5	
	-20°C	40°C	-20°C	40°C
His	98.1	92.8	98.5	96.9
His + NaCl	98.4	95.0	98.4	97.7
Citr	97.5	96.0	99.0	97.5
Citr + NaCl	98.4	96.7	98.7	98.4

[0091] These results indicate that NA-1 solution stability is best maintained at pH 6.5, and the addition of NaCl may offer a slight improvement in stability (Figures 2A and 2B). Due to improved buffering capacity and comparable stability of the histidine buffer, especially when the contaminant migrating with a relative retention time (RRT) of 0.28 is excluded (contaminant area included in the table above, resulting in a lower stability value for the NA-1 peak area), the histidine buffering species at pH 6.5 is the best formulation to move into lyophilization studies.

[0092] Vehicles at pH 6.5 are well tolerated for administration by IV.

#### **Example 4: Selection of bulking agents for NA-1 to form a stable lyophilized cake**

[0093] To identify bulking agents that would generate a nice cake upon lyophilization and improve stability, we compounded several 20 mg/mL NA-1 solutions in 50 mM histidine buffer,

bulking agent and NaCl as outlined in Table 8. To simulate the time and handling temperatures that NA-1 formulations may be exposed to during the lyophilization process, these samples were stored at -20°C (control) and 40°C/75%RH (test), and were analyzed after one week of storage for purity by HPLC (MSA method) and pH. Results for pH stability are outlined in Table 9, and results for the stability of NA-1 in the different liquid formulations are shown in Table 10 and Figure 3.

**Table 8. Bulking Agent Sample Matrix**

Vehicle #	Buffer	Bulking Agent	NaCl
1	50 mM histidine, pH 6.5	120 mM Mannitol	
2	50 mM histidine, pH 6.5	120 mM Mannitol	75 mM
3	50 mM histidine, pH 6.5	120 mM Trehalose	
4	50 mM histidine, pH 6.5	120 mM Trehalose	75 mM
5	50 mM histidine, pH 6.5	5% Dextran-40	
6	50 mM histidine, pH 6.5	5% Dextran-40	75 mM

**Table 9. pH, Bulking Agent Samples**

Vehicle	Target pH	pH, -20°C	pH, 40°C
Mannitol	6.5	6.5	6.5
Mannitol + NaCl	6.5	6.5	6.5
Trehalose	6.5	6.5	6.4
Trehalose + NaCl	6.5	6.5	6.4
Dextran-40	6.5	6.5	6.3
Dextran-40 + NaCl	6.5	6.5	6.4

**Table 10. Purity by % Area of NA-1 Peak, MSA Method**

Vehicle #	Vehicle	% Area of NA-1 Peak	
		-20°C	40°C
1	Mannitol	99.2	98.5
2	Mannitol + NaCl	99.4	98.6
3	Trehalose	99.1	98.5
4	Trehalose + NaCl	99.3	98.3
5	Dextran-40	99.2	97.6
6	Dextran-40 + NaCl	99.0	97.7



*Results of bulking agent liquid formulations on NA-1 stability*

[0094] Mannitol, Trehalose and Dextran-40 maintain the pH at 6.5 well (Table 9) and there is approximately a 1% decrease in purity (Table 10) over 1 week as a liquid formation when stored at high temperature. In terms of the chemical stability of the NA-1 lyophilization fill solution, mannitol and trehalose are preferred bulking agents as they confer better stability to NA-1 than the dextran-40 solutions (Figure 3).

**Example 5: Thermal analysis of bulking agents to facilitate design of lyophilization cycles**

[0095] As part of the lyophilization cycle development for NA-1 lyophilized drug product, proposed fill solutions from the bulking agent sample matrix (Table 8) were evaluated by Differential Scanning Calorimetry (DSC) for thermal characteristics including glass transition (T<sub>g</sub>) in the formulation. Results are listed in Table 11 and DSC traces are included in Figures 4A-6B.

**Table 11. Glass Transitions of NA-1 Lyophilization Fill Solutions**

Vehicle	T <sub>g</sub>
50 mM histidine, pH 6.5, 120 mM Mannitol	-37.25°C
50 mM histidine, pH 6.5, 120 mM Mannitol, 75 mM NaCl	-42.51°C
50 mM histidine, pH 6.5, 120 mM Trehalose	-28.25°C
50 mM histidine, pH 6.5, 120 mM Trehalose, 75 mM NaCl	-35.74°C
50 mM histidine, pH 6.5, 5% Dextran-40	-17.09°C
50 mM histidine, pH 6.5, 9% Dextran-40, 75 mM NaCl	-22.49°C

[0096] At a NA-1 concentration of 20 mg/mL, tested NA-1 formulations showed a thermal profile characterized by a broad melting event with onset at a low temperature. This extended melt masked the crystallization event typically seen in mannitol formulations, and may indicate that a robust freeze drying cycle must be performed where the product never exceeds the glass transition temperature. In this case, based on the observed glass transitions of the NA-1 drug product fill solution, the use of mannitol as a bulking agent would require a primary drying temperature lower than -40°C, the typical limit of feasibility for a scalable cycle. In terms of thermal profiles, Trehalose and Dextran-40 are superior for use as a bulking agent. However, given that the stability of NA-1 in the liquid formulations containing trehalose was superior to

those containing Dextran, trehalose would be the preferred bulking agent of those tested.

[0097] Due to the relatively low T<sub>g</sub> temperatures that would likely require a longer lyophilization cycle to dry, we looked at a wider range of standard bulking agents and looked to reduce the fill volume into the container closure system so that there would be a reduced volume of liquid to lyophilize. In an effort to decrease the fill volume and maintain 270 mg/vial, a solubility study of NA-1 in Histidine, pH 6.5 and in Histidine + Trehalose, pH 6.5 was performed. Samples were visually analyzed at 35, 50, 75 and 100 mg/mL. All solutions were clear at t=0 and t=24 hours. Based on this data, we could use fill volume lower than 3 mL, which using a 90 mg/mL NA-1 formulation would give provide 270 mg in a target vial. A wide range of quantities may be required in a vial, but 270 mg would provide a 2.6 mg/kg dose for a 100 kg patient. Assuming the target reconstitution concentration for patient administration is still 20 mg/mL (but can be from 1 mg/ml to 100 mg/ml), then a 20-mL lyophilization vial containing 270 mg of NA-1 can be used with a reconstitution volume of 13.5 mL. Therefore, optimal volumes of liquid for lyophilization of NA-1 in the vial would be between 2.5mL and 10 mL.

[0098] A wider range of bulking agents were tested prior to advancing into lyophilization development, and the T<sub>g</sub>'s are shown in Table 12. The 100 mg/mL NA-1 in histidine, pH 6.5 was also evaluated by DSC and the data is included in Table 12.

**Table 12. DSC Data, Formulations**

Formulation	Bulking Agent	Vehicle		DP Fill Solution
		T <sub>g</sub>	Temp of Crystallization	T <sub>g</sub>
1	Sorbitol	-41.03°C		
2	Dextrose	-38.78°C		
3	Sucrose	-31.09°C		
4	Mannitol	-37.38°C	-22.81°C	-35.49°C
5	Trehalose	-29.93°C		-28.25°C
6	Lactose	-27.93°C		
7	75:25 Trehalose:Dextran-40	-25.07°C		-25.07°C
8	50:50 Trehalose:Dextran-40	-22.60°C		-22.60°C
9	25:75 Trehalose:Dextran-40	-18.55°C		-18.55°C
10	Dextran-40			-17.09°C
11	100 mg/ml NA-1 in Histidine, pH 6.5			-21.67°C

[0099] Based on the DSC data in Table 12, there are several formulation options for both active

and placebo drug products. In general, formulations 5 and 11 are the most promising for the active product with respect to the Tg. Any bulking agent may be suitable for use in a placebo product, but Formulation 4 (Mannitol) will have the shortest cycle length if annealed, and may be the most desirable should the appearance match the active.

**[00100]** As we determine an optimal active formulation, it is important to consider solution stability, lyophilization cycle robustness, and chemical stability. Formulation 5 from Table 12 (Trehalose) demonstrated good solution stability and lyophile chemical stability at accelerated conditions (data shown subsequently), but requires a longer lyophilization cycle at a fill configuration of 13.5 mL. This longer cycle length may not be ideal for commercial manufacture in the future, where a shorter cycle is desirable. Formulation 11 from Table 12 (without a bulking agent, at 100 mg/mL NA-1) has a higher glass transition temperature than Formulation 5, allowing for a warmer, shorter cycle. In addition, a decreased fill volume will significantly shorten the run time as there will be less ice to sublime from each vial.

#### **Example 6: Stability of NA-1 with varying bulking agents, scales and lyophilization conditions**

##### *Bulking Agent Accelerated Stability*

**[00101]** A small batch of NA-1 drug product was lyophilized to evaluate solid state stability after 1 week at 25°C, 40°C, and 60°C. NA-1 was compounded at an active concentration of 20 mg/mL in three different vehicles. Samples were evaluated for appearance, reconstitution, pH, amount and purity by HPLC (MSA method) at t=0 and t=1 week. Water content was evaluated at t=0 only.

**[00102]** All NA-1 drug products appeared as white, lyophilized cakes and reconstituted in less than 10 seconds at t=0 and t=1 week.

**[00103]** The drug product vehicles are described in Table 13 and are listed with the respective glass transition temperature and water content results. The pH, NA-1 amount and NA-1 purity results are described in Tables 14-16.

**Table 13. Bulking Agent Sample Matrix: T<sub>g</sub> and % Water Content**

Vehicle #	Vehicle	T <sub>g</sub>	t=0 % Water Content
1	50 mM His, pH 6.5+ 120 mM Trehalose	-29.93°C -28.25°C w/NA-1	0.29%
2	50 mM His, pH 6.5+ 5% Dextran-40	-22.60°C	0.05%
3	50 mM His, pH 6.5 + 1:1 120 mM Trehalose: 5% Dextran-40	-17.09°C w/NA-1	0.10%

**Table 14. pH, Bulking Agent Lyo Small Scale #1**

Bulking Agent	Theoretical pH	Measured pH			
		t=0	t=1wk 25°C	t=1wk 40°C	t=1wk 60°C
Trehalose	6.5	6.4	6.4	6.4	6.4
Dextran-40	6.5	6.4	6.3	6.3	6.3
1:1 Trehalose:Dextran	6.5	6.4	6.3	6.4	6.4

**Table 15. Amount (mg/vial), Bulking Agent Lyo Small Scale #1**

Bulking Agent	t=0	t=1 week 25°C	t=1 week 40°C	t=1 week 60°C
Trehalose	20.6	20.3	20.7	20.7
Dextran-40	19.4	19.8	19.5	19.1
1:1 Trehalose:Dextran-40	20.3	20.8	20.2	20.2

**Table 16. Purity (% Area by HPLC), Bulking Agent Lyo Small Scale #1**

Bulking Agent	t=0	t=1 week 25°C	t=1 week 40°C	t=1 week 60°C
Trehalose	98.8	98.8	98.8	98.4
Dextran-40	98.9	98.9	98.6	96.5
1:1 Trehalose:Dextran-40	98.9	98.8	98.6	97.5

[00104] All three bulking agents, Trehalose, Dextran-40 and Trehalose:Dextran-40, maintain the pH at 6.5 (Table 14) and there is a range of 0.5-2.5% decrease in purity at 60°C after 1 week (Table 15). Both drug products containing dextran-40 and stored at 60°C showed growth in related substances at a retention time (RT) ~6.0. These related substances were not present in the

trehalose samples, suggesting that trehalose has a stabilizing effect in the lyophilized drug product and dextran-40 can cause a specific degradation product.

[00105] The inclusion of dextran-40 in the bulking agent allows for a warmer primary drying temperature, but dextran-40 as a bulking agent demonstrated the poorest stability. The combination of trehalose and dextran-40 (1:1) results in a glass transition temperature that is approximately 10°C warmer than trehalose alone. However, it appears that the 60°C stability is intermediate to the trehalose and dextran alone samples, so that trehalose is a preferred bulking agent.

#### *Lyophilized NA-1 Formulation Development: Small Scale Experiment #2*

[00106] A small batch of NA-1 drug product was lyophilized to evaluate setting the shelf temperature at 5°C during primary drying. NA-1 was compounded at an active concentration of 27 mg/mL in 50 mM Histidine, pH 6.5 and 120 mM Trehalose. The cycle parameters are outlined in Table 17. Four 20-mL glass lyophilization vials were filled with 10 mL. Two vials were probed with temperature probes.

**Table 17. Small Scale 2, NA-1 Formulation Development**

Function	Temperature (°C)	Hold/Rate	Rate (°C/minute)	Time (minutes)	Pressure (mTorr)
Load	5	Hold	-	0	Ambient
Equilibration	5	Hold	-	120	Ambient
Freeze	-40	Rate	0.5	90	Ambient
Freeze	-40	Hold	-	240	Ambient
Primary Drying <sup>1</sup>	5	Rate	0.25	180	225
Primary Drying <sup>1</sup>	5	Hold	-	2050	50
Secondary Drying	25	Rate	0.1	200	50
Secondary Drying	25	Hold	-	1440	50
Stopper	20	Hold	-	-	Nitrogen/Ambient
Unload	20	Hold	-	-	Ambient

<sup>1</sup>Primary drying temperature based on large vial size and fill volume, not directly related to glass transition temperature.

[00107] Due to the large fill volume, it is necessary to set the shelf temperature considerably warmer than the glass transition temperature in order to compensate for evaporative cooling. The solution temperature during primary drying was at -29°C, which is near the glass transition

temperature of -28°C from the DSC thermal analysis.

*90 mg/mL NA-1 Lyophile Accelerated Stability (Small Scale 3)*

[00108] Prior to compounding the small scale 3 fill solution, a 90 mg/mL NA-1 in buffer (50 mM Histidine, pH 6.5) was evaluated for pH. The pH of the solution was 6.04. It was determined that with the increased concentration of NA-1, the buffering strength also needed to increase.

Solutions were prepared at 150mM, 100 mM, 75 mM, and water and evaluated for pH. The pHs are listed in Table 18. Small scale 3 was compounded in a 100 mM Histidine buffer at pH 6.5, and the pH was re-adjusted to 6.5 after addition of NA-1.

**Table 18. pH, 90 mg/mL NA-1 in Histidine Buffers, pH 6.5**

Buffer	pH
Water	5.39
50mM	6.04
75mM	6.04
100mM	6.29
150mM	6.14

[00109] A small batch of NA-1 drug product was lyophilized to evaluate solid state stability after 1 week storage at 25°C and 60°C. Two 90 mg/mL NA-1 formulations were compounded (buffer and buffer with trehalose). Samples were evaluated for appearance, reconstitution, water content and purity by HPLC (MSA method) at t=0 and t=1 week.

[00110] All NA-1 drug products appeared as white, lyophilized cakes. Some cakes were cracked. Placebo formulations were visually similar to the active formulations.

[00111] Reconstitution time was approximately 1.5 minutes compared to less than 10 seconds in the previous formulations. The increased reconstitution time is most likely due to the increased concentrations of NA-1 and histidine. Further tests showed good stability of NA-1 with histidine buffer concentrations of 50 and 75 mM, with shortened resuspension times. Also, due to the 2-mL vial size used for this study, only 1 mL of water was added to the lyophile. The actual reconstitution volume is 4.5 mL in this small scale configuration. The reconstitution time will most likely improve when a larger volume of diluent is used.

[00112] Vials were placed on stability at 25°C and 60°C and tested after 1 week of storage.

[00113] Based on the visual appearance data, the trehalose sample gave a more elegant cake. The

lyophilization cycle was run conservatively over 5 days, with a primary drying temperature of -32°. Based on the temperature probe data, the cycle can be shortened, demonstrating that with the higher concentration and lower fill volume the optimized cycle will be shorter.

[00114] Purity results are outlined in Table 19.

**Table 19. Purity (% Area by HPLC), Small Scale 3**

Formulation	Composition	Fill Solution	t=0	t=1 week, 25°C/60%RH	t=1 week, 60°C
1	100 mM His, pH 6.5	99.2	99.3	99.3	97.8
2	100 mM His, pH 6.5 + 120 mM Trehalose	99.2	99.3	99.2	98.7

[00115] Based on this accelerated stability data, trehalose demonstrates a stabilizing effect on the NA-1 formulation which improves the chemical stability of the lyophile. It is surprising that trehalose is able to confer this stabilizing effect while other standard bulking agents such as dextran and mannitol used for other peptides do not.

[00116] The reduced fill volume minimizes the competing evaporative cooling of the surrounding vials and minimizes the resistance to the sublimating water.

*Lyophilization Cycle Development – Small Scale 4 (Placebo and Active)*

[00117] Small scale 4 of the lyophilization cycle development was initiated to test the cake appearance and lyophilization conditions for a 3 mL fill. Samples tested were 100 mM His, pH 6.5 with 120 mM Trehalose and 90 mg/kg NA-1 or an identical sample removing the Trehalose. A conservative, 4 day cycle was ran as described in Table 20. Placebo and active vials were included with a fill configuration of 3 mL into a 20-mL glass lyophilization vial instead of the small vials used for the previous experiments. An active temperature probe was used to confirm temperature during the lyophilization cycle. The resulting active vials is shown in Figure 7A.

**Table 20. Lyophilization Parameters for Small Scale 4**

Function	Temperature (°C)	Hold/Rate	Rate (°C/minute)	Time (minutes)	Pressure (mTorr)
Load	5	Hold	-	0	Ambient
Equilibration	5	Hold	-	120	Ambient
Freeze	-40	Rate	0.5	90	Ambient
Freeze	-40	Hold	-	120	Ambient
Primary Drying	-30	Rate	0.25	40	225
Primary Drying	-30	Hold	-	3400	50
Secondary Drying	25	Rate	0.1	550	50
Secondary Drying	25	Hold	-	1440	50
Stopper	20	Hold	-	-	Nitrogen/Ambient
Unload	20	Hold	-	-	Ambient

[00118] Formulation at 90 mg/ml in a 20 mL vial formed an elegant cake on a 4 day cycle, and temperature probe data suggested that the cycle could be shortened to 3 days.

[00119] Water content for the placebo and active was 0.01% and 0.00%.

#### **Lyophilization Cycle Development – Small Scale 5 (Placebo and Active)**

[00120] Small Scale 5 was performed to look at developing a matching placebo vial for clinical trials and to look at resuspension times for formulations at a potential commercial scale (270 mg/vial). 10 placebo formulations and 1 active formulation were evaluated for appearance and reconstitution time. The active cakes were elegant, white cakes with minor shrinkage resulting in a crack around the surface of the vial wall. The placebo cakes were white with more cracks in cakes containing increasing amounts of trehalose.

[00121] Vials were reconstituted with 13.5 mL of water. The time to dissolve is listed in Table 21. The active lyophile re-suspended immediately, but was cloudy for 17.6 sec before becoming a clear, colorless solution. All placebos were a clear, colorless solution.



**Table 21. Reconstitution of Placebo and Active (SS5)**

Formulations				Reconstitution Time (min)	
Placebo Formulation #	Trehalose, mM	Histidine, mM	Total, mg/vial	Vial #1	Vial #2
1 (Control)	120	100	170	< 10 sec	< 10 sec
2	200	100	252	< 10 sec	< 10 sec
3	300	100	355	< 10 sec	< 10 sec
4	400	100	457	< 10 sec	< 10 sec
5	500	100	560	< 10 sec	< 10 sec
6	120	20	133	< 10 sec	< 10 sec
7	200	20	215	< 10 sec	< 10 sec
8	300	20	317	< 10 sec	< 10 sec
9	400	20	420	< 10 sec	< 10 sec
10	500	20	523	< 10 sec	< 10 sec
Active Formulation #	Trehalose, mM	Histidine, mM	NA-1, mg	Vial #1	Vial #2
1 (Control)	120	100	90	17.6 sec	NA

[00122] Based on the stability, resuspension times, and lyophilization times, a preferred commercial formulation for NA-1, prelyophilization, would be 20-100 mM Histidine, 120 mM Trehalose pH 6.5. Trehalose concentrations can be increased without a loss of stability or cake elegance but resuspension times.

*Examination of increased Trehalose in cake formation and placebo matching by visual appearance and resuspension time.*

[00123] To better match a placebo, varying concentrations of Trehalose were tested with and without NA-1, and at either 3 or 5 mL fill volumes.

[00124] First, the active formulations and the placebo formulations will be summarized. Then the lead visual matches for the 3-mL fill and the 5-mL fill will be highlighted. Analytical samples (fill solution and one potency sample) are currently being analyzed. Tables 22 and 23 show a subset of the formulations tested.

**Table 22. Active Formulations**

Formulation	Fill Volume	Composition
1	3-mL	270 mg/vial in 120 mM Trehalose + 100 mM Histidine, pH 6.5
2	3-mL	270 mg/vial in 500 mM Trehalose + 20 mM Histidine, pH 6.5
3	5-mL	270 mg/vial in 120 mM Trehalose + 50 mM Histidine, pH 6.5

Figure 7B shows the appearance of the active formulations listed above.

**Table 23. Placebo Formulations**

Placebo Formulations	Active Formulations (~270 mg/vial)
500 mM Trehalose + 20 mM Histidine (n=7)	500 mM Trehalose + 20 mM Histidine (n=2)
400 mM Trehalose + 20 mM Histidine (n=3)	400 mM Trehalose + 20 mM Histidine (n=1)
300 mM Trehalose + 20 mM Histidine (n=3)	300 mM Trehalose + 20 mM Histidine (n=1)

Table 24 shows the lyophilization cycle conditions for the above samples

**Table 24. Cycle Parameters**

Function	Temperature (°C)	Hold/Rate	Rate (°C/minute)	Time (minutes)	Pressure (mTorr)
Load	5	Hold	-	0	Ambient
Equilibration	5	Hold	-	120	Ambient
Freeze	-40	Rate	0.25	180	Ambient
Freeze	-40	Hold	-	120	Ambient
Anneal	-27	Rate	0.25	52	Ambient
Anneal	-27	Hold	-	120	Ambient
Freeze	-40	Rate	0.25	52	Ambient
Freeze	-40	Hold	-	120	Ambient
Primary Drying	-30	Rate	0.25	40	225
Primary Drying	-30	Hold	-	4405	50
Secondary Drying	25	Rate	0.1	550	50
Secondary Drying	25	Hold	-	1440	50
Stopper	20	Hold	-	-	Nitrogen/Ambient
Unload	20	Hold	-	-	Ambient

**Table 25. Summary of Lead Matches**

Sample Name	Color & Finish: (Shine or Matte)	Topography: Ex. Skin, bumps, cracks, peak, curls	Structure: Dense or porous	Shrinkage	Friability	Reconstitution Time
SS6 - 3 ml. Fills						
Active #2 500 mg Trehalose 20 mM Histidine	off white matte	thin cracks	dense	minimal	see photo	2 min 30 sec
Placebo #2 500 mg Trehalose 20 mM Histidine	off white matte	cracked	dense	minimal		1 min
SS7 - 5 ml. Fills						
Active 500 mg Trehalose 20 mM Histidine	off white matte w/shiny specks	cracked pocked bottom	semi-dense, layered	yes, base of cake	NT	1 min
Placebo 500 mg Trehalose 20 mM Histidine	off white matte w/shiny specks	cracked pocked bottom	semi-dense, more porous than active	minimal		20 sec unannealed: 33 sec
NT = not tested						

NT = not tested

**Example 7: Stability of lyophilized 270 mg NA-1 in 20 mM Histidine buffer pH 6.5 and 120 mM trehalose***Preparation of the lyophilized drug product*

**[00125]** A small batch of NA-1 drug product was formulated at 90 mg/mL in 20 mM Histidine pH 6.5 and 120 mM trehalose and lyophilized to evaluate solid state stability after 4 weeks at -20°C, 40°C, and 60°C. Table 25 shows the lyophilization conditions.

Table 25: Lyophilization cycle conditions for Example 7

Function	Temperature (°C)	Hold/Rate	Rate (°C/minute)	Time (minutes)	Pressure (mTorr)
Load	5	Hold	-	0	Ambient
Equilibration	5	Hold	-	120	Ambient
Freeze	-40	Rate	0.5	90	Ambient
Freeze	-40	Hold	-	120	Ambient
Primary Drying	-28	Rate	0.25	45	225
Primary Drying	-28	Hold	-	3412	50
Secondary Drying	25	Rate	0.1	530	50
Secondary Drying	25	Hold	-	1440	50
Stopper	20	Hold	-	-	Nitrogen/Ambient
Unload	20	Hold	-	-	Ambient

[00126] Samples were stored in constant temperature ovens with and the purity, potency, and reconstitution time in 13.2 mL (for 13.5 final volume) were assessed at 0, 1, 2 and 4 weeks. The data for each storage temperature and time is presented in Tables 26A-C.

Table 26A: Stability at -20°C

Parameter	t = 0		t = 4 weeks	
Appearance	Dense white cake		Dense white cake	
Reconstitution Time	~60 sec		~60 sec	
pH	6.32		TBD	
Water Content	0.02%		NT	
% Label Claim, TFA Method	99.0%		101.3%	
Total Purity, MSA Method (% Area)	99.7%		99.7%	
Individual Impurities	RRT	% Area	RRT	% Area
	0.59	0.02%	0.59	0.02%
	ND	ND	0.95	0.01%
	0.97	0.26%	0.98	0.21%
	1.04	0.26%	1.05	0.32%
	1.07	0.09%	1.09	0.04%
	1.10	0.13%	1.11	0.12%
	ND	ND	1.14	0.03%
	1.15	0.02%	1.16	0.02%
Deamidated NA-1, SCX Method (% Area)	<0.05%		TBD	

Table 26B: Stability at 40°C

Parameter	t = 0		t = 1 month		t = 2 months		t = 4 weeks	
Appearance	Dense white cake		Dense white cake		Dense white cake		Dense white cake	
Reconstitution Time	~60 sec		~60 sec		~60 sec		~60 sec	
pH	6.32		6.35		6.33		7.80	
Water Content	99.8%		NT		NT		NT	
% Label Claim, USP Method	99.8%		97.5%		100.5%		100.4%	
Total Purity, USP Method (% Area)	99.2%		99.1%		99.9%		99.6%	
Individual Impurities	RRT	% Area	RRT	% Area	RRT	% Area	RRT	% Area
	0.59	0.00%	0.59	0.00%	0.59	0.00%	0.59	0.00%
	0.97	0.00%	0.97	0.00%	0.95	0.00%	0.95	0.00%
	1.04	0.00%	1.04	0.00%	0.98	0.00%	0.97	0.00%
	1.07	0.00%	1.07	0.00%	1.05	0.00%	1.05	0.00%
	ND	ND	ND	ND	ND	ND	ND	ND
	1.10	0.00%	1.10	0.00%	1.10	0.00%	1.10	0.00%
	ND	ND	1.18	0.00%	1.18	0.00%	1.18	0.00%
	1.15	0.00%	1.15	0.00%	1.15	0.00%	1.15	ND
	ND	ND	ND	ND	1.17	0.00%	1.16	0.00%
	1.26	0.00%	1.26	0.00%	1.26	0.00%	1.26	0.00%
	1.29	0.00%	1.29	0.00%	1.31	0.00%	1.30	0.00%
Decomposed NA-1, USP Method (% Area)	<0.05%		NT		NT		TBD	

Table 26C: Stability at 60°C

Parameter	t = 0		t = 1 month		t = 2 months		t = 4 weeks	
Appearance	Dense white cake		Dense white cake		Dense white cake		Dense white cake	
Reconstitution Time	~60 sec		~60 sec		~60 sec		~60 sec	
pH	6.32		6.45		6.28		6.29	
Water Content	99.8%		NT		NT		NT	
% Label Claim, USP Method	99.8%		97.5%		101.5%		101.5%	
Total Purity, USP Method (% Area)	99.2%		98.4%		98.3%		98.0%	
Individual Impurities	RRT	% Area	RRT	% Area	RRT	% Area	RRT	% Area
	ND	ND	0.59	0.00%	0.57	0.00%	0.53	0.00%
	0.59	0.00%	0.59	0.00%	0.60	0.00%	0.59	0.00%
	ND	ND	0.91	0.00%	0.93	0.00%	0.91	0.00%
	ND	ND	0.95	0.00%	0.95	0.00%	0.95	0.00%
	0.97	0.00%	0.97	0.00%	0.99	0.00%	0.97	0.00%
	1.04	0.00%	1.04	0.00%	1.09	0.00%	1.08	0.00%
	1.07	0.00%	1.07	0.00%	1.07	0.00%	1.08	0.00%
	1.10	0.00%	1.10	0.00%	1.09	0.00%	1.10	0.00%
	ND	ND	1.13	0.00%	1.13	0.00%	1.13	0.00%
	1.15	0.00%	1.15	0.00%	1.13	0.00%	1.15	0.00%
	ND	ND	ND	ND	1.17	0.00%	1.17	0.00%
Decomposed NA-1, USP Method (% Area)	<0.05%		NT		NT		TBD	

<sup>1</sup> Lactose in reconstitution around main peak.

**[00127]** This formulation of NA-1 is stable at -20°C. For storage temperatures of 40°C and 60°C, potential impurities with relative retention times (RRT) of 1.07, 1.1 and 1.29 increased slowly using the MSA HPLC assay, with the largest growth appearing at 1.07 RRT. For the 40°C storage temperature, the impurity increases from 0.09% to 0.27% over 1 month, and for the 60°C storage temperature the impurity increases from 0.09% to 0.59%. No impurity was observed at -20°C. Impurities interpolated using the Arrhenius equation are less than 0.5% after 16 months at 25°C or 123 months at 5°C, and less than 2% for >60 months at room temperature and many years at 5°C. Thus, this and related formulations are suitable for room temperature storage of lyophilized drug product.

*Overall conclusions*

**[00128]** Based on the stability, resuspension times, and lyophilization times, a preferred commercial formulation for NA-1 is 20-100 mM Histidine, 120 mM Trehalose pH 6.5. Trehalose concentrations can be increased without a loss of stability or cake elegance but resuspension times increase with increased trehalose concentration.

**What is claimed is:**

1. A prelyophilized formulation comprising TAT-NR2B9c (SEQ ID NO:6), histidine and trehalose at a pH of 6-7, wherein the TAT-NR2B9c is at a concentration of 70-120 mg/ml, the histidine is at a concentration of 15-100 mM, and the trehalose is at a concentration of 80-160 mM.
2. The prelyophilized formulation of claim 1, wherein the TAT-NR2B9c is at a concentration of 70-120 mg/ml, the histidine is at a concentration of 20-100 mM, and the trehalose is at a concentration of 100-140 mM.
3. The prelyophilized formulation of claim 1, wherein the Tat-NR2B9c is at a concentration of 70-120 mg/ml, the concentration of histidine 20-50 mM, and the concentration of trehalose is 100-140 mM.
4. The prelyophilized formulation of claim 1, wherein the concentration of histidine is 20 mM, the concentration of trehalose is 100-120 mM and the concentration of TAT-NR2B9c is 90 mg/ml.
5. The prelyophilized formulation of claim 4, wherein the concentration of trehalose is 120 mM.
6. A lyophilized formulation prepared by lyophilizing the prelyophilized formulation of any one of claims 1 to 5.
7. A reconstituted formulation prepared by combining the lyophilized formulation of claim 6 with an aqueous solution.
8. The reconstituted formulation of claim 7, wherein the aqueous solution is water or normal saline.
9. The reconstituted formulation of claim 7 or 8, wherein the volume of the reconstituted formulation is 3-6 times the volume of the prelyophilized formulation.
10. A reconstituted formulation comprising TAT-NR2B9c at concentration of 15-25 mg/ml, histidine at a concentration of 4-20 mM and trehalose at a concentration of 20-30 mM at pH 6-7.

11. A prelyophilized formulation comprising a peptidomimetic of TAT-NR2B9c (SEQ ID NO:6), which differs from SEQ ID NO:6 in that one or more amino acids are D-amino acids, histidine and trehalose at a pH of 6-7, wherein the peptidomimetic of TAT-NR2B9c is at a concentration of 70-120 mg/ml, the histidine is at a concentration of 15-100 mM, and the trehalose is at a concentration of 80-160 mM.
12. A formulation as defined in any one of claims 1 to 11 for use in treating stroke or traumatic injury to the CNS.
13. A formulation as defined in any of claims 1 to 11 for treating subarachnoid hemorrhage.
14. A formulation as defined in any one of claims 1 to 11 for treating a patient undergoing endovascular repair for an aneurysm.
15. A method of preparing a formulation, comprising storing a lyophilized formulation according to claim 6 for at least a week at room temperature; and reconstituting the lyophilized formulation.
16. The method of claim 15, wherein the lyophilized sample is stored in an ambulance.
17. Use of formulation as defined in any one of claims 1 to 11 in treating stroke or traumatic injury to the CNS.
18. Use of formulation as defined in any one of claims 1 to 11 in preparation of a medicament for treating stroke or traumatic injury to the CNS.
19. Use of formulation as defined in any of claims 1 to 11 for treating subarachnoid hemorrhage.
20. Use of formulation as defined in any of claims 1 to 11 in preparation of a medicament for treating subarachnoid hemorrhage.
21. Use of formulation as defined in any one of claims 1 to 11 for treating a patient undergoing endovascular repair for an aneurysm.
22. Use of formulation as defined in any one of claims 1 to 11 in preparation of a medicament for treating a patient undergoing endovascular repair for an aneurysm.



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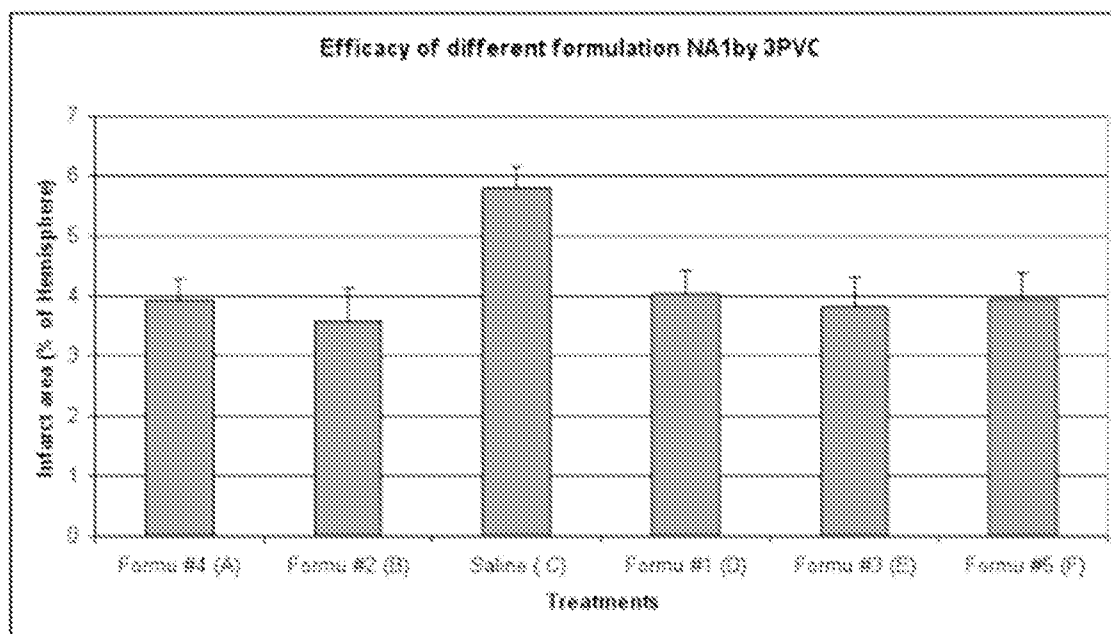


FIG. 1

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A

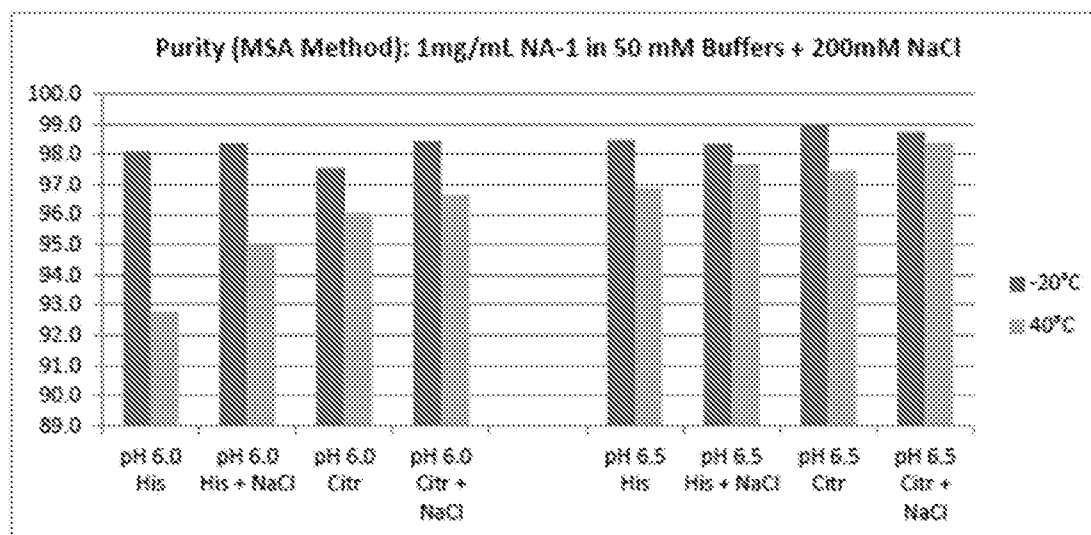


Figure B. Plot of Purity Results, MSA Method (Ordered by pH)

B

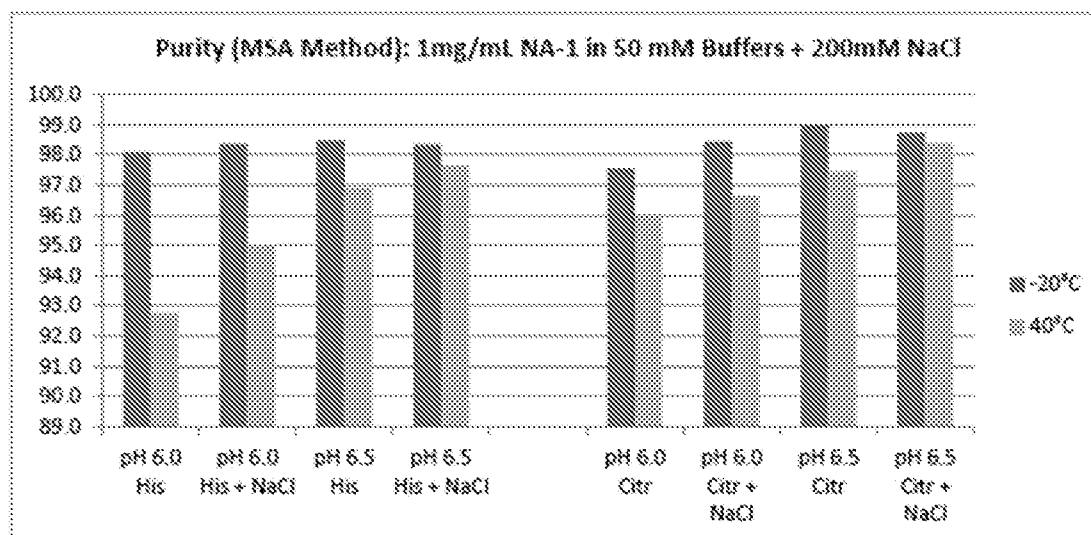


FIG. 2A, B

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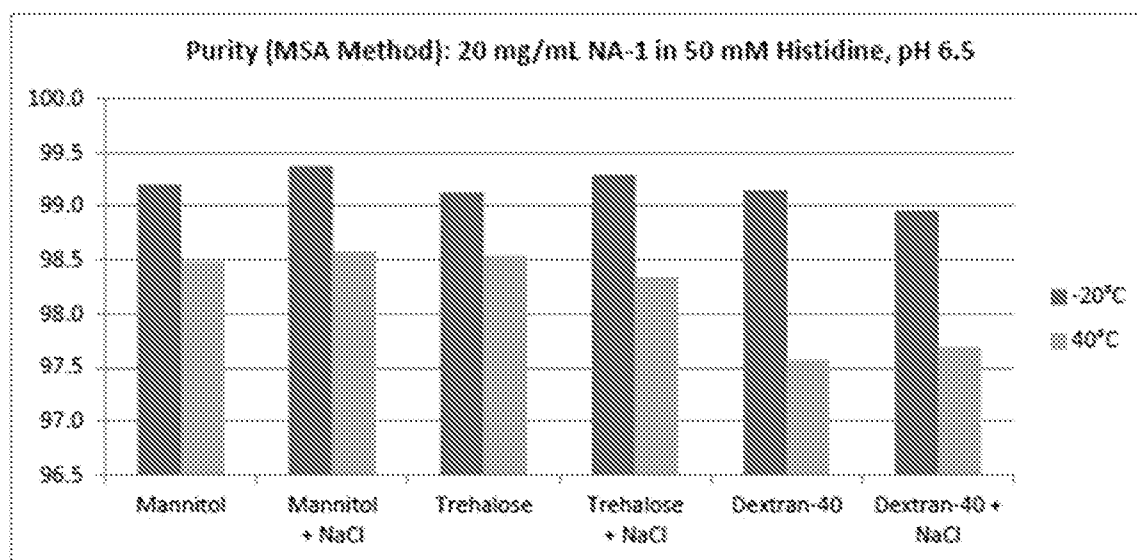
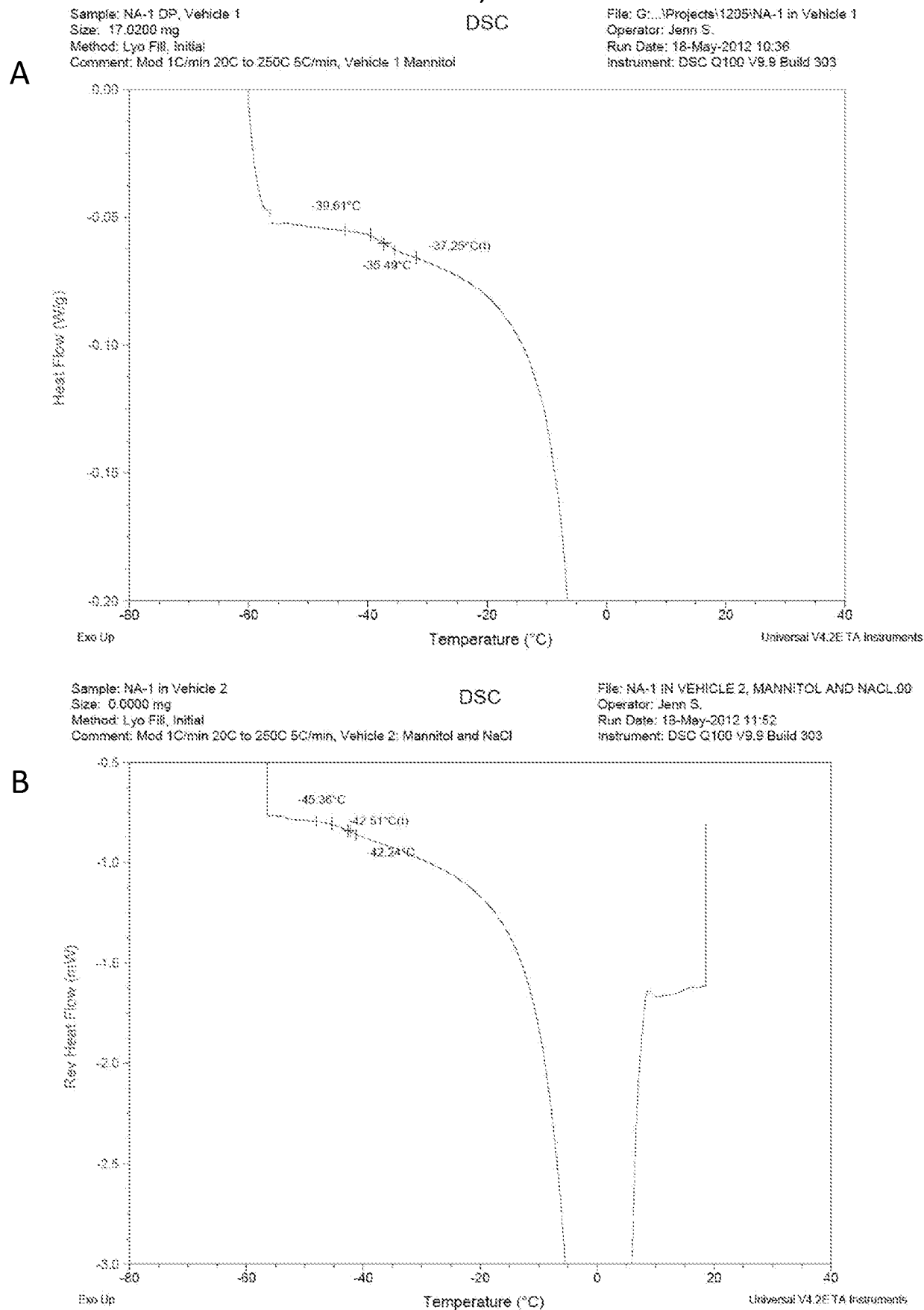


FIG. 3

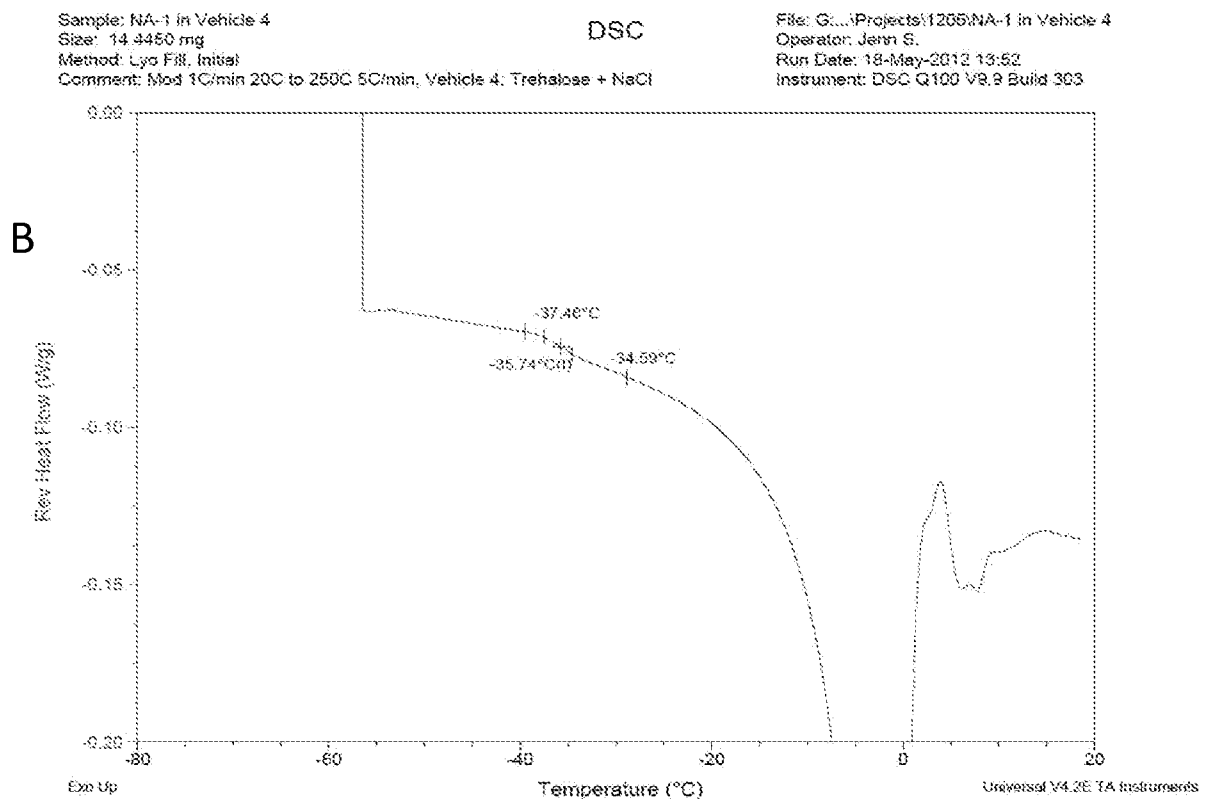
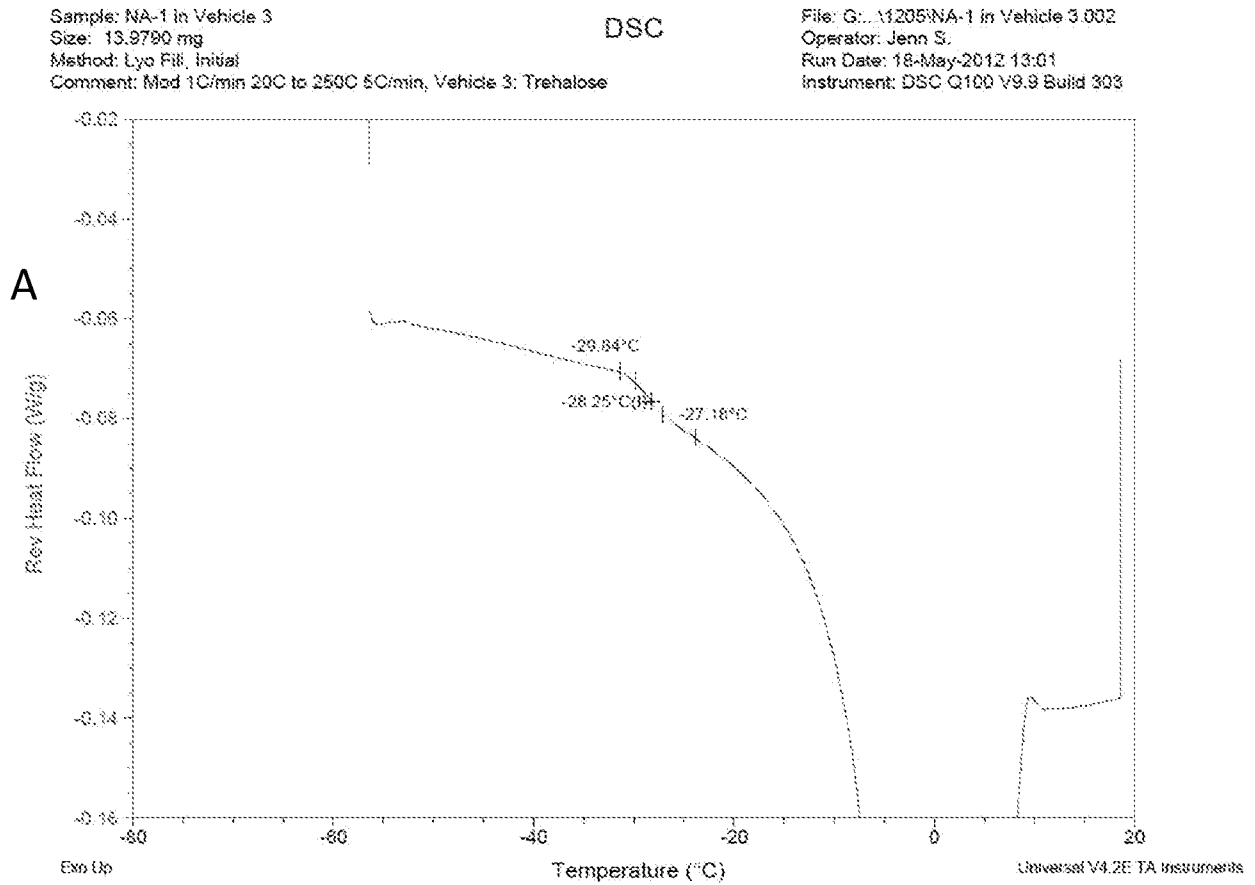
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FIG. 4A, B



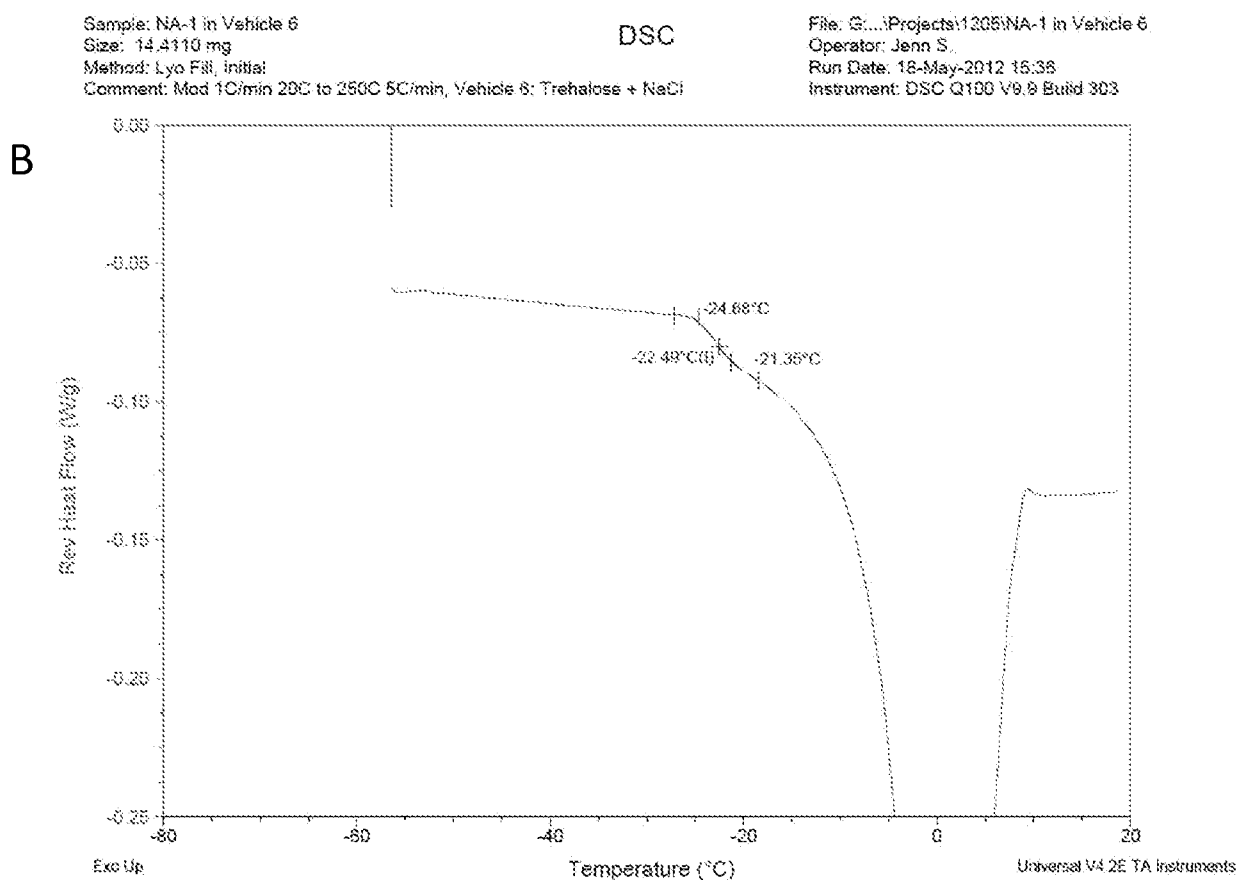
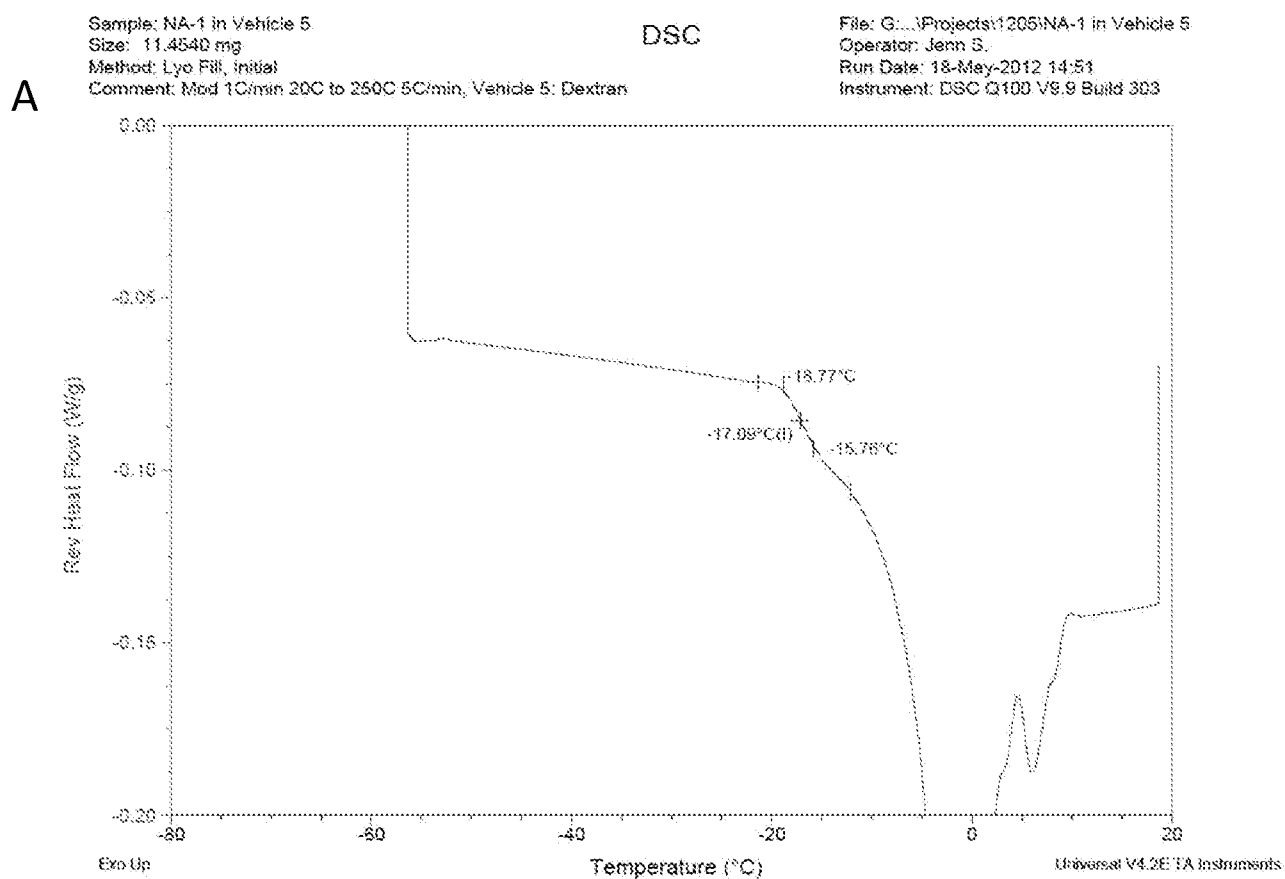
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FIG. 5A, B



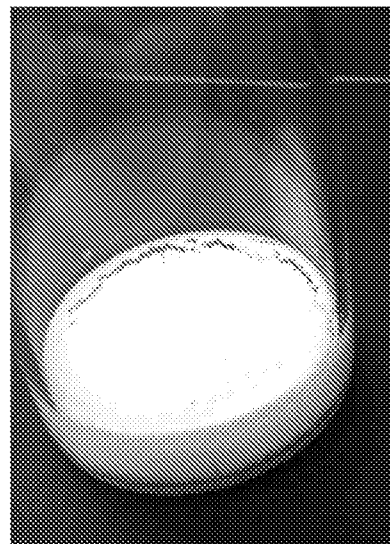
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FIG. 6A, B



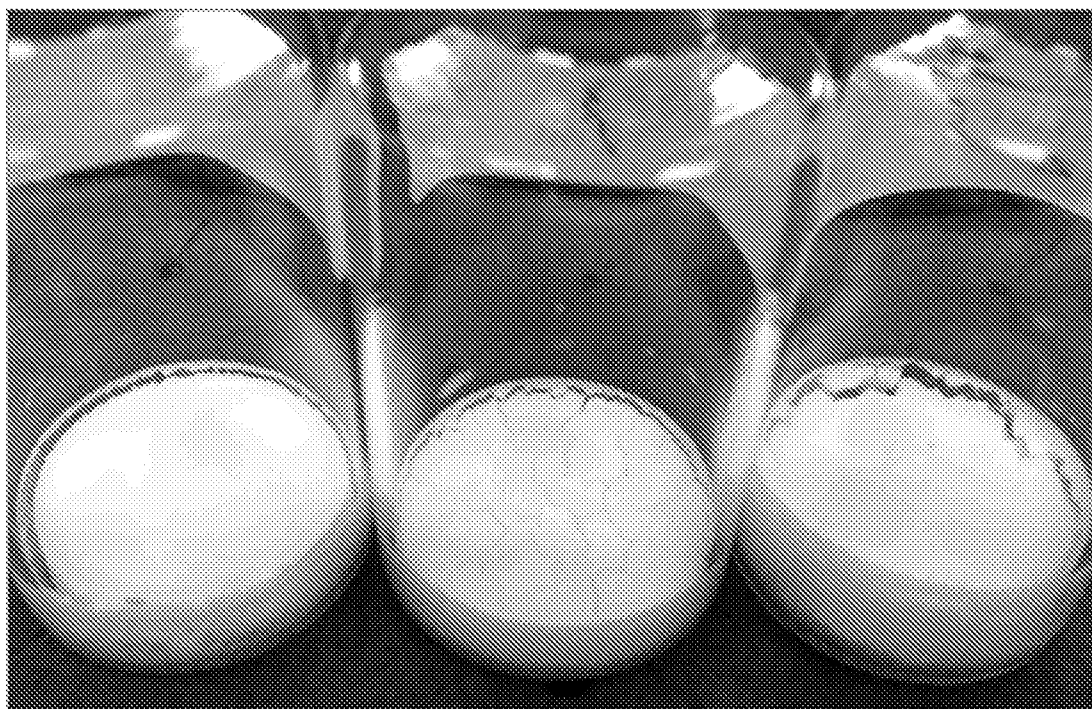
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A



Small scale 4 NA-1

B



Formulations 1-3 from small scale 6

FIG. 7A, B

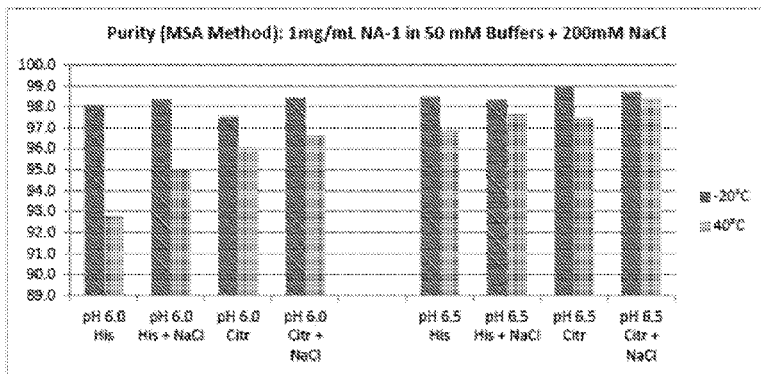


Figure B. Plot of Purity Results, MSA Method (Ordered by pH)