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1A

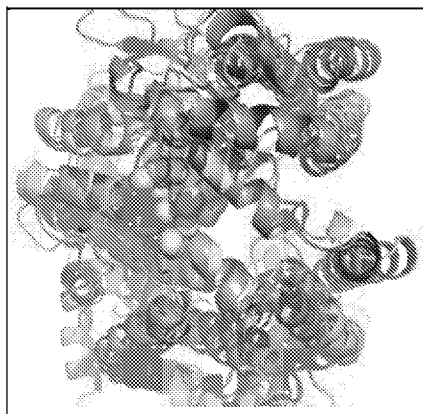


FIG. 1A

(57) Abstract: The present invention includes a composition and method of allosterically potentiating the activity of neurolysin comprising contacting the neurolysin with an amount of a histidine-containing dipeptide that is an allosteric that increases the activity of neurolysin.



ENHANCERS OF NEUROLYSIN ACTIVITY**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] None.

STATEMENT OF FEDERALLY FUNDED RESEARCH

5 [0002] None.

TECHNICAL FIELD OF THE INVENTION

[0003] The present invention relates in general to the field of molecules that act as neurolysin enhancers, which can be used as therapeutic agents.

BACKGROUND OF THE INVENTION

10 [0004] Without limiting the scope of the invention, its background is described in connection with stroke and neurolysin.

[0005] Stroke is a condition where blood flow to the brain is severely reduced or interrupted. Approximately 795,000 people in the United States suffer from a stroke each year, making stroke the fifth leading cause of death in the United States. Stroke is divided into two categories: ischemic and hemorrhagic. Ischemic stroke makes up about 87% of stroke, making it the most common type of stroke. Ischemic stroke occurs by either
15 narrowed or blocked arteries in the brain, causing severely reduced blood flow to brain cells. The reduced blood flow in the brain prevents oxygen and nutrients from reaching brain cells, leading to cell damage or cell death.

[0006] Currently, there are only two treatment options for stroke. The first option is the only FDA approved
20 treatment for ischemic stroke, which uses tissue plasminogen activator (tPA)—a thrombolytic drug designed to break up blood clots. The clinical administration of tPA is effective only within 4.5 hours after ischemic stroke symptoms appear. The second treatment option is a surgical procedure to remove the blood clot. About 90% of patients suffering from stroke are not eligible for either treatment, making it crucial to quickly recognize ischemic stroke and have effective therapy for those non-eligible patients.

25 [0007] Neuropeptides are the largest and most diverse signaling molecules in the mammalian brain that function as neurotransmitters, modulators of neurotransmission, autocrine or paracrine regulators that act within a close cellular environment, or hormones/trophic factors which reach to the site of action far from release (1). These properties of neuropeptides make them one of the most critical classes of bioactive molecules involved in response of brain to stress and adaptation to various challenges (e.g.,

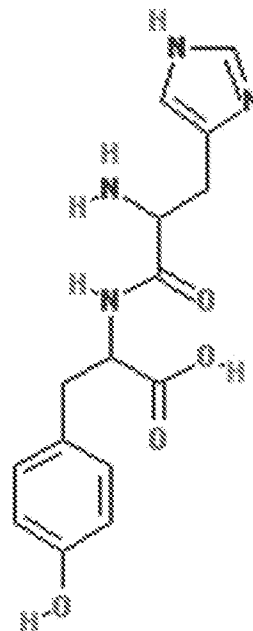
stroke, nerve injury, seizure, and other neurological disorders) (2,3). Actions of neuropeptides are tightly linked to hydrolytic enzymes known as peptidases, which are involved in processing (formation and degradation) of bioactive peptides and are key regulators of neuropeptide function (4-7).

[0008] Altered expression and function of several peptidases and related peptidergic systems have been documented in a number of neurological disorders making them potential pharmacological targets for drug development (8-13). The conventional strategy for therapeutic targeting of peptidases and proteases, enzymes hydrolyzing peptide bonds in proteins, has been identification of a specific inhibitor, generally a small molecule, which blocks the active site (14,15). The main pitfall of such approach, which is also the fundamental challenge in developing protease inhibitors into drugs, is the difficulty to achieve target specificity with such inhibitors. The latter is because peptidases and proteases commonly comprise family of proteins with close homologues that have an identical catalytic mechanism and similar substrate specificity profiles (14). Currently, among most promising emerging approaches used to overcome this problem is development of biological or small-molecule allosteric modulators (16-18). The uniqueness of allosteric modulators is in high specificity of such molecules towards the target enzyme because allosteric binding sites are less conserved and exhibit a greater structural diversity than substrate binding sites. Another important feature of allosteric modulators is that they can potentially act as catalytic enhancer, instead of inhibitors, of the target enzyme. Availability of allosteric enhancers as drugs could therapeutically be very valuable if the function of the target peptidase is directed towards protection of the brain and/or its recovery after an insult. Examples of such peptidases include neprilysin, endothelin converting enzymes and insulin degrading enzyme which are the most studied members of amyloid-degrading enzymes involved in clearance of amyloid- β peptides and hence in halting development of Alzheimer's disease (19-22). Angiotensin converting enzyme 2, which is the main peptidase responsible for generation of angiotensin-(1-7), is another example with documented function in protecting brain from stroke injury (23,24).

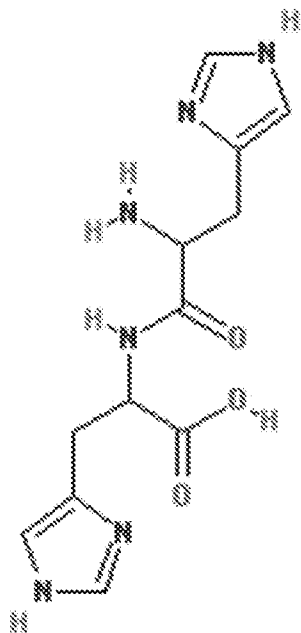
[0009] Thus, a need remains for the development of improved therapies that reduce the effects of ischemic trauma.

SUMMARY OF THE INVENTION

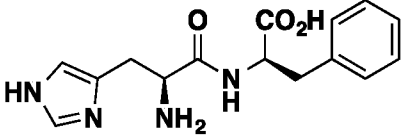
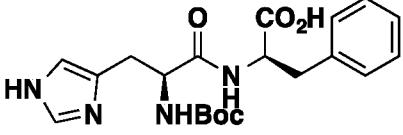
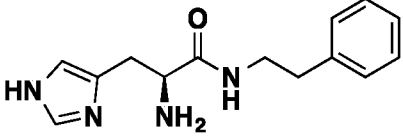
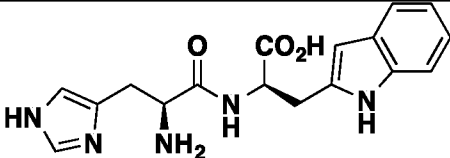
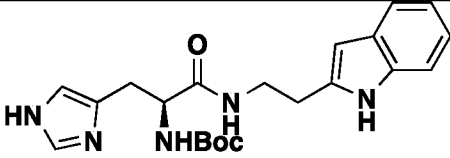
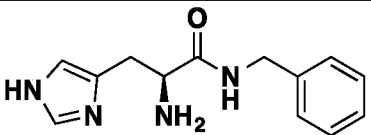
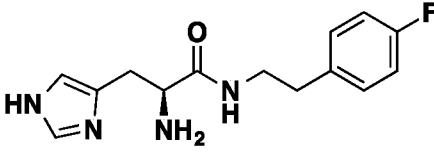
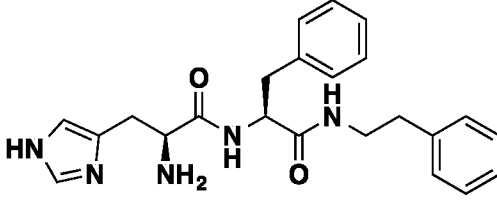
[0010] In one embodiment, the present invention includes a method of allosterically potentiating the activity of neurolysin comprising: contacting the neurolysin with an amount of a small molecule that allosterically increases the activity of neurolysin. In one aspect, the small molecule is a histidine-containing dipeptide. In another aspect, the neurolysin is murine or human. In another aspect, the histidine-containing dipeptide reduces the K_m and increased V_{max} values for hydrolysis of one or more neurolysin substrates. In another

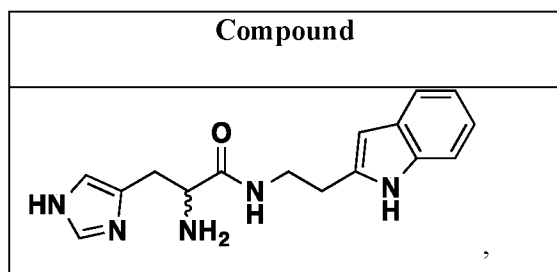


aspect, the histidine-containing dipeptide is selected from



Compound

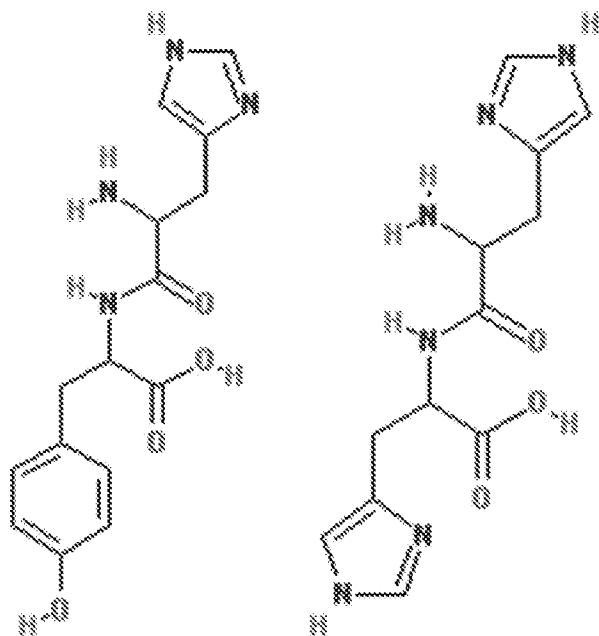
Compound








OR

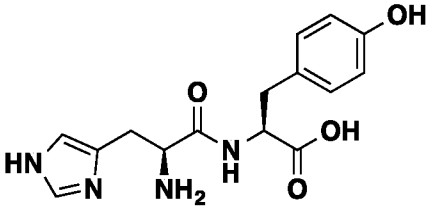
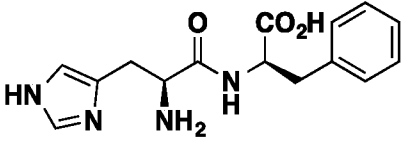
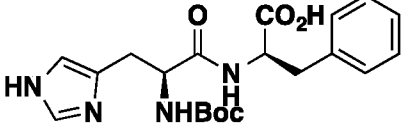
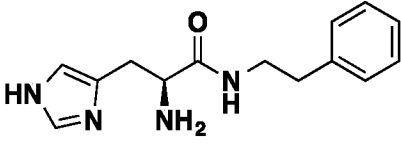
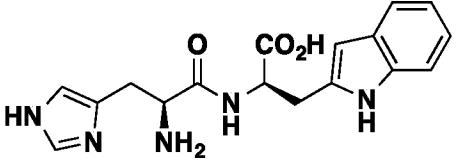
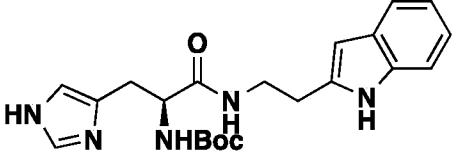
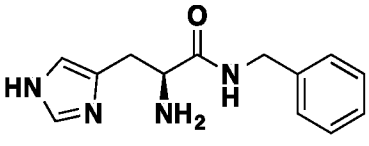
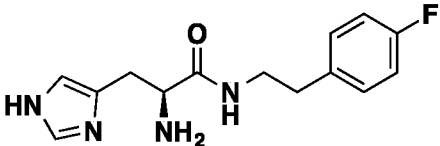


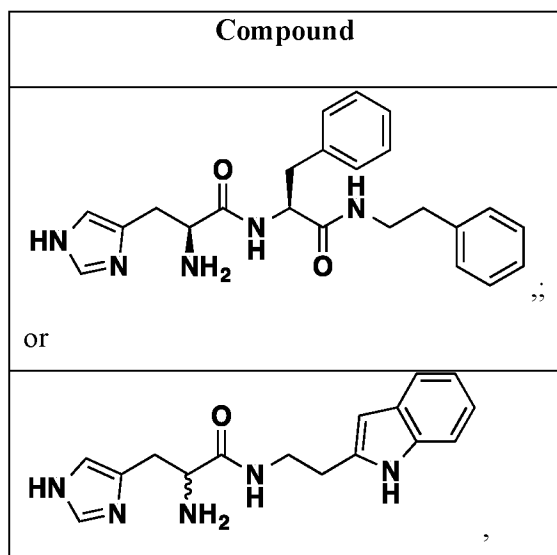
derivatives, salts, or enantiomers thereof.

[0011] In another aspect, the histidine-containing dipeptide does not bind at a substrate binding pocket or blocks the binding of a neurolysin substrate. In another aspect, the one or more neurolysin substrates are selected from at least one of neurolysin substrates, neurotensin, bradykinin, angiotensin I, substance P, hemopressin, dynorphin A(1-8), metorphamide, or somatostatin. In another aspect, the histidine-containing dipeptide increases the activity of neurolysin, but does not affect the activity of thimet oligopeptidase (TOP), angiotensin converting enzyme (ACE), angiotensin converting enzyme 2 (ACE2), or neprilysin (NEP). In another aspect, the small molecule that allosterically increases the activity of neurolysin is provided to a subject in an amount sufficient to treat a peripheral inflammatory disorder selected from ischemic stroke, traumatic brain injury, autism, Alzheimer's Disease, dementias or Parkinson's Disease.

[0012] Yet another embodiment of the present invention includes an allosteric activator of neurolysin selected from:

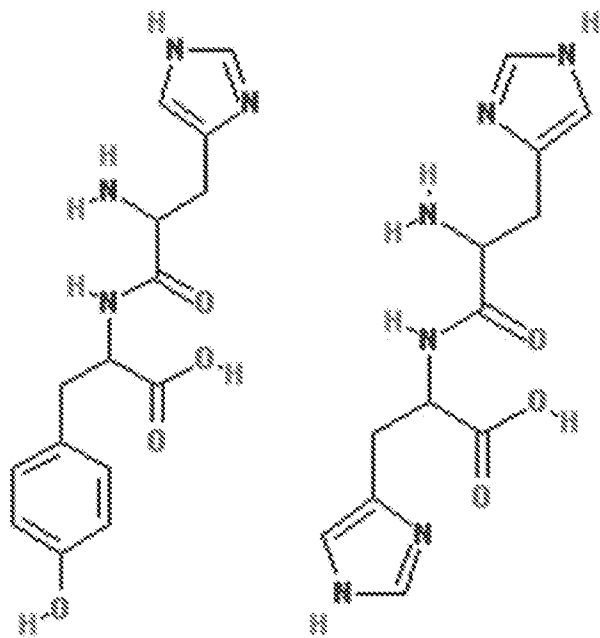


Compound
 <chem>Nc1cc[nH]1CC[C@H](N)C(=O)N[C@@H](Cc1ccc(O)cc1)C(=O)O</chem>
 <chem>Nc1cc[nH]1CC[C@H](N)C(=O)N[C@@H](Cc1ccccc1)C(=O)O</chem>
 <chem>Nc1cc[nH]1CC[C@H](N)C(=O)N[C@@H](Cc1ccccc1)C(=O)OC(=O)C(C)(C)C</chem> ;
 <chem>Nc1cc[nH]1CC[C@H](N)C(=O)NCCc1ccccc1</chem> ;
 <chem>Nc1cc[nH]1CC[C@H](N)C(=O)N[C@@H](Cc1c[nH]c2ccccc12)C(=O)O</chem> ;
 <chem>Nc1cc[nH]1CC[C@H](N)C(=O)N[C@@H](Cc1c[nH]c2ccccc12)C(=O)OC(=O)C(C)(C)C</chem> ;
 <chem>Nc1cc[nH]1CC[C@H](N)C(=O)NCCc1ccccc1</chem> ;
 <chem>Nc1cc[nH]1CC[C@H](N)C(=O)NCCc1ccc(F)cc1</chem> ;

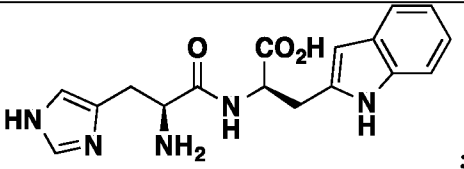
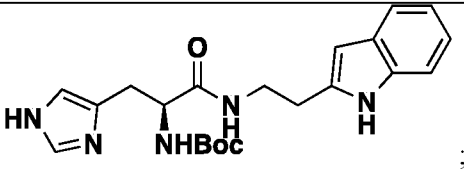
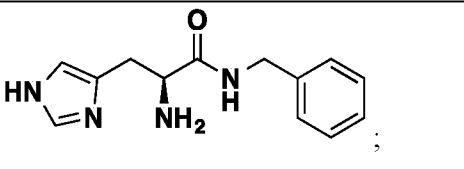
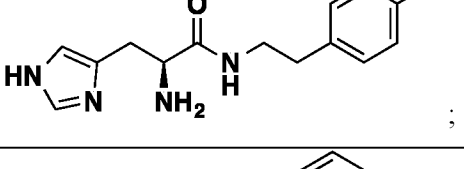
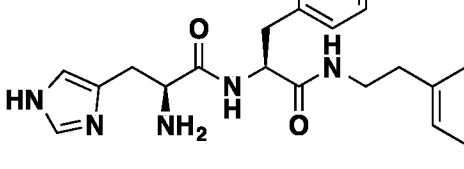
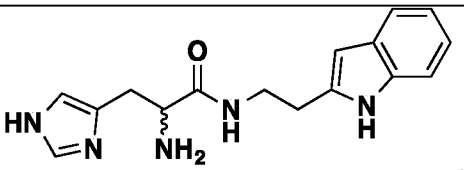


derivatives, salts, or enantiomers thereof. In one aspect, the activator is adapted for oral, intraperitoneal, intradermal, subcutaneous, intravenous, enteral, parental, or pulmonary administration. In another aspect, the activator is combined with one or more excipients, buffers, fillers, or detergents. In another aspect, the activator is adapted for at least one of immediate release, delayed release, or prolonged release. In another aspect, the activator comprises a single enantiomer.

[0013] Yet another embodiment of the present invention includes a method of treating the symptoms of peripheral inflammatory disorder comprising: identifying a subject in need of treatment for ischemic stroke; and providing the subject with an amount of an allosteric potentiator of neurolysin sufficient to increase the activity of neurolysin. In one aspect, the peripheral inflammatory disorder is selected from ischemic stroke, traumatic brain injury, autism, Alzheimer's Disease, dementias or Parkinson's Disease. In another aspect, the neurolysin is murine or human. In another aspect, the allosteric potentiator is a histidine-containing dipeptide. In another aspect, the allosteric potentiator reduced the K_m and increased V_{max} values for hydrolysis of one or more neurolysin substrates. In another aspect, the allosteric potentiator of neurolysin is selected from



Compound

Compound





OR


derivatives, salts, or enantiomers thereof. In another aspect, the allosteric potentiator of neurolysin does not bind at a substrate binding pocket or blocks the binding of a neurolysin substrate. In another aspect, the one or more neurolysin substrates are selected from at least one of neurolysin susbtreates, neurotensin, bradykinin, angiotensin I, substance P, hemopressin, dynorphin A(1-8), metorphamide, or somatostatin. In another aspect, the histidine-containing dipeptide increases the activity of neurolysin, but does not affect the activity of thimet oligopeptidase (TOP), angiotensin converting enzyme (ACE), angiotensin converting enzyme 2 (ACE2), or neprilysin (NEP).

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

[0015] FIGS. 1A and 1B, show the surface pocket in the hinge region selected for molecular docking.

5 FIG. 1A: superposition of ribbon diagrams of ACE2 (cyan) and neurolysin (orange) and their hinge regions. Open conformations of both peptidases were used based on their published crystal structures (33, 41, relevant portions incorporated by reference). The yellow spheres represent the site in the hinge region of neurolysin selected for molecular docking. FIG. 1B: the crystal structure of neurolysin shown in the same orientation as in the left panel. The molecular surface is colored gold and red for carbon, and blue for nitrogen. Yellow spheres depict sites for interaction of potential ligand atoms used in molecular docking orientations. The box in magenta represents the boundaries of the scoring grid used to generate scores that consider electrostatic (polar) and van der Waals (non-polar) interactions.

[0016] FIGS. 2A to 2C show the effect of compounds NlnA#1 and NlnA#2 on catalytic activity of rat recombinant neurolysin. FIGS. 2A and 2B: representative reaction progress curves of QFS hydrolysis (25 μ M) by rat recombinant neurolysin (0.3 nM) in the presence of different concentrations of NlnA#1 and NlnA#2. FIG. 2C: concentration-dependent effect of both compounds on hydrolysis of QFS under the same experimental conditions ($n = 3$, mean $\pm$ SD are presented). Note that the initial velocity of the hydrolysis in the absence of either compound corresponds to 100% on the vertical axis and to -13 on the horizontal axis. Calculated EC₅₀ value for NlnA#1 is 45.4 μ M (95% confidence intervals 29.9 to 68.9 μ M), and E_{max} is 234.7% (95% confidence intervals 219.0 to 250.4%). Calculated EC₅₀ value for NlnA#2 is 45.8 μ M (95% confidence intervals 32.0 to 65.6 μ M), and E_{max} is 202.8% (95% confidence intervals 192.2 to 213.4%).

[0017] FIG. 3 shows the effect of compounds NlnA#1 and NlnA#2 on the potency of dynorphin A (1-13). Concentration-dependent inhibitory effect of dynorphin A (1-13) on hydrolysis of QFS (15 μ M) by rat recombinant neurolysin (rNln, 0.3 nM) in the absence or presence of NlnA#1 or NlnA#2 (100 μ M) is presented ($n = 3$, mean $\pm$ SD are presented, FLU – fluorescence unit). Note that the initial velocity of the hydrolysis in the absence of dynorphin A (1-13) corresponds to -13 on the horizontal axis. Calculated IC₅₀ values for dynorphin A (1-13) are: 1.52 μ M (95% CI: 0.9 – 2.5 μ M) in rNln, 1.40 μ M (95% CI: 1.1 – 1.8 μ M) in rNln+NlnA#1, and 1.20 μ M (95% CI: 0.91 – 1.5 μ M) in rNln+NlnA#2.

30 [0018] FIGS. 4A and 4B show the effect of dynorphin A (1-13) on the potency of compounds NlnA#1 (FIG. 4A) and NlnA#2 (FIG. 4B). Concentration-dependent effect of NlnA#1 and NlnA#2 on hydrolysis of QFS (15 μ M) by rat recombinant neurolysin (rNln, ~0.3 nM) in the absence or presence of dynorphin A

(1-13) (1 μ M) (n = 3, mean $\pm$ SD are presented, FLU – fluorescence unit). Note that the initial velocity of the hydrolysis in the absence of NlnA#1 or NlnA#2 corresponds to -13 on the horizontal axis in both panels. Calculated EC₅₀ values for NlnA#1 are 42.3 μ M (95% CI: 24.0 – 74.8 μ M) and 58.6 μ M (95% CI: 37.5 – 91.6 μ M) in the absence and presence of dynorphin A (1-13), respectively. Corresponding E_{max} values for NlnA#1 are 292.4% (95% confidence intervals 265.7 to 319.0%) and 238.3% (95% confidence intervals 222.2 to 254.3%). Calculated EC₅₀ values for NlnA#2 are 23.3 μ M (95% CI: 6.7 – 81.2 μ M) and 24.9 μ M (95% CI: 8.4 – 73.5 μ M) in the absence and presence of dynorphin A (1-13), respectively. Corresponding E_{max} values for NlnA#2 are 254.7% (95% confidence intervals 226.3 to 283.1%) and 198.3% (95% confidence intervals 179.5 to 217.0%).

[0019] FIG. 5A and 5B show the effect of compounds NlnA#1 (FIG. 5A) and NlnA#2 (FIG. 5B) on catalytic efficiency of neurolysin. Hydrolysis of different concentrations of QFS by rat recombinant neurolysin (0.3 nM) in the absence or presence of NlnA#1 and NlnA#2 (40 and 100 μ M) is presented (n = 16 for 0 μ M (neurolysin alone), n = 4 for all other groups, mean $\pm$ SD are presented, FLU – fluorescence unit).

[0020] FIG. 6A and 6B show the effect of compounds NlnA#1 (FIG. 6A) and NlnA#2 (FIG. 6B) on catalytic activity of human recombinant and mouse brain neurolysin. Both panels document concentration-dependent effect of both compounds on hydrolysis of QFS at 25 μ M (n = 3, mean $\pm$ SD are presented). Note that the initial velocity of the hydrolysis in the absence of either compound corresponds to 100% on the vertical axis and to -13 on the horizontal axis. In human recombinant neurolysin the EC₅₀ for NlnA#1 is 23.6 μ M (95% confidence intervals 16.5 to 33.7 μ M), and E_{max} is 203.5 (95% confidence intervals 193.7 to 213.4%). The EC₅₀ value for NlnA#2 is 69.5 μ M (95% confidence intervals 56.0 to 86.2 μ M), and E_{max} is 214.3% (95% confidence intervals 205.7 to 222.9%). In mouse brain neurolysin the EC₅₀ for NlnA#1 is 9.2 μ M (95% confidence intervals 3.1 to 26.9 μ M), and E_{max} is 190% (95% confidence intervals 169.2 to 210.7%). The EC₅₀ value for NlnA#2 is 17.1 μ M (95% confidence intervals 6.0 to 49.1 μ M), and E_{max} is 258% (95% confidence intervals 232.7 to 283.8%). Note that in the mouse brain neurolysin, the initial velocity of QFS hydrolysis was consistently lower in the presence of 300 μ M NlnA#1 compared to that of 100 μ M (300 μ M concentration was not included in the analysis for determination of the EC₅₀ value).

[0021] FIGS. 7A to 7D show the effect of compounds NlnA#1 and NlnA#2 on catalytic activity of human recombinant peptidases. All panels document concentration-dependent effect of NlnA#1 and NlnA#2 on hydrolysis of a respective quenched fluorescent substrate (n = 3, mean $\pm$ SD are presented): Mca-Pro-Leu-Gly-Pro-D- Lys(DNP)-OH at 25 μ M for thimet oligopeptidase (TOP)(FIG. 7A), Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe- Lys(Dnp)-OH at 10 μ M for angiotensin converting enzyme (ACE) (FIG. 7B) and

neprilysin (NEP) (FIG. 7D), and Mca-Ala- Pro-Lys-(Dnp)-OH at 10 μ M for angiotensin converting enzyme 2 (ACE2) (FIG. 7C). In all panels, the initial velocity of the hydrolysis in the absence of either compound corresponds to 100% on the vertical axis and to -13 on the horizontal axis.

[0022] FIG. 8 shows the effect of compounds NlnA#1 and NlnA#2 on hydrolysis of endogenous substrates by neurolysin. Rat recombinant neurolysin (rNln, 2 nM) was incubated with angiotensin I, bradykinin or neurotensin (20 μ M) in the absence or presence of NlnA#1 or NlnA#2 (100 μ M), and generation of angiotensin-(1-7) (Ang-(1-7)), bradykinin-(1-5) (BK-(1-5)) and neurotensin-(1-10) (NT-(1-10)) was documented by mass spectrometry analysis, respectively (n = 2, mean $\pm$ SD are presented). Column 1 in all panels represents the amount of respective peptide fragment, i.e. product of hydrolysis, in the absence of rNln; column 2 – the amount of respective peptide fragment generated in the presence of rNln alone; columns 3 and 4 – the amount of respective peptide fragment generated in the presence of rNln plus NlnA#1 or NlnA#2, respectively.

[0023] FIGS. 9A and 9B show the docking of NlnA#1 (FIG. 9A) and NlnA#2 (FIG. 9B) into the selected surface pocket of neurolysin in its hinge region. *Top panels*: chemical structure of the compounds. NlnA#1, NSC 374121, L-histidyl-L-tyrosine. NlnA#2, NSC 523374, D,L-histidyl-D,L-histidine. *Bottom panels*: close-up view of the crystal structure of neurolysin in its open conformation shown in FIG. 1B together with a molecule of NlnA#1 or NlnA#2 bound to the selected surface pocket, i.e. the hypothesized allosteric binding site. The molecular surface of the protein is colored gold for carbon, blue for nitrogen, red for oxygen. In the compounds, yellow for carbon, red for oxygen, and blue for nitrogen.

[0024] FIGS. 10A and 10B show the effect of compounds NlnA#1 and NlnA#2 on catalytic activity of neurolysin in the presence of CHAPS (FIG. 10A) and BSA (FIG. 10B). Both panels document representative concentration- dependent effect of the compounds on hydrolysis of QFS (25 μ M) by rat recombinant neurolysin (0.3 nM). The initial velocity of the hydrolysis in the absence of either compound corresponds to 100% on the vertical axis and to -13 on the horizontal axis. In the presence of 0.1% CHAPS, EC50 value for NlnA#1 is 36.2 μ M (95% confidence intervals 22.2 to 59.3 μ M), whereas for NlnA#2 it is 34.5 μ M (95% confidence intervals 24.3 to 49.2 μ M). In the presence of 0.01 mg/ml BSA, EC50 value for NlnA#1 is 20.5 μ M (95% confidence intervals 9.0 to 47.1 μ M), whereas for NlnA#2 it is 30.6 μ M (95% confidence intervals 10.6 to 87.8 μ M).

[0025] FIGS. 11A and 11B show the effect of compounds NlnA#1 (FIG. 11A) and NlnA#2 (FIG. 11A) on fluorescence signal of Mca-Pro-Leu-OH. Both panels document representative concentration-dependent effect of the compounds on fluorescence signal of Mca-Pro-Leu-OH, the product of QFS hydrolysis by neurolysin, under the same assay conditions as presented in FIGS. 2A-C. The only difference was that Mca-Pro-Leu-OH, instead of QFS, was present in the assay at 2 μ M final concentration. Each data point

represents the average fluorescence signal measured every minute for duration of 10 min. Note that -13 on the horizontal axis corresponds to the condition where neither NInA#1 nor NInA#2 was present.

[0026] FIG. 12 shows a mass spectrometry analysis of angiotensin I hydrolysis by recombinant neurolysin. Top and middle panels demonstrate representative mass spectra of neurolysin substrate angiotensin I and the product of its hydrolysis angiotensin-(1-7), respectively. Bottom panel demonstrates representative LC-MS chromatograms of experimental samples in which angiotensin-(1-7) ([DRVYIHP+3H]³⁺, m/z = 300.4960) was measured for quantification.

DETAILED DESCRIPTION OF THE INVENTION

[0027] While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

[0028] To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as “a”, “an” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not limit the invention, except as outlined in the claims.

[0029] The present inventors describe the discovery of a group of small molecules that specifically enhance the catalytic efficiency of peptidase neurolysin. A computational approach was used to explore the structure of neurolysin and identify a druggable surface pocket in its hinge region, followed by docking and ranking of 139,735 molecules from the NCI Developmental Therapeutics Program database. Top ranking compounds were subjected to pharmacological evaluation to identify an enhancer of neurolysin activity. Two structurally related compounds were identified which enhanced the rate of substrate hydrolysis by recombinant (human and rat) and mouse brain-purified neurolysins in a concentration- dependent manner. Neither the identified modulators nor dynorphin A(1-13), a competitive inhibitor, affected each other's affinity in modulating activity of neurolysin, suggesting that the modulators do not bind to the substrate binding site. Both modulators reduced K_m and increased V_{max} values for hydrolysis of the synthetic substrate by neurolysin in a concentration-dependent manner. The modulators had negligible effect on catalytic activity of thimet oligopeptidase, neprilysin, angiotensin converting enzyme (ACE) and ACE2, indicating that they are specific to neurolysin. Both modulators also enhanced hydrolysis of endogenous substrates, suggesting

independence of their effect from the synthetic substrate. The identified molecules could be developed into research tools for evaluation of the (patho)physiological function of neurolysin, and may aim development of a new drug class. This study is one of few utilizing a structure- based drug-discovery approach for rational identification of enzyme activators, and by that it demonstrates applicability of this methodology for identification of allosteric modulators of other enzymes.

[0030] The present inventors have previously suggested that peptidase neurolysin plays a role in processes modulating the brain's response to stroke and its recovery after stroke (25). Neurolysin (EC 3.4.24.16) is a zinc endopeptidase belonging to the thermolysin-like mammalian family of peptidases (26), which are maximally active at neutral pH and comprise the most studied group of peptidases involved in hydrolytic processing of bioactive peptides in the extracellular environment (5). The hypothesized role of brain neurolysin in response to stroke is based on observations indicating sustained functional up-regulation of this peptidase in mouse brain for at least 7 days after stroke, and diversity of its endogenous substrates with well-documented role in pathogenesis of stroke (25). To develop novel approaches to aid in the treatment of ischemic trauma, and to aid in the understanding of the (patho)physiological function of neurolysin in stroke and other neurological disorders, the present invention was developed.

[0031] The present inventors describe herein the discovery of small molecules that enhance catalytic efficiency of neurolysin. For this purpose a computational approach was used to explore structure of neurolysin and identify a specific, druggable allosteric surface pocket, followed by molecular docking and ranking of 139,735 drug-like small molecules from the National Cancer Institute Developmental Therapeutics Program (NCI DTP) database. Top ranking compounds were received from NCI DTP and subjected to unbiased pharmacological evaluation to identify a molecule specifically enhancing activity of neurolysin. Two structurally similar compounds were identified which increased the rate of substrate hydrolysis by recombinant (human and rat) and mouse brain-purified neurolysins in a concentration-dependent manner. Concentration-response experiments involving the identified modulators and dynorphin A(1-13), a competitive inhibitor of neurolysin, revealed that neither the compounds, nor dynorphin A(1-13), affected each other's affinity in modulating activity of neurolysin. At two different concentrations, both modulators reduced K_m and increased V_{max} values (i.e., increased V_{max}/K_m ratio) for hydrolysis of the synthetic substrate by neurolysin. Enhanced activity of neurolysin was observed not only with use of the synthetic substrate but also with three endogenous substrates of the peptidase. Both modulators had either no, or marginal, effects on catalytic activity closely related peptidases, including thimet oligopeptidase, neprilysin, angiotensin converting enzyme (ACE) and ACE2. This is the first report in scientific literature describing the discovery of specific, small molecule enhancers of neurolysin activity. The identified molecules can be used to target neurolysin in pathogenesis of stroke and other neurological disorders, and may serve as starting structures for development of a new class of drugs.

[0032] Molecular docking – Candidate small molecule modulators of neurolysin were selected based on the strategy used for discovery of allosteric enhancers of angiotensin converting enzyme 2 (ACE2) (27,28). In the current study, structural analysis of the hinge region of neurolysin in its open conformation was carried out using DSSP (29) and castP (30) to identify unique structural pockets with adequate solvent accessible volumes and chemical characteristics for binding of drug-like small molecules (molecular weight: <500; octanol/water partition coefficients: <5; H-bond donors: <5; H-bond acceptors: <10 (31)). One potentially druggable site was found unique to the open conformation of the neurolysin hinge region. Programs implemented in the DOCK program package (UCSF) were used to generate files used for molecular docking. SPHGEN was used to position spheres in the selected hinge site, GRID was used to calculate scoring grids with a box size 5 angstrom from the selected spheres. DOCK 6.5 (UCSF) was used to dock and rank 139,725 compounds from the NCI DTP repository (zinc.docking.org/catalogs/ncip). The job was run on the University of Florida High Performance Computing Facility by parallel processing. Compounds were selected to include protonation variants at medium pH (5.75 - 8.25). Each molecule was positioned into the selected surface pocket in 1000 different orientations and scored with rank based on the predicted polar (H-bonding) and nonpolar (van der Waals) interactions. Top scoring 40 compounds (0.03 % of the chemical library screened) were received from NCI DTP and used for pharmacological evaluation (see below).

[0033] Rat recombinant neurolysin – N-terminal polyhistidine tagged recombinant rat neurolysin was produced in Escherichia coli using a plasmid construct in pBAD/His vector system (Invitrogen) kindly provided by Dr. David W. Rodgers (University of Kentucky) (32,33). Bacterial culture was carried out in Terrific Broth in 2 L Erlenmeyer flasks using an incubator shaker (I-2400, New Brunswick Scientific). Expression was induced by addition of arabinose (1 mM final concentration), the cells were pelleted 3 h after induction and stored at -80oC freezer. Bacterial pellets were resuspended and lysed in phosphate buffer (20 mM NaH₂PO₄, 500 mM NaCl, 0.05% Tween-20, pH 8.0) containing 0.25 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, 0.05 mg/ml DNase and 0.02 mg/ml RNase, followed by brief sonication. After centrifugation (40,000 x g, 20 min at 4°C) the supernatant was applied to a Ni-NTA Superflow cartridge (Qiagen), followed by washing and elution of recombinant neurolysin with phosphate buffer containing 25 and 250 mM imidazole, respectively. The eluted protein was further purified by size exclusion chromatography using a TSK gel BioAssist G2SWxl column (Tosoh bioscience) in AKTA Purifier FPLC system (GE Healthcare). Purity of recombinant neurolysin (≥ 95%) was verified by SDS-PAGE and size exclusion chromatography, whereas identity was confirmed by Western blotting using a specific anti-neurolysin antibody (TriplePointBiologics; product number RP3- Neurolysin) (25,34). Specific activity of recombinant neurolysin was determined by an enzymatic assay described below.

[0034] Human recombinant peptidases – recombinant human neurolysin (product number 3814-ZN), thimet oligopeptidase (TOP; product number 3439-ZN), neprilysin (NEP; product number 1182-ZNC), angiotensin

converting enzyme (ACE; product number 929-ZN), and ACE2 (product number 933-ZN) were purchased from R & D systems.

[0035] Mouse brain neurolysin – P10 mouse forebrains were used for purification of native neurolysin as expression of the peptidase is about 5 times higher in comparison to adult mouse forebrain (34,35). In brief, forebrains were homogenized in hypotonic buffer (20 mM NaPO₄, pH 7.2) followed by gentle sonication and centrifugation (30 min at 48,000 x g at 4°C). The resulting supernatant was filtered through a 100 kDa cut-off centrifugal filtering unit and concentrated in a 30 kDa cut-off centrifugal filtering unit (Amicon Ultra; Millipore). Neurolysin was semi-purified from the concentrate by size exclusion chromatography using a Superdex 200 Increase 10/300 GL column (GE Healthcare) in AKTA Purifier FPLC system (GE Healthcare). Elution of neurolysin was tracked by the enzymatic assay described below, and the fraction with the highest neurolysin activity was directly used for the experiments.

[0036] Enzymatic assays – activity of neurolysin was measured in a continuous assay by documenting the increase in fluorescence occurring upon cleavage of a quenched fluorescent substrate (QFS) Mca-Pro-Leu-Gly-Pro-D-Lys(DNP)-OH (Bachem) (25,36). In brief, a fixed concentration of recombinant or mouse brain purified neurolysin was incubated with 25 µM (for primary screening and determination of EC₅₀ values) or 2.5 to 75 µM (for determination of K_m and V_{max} values) QFS in artificial cerebrospinal fluid (NaCl 126 mM, NaHCO₃ 26 mM, KCl 3 mM, KH₂PO₄ 1.4 mM, HEPES 25 mM, glucose 4 mM, MgCl₂ 1.3 mM, CaCl₂ 1.4mM, ZnSO₄ 0.0002 mM, pH 7.2) containing 0.01% final assay concentration of Triton X-100 at 37°C. In studies involving dynorphin A(1-13), a fixed concentration of rat recombinant neurolysin was incubated with 15 µM QFS and varying concentrations of dynorphin A(1-13) in the absence or presence of one of the modulators at 100 µM. In the reverse experiment, a fixed concentration of rat recombinant neurolysin was incubated with 15 µM QFS and varying concentrations of one of the modulators in the absence or presence of dynorphin A(1-13) at 1 µM. All assays were initiated by addition of QFS (100 µl assay volume in 96-well plates), and each experimental sample was present in duplicate. Generation of the fluorescent product (Mca-Pro-Leu-OH; λ_{ex} = 320, λ_{em} = 405) was documented every 1 min in a plate reader (SynergyMX; Biotek) at initial velocity conditions where less than 20% of the substrate was metabolized. For mouse brain purified neurolysin, Pro-Ile-inhibited (10 mM final assay concentration) fraction of substrate hydrolysis was considered neurolysin specific.

[0037] Activity of recombinant human TOP was assessed in the same way as for neurolysin, except that the assay was carried out in the presence of 0.1 mM dithiothreitol (37). Likewise, activities of recombinant human ACE, NEP and ACE2 were measured similar to neurolysin, except that quenched fluorescent substrate Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH (SEQ ID NO:1) was used for ACE and NEP

(38,39), and Mca-Ala-Pro-Lys-(Dnp)-OH (SEQ ID NO:2) was used for ACE2 (40) (both substrates at 10 μ M final assay concentration, obtained from Enzo Life Sciences).

[0038] Screening of test compounds – primary screening of top-ranked 40 compounds (received from NCI DTP) was carried out at 10 and 100 μ M final assay concentrations. Determination of EC50 values was conducted in the presence of 0.1 to 300 μ M final assay concentrations of the two identified activators. In all experiments, test compounds were incubated with neurolysin for 10 min at 37°C before addition of the substrate to start the reaction. All test compounds were dissolved in DMSO at 10 to 50 mM stock concentrations. Presence of DMSO (up to 3.5%) did not affect activity of the enzyme.

[0039] Endogenous substrates of neurolysin and mass spectrometry analysis – hydrolysis of neurotensin, bradykinin and angiotensin I (20 μ M; obtained from American Peptide Company) by recombinant rat neurolysin (2 nM in artificial cerebrospinal fluid containing 0.01% Triton X-100) was carried out in the absence and presence of the two identified modulators (100 μ M) at 37°C for 20 min. The reaction was stopped with HCl (~32 mM final concentration) followed by freezing at -80°C.

[0040] One pmol of each sample was subjected to LC-MS/MS analysis using a Dionex 3000 Ultimate nano-LC system (Dionex, Sunnyvale, CA) and a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA). Samples were online-purified using Acclaim PepMap100 C18 pre-column (75 μ m x 2 cm, 3 μ m, 100Å, Dionex) and separated using Acclaim PepMap100 C18 RSLC column (75 μ m x 15 cm, 2 μ m, 100Å, Dionex). A 30 min LC elution gradient was employed at 350 nL/min flow rate and 29.5°C column compartment temperature. The elution gradient of solvent B was: 5% over 10 min, 5% - 90% over 10 min and 90% over 10 min. Solvent B consisted of 100% ACN containing 0.1% formic acid, while solvent A composed of 98% HPLC water containing 0.1% formic. Eluting peptides were ionized by electrospray ionization at 1.5kV. The LTQ Orbitrap Velos mass spectrometer was operated in data-dependent acquisition mode comprised of two scan events. The first scan event was a full MS scan of 300-2000 m/z at a mass resolution of 60,000. The precursor ions with charge state +2 or higher were subjected to the second scan, which was a collision induced dissociation (CID) MS/MS scan. The isolation width was set to 3.0 m/z, the normalized collision energy was set to 35%, and the activation Q value was set to 0.250. The top 5 most intense ions observed in the MS scan event were subjected to the CID MS/MS scan. The dynamic exclusion was set to have repeat count of 2, repeat duration of 30s, exclusion list size of 200 and exclusion duration of 90s. The relative quantitation of each peptide was achieved by summing up the extracted peak area of all the observed protonated precursor ions corresponding to the certain peptide.

[0041] Statistical Analyses – statistical analyses and curve fitting were conducted with GraphPad Prism 6.0 software. For each enzymatic reaction, slope of the line which represents the initial velocity (V_o) for the reaction progress curve was calculated using the liner regression model of the software (V_o = Slope = Δ

fluorescent intensity of the reaction product/ Δ time). EC₅₀ values for the modulators were calculated by fitting initial velocity values for hydrolysis of QFS by neurolysin in the presence of varying concentrations of a modulator into a nonlinear regression model for the three-parameter log(stimulator) vs. response equation $[Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X))})]$. IC₅₀ values for dynorphin A(1-13) were calculated by fitting initial velocity values for hydrolysis of QFS by neurolysin in the presence of varying concentrations of dynorphin A(1-13) into a nonlinear regression model for the three-parameter log(inhibitor) vs. response equation $[Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((X - \text{LogIC}_{50}))})]$. K_i values were determined using the Cheng-Prusoff equation: $K_i = \text{IC}_{50} / (1 + S/K_m)$ where S is the substrate concentration (15 μM QFS in our experiments), and K_m is the K_m value for the substrate (15 μM for QFS, see Table 3). K_m and V_{max} values were calculated by fitting initial velocity values for hydrolysis of varying concentrations of QFS by neurolysin in the absence or presence of 40 or 100 μM of each modulator into Michaelis-Menten equation $[Y = V_{\text{max}} * X / (K_m + X)]$. Data are presented as mean with 95% confidence intervals or mean $\pm$ S.D.

[0042] Molecular docking – In this study, a structural analysis of the hinge region of neurolysin in its open conformation was carried out to identify a unique surface pocket. The search for an allosteric site was carried out in the hinge region because crystal structures of neurolysin demonstrates that catalysis is accompanied by large a hinge-bending motion (33,41). The hinge region can affect the distance between the two structural domains and by that modulate substrate binding and catalysis. Moreover, the equilibrium between open and closed conformations can be perturbed with drug-like small molecules to enhance or inhibit catalytic activity. Lastly, peptidases are sensitive to activation by monovalent anions.

[0043] The hinge region in neurolysin showed presence of several surface pockets with adequate solvent accessible volumes (DSSP (29)) and castP (30)). One of these pockets was selected as a potential allosteric site (FIGS. 1A and 1B) because it is located at a position analogous to the ACE2 site used for selection of small molecule enhancers of catalytic activity.

[0044] The selected surface pocket was used for molecular docking and virtual, high-throughput screening of drug-like compounds from NCI DTP (FIGS. 1A and 1B). As a result of *in silico* screening the compounds were ranked according to their combined energy scores for hydrogen bonding and van der Waals contact interactions with the selected surface pocket (Table 1).

[0045] Table 1. Top ten scoring compounds for the selected surface pocket, i.e. the hypothesized allosteric binding site, on the hinge region of neurolysin.

Rank	ID	LogP	H-	H-	MW	VDW	ES	Score
1	NSC 42215*	2.41	0	3	184.234	19.338	-108.107	-88.767
2	NSC 353874	1.24	1	3	196.245	-9.700	-46.004	-55.705
3	NSC 128977	-0.03	2	6	272.174	8.901	-59.141	-50.239

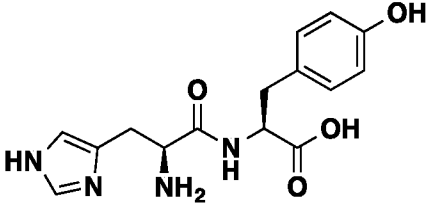
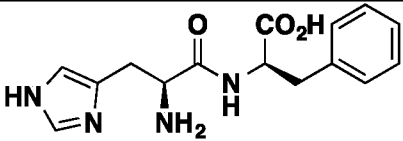
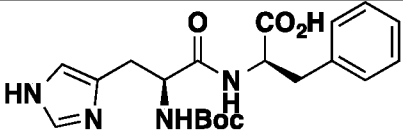
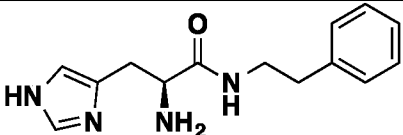
5	NSC 359097	-1.65	2	3	169.178	3.290	-50.315	-47.025
6	NSC 14541	-0.52	2	4	228.38	-26.993	-19.369	-46.363
7	NSC	-1.71	5	8	292.296	-32.834	-11.721	-44.555
8	NSC 155877	-1.48	0	3	334.655	-23.804	-19.725	-43.529
10	NSC 302851	-5.51	4	6	240.291	-26.927	-15.557	-42.484
11	NSC 600947	1.72	1	3	239.403	-30.350	-12.113	-42.463
12	NSC 163084	-0.59	4	6	352.404	-34.094	-8.321	-42.416

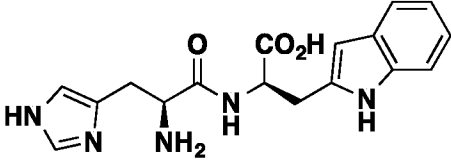
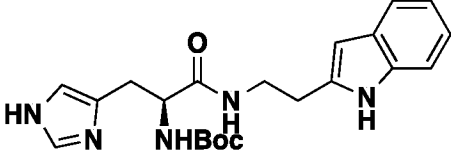
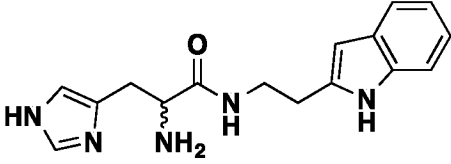
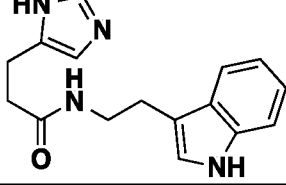
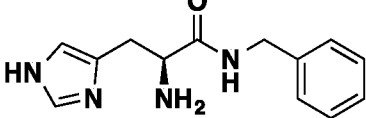
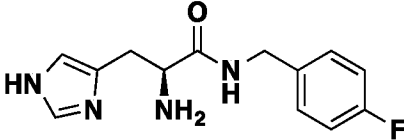
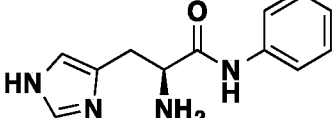
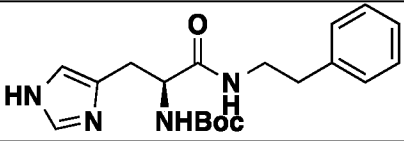
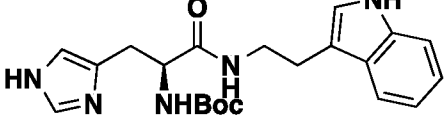
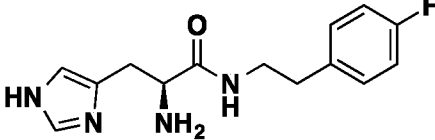
VDW – van der Waals interactions; ES – electrostatic interactions

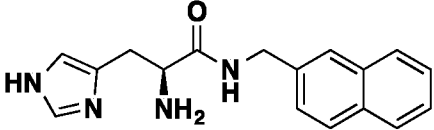
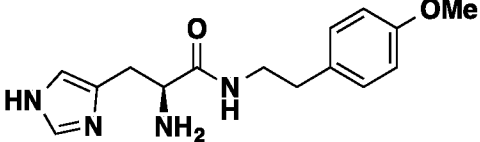
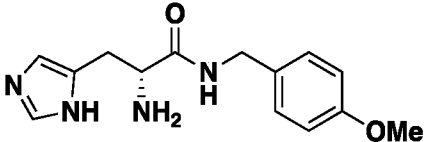
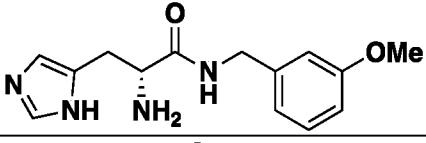
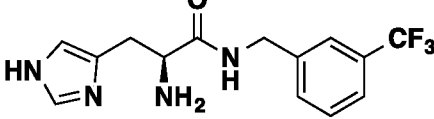
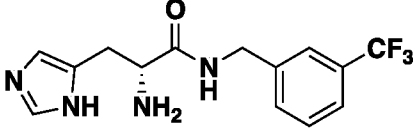
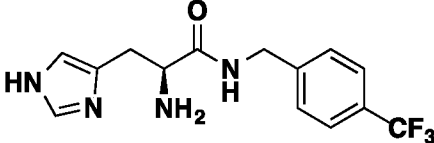
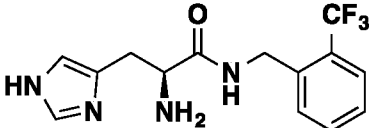
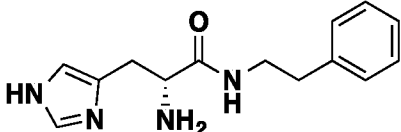
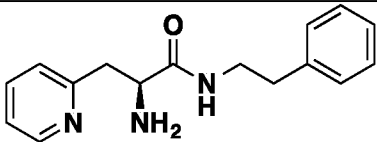
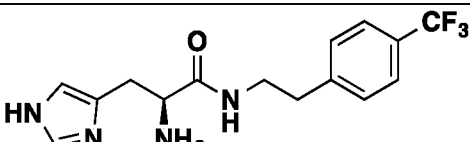
*NSC 42215 was also ranked # 4 with combined score of -48.091 (was not used in the enzymatic assays as it was not available from NCI DTP or from a commercial vendor); **NSC 523374 was also ranked # 9 with combined score of -43.371. Note that ranking of a compound more than once indicates that it can bind to neurolysin in more than one orientation.

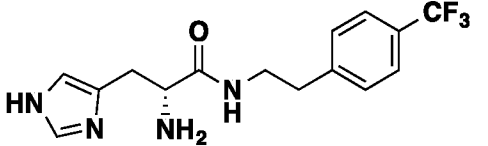
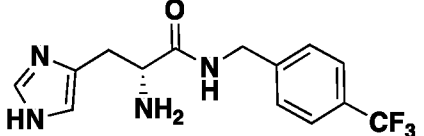
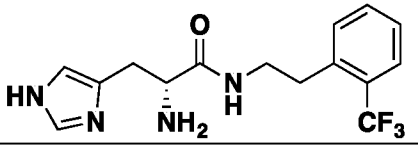
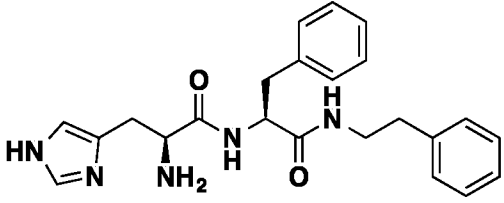
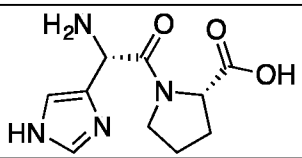
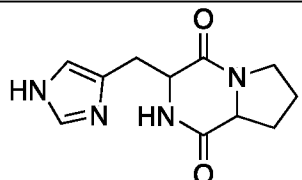
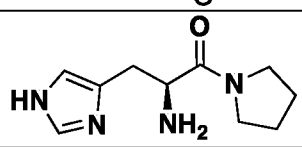
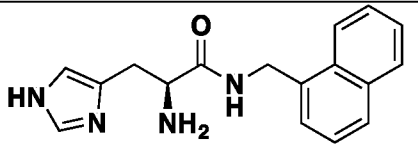
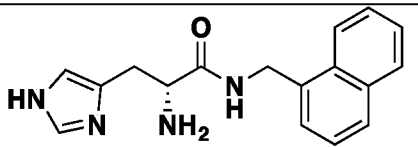
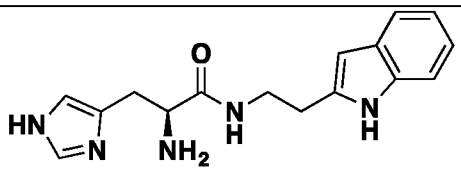
[0046] Primary and secondary screens – Top ranking 40 compounds identified in the virtual screen were obtained from NCI DTP and used at 10 and 100 μ M concentrations to observe their effects on ability of recombinant rat neurolysin to hydrolyze QFS. Table 2 summarizes the effects of all compounds on activity of the peptidase at both concentrations. Among them there were more than a handful of compounds showing inhibition of the peptidase at both concentrations, and two compounds (NSC 374121 and NSC 523374), which showed robust, concentration-dependent activation of neurolysin.

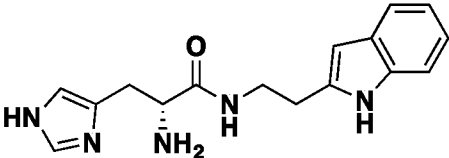
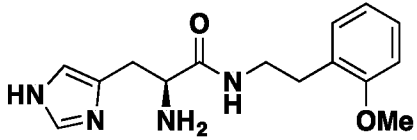
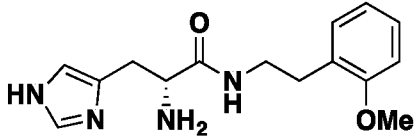
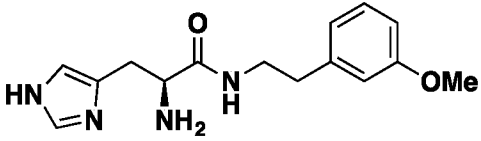
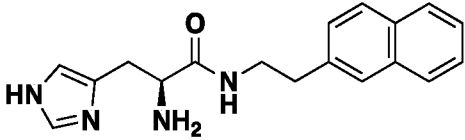
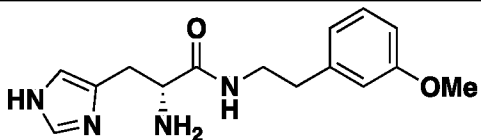
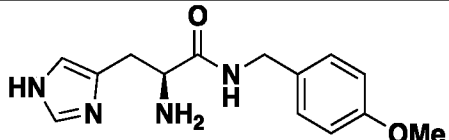
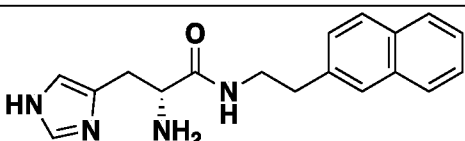
[0047] Table 2. Structure, A_{50} and A_{max} activity data for synthesized dipeptide Neurolysin (Nln) activators.

Compound	Code	A_{50} (μ M; 95% CI)	A_{max} (%; 95% CI)
	His-Tyr	37.7 (24.5 to 58.6)	467 (424.9 to 516.9)
	His-Phe	130.2 (113.5 to 150.1)	573 (547.9 to 602.7)
	NK-61-2A	Inactive	Inactive
	NK-61-4A	9.46 (5.9 to 15)	231 (216.2 to 248.9)

	His-Trp	34 (24 to 48.12)	440.3 (414.3 to 467.9)
	PT-1-172	Inactive	Inactive
	(±)PT-1-173 RACEMIC	4.3 (3.4 to 5.4)	311 (298.6 to 325.7)
	PT-1-177	Inactive	Inactive
	DF-3-2B	24.25 (7.85 to 71.4)	152.2 (137.3 to 171.5)
	DF-6-2A	24.9 (15.7 to 39.5)	252 (235.6 to 270.5)
	DF-5-2A	7.743 (0.66 to 46.8)	139.8 (124.7 to 158.4)
		Inactive	Inactive
		Inactive	Inactive
	DF-2-2B	27.89 (22.4 to 34.8)	323.1 (311.4 to 335.6)

	DF-9-2	12.27 (7.6 to 19.7)	262.3 (246.7 to 279.4)
	AB-1-4-2	52.5 (34.5 to 81.3)	544 (492.2 to 609.4)
	AB-1-16-2	226.8 (146 to 392.4)	703.5 (588.9 to 915.6)
	AB-1-14-2	61.83 (46.5 to 83)	477.1 (444 to 516)
	AB-1-6-2	16 (13 to 19.7)	494.4 (476.7 to 512.9)
	AB-1-9-2	11.99 (4.9 to 27.3)	184.4 (169.4 to 201.5)
	DF-8-2	36.31 (25.3 to 52.5)	305.2 (285.3 to 328)
	DF-10-2	40.06 (29.4 to 55.12)	334 (313.7 to 357.2)
	AB-2-7-2	60.4 (46.2 to 79.8)	407 (382.7 to 434.9)
	AB-1-3-2	Inactive	Inactive
	AB 3-2-2	42.7 (21 to 89.8)	438.4 (379.4 to 520.4)

	AB-1-8-2	157.6 (122.4 to 207.2)	643 (588.8 to 714)
	AB1-17-2	139.5 (98.15 to 206.3)	598.8 (532.8 to 692.9)
	HP2CF3	23.08 (19.6 to 27.2)	396.2 (384.5 to 408.5)
	AB-2-5-4	5.4 (1.45 to 17.2)	177.6 (158.7 to 198.1)
	His-Pro	5.6 (4.7 to 6.7)	502 (488.4 to 515.7)
	Cyclo(His-Pro)	Inactive	Inactive
	SR-5-3d	53 (28.5 to 106)	173 (158.5 to 194.1)
	SR-9-1d	4.2 (3.17 to 5.6)	264 (256.3 to 272.6)
	SR-10-1d	15.37 (10.9 to 21.5)	343 (325.2 to 363.5)
	SR-7-2d	6.1 (3.7 to 10.12)	259 (240.7 to 281)

	SR-8-2d	1.0 (0.16 to 4.4)	132 (122.5 to 143.6)
	SR11-1d	31.8 (21 to 48.7)	319 (296.1 to 345.4) 5
	SR12-1d	30 (24 to 37.5)	328 (315.4 to 342)
	SR13-1d	29.6 (22.7 to 38.8)	367 (349.7 to 387.1)
	SR18-1	13.6 (10.1 to 18.2)	323.7 10 (310.3 to 337.9)
	SR16-1d	43.2 (30.9 to 61.5)	324.9 (304 to 349)
	SR15-1d	19.8 (9.5 to 40.6)	255.3 (230.5 to 284.8)
	SR20-1d	221 (149 – 354)	428.8 (373 to 523.5)
	SR19-2d	25.2 (19.6 to 32.4)	339.8 (325.4 to 355.4) 20

[0048] Table 3. The effect of top-ranking 40 compounds on catalytic activity of rat recombinant neurolysin.

NSC #	608820	615399	639022	640354	659264	296961	302851	332636	333568	339919
Score ranking	13, 41,	34	54	32	49	48	10, 27, 56	46	23, 37	57
% activity at 10	102	108	91	79	104	106	104	103	106	100
% activity at 100	108	108	30	76	107	127	80	64	77	63

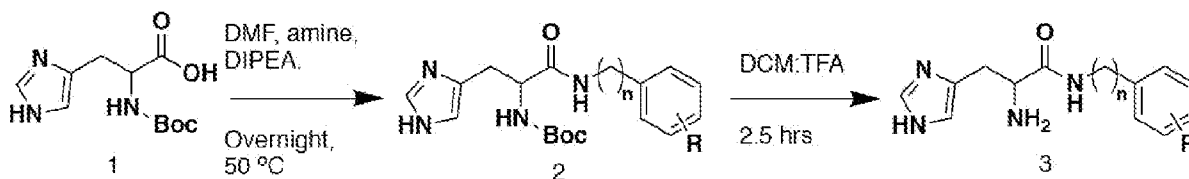
NSC #	359097	374121	400844	523374	600947	353874	211002	266752	281707	282137
Score ranking	5	35	30	7, 9, 22,	11, 45,	2	15	59, 83	14	36
% activity at 10	87	121	104	122	107	122	104	107	109	106
% activity at 100	92	226	84	206	105	80	100	97	113	94

NSC #	343659	14541	15180	47096	134514	155877	163084	203396	210826	48778
Score ranking	20	6	42	43, 99	21, 25, 40,	8	12	16	53	18, 73
% activity at 10	105	97	102	101	92	82	103	102	101	89
% activity at 100	106	103	95	111	9	4	102	106	105	84

NSC #	82001	88659	89624	92597	121184	128977	131922	134119	340049	343028
Score ranking	17	50	19, 38	51, 64	24	3	26, 39, 55,	31	33, 52	28, 29
% activity at 10	105	100	102	105	77	80	96	83	94	98
% activity at 100	100	100	38	98	18	68	28	41	52	73

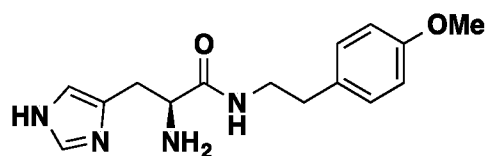
*, compared to vehicle control.

5 Synthesis Scheme:



[0049] General Synthetic Procedures. All reactions were carried out in oven dried glassware under positive nitrogen pressure. Reaction progress was monitored by thin-layer chromatography (TLC) and visualized by using UV lamp (254 nm), or by ninhydrin, potassium permanganate or phosphomolybdic acid solutions as indicator. Column chromatography was performed with silica gel using the mobile phase indicated. Commercial grade solvents and reagents were purchased from Fisher Scientific or Sigma-Aldrich and were used without further purification except as indicated. Anhydrous solvents were purchased from Across Organics and stored under an atmosphere of dry nitrogen over molecular sieves. ¹H and ¹³C NMR spectra were recorded in the indicated solvent on a Bruker 400 MHz Avance III HD spectrometer at 400 and 100 MHz for ¹H and ¹³C, respectively, with deuterated solvent peaks as internal standard. Multiplicities are indicated by s (single), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad). Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J), in hertz. High-pressure liquid chromatography was performed on a Gilson HPLC system, equipped with 254 UV detector using Trilution software v.2.1 with an ACE Equivalence 3 (C18, 3 mm, 4.6 mm x 150 mm) column. All samples were determined to possess >95% purity, except where indicated otherwise.

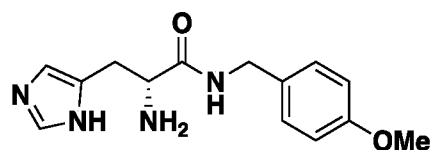
[0050] General method to synthesize di- and tripeptides: To a solution of N-Boc-L-Histidine (1 mmol) in DMF (6 mL) was added BOP (1 mmol) and the solution stirred at for 30 minutes at 50 °C under an atmosphere of nitrogen. In a separate vessel, DIPEA (1 equiv.) was added dropwise to a solution of the appropriate amine (1 equiv.) in DMF (4 mL) and the mixture stirred at room temperature for 30 minutes under an atmosphere of nitrogen. The contents of this vessel were added dropwise to the mixture of N-Boc-L-Histidine (1 equiv.) and BOP and the reaction stirred for 30 minutes at 50 °C. The reaction was extracted with EtOAc and washed with water. The organic phase was dried (Na₂SO₄), evaporated in vacuo and purified by column chromatography (1%-7% MeOH : DCM). The crude product was dissolved in 20% TFA in DCM (20 mL) and stirred for 3 hours at room temperature. The solvent was evaporated in vacuo to yield the title compound.



[0051] AB-1-4-2

[0052] ¹H (DMSO-d₆, 400 Hz) δ_H: 2.61 (2H, t, *J* = 7.2 Hz, CH₂), 3.10-3.14 (2H, m, CH₂), 3.24-3.27 (1H, m, CH), 3.33-3.38 (1H, m, CH), 2.95 (3H, s, OCH₃), 4.02-4.08 (1H, m, CH), 6.85 (2H, d, *J* = 8.4 Hz, Ar-H), 7.10 (2H, d, *J* = 8.0 Hz, Ar-H), 7.39 (1H, s, CH), 8.34-8.41 (2H, br. s, NH₂), 8.54 (1H, t, *J* = 5.4 Hz, NH), 9.01 (1H, s, CH), 14.31-14.80 (1H, br. s, NH).

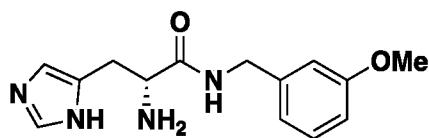
¹³C (DMSO-d₆, 100 Hz) δ_C: 27.0, 34.3, 41.1, 51.8, 55.4, 114.2, 118.2, 127.5, 129.9, 131.2, 134.9, 158.2, 167.4



[0053] AB-1-16-2

[0054] ¹H (DMSO-d₆, 400 Hz) δ_H: 3.11-3.23 (2H, m, CH₂), 3.74 (3H, s, OCH₃), 4.09 (1H, t, *J* = 7.2 Hz, CH), 4.19-4.29 (2H, m, CH₂), 6.88 (2H, d, *J* = 8.8 Hz, Ar-H), 7.09 (2H, d, *J* = 8.0 Hz, Ar-H), 7.41 (1H, s, CH), 8.39-8.45 (2H, br. s, NH₂), 8.86 (1H, t, *J* = 5.6 Hz, NH), 9.01 (1H, s, CH), 14.3-14.62 (1H, br. s, NH).

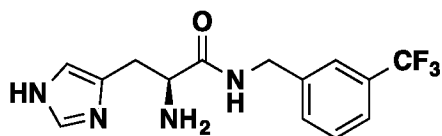
[0055] ¹³C (DMSO-d₆, 100 Hz) δ_C: 26.9, 42.3, 51.8, 55.5, 114.1, 118.3, 129.1, 130.4, 134.8, 158.8, 167.3.



[0056] AB-1-14-2

[0057] ^1H (DMSO- d_6 , 400 Hz) δ_{H} : 3.14-3.26 (2H, m, CH_2), 3.73 (3H, s, OCH_3), 4.17 (1H, t, $J = 6.2$ Hz, CH), 4.28-4.31 (2H, m, CH_2), 6.73-6.84 (3H, m, Ar-H), 7.23 (1H, t, Ar-H), 7.42 (1H, s, CH), 8.45-8.47 (2H, br. s, NH_2), 8.98 (1H, t, $J = 5.6$ Hz, NH), 9.04 (1H, s, CH), 14.55-14.66 (1H, br. s, NH).

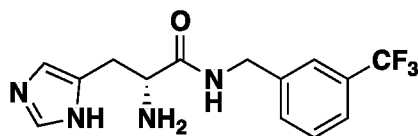
[0058] ^{13}C (DMSO- d_6 , 100 Hz) δ_{C} : 26.8, 42.8, 51.8, 55.4, 112.8, 113.6, 118.3, 119.8, 127.7, 129.9, 134.8, 140.1, 159.7, 167.5.



[0059] AB-1-6-2

[0060] ^1H (DMSO- d_6 , 400 Hz) δ_{H} : 3.15-3.28 (2H, m, CH_2), 4.20 (1H, t, $J = 6.8$ Hz, CH), 4.35-4.50 (2H, m, CH_2), 7.42 (1H, s, CH), 7.50 (1H, d, $J = 7.6$ Hz, Ar-H), 7.55-7.64 (3H, m, Ar-H), 8.45-8.48 (2H, br. s, NH_2), 9.03 (1H, s, CH), 9.10 (1H, t, $J = 5.6$ Hz, NH), 14.49-14.67 (1H, br. s, NH).

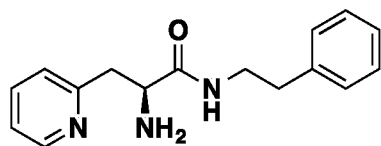
[0061] ^{13}C (DMSO- d_6 , 100 Hz) δ_{C} : 26.9, 42.5, 51.9, 118.2, 124.2, 127.5, 129.3, 129.8, 130.1, 131.9, 134.9, 140.2, 159, 167.8



[0062] AB-1-9-2

[0063] ^1H (DMSO- d_6 , 400 Hz) δ_{H} : 3.16-3.29 (2H, m, CH_2), 4.21 (1H, t, $J = 6.6$ Hz, CH), 4.35-4.49 (2H, m, CH_2), 7.42 (1H, s, CH), 7.49 (1H, d, $J = 7.6$ Hz, Ar-H), 7.54-7.63 (3H, m, Ar-H), 8.48-8.52 (2H, br. s, NH_2), 9.03 (1H, s, CH), 9.14 (1H, t, $J = 5.8$ Hz, NH), 14.69-14.81 (1H, br. s, NH).

[0064] ^{13}C (DMSO- d_6 , 100 Hz) δ_{C} : 26.8, 42.5, 51.8, 118.3, 124.4, 127.2, 129.8, 130.1, 131.8, 133.6, 134.9, 135.8, 140.2, 159.1, 167.8.

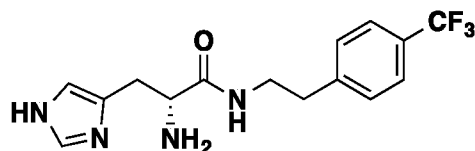


[0065] AB-1-3-2

- 5 [0066] ¹H (DMSO-d₆, 400 Hz) δ_H: 2.66 (2H, t, *J* = 7.4 Hz), 3.22-3.40 (4H, m, 2xCH₂), 4.23 (1H, t, *J* = 6.8 Hz, CH), 7.16-7.22 (4H, m, Ar-H), 7.26-7.30 (2H, m, Ar-H), 7.52 (1H, d, *J* = 8.0 Hz, Ar-H), 7.61 (1H, t, *J* = 6.0 Hz, Ar-H), 8.10 (1H, t, *J* = 7.2 Hz, NH), 8.48-8.49 (2H, br. s, NH₂), 8.68 (1H, d, *J* = 4.0 Hz, Ar-H).

[0067] ¹³C (DMSO-d₆, 100 Hz) δ_c: 35.5, 42.3, 54.9, 60.2, 122, 124.3, 126.5, 128.7, 136.6, 139.8, 149.3, 158.7, 170.7.

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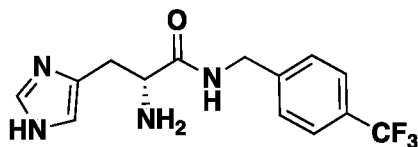


[0068] AB-1-8-2

- 15 [0069] ¹H (DMSO-d₆, 400 Hz) δ_H: 2.79 (2H, t, *J* = 7.2 Hz, CH₂), 3.03-3.16 (2H, m, CH₂), 3.31-3.47 (2H, m, CH₂), 4.04 (1H, t, *J* = 6.8 Hz, CH), 7.37 (1H, s, CH), 7.40 (2H, d, *J* = 8.0 Hz, Ar-H), 7.63 (2H, d, *J* = 8.0 Hz, Ar-H), 8.28-8.43 (2H, br. s, NH₂), 8.58 (1H, t, *J* = 5.6 Hz, NH), 8.91 (1H, s, CH), 14.22-14.60 (1H, br. s, NH).

[0070] ¹³C (DMSO-d₆, 100 Hz) δ_c: 27.1, 34.9, 51.8, 118.1, 125.5, 127.3, 127.5, 129.9, 135, 144.4, 158.9, 167.6.

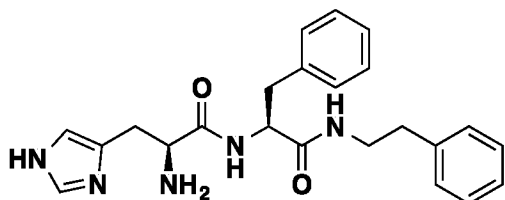
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[0071] AB-17-2

- 25 [0072] ¹H (DMSO-d₆, 400 Hz) δ_H: 3.14-3.26 (2H, m, CH₂), 4.21 (1H, t, *J* = 6.8 Hz, CH), 4.43-4.54 (2H, m, CH₂), 7.36 (1H, d, *J* = 8.0 Hz, Ar-H), 7.42 (1H, s, CH), 7.50 (1H, t, *J* = 7.6 Hz, Ar-H), 7.64 (1H, t, *J* = 7.6 Hz, Ar-H), 7.73 (1H, d, *J* = 7.6 Hz, Ar-H), 8.48-8.56 (2H, br. s, NH₂), 8.94 (1H, s, CH), 9.06 (1H, t, *J* = 5.6 Hz, NH), 14.34-14.59 (1H, br. s, NH).

[0073] ^{13}C (DMSO- d_6 , 100 Hz) δ_c : 27, 52, 118.2, 126.2, 126.6, 128.3, 129.4, 130.8, 133.1, 135, 136.6, 158.6, 167.9.



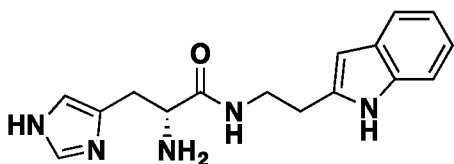
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[0074] AB-2-5-4

[0075] ^1H (DMSO- d_6 , 400 Hz) δ_H : 2.67 (2H, t, $J = 7.4$ Hz, CH_2), 2.79-2.84 (1H, m, CH), 2.93-2.98 (1H, m, CH), 3.07-3.10 (1H, m, CH), 3.20-3.27 (2H, m, CH_2), 3.27-3.34 (1H, m, CH), 4.14 (1H, s, CH), 4.50 (1H, q, $J = 8.4$ Hz, CH), 7.19-7.31 (10H, m, Ar-H), 7.42 (1H, s, CH), 8.25-8.28 (2H, br. s, NH_2), 8.46 (1H, t, $J = 5.1$ Hz, NH), 8.73 (1H, d, $J = 7.6$ Hz, NH), 9.04 (1H, s, CH), 14.32-14.41 (1H, br. s, NH).

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[0076] ^{13}C (DMSO- d_6 , 100 Hz) δ_c : 26.8, 35.3, 37.9, 46.2, 51.4, 55.2, 118.7, 126.6, 127, 128.6, 128.7, 129.1, 129.5, 134.9, 137.6, 139.6, 167.7, 171.4.



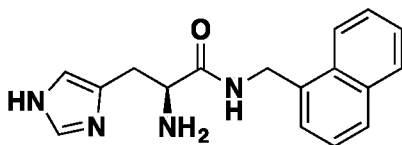
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[0077] SR 8-2d

[0078] ^1H NMR (400 MHz, MeOH- d_4) δ_H : 2.82-3.07 (m, 2H), 3.10-3.30 (m, 2H), 3.52 (dt, $J = 13.4, 6.8$ Hz, 1H), 3.58-3.74 (m, 1H), 4.00-4.21 (m, 1H), 6.89-7.05 (m, 1H), 7.05-7.16 (m, 2H), 7.16-7.29 (m, 1H), 7.30-7.42 (m, 1H), 7.48-7.70 (m, 1H), 8.69 (br. s, 1H).

[0079] ^{13}C NMR (100 MHz, MeOH- d_4) δ_c : 13.05, 19.47, 24.59, 40.05, 51.91, 60.15, 110.95, 111.31, 117.80, 118.30, 121.06, 122.19, 127.24, 134.30, 136.74, 167.03.

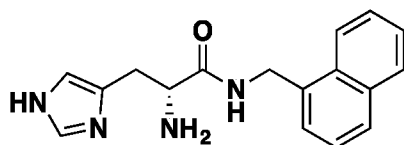
20



[0080] SR 9-1d

[0081] ^1H NMR (400 MHz, MeOH- d_4) δ_{H} : 3.29 (dd, $J = 7.0, 2.9$ Hz, 2 H), 4.21 (t, $J = 7.1$ Hz, 1H), 4.78 (d, $J = 14.67$ Hz, 1H), 7.20 (s, 1H), 7.40-7.50 (m, 2H), 7.50-7.62 (m, 2H), 7.82-7.89 (m, 1H), 7.89-7.96 (m, 1H), 7.96-8.05 (m, 1H), 8.62 (s, 1H).

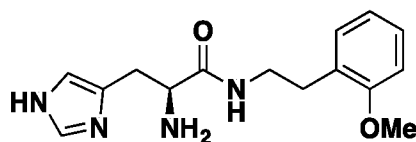
[0082] ^{13}C NMR (100 MHz, MeOH- d_4) δ_{C} : 26.47, 41.00, 51.99, 117.67, 122.92, 125.05, 125.64, 126.17,
5 126.62, 128.30, 128.52, 131.14, 132.85, 133.97, 134.21, 166.76.



[0083] SR 10-1d

[0084] ^1H NMR (400 MHz, MeOH- d_4) δ_{H} : 3.24-3.31 (m, 2H), 4.23 (t, $J = 6.8$ Hz, 1H), 4.77 (d, $J = 14.7$ Hz, 1H), 4.96 (d, $J = 14.7$ Hz, 2H), 7.19 (s, 1H), 7.38-7.48 (m, 2H), 7.48-7.57 (m, 2H), 7.78-7.85 (m, 1H), 7.95-8.02 (m, 1H), 8.61 (s, 1H).

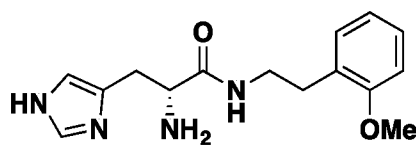
[0085] ^{13}C NMR (100 MHz, MeOH- d_4) δ_{C} : 26.32, 40.98, 51.94, 117.83, 122.92, 125.05, 125.63, 126.17, 126.59, 126.83, 128.27, 128.49, 131.13, 132.86, 133.95, 134.10, 166.76.



[0086] SR 11-1d

[0087] ^1H NMR (400 MHz, MeOH- d_4) δ_{H} : 2.57-2.79 (m, 2H), 3.03-3.20 (m, 2H), 3.23-3.31 (m, 1H), 3.35-3.47 (m, 1H), 3.72 (s, 3H), 4.03 (t, $J = 6.7$ Hz, 1H), 6.74 (t, $J = 7.4$ Hz, 1H), 6.82 (d, $J = 8.2$ Hz, 1H), 6.99 (d, $J = 7.2$ Hz, 1H), 7.09 (t, $J = 7.3$ Hz, 1H), 7.20 (s, 1H) 8.74 (s, 1H).

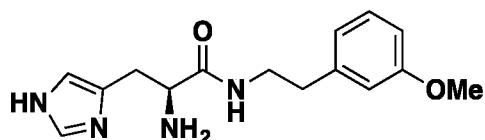
[0088] ^{13}C NMR (100 MHz, MeOH- d_4) δ_{C} : 26.38, 29.81, 39.19, 51.94, 54.38, 69.94, 110.17, 118.00, 120.11, 126.53, 126.98, 127.71, 130.00, 134.44, 157.68, 166.98.



[0089] SR 12-1d

[0090] ^1H NMR (400 MHz, MeOH-d_4) δ_{H} : 2.57-2.77 (m, 2H), 3.03-3.20 (m, 2H), 3.24-3.35 (m, 1H), 3.35-3.46 (m, 1H), 3.67-3.76 (m, 3H), 4.02 (t, $J = 6.9$ Hz, 1H), 6.68-6.78 (m, 1H), 6.83 (d, $J = 8.1$ Hz, 1H), 6.95-7.03 (m, 1H), 7.04-7.15 (m, 1H), 7.20 (s, 1H), 8.75 (s, 1H).

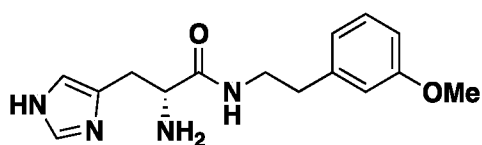
[0091] ^{13}C NMR (100 MHz, MeOH-d_4) δ_{C} : 26.47, 29.81, 39.19, 51.98, 54.37, 110.17, 117.90, 120.11,
5 126.52, 127.18, 127.71, 130.00, 134.48, 157.67, 167.01.



[0092] SR 13-1d

[0093] ^1H NMR (400 MHz, MeOH-d_4) δ_{H} : 2.58-2.72 (m, 2H), 3.01-3.18 (m, 2H), 3.27-3.48 (m, 2H), 3.67
10 (s, 4H), 4.02 (t, $J = 6.9$ Hz, 1H), 6.60-6.72 (m, 3H), 7.01-7.15 (m, 1H), 7.21 (s, 1H), 8.68-8.77 (m, 1H).

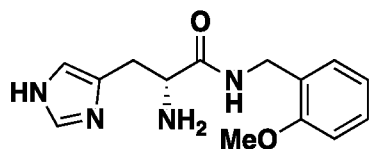
[0094] ^{13}C NMR (100 MHz, MeOH-d_4) δ_{C} : 26.48, 34.79, 40.57, 51.96, 54.21, 111.43, 114.12, 117.88,
120.63, 127.17, 129.19, 134.50, 140.17, 159.93, 167.08.



[0095] SR 15-1d

[0096] ^1H NMR (400 MHz, MeOH-d_4) δ_{H} : 2.67-2.85 (m, 2H), 3.28 (dd, $J = 6.7, 3.7$ Hz, 2H), 3.44 (dt, $J =$
13.5, 6.9 Hz, 1H), 3.55 (dd, $J = 14.2, 6.9$ Hz, 1H), 3.81 (s, 3H), 4.17 (t, $J = 6.9$ Hz, 1H), 6.74-6.89 (m, 3H),
7.17-7.32 (m, 2H), 8.66-8.75 (m, 1H).

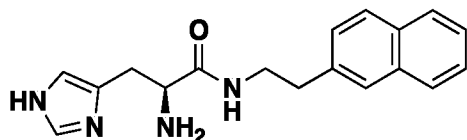
[0097] ^{13}C NMR (100 MHz, MeOH-d_4) δ_{C} : 26.42, 34.79, 40.57, 51.91, 54.19, 111.42, 114.09, 117.94,
20 120.63, 127.03, 129.19, 134.49, 140.17, 159.93, 167.07.



[0098] SR 16-1d

[0099] ^1H NMR (400 MHz, MeOH- d_4) δ_{H} : 3.02-3.31 (m, 2H), 3.78-3.89 (m, 3H), 4.24 (d, $J = 14.6$ Hz, 2H), 4.46 (d, $J = 14.4$ Hz, 1H), 6.95 (t, $J = 7.3$ Hz, 1H), 7.01 (d, $J = 8.2$ Hz, 1H), 7.10-7.22 (m, 2H), 7.34 (t, $J = 7.8$ Hz, 1H), 8.49 (br. s, 1H).

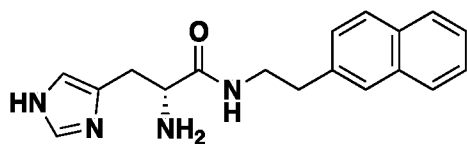
[0100] ^{13}C NMR (100 MHz, MeOH- d_4) δ_{C} : 26.39, 38.74, 52.10, 54.91, 110.59, 117.78, 120.34, 124.97, 129.29, 129.36, 134.07, 157.32, 167.16.



[0101] SR 18-1d

[0102] ^1H NMR (400 MHz, MeOH- d_4) δ_{H} : 2.88-3.08 (m, 2H), 3.09-3.31 (m, 2H), 3.47-3.75 (m, 3H), 4.15 (br. s, 1H), 7.24 (s, 1H), 7.32-7.53 (m, 3H), 7.59-7.72 (m, 1H), 7.72-7.88 (m, 3H), 8.70 (s, 1H).

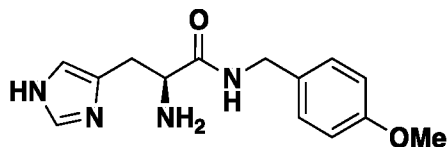
[0103] ^{13}C NMR (100 MHz, MeOH- d_4) δ_{C} : 26.42, 34.92, 40.49, 51.81, 117.84, 125.17, 125.76, 126.76, 126.78, 127.06, 127.23, 127.80, 132.35, 133.61, 134.33, 136.17.



[0104] SR 19-2d

[0105] ^1H NMR (400 MHz, MeOH- d_4) δ_{H} : 2.91-3.05 (m, 2H), 3.06-3.26 (m, 2H), 3.49-3.73 (m, 2H), 4.11 (t, $J = 6.6$ Hz, 1H), 7.19 (s, 1H), 7.34-7.42 (m, 1H), 7.42-7.48 (m, 2H), 7.69 (s, 1H), 7.74-7.86 (m, 3H), 8.55 (s, 1H).

[0106] ^{13}C NMR (100 MHz, MeOH- d_4) δ_{C} : 26.33, 34.91, 40.49, 51.77, 117.92, 125.17, 125.75, 126.69, 126.76, 126.78, 127.06, 127.23, 127.80, 132.34, 133.61, 134.27, 136.17, 167.09.



[0107] SR 20-1d

[0108] ^1H NMR (400 MHz, MeOH-d_4) δ_{H} : 3.24-3.31 (m, 2H), 3.77-3.84 (m, 3H), 4.14 (t, $J = 7.0$ Hz, 1H), 4.27 (d, $J = 14.6$ Hz, 1H), 4.38 (d, $J = 14.4$ Hz, 1H), 6.89 (m, 2H), 7.17 (m, 2H), 7.30 (s, 1H), 8.73 (s, 1H).

[0109] ^{13}C NMR (100 MHz, MeOH-d_4) δ_{C} : 26.43, 42.47, 52.08, 54.29, 113.58, 117.88, 127.21, 128.89, 129.66, 134.39, 159.26, 166.71.

5 [0110] Based on the results of the primary screen, compounds NSC 374121 (referred as NlnA#1) and NSC 523374 (referred as NlnA#2) were subjected to further pharmacological evaluation. Concentration-dependent effect of both compounds on initial velocity of hydrolysis of QFS by neurolysin is presented in FIGS. 2A to 2C. For NlnA#1 (FIG. 2A) the calculated average EC_{50} was 45.4 μM (95% confidence intervals 29.9 to 68.9 μM), and E_{max} was 234.7% (95% confidence intervals 219.0 to 250.4%). For NlnA#2 (FIG. 2B) the
10 calculated average EC_{50} was 45.8 μM (95% confidence intervals 32.0 to 65.6 μM), and E_{max} was 202.8% (95% confidence intervals 192.2 to 213.4%).

[0111] Notably, both the primary screen and the concentration-response experiments were carried out in an assay buffer containing 0.01% Triton X-100, as recommended by Feng B. and colleagues (44,45), to avoid identification of promiscuous modulators of enzymes. In addition, the concentration-response experiment
15 was also conducted in the presence of 0.1% CHAPS or 0.01 mg/ml bovine serum albumin (BSA), as alternatives to Triton X-100, to prevent identification of non-specific enzyme modulators (46). In the latter experiments, both compounds demonstrated concentration- dependent effect on activity of neurolysin similar to the condition where Triton X-100 was present in the assay buffer (FIGS. 10A and 10B). The inherent fluorescent enhancing or quenching properties of NlnA#1 and NlnA#2 were also verified to avoid false
20 conclusions. In this set of experiments it was determined that the compounds had negligible or no effect of on the fluorescence signal documented from the hydrolysis product of QFS (Mca-Pro-Leu-OH; (36)) under the same experimental conditions (FIGS. 11A and 11B). Lastly, activity of neurolysin could be enhanced by NlnA#1 and NlnA#2 purchased from a commercial vendor (AnaSpec), as well as in several independently produced and isolated batches of the rat recombinant neurolysin (data not shown). Based on these results it
25 was concluded that enhancement of neurolysin activity by NlnA#1 and NlnA#2 was not an artifact and pharmacological studies were continued.

[0112] The modulatory site on neurolysin is different from the substrate binding site – To confirm that NlnA#1 and NlnA#2 were interacting with a binding site on neurolysin that was different from the substrate binding site, a set of experiments were carried out using a competitive inhibitor of neurolysin dynorphin A(1-
30 13) (47). In the first experiment, the effect of a fixed concentration of NlnA#1 and NlnA#2 on the affinity of dynorphin A(1-13), i.e. K_i value, in inhibiting activity of neurolysin was determined (FIG. 3). As expected, both NlnA#1 and NlnA#2 enhanced activity of neurolysin, and this effect was also observed in the presence of dynorphin A(1-13) at concentrations close to its IC_{50} value and below. However, K_i values for dynorphin

A(1-13) did not differ significantly in the absence and presence of the modulators (FIG. 3): K_i value was $0.76 \mu\text{M}$ (95% CI: $0.45 - 1.25 \mu\text{M}$) in the absence of any of the modulators, it was $0.7 \mu\text{M}$ (95% CI: $0.55 - 0.9 \mu\text{M}$) in the presence of $100 \mu\text{M}$ NlnA#1, and $0.6 \mu\text{M}$ (95% CI: $0.45 - 0.75 \mu\text{M}$) in the presence of $100 \mu\text{M}$ NlnA#2. In a reverse experiment, the concentration-response effect of NlnA#1 and NlnA#2 on activity of neurolysin were studied in the absence and presence of a fixed concentration of dynorphin A(1-13) (FIGS. 4A and 4B). In these experiments, dynorphin A(1-13) inhibited activity of neurolysin and decreased the E_{max} values of NlnA#1 (FIG. 4A) and NlnA#2 (FIG. 4B). However, it did not significantly affect the EC_{50} values of the modulators (FIGS. 4A and 4B): EC_{50} value for NlnA#1 was $42.3 \mu\text{M}$ (95% CI: $24.0 - 74.8 \mu\text{M}$) and $58.6 \mu\text{M}$ (95% CI: $37.5 - 91.6 \mu\text{M}$) in the absence and presence of dynorphin A (1-13), respectively. EC_{50} value for NlnA#2 was $23.3 \mu\text{M}$ (95% CI: $6.7 - 81.2 \mu\text{M}$) and $24.9 \mu\text{M}$ (95% CI: $8.4 - 73.5 \mu\text{M}$) in the absence and presence of dynorphin A(1-13), respectively. The observations made in this set of experiments suggest that dynorphin A(1-13) and NlnA#1 or NlnA#2 interact with different binding sites on neurolysin, because they did not affect each other's affinity for the peptidase (FIGS. 3 and 4A/4B). Although, the EC_{50} value is a not a direct indicator of the affinity of a ligand, in these experiments where all variables were maintained unchanged (concentrations of neurolysin, substrate, NlnA#1, NlnA#2, etc.) the documented EC_{50} values of the modulators in the absence and presence of dynorphin A(1-13) suggest that affinity of the modulators for neurolysin remained unaffected. Considering that dynorphin A(1-13) is a competitive inhibitor of neurolysin (47), these data also suggest that the binding site of the modulators is different from the substrate binding site.

[0113] Effect of the modulators on catalytic efficiency of neurolysin – To determine whether the increased initial velocity of QFS hydrolysis by neurolysin in the presence of NlnA#1 or NlnA#2 translates into increased catalytic efficiency of the peptidase, in this set of experiments the effect of both modulators on hydrolysis of different concentrations of the substrate (spanning from ~6-fold less to ~5-fold more of its K_m value) was studied. In the presence of NlnA#1 at a concentration close to its EC_{50} value ($40 \mu\text{M}$) maximal velocity (V_{max}) of the hydrolysis was increased by ~29%, whereas K_m value was decreased by ~43% resulting in more than doubling of V_{max}/K_m ratio (FIGS. 5A and 5B and Table 3). Likewise, in the presence of NlnA#2 ($40 \mu\text{M}$) V_{max} value was increased by ~16.5%, whereas K_m value was decreased by ~42.4%, again resulting in doubling of the V_{max}/K_m ratio (FIGS. 5A and 5B and Table 3). The same trend was observed in the presence of $100 \mu\text{M}$ concentration of either modulators resulting in more than tripling of the V_{max}/K_m ratio (FIGS. 5A and 5B and Table 3). These data suggest that both NlnA#1 and NlnA#2 increased catalytic efficiency of neurolysin. These experiments also allowed us to define the kinetic mechanism of neurolysin activation by NlnA#1 and NlnA#2. Since catalysis of the reaction by neurolysin occurs both in the absence and presence of the modulators, NlnA#1 and NlnA#2 can be classified as non-essential activators (48). Considering that the modulators affect both K_m and V_{max} values of the substrate hydrolysis by

neurolysin, they can be classified as allosteric effectors of the “K system” and “V system”, which may indicate that both substrate-binding affinity (“K-systems”) and the rate of substrate conversion into product (“V-systems”) are altered (49).

[0114] Table 4. Calculated Vmax and Km values for studies presented in FIGS. 5A and 5B.

	Vmax	Km	Vmax/Km	Fold change
rNln	96.6 (91.3 - 101.8)	13.9 (12.0 - 15.8)	6.9	---
rNln + 40μM NlnA#1	124.2*** (112.2 - 136.2)	7.9*** (5.7 - 10.1)	15.7	2.3
rNln + 100μM NlnA#1	164.8*** (147.2 - 182.4)	6.4*** (4.3 - 8.5)	25.8	3.7
rNln + 40μM NlnA#2	112.5* (102.7 - 122.3)	8.0*** (6.0 - 10.0)	14.0	2.0
rNln + 100μM NlnA#2	148.2*** (133.0 - 163.3)	6.1*** (4.1 - 8.0)	24.4	3.5

Vmax unites are in FLU/ng protein per min, Km units are in μM (FLU – fluorescent unit). Data are presented as average values together with 95% confidence intervals in parenthesis (n = 16 for recombinant neurolysin (rNln), n = 4 for all other groups; *p<0.05; ***p<0.001 in comparison to rNln values).

[0115] Species selectivity of the identified modulators – To determine whether the observed effects of NlnA#1 and NlnA#2 are only limited to the rat neurolysin or not, concentration response studies were also carried out with human recombinant and mouse brain- purified neurolysin. Concentration-dependent effects of both compounds on initial velocity of hydrolysis of QFS by human and mouse neurolysin were similar to that of the rat peptidase, and are presented in FIGS. 6A and 6B. With human recombinant neurolysin the calculated average EC50 for NlnA#1 (FIG. 6A) was 23.6 μM (95% confidence intervals 16.5 to 33.7 μM), and Emax was 203.5% (95% confidence intervals 193.7 to 213.4%). For NlnA#2 (FIG. 6B) the calculated average EC50 was 69.5 μM (95% confidence intervals 56.0 to 86.2 μM), and Emax was 214.3% (95% confidence intervals 205.7 to 222.9%). With mouse brain neurolysin the calculated average EC50 for NlnA#1 was 9.2 μM (95% confidence intervals 3.1 to 26.9 μM), and Emax was 190% (95% confidence intervals 169.2 to 210.7%). For NlnA#2 the calculated average EC50 was 17.1 μM (95% confidence intervals 6.0 to 49.1 μM), and Emax was 258% (95% confidence intervals 232.7 to 283.8%). These observations indicate that both modulators enhance activity of human recombinant and mouse brain neurolysins similar to that of the rat recombinant peptidase, with NlnA#1 being somewhat more potent than NlnA#2. Importantly, the ability of the modulators to enhance activity of native, i.e., mouse brain-purified,

neurolysin also indicates that our observations are not limited to the recombinantly produced peptidase and that the allosteric site is the same/similar in these species.

[0116] Peptidase selectivity of the identified modulators – In this set of experiments concentration-dependent effects of NlnA#1 and NlnA#2 on activity of peptidases related to neurolysin were studied (FIGS. 7A to 7D). Overall, both modulators demonstrated negligible effect on activity of thimet oligopeptidase (TOP, FIG. 7A), angiotensin converting enzyme (ACE, FIG. 7B), ACE2 and neprilysin (NEP, FIG. 7D) at concentrations up to 300 μ M. At 300 μ M concentration NlnA#1 inhibited activity of TOP by $25.9 \pm 0.7\%$, ACE by $22.6 \pm 2.5\%$, ACE2 (FIG. 7C) by $13.4 \pm 2.9\%$, and NEP by $2.9 \pm 4.7\%$ ($n = 3$, mean $\pm$ SD are presented). Under the same experimental conditions, NlnA#2 (at 300 μ M) inhibited activity of TOP by $15.6 \pm 2.6\%$, ACE by $12.2 \pm 1.8\%$, ACE2 by $10.9 \pm 2.9\%$, and NEP by $1.6 \pm 2.0\%$ ($n = 3$, mean $\pm$ SD are presented). Although, the list of tested peptidases was not exhaustive and it did not include many other pharmacological targets, the negligible effect of NlnA#1 and NlnA#2 on activity of four closely related peptidases, including TOP – the closest homolog to neurolysin (50), suggests that the modulators possessed excellent selectivity towards neurolysin and did not promiscuously enhance activity of peptidases.

[0117] Effect of modulators on hydrolysis of endogenous substrates by neurolysin – The use of synthetic substrates (usually with fluorescent properties) in enzymatic assays to identify and characterize modulators is very convenient as they allow easy tracking of the reaction progress (17). However, observations made with such substrates cannot be translated a priori to the endogenous substrates, as there are examples in the scientific literature describing compounds which could modulate the target enzyme only when a synthetic substrate was used (51,52). To avoid such artifacts, in this set of studies the effect of NlnA#1 and NlnA#2 on hydrolysis of three endogenous substrates of neurolysin (angiotensin I, bradykinin and neurotensin (26,47)) were studied, and mass-spectrometry was used to document the reaction (FIG. 8 and Supplementary FIG. 3). In the presence of both modulators formation of angiotensin-(1-7) from angiotensin I was increased by ~ 6 -fold, formation of bradykinin-(1-5) from bradykinin by ~ 2 -fold, and formation of neurotensin-(1-10) from neurotensin by ~ 3 -fold. These results provide additional evidence that catalytic activity of neurolysin can be enhanced by NlnA#1 and NlnA#2, and importantly, that this phenomenon is not limited to QFS but is also observed with endogenous substrates of the peptidase.

[0118] Structural analysis – Comparison of the structures of the identified modulators suggest chemical groups functionally relevant to the activity modulating mechanism. As shown in FIGS. 9A and 9B, top panels, NlnA#1 (L-histidyl-L-tyrosine) and NlnA#2 (D,L-histidyl-D,L-histidine) are largely identical, with common core structures linked to an imidazole ring. These common elements have the potential to either interact with the hinge region of neurolysin (and other sites) or be solvent accessible. Molecular docking of the modulators shows possible binding interactions with the hinge region of neurolysin (FIGS. 9A and 9B,

bottom panels). The top scoring orientations of these compounds, as predicted by molecular docking, show common binding modes in which the common carboxylate groups have the potential to form H bonds with neurolysin. The predicted binding modes suggest that the imidazole group may be solvent accessible and available to participate in interactions with neighboring hinge residues.

5 [0119] The present inventors identified and characterized two structurally related compounds that enhance the catalytic efficiency of metallopeptidase neurolysin. To accomplish this a rational, structure-based drug-discovery approach was used involving structural analysis of the hinge region of neurolysin to identify a potential allosteric binding site with druggable properties, followed by docking and in silico screening of ~140,000 drug-like compounds from NCI DTP. The compounds were ranked based on their combined
10 energy scores for hydrogen bonding and van der Waals contact interactions with the hypothesized allosteric binding site, and the highest ranking 40 compounds were subjected to thorough in vitro pharmacological evaluation. In the primary screen the effect of these compounds on activity of rat recombinant neurolysin was evaluated at 10 and 100 μM concentrations, resulting in identification of two modulators, which can increase the rate of hydrolysis of a synthetic substrate by neurolysin. Potencies of the identified modulators, i.e., EC_{50}
15 values determined from concentration-response studies, were $\sim 45 \mu\text{M}$ with E_{max} values $\geq 200\%$ for hydrolysis of the synthetic substrate by rat recombinant neurolysin. The observed effects were reproducible in an assay buffer supplemented with 0.01% Triton X-100, or 0.1% CHAPS or 0.01 mg/ml BSA, all of which are methodological tactics recommended by different investigators as a way to prevent identification of promiscuous modulators of enzymes in high or low-throughput screening studies. Notably, the observation
20 was reproduced in different isolates of the rat recombinant neurolysin and with the identified modulators purchased from a commercial vendor instead of the stock obtained from NCI DTP. Both modulators were also capable of enhancing activity of human recombinant and mouse brain purified neurolysin, indicating that the effect of modulators was not limited to one species or to a recombinantly produced peptidase. To verify whether the identified modulators interacted with a binding site different from the substrate binding site or
25 not, concentration-response experiments were carried out involving the modulators and dynorphin A(1-13), a competitive inhibitor of neurolysin. These studies show that neither the modulators nor dynorphin A(1-13) affected each other's affinity in modulating activity of neurolysin, providing evidence that the modulators bind to a site which is different from the substrate binding site. The effect of identified modulators on catalytic efficiency of neurolysin was determined using ranging concentrations of the synthetic substrate.
30 Both modulators reduced K_m and increased V_{max} values, thus increasing V_{max}/K_m ratio, in a concentration-dependent manner, indicating that catalytic efficiency of neurolysin is enhanced by the modulators. On the contrary, the modulators had negligible or no effect on catalytic activity of peptidases closely related to neurolysin, including thimet oligopeptidase, neprilysin, angiotensin converting enzyme (ACE) and ACE2, indicating that the effect of the identified molecules is specific to neurolysin. Lastly, to

determine whether the effect of the identified modulators on activity of neurolysin was observed only with the synthetic substrate or not, hydrolysis of three endogenous substrates of the peptidase was studied. These studies demonstrate that both modulators enhanced hydrolysis of neurotensin, bradykinin and angiotensin I by neurolysin, showing that the effect of the identified modulators was specific to the peptidase and not linked to the synthetic substrate only.

[0120] This is the first report describing the discovery of specific, small molecule enhancers of neurolysin activity. These molecules can be used to target neurolysin in different (patho)physiological states. The structures are the lead molecules for development of an entirely new class of drugs, i.e., neurolysin potentiators/activators. The latter can be used for the (patho)physiological effects of endogenous substrates of neurolysin, among which the most characterized ones are neurotensin, bradykinin, angiotensin I, substance P, hemopressin, dynorphin A(1-8), metorphamide, and somatostatin (47,53-55). Neurolysin hydrolyzes and inactivates all of these peptides except angiotensin I, dynorphin A(1-8), and metorphamide. Angiotensin I, the main inactive precursor peptide of the renin-angiotensin system, is converted into a bioactive peptide angiotensin-(1-7) by neurolysin. Dynorphin A(1-8), a kappa-opioid receptor agonist, and metorphamide, a kappa- and mu-opioid receptor agonist, are converted into delta-opioid receptor agonists Leu- and Met-enkephalins, respectively, by neurolysin. All of these bioactive peptides are critically involved in various brain functions (56-61). Their role in pathogenic mechanisms responsible for ischemic brain injury and several other neurological disorders has also been documented in multiple studies (62-67). If in a given pathophysiological condition several or most of the peptides inactivated by neurolysin have deleterious actions, then its activation could be beneficial to reduce levels of these peptides and halt or reverse progression of the disease. Likewise, if the bioactive peptides generated in the result of neurolysin's activity are protective, increased activity of the peptidase could again be beneficial. From a pharmaceutical standpoint, using one small molecule potentiator of neurolysin would be much more manageable and desirable for modulation of the function of these peptide systems, rather than using multiple small molecule receptor antagonists or agonists to target each peptide system separately.

[0121] It is important to note that the modulators identified in this study are histidine-containing dipeptides, L-histidyl-L-tyrosine (NlnA#1) and D,L-histidyl-D,L-histidine (NlnA#2). Interestingly, the first specific inhibitors of neurolysin were proline-containing dipeptides (68), of which L-prolyl-L-isoleucine is the most potent inhibitor in use as a research tool. Based on the data presented in this study, it is reasonable to predict (but not a limitation of the present invention) that N-terminal histidine, or part of its molecule, is the main pharmacophore in both modulators. However, the role of the C-terminal amino acid in binding of the molecule to the allosteric site of neurolysin and modulation of its activity cannot be excluded. It is important to note that NlnA#2 used in this study was a mixture of enantiomers (D- and L-histidine).

[0122] The most important findings of this study are that: (a) activity of neurolysin can be enhanced by small molecules; (b) allosteric binding site appears to be the same/very similar in neurolysins of rodent and human origin; and (c) identified enhancers are specific to neurolysin and largely inert to other homologous peptidases. Based on these key findings rational medicinal-chemical approaches could be applied to design molecules that possess high potency and other pharmacological properties (e.g., systemic and brain bioavailability) desirable for new research tools and drug leads.

[0123] In summary, this invention is the first to demonstrate that activity of neurolysin can be enhanced and describes identification of two small molecules that possess such properties. The molecules identified in this study could be developed into research tools for evaluation of the functional significance of neurolysin in pathogenesis of stroke and other neurological disorders, and may serve as starting structures for development of new class of drugs. Notably, the approach utilized in this study to identify allosteric modulators of neurolysin is largely unrecognized, despite being more efficient and economical than random screening approaches in yielding hit rates (27,46).

[0124] It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, kit, reagent, or composition of the invention, and vice versa. Furthermore, compositions of the invention can be used to achieve methods of the invention.

[0125] It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.

[0126] All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

[0127] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.” The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0128] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. In embodiments of any of the compositions and methods provided herein, “comprising” may be replaced with “consisting essentially of” or “consisting of”. As used herein, the phrase “consisting essentially of” requires the specified integer(s) or steps as well as those that do not materially affect the character or function of the claimed invention. As used herein, the term “consisting” is used to indicate the presence of the recited integer (e.g., a feature, an element, a characteristic, a property, a method/process step or a limitation) or group of integers (e.g., feature(s), element(s), characteristic(s), property(ies), method/process steps or limitation(s)) only.

[0129] The term “or combinations thereof” as used herein refers to all permutations and combinations of the listed items preceding the term. For example, “A, B, C, or combinations thereof” is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0130] As used herein, words of approximation such as, without limitation, “about”, “substantial” or “substantially” refers to a condition that when so modified is understood to not necessarily be absolute or perfect but would be considered close enough to those of ordinary skill in the art to warrant designating the condition as being present. The extent to which the description may vary will depend on how great a change can be instituted and still have one of ordinary skilled in the art recognize the modified feature as still having the required characteristics and capabilities of the unmodified feature. In general, but subject to the preceding discussion, a numerical value herein that is modified by a word of approximation such as “about” may vary from the stated value by at least ± 1 , 2, 3, 4, 5, 6, 7, 10, 12 or 15%.

[0131] All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

- [0132] Burbach, J. P. (2010) Neuropeptides from concept to online database www.neuropeptides.nl. European journal of pharmacology 626, 27-48
- [0133] Hokfelt, T., Bartfai, T., and Bloom, F. (2003) Neuropeptides: opportunities for drug discovery. The Lancet. Neurology 2, 463-472
- [0134] Burbach, J. P. (2011) What are neuropeptides? Methods in molecular biology 789, 1-36
- [0135] Lopez-Otin, C., and Bond, J. S. (2008) Proteases: multifunctional enzymes in life and disease. The Journal of biological chemistry 283, 30433-30437
- [0136] Shrimpton, C. N., Smith, A. I., and Lew, R. A. (2002) Soluble metalloendopeptidases and neuroendocrine signaling. Endocrine reviews 23, 647-664
- [0137] Karamyan, V. T., and Speth, R. C. (2007) Enzymatic pathways of the brain renin-angiotensin system: unsolved problems and continuing challenges. Regul Pept 143, 15-27
- [0138] Davis, T. P., and Konings, P. N. (1993) Peptidases in the CNS: formation of biologically active, receptor-specific peptide fragments. Crit Rev Neurobiol 7, 163-174
- [0139] Kim, M., Hersh, L. B., Leissring, M. A., Ingelsson, M., Matsui, T., Farris, W., Lu, A., Hyman, B. T., Selkoe, D. J., Bertram, L., and Tanzi, R. E. (2007) Decreased catalytic activity of the insulin-degrading enzyme in chromosome 10-linked Alzheimer disease families. The Journal of biological chemistry 282, 7825-7832
- [0140] Huang, S. M., Mouri, A., Kokubo, H., Nakajima, R., Suemoto, T., Higuchi, M., Staufenbiel, M., Noda, Y., Yamaguchi, H., Nabeshima, T., Saido, T. C., and Iwata, N. (2006) Neprilysin-sensitive synapse-associated amyloid-beta peptide oligomers impair neuronal plasticity and cognitive function. The Journal of biological chemistry 281, 17941-17951
- [0141] Savolainen, M. H., Yan, X., Myohanen, T. T., and Huttunen, H. J. (2015) Prolyl oligopeptidase enhances alpha-synuclein dimerization via direct protein-protein interaction. The Journal of biological chemistry 290, 5117-5126
- [0142] Basurto-Islas, G., Grundke-Iqbal, I., Tung, Y. C., Liu, F., and Iqbal, K. (2013) Activation of asparaginyl endopeptidase leads to Tau hyperphosphorylation in Alzheimer disease. The Journal of biological chemistry 288, 17495-17507

- [0143] Rohnert, P., Schmidt, W., Emmerlich, P., Goihl, A., Wrenger, S., Bank, U., Nordhoff, K., Tager, M., Ansorge, S., Reinhold, D., and Striggow, F. (2012) Dipeptidyl peptidase IV, aminopeptidase N and DPPIV/APN-like proteases in cerebral ischemia. *J Neuroinflammation* 9, 44
- [0144] Simoes, P. S., Visniauskas, B., Perosa, S. R., Yacubian, E. M., Centeno, R., Canzian, M., Lopes-Cendes, I., Maurer Morelli, C. V., Carrete, H., Jr., Cavaleiro, E. A., Tufik, S., Chagas, J. R., and Mazzacoratti Mda, G. (2014) Expression and activity of thimet oligopeptidase (TOP) are modified in the hippocampus of subjects with temporal lobe epilepsy (TLE). *Epilepsia* 55, 754- 762
- [0145] Drag, M., and Salvesen, G. S. (2010) Emerging principles in protease-based drug discovery. *Nat Rev Drug Discov* 9, 690-701
- 10 [0146] Deu, E., Verdoes, M., and Bogoy, M. (2012) New approaches for dissecting protease functions to improve probe development and drug discovery. *Nat Struct Mol Biol* 19, 9-16
- [0147] Shen, A. (2010) Allosteric regulation of protease activity by small molecules. *Mol Biosyst* 6, 1431-1443
- [0148] Novinec, M., Korenc, M., Caflisch, A., Ranganathan, R., Lenarcic, B., and Baici, A. (2014) A novel
15 allosteric mechanism in the cysteine peptidase cathepsin K discovered by computational methods. *Nat Commun* 5, 3287
- [0149] Hines, C. S., Ray, K., Schmidt, J. J., Xiong, F., Feenstra, R. W., Pras-Raves, M., de Moes, J. P., Lange, J. H., Melikishvili, M., Fried, M. G., Mortenson, P., Charlton, M., Patel, Y., Courtney, S. M., Kruse, C. G., and Rodgers, D. W. (2014) Allosteric inhibition of the neuropeptidase neurolysin. *The Journal of biological chemistry* 289, 35605-35619
- 20 [0150] Spencer, B., Verma, I., Desplats, P., Morvinski, D., Rockenstein, E., Adame, A., and Masliah, E. (2014) A neuroprotective brain-penetrating endopeptidase fusion protein ameliorates Alzheimer disease pathology and restores neurogenesis. *The Journal of biological chemistry* 289, 17917- 17931
- [0151] Eckman, E. A., Reed, D. K., and Eckman, C. B. (2001) Degradation of the Alzheimer's amyloid beta peptide by endothelin-converting enzyme. *The Journal of biological chemistry* 276, 24540- 24548
- 25 [0152] Qiu, W. Q., Walsh, D. M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M. B., Rosner, M. R., Safavi, A., Hersh, L. B., and Selkoe, D. J. (1998) Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. *The Journal of biological chemistry* 273, 32730-32738
- [0153] Leissring, M. A. (2008) The AbetaCs of Abeta-cleaving proteases. *The Journal of biological chemistry* 283, 29645-29649
- 30

- [0154] Chen, J., Zhao, Y., Chen, S., Wang, J., Xiao, X., Ma, X., Penchikala, M., Xia, H., Lazartigues, E., Zhao, B., and Chen, Y. (2014) Neuronal over-expression of ACE2 protects brain from ischemia- induced damage. *Neuropharmacology* 79, 550-558
- [0155] Bennion, D. M., Haltigan, E. A., Irwin, A. J., Donnangelo, L. L., Regenhardt, R. W., Pioquinto, D. J.,
5 Purich, D. L., and Sumners, C. (2015) Activation of the Neuroprotective Angiotensin- Converting Enzyme 2 in Rat Ischemic Stroke. *Hypertension* 66, 141-148
- [0156] Rashid, M., Wangler, N. J., Yang, L., Shah, K., Arumugam, T. V., Abbruscato, T. J., and Karamyan, V. T. (2014) Functional upregulation of endopeptidase neurolysin during post-acute and early recovery phases of experimental stroke in mouse brain. *Journal of neurochemistry* 129, 179-189
- [0157] Dauch, P., Vincent, J. P., and Checler, F. (1995) Molecular cloning and expression of rat brain endopeptidase 3.4.24.16. *J Biol Chem* 270, 27266-27271
- [0158] Hernandez Prada, J. A., Ferreira, A. J., Katovich, M. J., Shenoy, V., Qi, Y., Santos, R. A., Castellano, R. K., Lampkins, A. J., Gubala, V., Ostrov, D. A., and Raizada, M. K. (2008) Structure-based
15 identification of small-molecule angiotensin-converting enzyme 2 activators as novel antihypertensive agents. *Hypertension* 51, 1312-1317
- [0159] Kulemina, L. V., and Ostrov, D. A. (2011) Prediction of off-target effects on angiotensin- converting enzyme 2. *J Biomol Screen* 16, 878-885
- [0160] Kabsch, W., and Sander, C. (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 22, 2577-2637
- [0161] Dundas, J., Ouyang, Z., Tseng, J., Binkowski, A., Turpaz, Y., and Liang, J. (2006) CASTp: computed atlas of surface topography of proteins with structural and topographical mapping of functionally annotated residues. *Nucleic acids research* 34, W116-118
- [0162] Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development
25 settings. *Adv Drug Deliv Rev* 46, 3-26
- [0163] Lian, W., Chen, G., Wu, D., Brown, C. K., Madauss, K., Hersh, L. B., and Rodgers, D. W. (2000) Crystallization and preliminary analysis of neurolysin. *Acta Crystallogr D Biol Crystallogr* 56, 1644-1646
- [0164] Brown, C. K., Madauss, K., Lian, W., Beck, M. R., Tolbert, W. D., and Rodgers, D. W. (2001) Structure of neurolysin reveals a deep channel that limits substrate access. *Proc Natl Acad Sci U S A* 98,
30 3127-3132

- [0165] Wangler, N. J., Santos, K. L., Schadock, I., Hagen, F. K., Escher, E., Bader, M., Speth, R. C., and Karamyan, V. T. (2012) Identification of Membrane-bound Variant of Metalloendopeptidase Neurolysin (EC 3.4.24.16) as the Non-angiotensin Type 1 (Non-AT1), Non-AT2 Angiotensin Binding Site. *J Biol Chem* **287**, 114-122
- 5 [0166] Rashid, M., Arumugam, T. V., and Karamyan, V. T. (2010) Association of the novel non-AT1, non-AT2 angiotensin binding site with neuronal cell death. *J Pharmacol Exp Ther* **335**, 754-761
- [0167] Dauch, P., Barelli, H., Vincent, J. P., and Checler, F. (1991) Fluorimetric assay of the neurotensin-degrading metalloendopeptidase, endopeptidase 24.16. *The Biochemical journal* **280** (Pt 2), 421-426
- 10 [0168] Shrimpton, C. N., Glucksman, M. J., Lew, R. A., Tullai, J. W., Margulies, E. H., Roberts, J. L., and Smith, A. I. (1997) Thiol activation of endopeptidase EC 3.4.24.15. A novel mechanism for the regulation of catalytic activity. *J Biol Chem* **272**, 17395-17399
- [0169] Miners, J. S., Verbeek, M. M., Rikkert, M. O., Kehoe, P. G., and Love, S. (2008) Immunocapture-based fluorometric assay for the measurement of neprilysin-specific enzyme activity in
15 brain tissue homogenates and cerebrospinal fluid. *Journal of neuroscience methods* **167**, 229-236
- [0170] Joyner, J. C., Hocharoen, L., and Cowan, J. A. (2012) Targeted catalytic inactivation of angiotensin converting enzyme by lisinopril-coupled transition-metal chelates. *J Am Chem Soc* **134**, 3396-3410
- [0171] Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., Tang, J., Godbout, K., Parsons, T.,
20 Baronas, E., Hsieh, F., Acton, S., Patane, M., Nichols, A., and Tummino, P. (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* **277**, 14838-14843
- [0172] Towler, P., Staker, B., Prasad, S. G., Menon, S., Tang, J., Parsons, T., Ryan, D., Fisher, M., Williams, D., Dales, N. A., Patane, M. A., and Pantoliano, M. W. (2004) ACE2 X-ray structures reveal a
25 large hinge-bending motion important for inhibitor binding and catalysis. *The Journal of biological chemistry* **279**, 17996-18007
- [0173] Ray, K., Hines, C. S., Coll-Rodriguez, J., and Rodgers, D. W. (2004) Crystal structure of human thimet oligopeptidase provides insight into substrate recognition, regulation, and localization. *J Biol Chem* **279**, 20480-20489
- 30 [0174] Oliveira, V., Gatti, R., Rioli, V., Ferro, E. S., Spisni, A., Camargo, A. C., Juliano, M. A., and Juliano, L. (2002) Temperature and salts effects on the peptidase activities of the recombinant

metallooligopeptidases neurolysin and thimet oligopeptidase. *European journal of biochemistry / FEBS* **269**, 4326-4334

[0175] Feng, B. Y., Shelat, A., Doman, T. N., Guy, R. K., and Shoichet, B. K. (2005) High-throughput assays for promiscuous inhibitors. *Nat Chem Biol* **1**, 146-148

5 [0176] Feng, B. Y., Simeonov, A., Jadhav, A., Babaoglu, K., Inglese, J., Shoichet, B. K., and Austin, C. P. (2007) A high-throughput screen for aggregation-based inhibition in a large compound library. *J Med Chem* **50**, 2385-2390

[0177] Goode, D. R., Totten, R. K., Heeres, J. T., and Hergenrother, P. J. (2008) Identification of promiscuous small molecule activators in high-throughput enzyme activation screens. *J Med Chem* **51**,
10 2346-2349

[0178] Rioli, V., Gozzo, F. C., Heimann, A. S., Linardi, A., Krieger, J. E., Shida, C. S., Almeida, P. C., Hyslop, S., Eberlin, M. N., and Ferro, E. S. (2003) Novel natural peptide substrates for endopeptidase 24.15, neurolysin, and angiotensin-converting enzyme. *J Biol Chem* **278**, 8547- 8555

[0179] Fontes, R., Ribeiro, J. M., and Sillero, A. (2000) Inhibition and activation of enzymes. The effect of
15 a modifier on the reaction rate and on kinetic parameters. *Acta Biochim Pol* **47**, 233-257

[0180] Monod, J., Wyman, J., and Changeux, J. P. (1965) On the Nature of Allosteric Transitions: A Plausible Model. *Journal of molecular biology* **12**, 88-118

[0181] Lim, E. J., Sampath, S., Coll-Rodriguez, J., Schmidt, J., Ray, K., and Rodgers, D. W. (2007) Swapping the substrate specificities of the neuropeptidases neurolysin and thimet oligopeptidase. *The*
20 *Journal of biological chemistry* **282**, 9722-9732

[0182] Pacholec, M., Bleasdale, J. E., Chrunk, B., Cunningham, D., Flynn, D., Garofalo, R. S., Griffith, D., Griffor, M., Loulakis, P., Pabst, B., Qiu, X., Stockman, B., Thanabal, V., Varghese, A., Ward, J., Withka, J., and Ahn, K. (2010) SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. *The Journal of biological chemistry* **285**, 8340-8351

25 [0183] Song, E. S., Juliano, M. A., Juliano, L., Fried, M. G., Wagner, S. L., and Hersh, L. B. (2004) ATP effects on insulin-degrading enzyme are mediated primarily through its triphosphate moiety. *The Journal of biological chemistry* **279**, 54216-54220

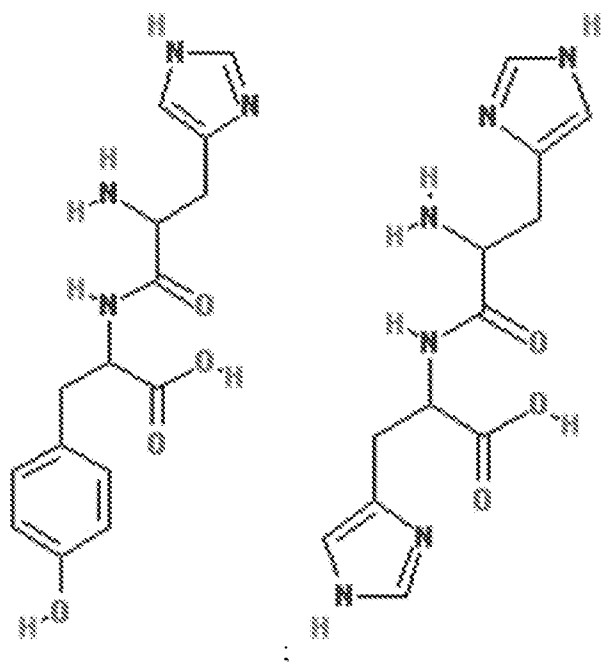
[0184] Dahms, P., and Mentlein, R. (1992) Purification of the main somatostatin-degrading proteases from rat and pig brains, their action on other neuropeptides, and their identification as endopeptidases
30 24.15 and 24.16. *Eur J Biochem* **208**, 145-154

- [0185] Vincent, B., Jiracek, J., Noble, F., Loog, M., Roques, B., Dive, V., Vincent, J. P., and Checler, F. (1997) Contribution of endopeptidase 3.4.24.15 to central neurotensin inactivation. *Eur J Pharmacol* **334**, 49-53
- [0186] Rioli, V., Kato, A., Portaro, F. C., Cury, G. K., te Kaat, K., Vincent, B., Checler, F., Camargo, A. C., Glucksman, M. J., Roberts, J. L., Hirose, S., and Ferro, E. S. (1998) Neuropeptide specificity and inhibition of recombinant isoforms of the endopeptidase 3.4.24.16 family: comparison with the related recombinant endopeptidase 3.4.24.15. *Biochem Biophys Res Commun* **250**, 5-11
- [0187] Walker, K., Perkins, M., and Dray, A. (1995) Kinins and kinin receptors in the nervous system. *Neurochemistry international* **26**, 1-16
- 10 [0188] Tyler-McMahon, B. M., Boules, M., and Richelson, E. (2000) Neurotensin: peptide for the next millennium. *Regulatory peptides* **93**, 125-136
- [0189] Olias, G., Viollet, C., Kusserow, H., Epelbaum, J., and Meyerhof, W. (2004) Regulation and function of somatostatin receptors. *Journal of neurochemistry* **89**, 1057-1091
- [0190] Heimann, A. S., Gomes, I., Dale, C. S., Pagano, R. L., Gupta, A., de Souza, L. L., Luchessi, A. D., Castro, L. M., Giorgi, R., Rioli, V., Ferro, E. S., and Devi, L. A. (2007) Hemopressin is an inverse agonist of CB1 cannabinoid receptors. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 20588-20593
- 15 [0191] Sargeant, T. J., Miller, J. H., and Day, D. J. (2008) Opioidergic regulation of astroglial/neuronal proliferation: where are we now? *Journal of neurochemistry* **107**, 883-897
- 20 [0192] Xia, H., and Lazartigues, E. (2008) Angiotensin-converting enzyme 2 in the brain: properties and future directions. *Journal of neurochemistry* **107**, 1482-1494
- [0193] Albert-Weissenberger, C., Siren, A. L., and Kleinschnitz, C. (2013) Ischemic stroke and traumatic brain injury: the role of the kallikrein-kinin system. *Progress in neurobiology* **101-102**, 65-82
- [0194] Turner, R., and Vink, R. (2007) Inhibition of neurogenic inflammation as a novel treatment for ischemic stroke. *Drug News Perspect* **20**, 221-226
- 25 [0195] Caceda, R., Kinkead, B., and Nemeroff, C. B. (2006) Neurotensin: role in psychiatric and neurological diseases. *Peptides* **27**, 2385-2404
- [0196] Sumners, C., Horiuchi, M., Widdop, R. E., McCarthy, C., Unger, T., and Steckelings, U. M. (2013) Protective arms of the renin-angiotensin-system in neurological disease. *Clin Exp Pharmacol Physiol* **40**, 580-588
- 30

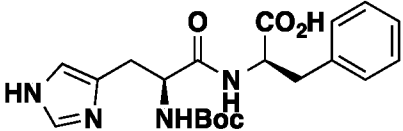
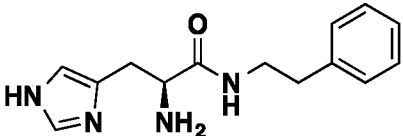
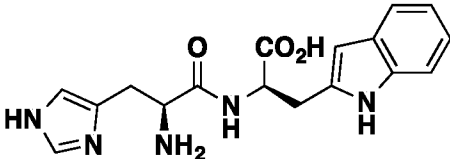
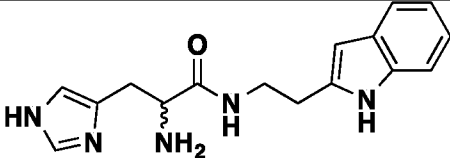
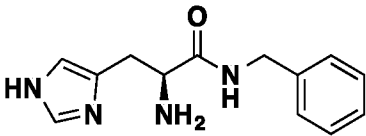
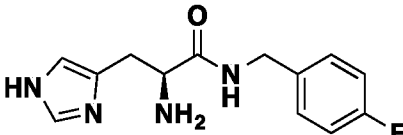
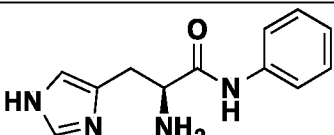
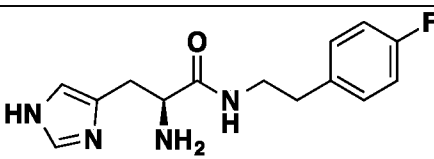
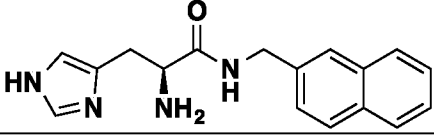
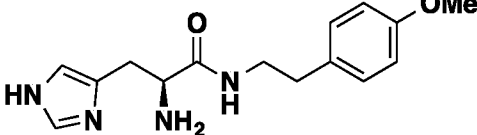
- [0197] Bisogno, T., and Di Marzo, V. (2010) Cannabinoid receptors and endocannabinoids: role in neuroinflammatory and neurodegenerative disorders. *CNS Neurol Disord Drug Targets* **9**, 564- 573
- [0198] Feng, Y., He, X., Yang, Y., Chao, D., Lazarus, L. H., and Xia, Y. (2012) Current research on opioid receptor function. *Curr Drug Targets* **13**, 230-246
- 5 [0199] Dauch, P., Vincent, J. P., and Checler, F. (1991) Specific inhibition of endopeptidase 24.16 by dipeptides. *Eur J Biochem* **202**, 269-276
- [0200] Cushman, D. W., and Ondetti, M. A. (1991) History of the design of captopril and related inhibitors of angiotensin converting enzyme. *Hypertension* **17**, 589-592
- [0201] Timmermans, P. B., Duncia, J. V., Carini, D. J., Chiu, A. T., Wong, P. C., Wexler, R. R., and
10 Smith, R. D. (1995) Discovery of losartan, the first angiotensin II receptor antagonist. *Journal of human hypertension* **9 Suppl 5**, S3-18

What is claimed is:

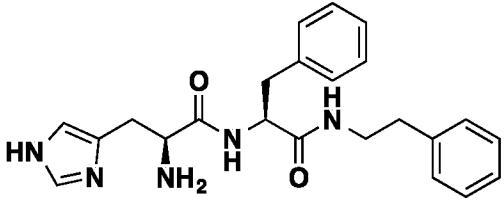
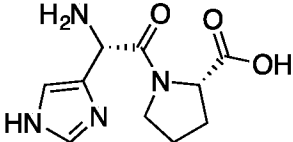
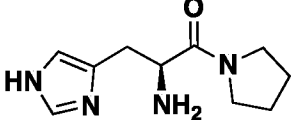
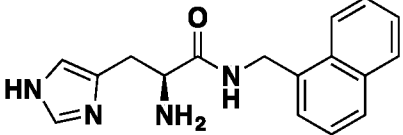
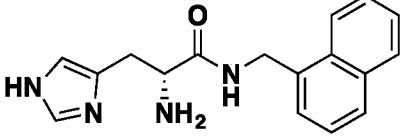
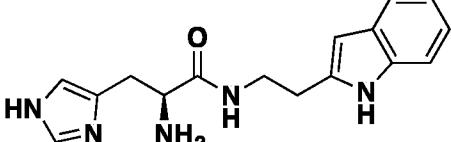
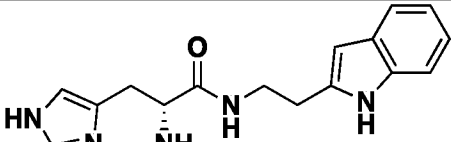
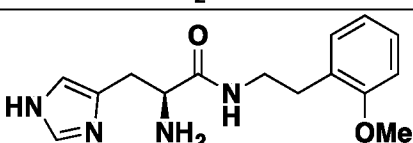
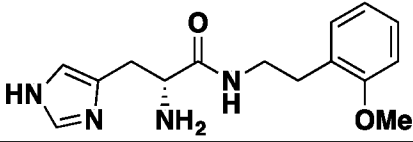
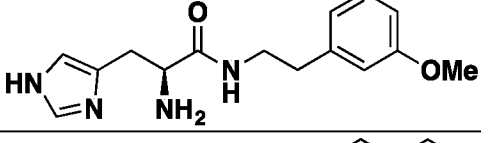
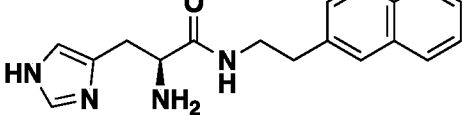
1. A method of allosterically potentiating the activity of neurolysin comprising:
contacting the neurolysin with an amount of a small molecule that allosterically increases the activity of neurolysin, wherein the small molecule is a histidine-containing dipeptide.
2. The method of claim 1, wherein the neurolysin is murine or human.
3. The method of claim 1, wherein the histidine-containing dipeptide reduces the K_m and increased V_{max} values for hydrolysis of one or more neurolysin substrates.
4. The method of claim 1, wherein the histidine-containing dipeptide is selected from:

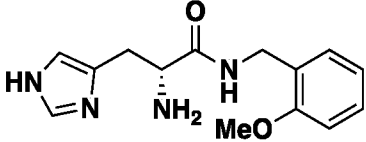
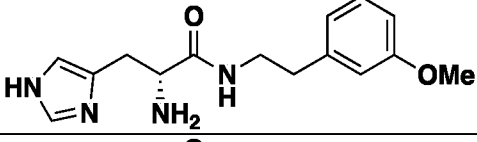
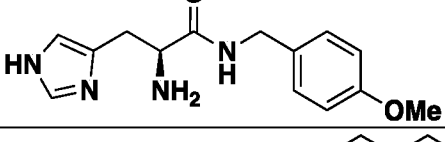
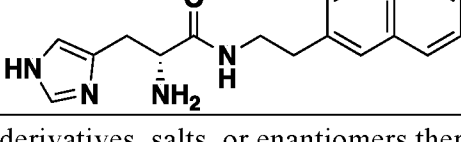


Compound	Code
	His-Tyr;
	His-Phe;

	NK-61-2A;
	NK-61-4A ;
	His-Trp;
	(±)PT-1-173 RACEMIC;
	DF-3-2B;
	DF-6-2A;
	DF-5-2A;
	DF-2-2B;
	DF-9-2;
	AB-1-4-2;

	AB-1-16-2;
	AB-1-14-2;
	AB-1-6-2;
	AB-1-9-2;
	DF-8-2;
	DF-10-2;
	AB-2-7-2;
	AB 3-2-2;
	AB-1-8-2;
	AB1-17-2;
	HP2CF3;

	AB-2-5-4;
	His-Pro;
	SR-5-3d;
	SR-9-1d;
	SR-10-1d;
	SR-7-2d;
	SR-8-2d;
	SR11-1d;
	SR12-1d;
	SR13-1d;
	SR18-1;

	SR16-1d;
	SR15-1d; 5
	SR20-1d;
	SR19-2d;

10 derivatives, salts, or enantiomers thereof.

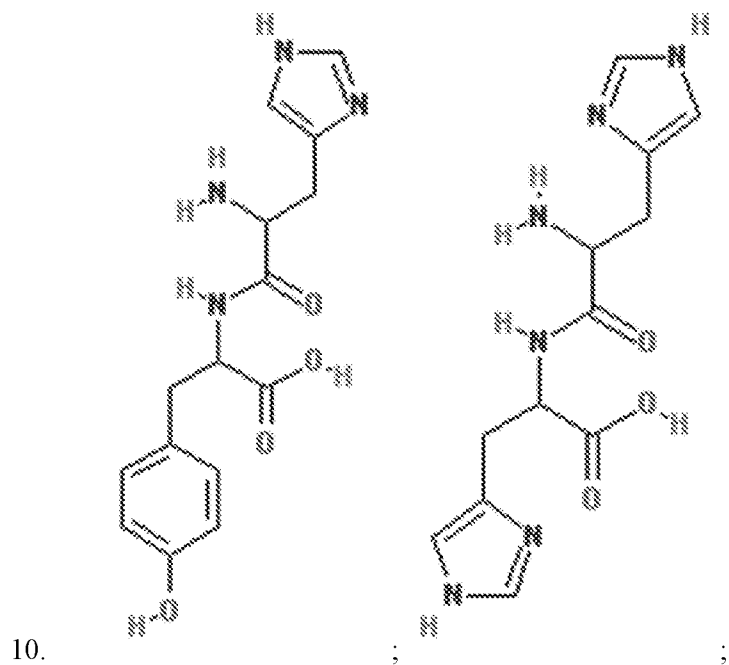
5. The method of claim 1, wherein the histidine-containing dipeptide does not bind at a substrate binding pocket or blocks the binding of a neurolysin substrate.

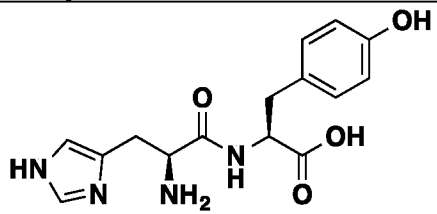
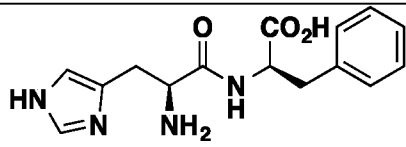
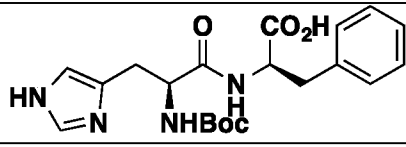
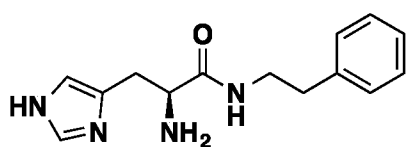
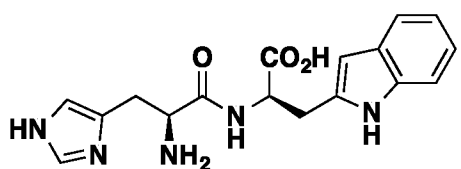
6. The method of claim 1, wherein the one or more neurolysin substrates are selected from at least one of neurolysin substrates, neurotensin, bradykinin, angiotensin I, substance P, hemopressin, dynorphin A(1-8),
15 metorphamide, or somatostatin.

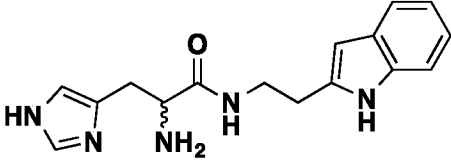
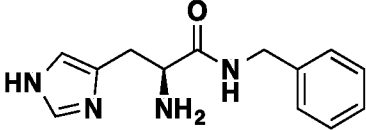
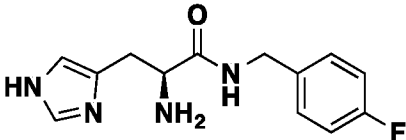
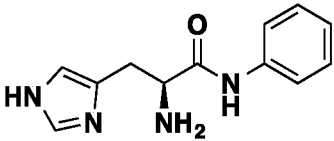
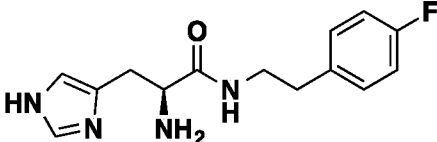
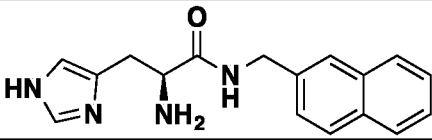
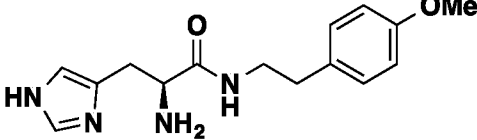
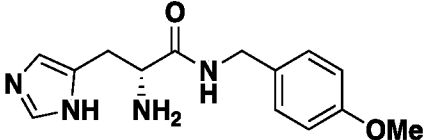
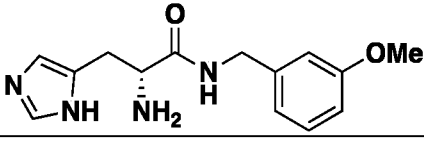
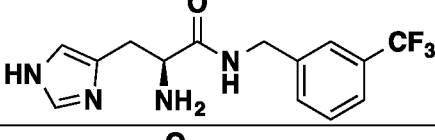
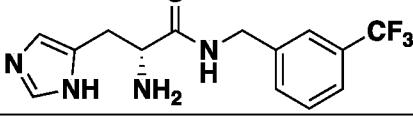
7. The method of claim 1, wherein the histidine-containing dipeptide increases the activity of neurolysin, but does not affect the activity of thimet oligopeptidase (TOP), angiotensin converting enzyme (ACE), angiotensin converting enzyme 2 (ACE2), or neprilysin (NEP).

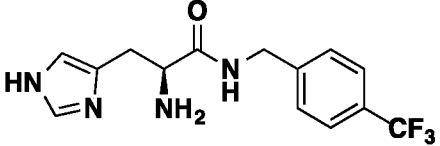
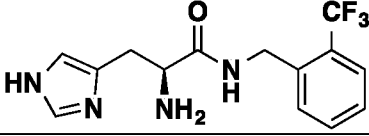
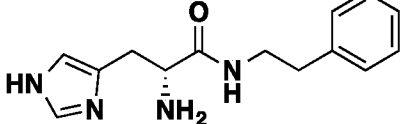
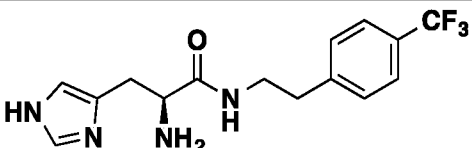
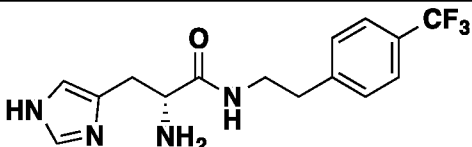
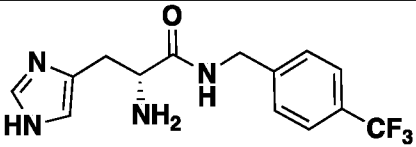
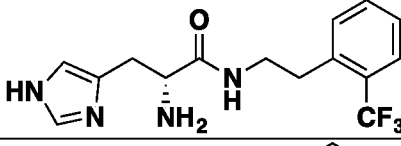
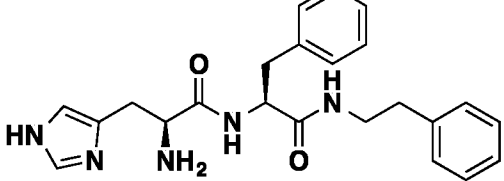
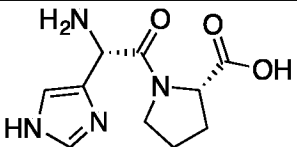
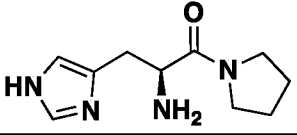
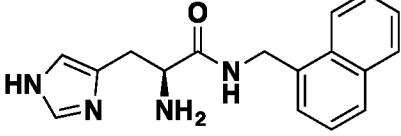
8. The method of claim 15, wherein the small molecule that allosterically increases the activity of
20 neurolysin is provided to a subject in an amount sufficient to treat a peripheral inflammatory disorder selected from ischemic stroke, traumatic brain injury, autism, Alzheimer's Disease, dementias or Parkinson's Disease.

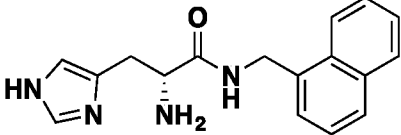
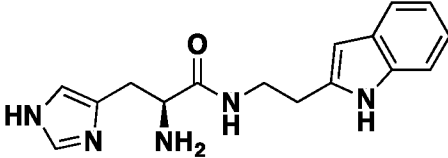
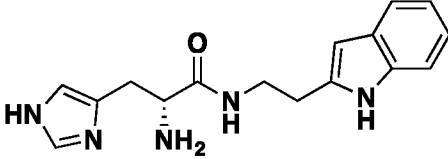
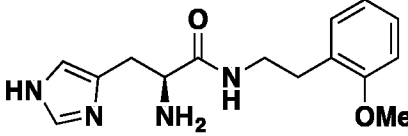
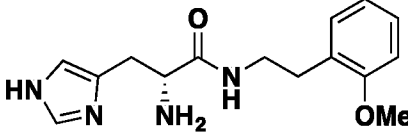
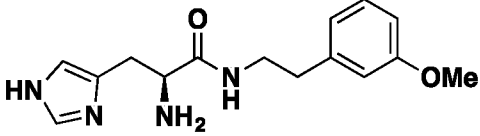
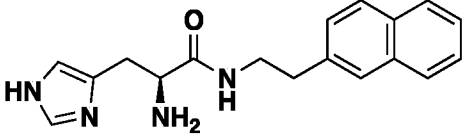
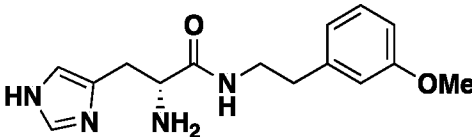
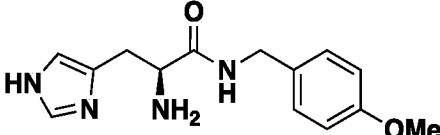
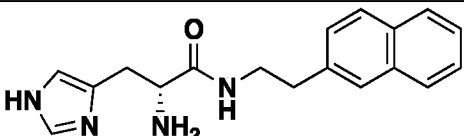
9. An allosteric activator of neurolysin selected from:



Compound	Code
	His-Tyr;
	His-Phe;
	NK-61-2A;
	NK-61-4A ;
	His-Trp;

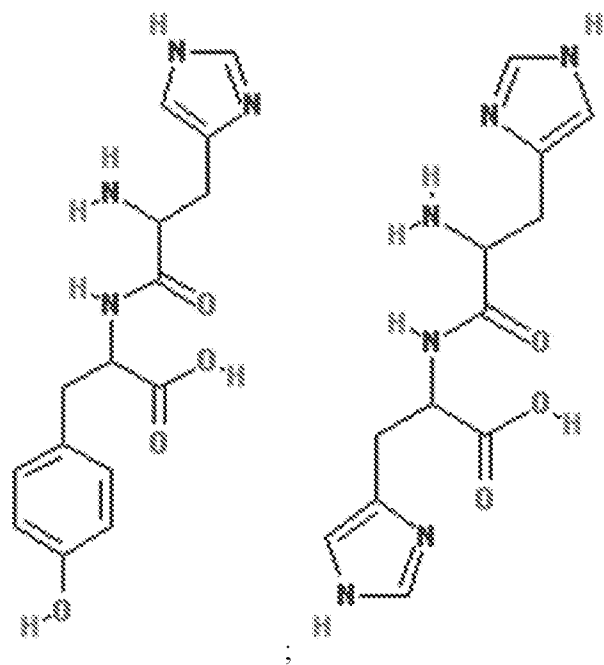
	(±)PT-1-173 RACEMIC;
	DF-3-2B;
	DF-6-2A;
	DF-5-2A;
	DF-2-2B;
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	AB-1-4-2;
	AB-1-16-2;
	AB-1-14-2;
	AB-1-6-2;
	AB-1-9-2;

	DF-8-2;
	DF-10-2;
	AB-2-7-2;
	AB 3-2-2;
	AB-1-8-2;
	AB1-17-2;
	HP2CF3;
	AB-2-5-4;
	His-Pro;
	SR-5-3d;
	SR-9-1d;

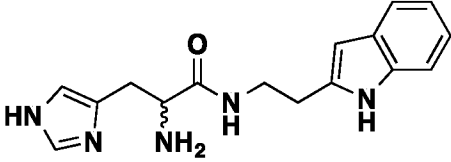
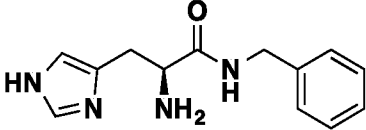
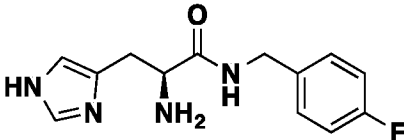
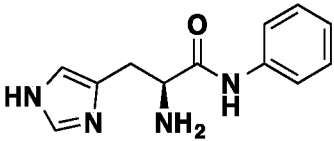
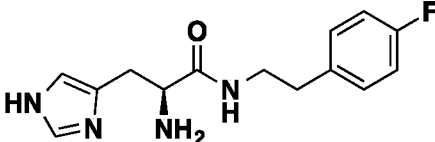
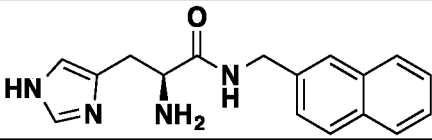
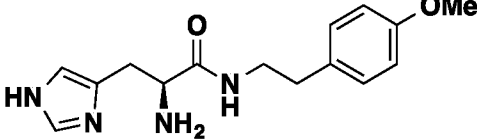
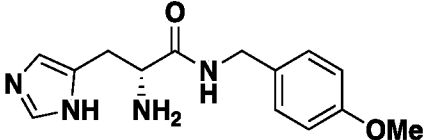
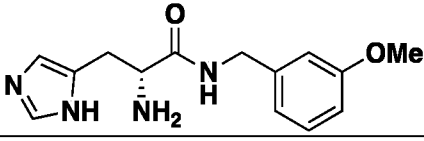
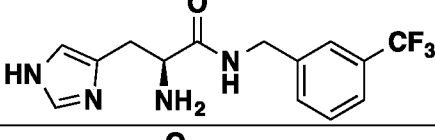
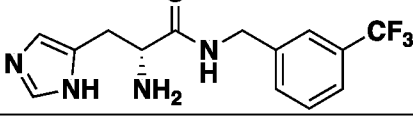
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	SR-8-2d;
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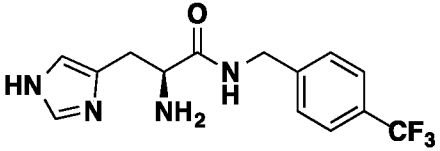
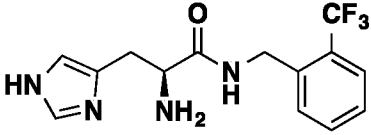
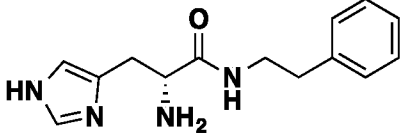
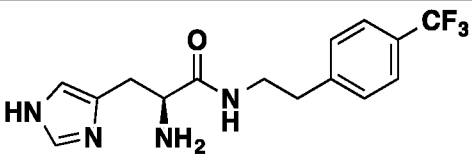
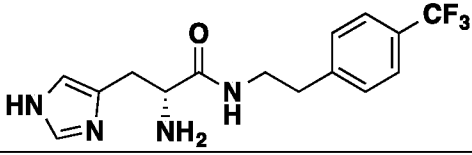
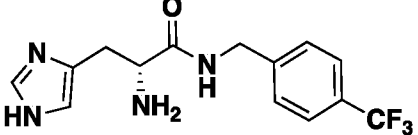
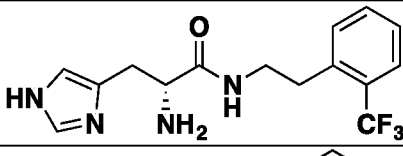
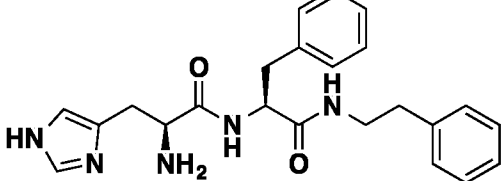
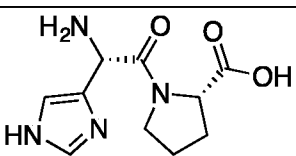
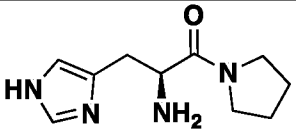
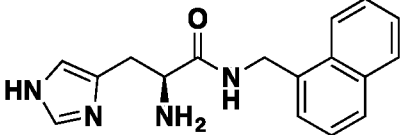
derivatives, salts, or enantiomers thereof.

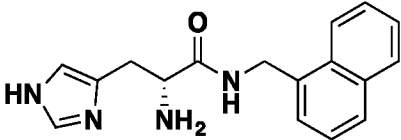
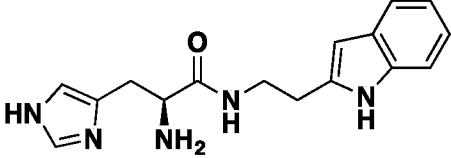
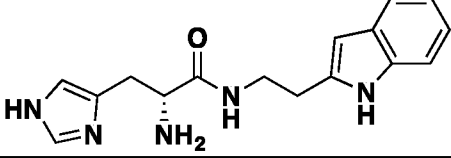
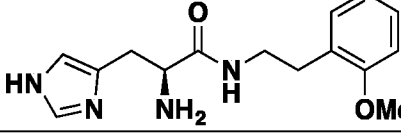
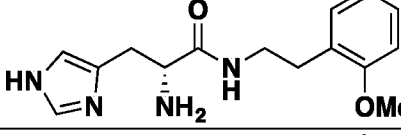
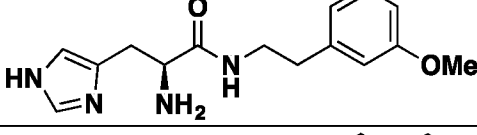
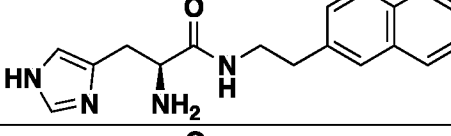
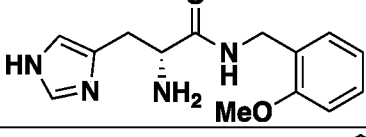
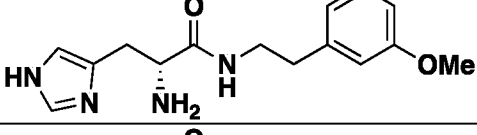
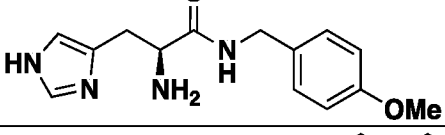
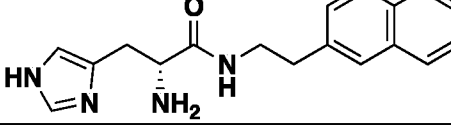
11. The activator of claim 9, wherein the inhibitor is adapted for oral, intraperitoneal, intradermal, subcutaneous, intravenous, enteral, parental, or pulmonary administration.
12. The activator of claim 9, wherein the inhibitor is combined with one or more excipients, buffers, fillers, or detergents.
- 5 13. The activator of claim 9, wherein the inhibitor is adapted for at least one of immediate release, delayed release, or prolonged release.
14. The activator of claim 9, wherein the activator comprises a single enantiomer.
15. A method of treating the symptoms of peripheral inflammatory disorder comprising:
identifying a subject in need of treatment for ischemic stroke; and
10 providing the subject with an amount of an allosteric potentiator of neurolysin sufficient to increase the activity of neurolysin.
16. The method of claim 15, wherein the peripheral inflammatory disorder is selected from ischemic stroke, traumatic brain injury, autism, Alzheimer's Disease, dementias or Parkinson's Disease.
17. The method of claim 15, wherein the neurolysin is murine or human.
- 15 18. The method of claim 15, wherein the allosteric potentiator is a histidine-containing dipeptide.
19. The method of claim 15, wherein the allosteric potentiator reduced the K_m and increased V_{max} values for hydrolysis of one or more neurolysin substrates.
20. The method of claim 15, wherein the allosteric potentiator of neurolysin has a reduced or not activity against at least one of thimet oligopeptidase, neprilysin, angiotensin converting enzyme (ACE), or ACE2.
- 20 21. The method of claim 15, wherein the allosteric potentiator of neurolysin is selected from:



Compound	Code
	His-Tyr;
	His-Phe;
	NK-61-2A;
	NK-61-4A ;
	His-Trp;

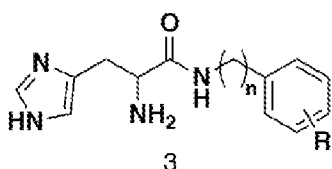
	(±)PT-1-173 RACEMIC;
	DF-3-2B;
	DF-6-2A;
	DF-5-2A;
	DF-2-2B;
	DF-9-2;
	AB-1-4-2;
	AB-1-16-2;
	AB-1-14-2;
	AB-1-6-2;
	AB-1-9-2;

	DF-8-2;
	DF-10-2;
	AB-2-7-2;
	AB 3-2-2;
	AB-1-8-2;
	AB1-17-2;
	HP2CF3;
	AB-2-5-4;
	His-Pro;
	SR-5-3d;
	SR-9-1d;

	SR-10-1d;
	SR-7-2d; 5
	SR-8-2d;
	SR11-1d; 10
	SR12-1d;
	SR13-1d;
	SR18-1; 15
	SR16-1d;
	SR15-1d; 20
	SR20-1d;
	SR19-2d; 25

derivatives, salts, or enantiomers thereof.

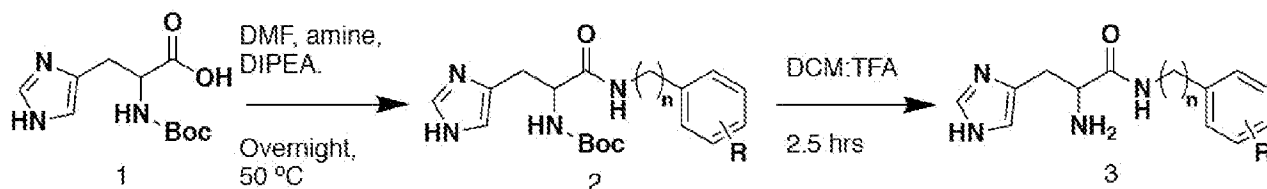
22. The method of claim 15, wherein the allosteric potentiator of neurolysin does not bind at a substrate binding pocket or blocks the binding of a neurolysin substrate.
23. The method of claim 15, wherein the one or more neurolysin substrates are selected from at least one of neurolysin substrates, neurotensin, bradykinin, angiotensin I, substance P, hemopressin, dynorphin A(1-8), metorphamide, or somatostatin.
24. The method of claim 15, wherein the histidine-containing dipeptide increases the activity of neurolysin, but does not affect the activity of thimet oligopeptidase (TOP), angiotensin converting enzyme (ACE), angiotensin converting enzyme 2 (ACE2), or neprilysin (NEP).
25. An allosteric potentiator of neurolysin having the formula:



wherein $n = 0-5$, and R is one or more electron-donating group(s) selected from the group consisting of: linear or branched alkyl groups, linear or branched alkenyl groups, linear or branched halogenoalkyl groups, cycloalkyl groups comprising 3 to 6 carbon atoms, a phenyl group, alkoxy groups of formula R_x-O- or thioether groups of formula R_x-S- , wherein R_x is a linear or branched alkyl group comprising 1 to 6 carbon atoms, or a phenyl group, groups of formula $-N-(R_x)_2$, wherein R_x groups, which are identical or different, represent a hydrogen atom, a linear or branched alkyl group comprising 1 to 6 carbon atoms, or a phenyl group, and a $-CF_3$ group; or

R is one or more electron-withdrawing group(s) selected from the group consisting of: NO_2 , F , Cl , CF_3 , CN , $COOX$, $CONHX$, $CONX$, COX , SO_2X , SO_2OX , SO_2NX_2 , PO_3X_2 , NO and Ar , wherein X is H or C_1-C_6 alkyl, and Ar is phenyl, pyridyl or naphthyl.

26. A method of making a compound that is an allosteric potentiator of neurolysin comprising:



1A

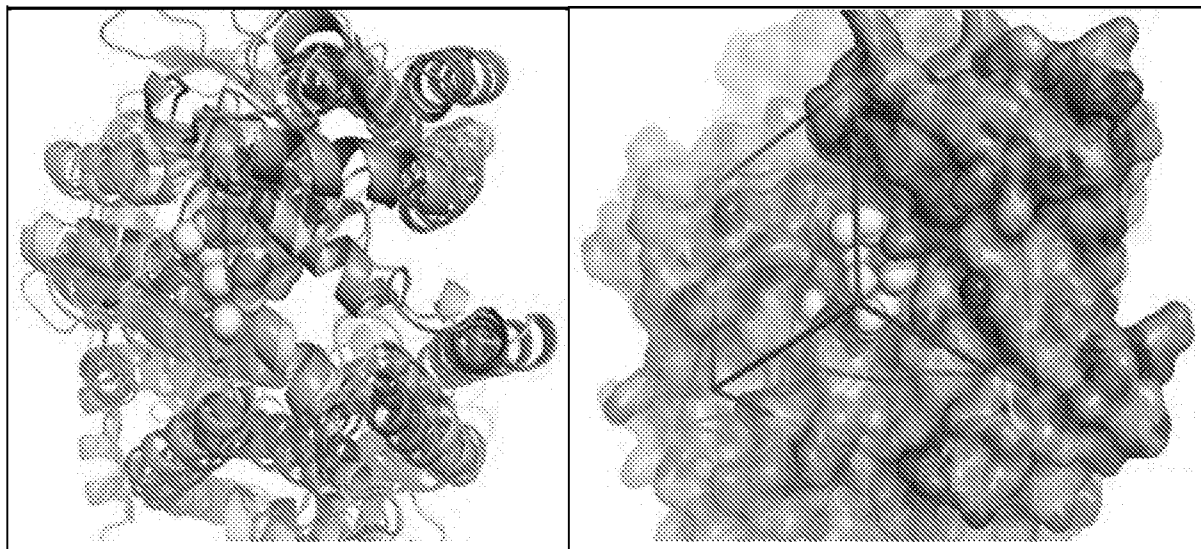


FIG. 1A

FIG. 1B

FIG. 2A

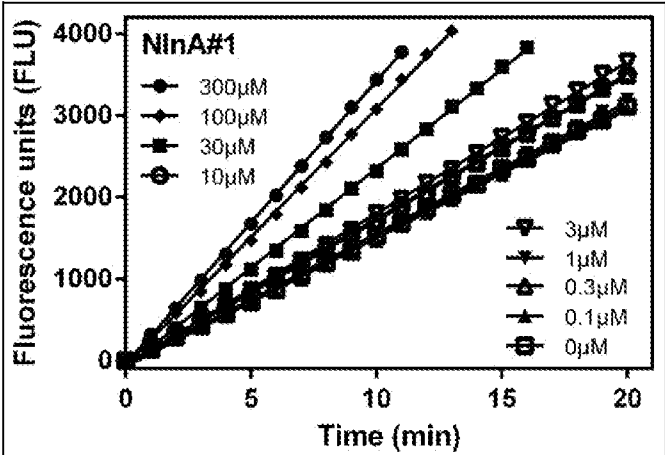


FIG. 2B

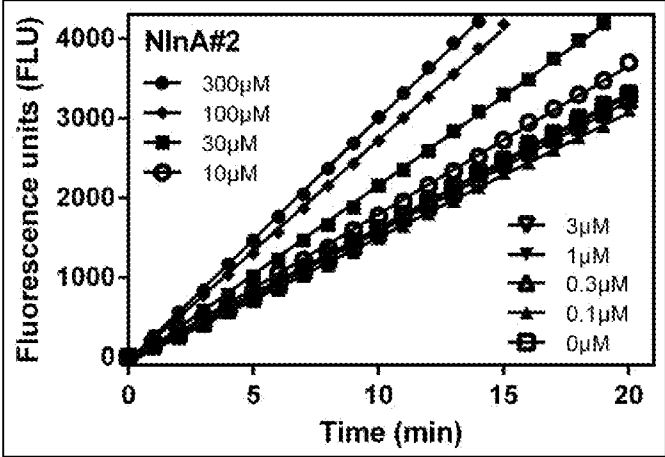
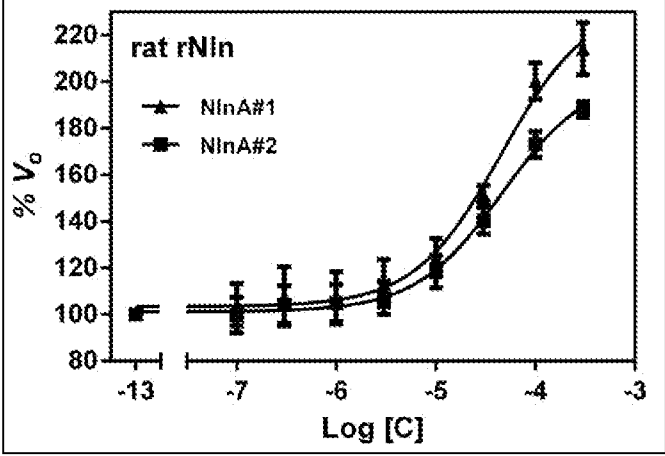


FIG. 2C



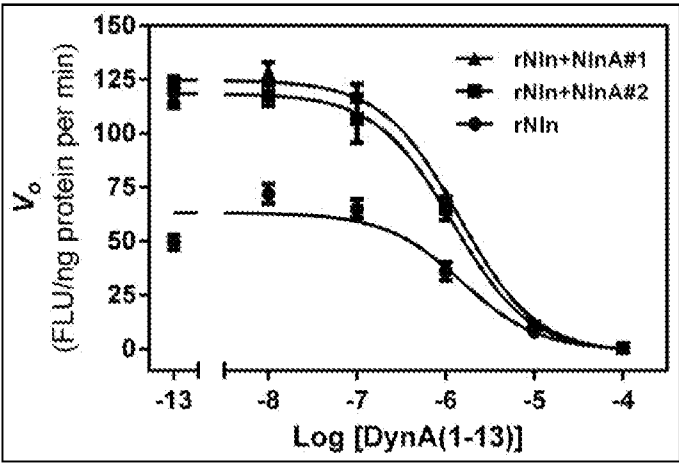


FIG. 3

FIG. 4A

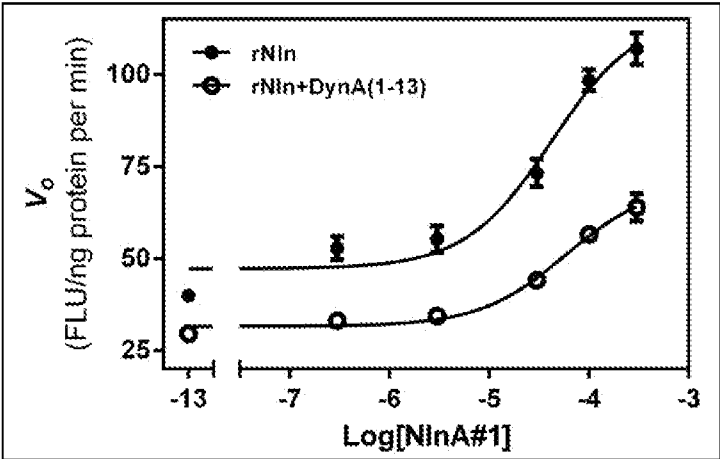


FIG. 4B

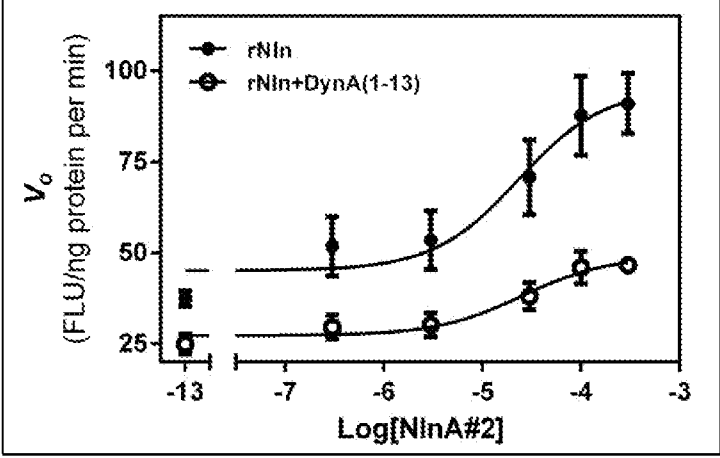


FIG. 5A

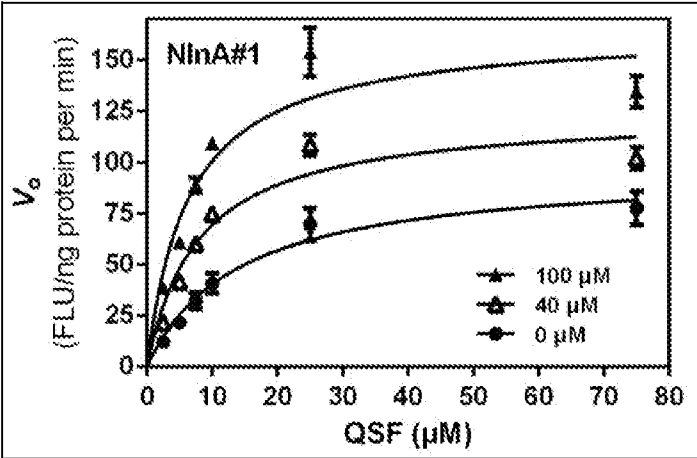


FIG. 5B

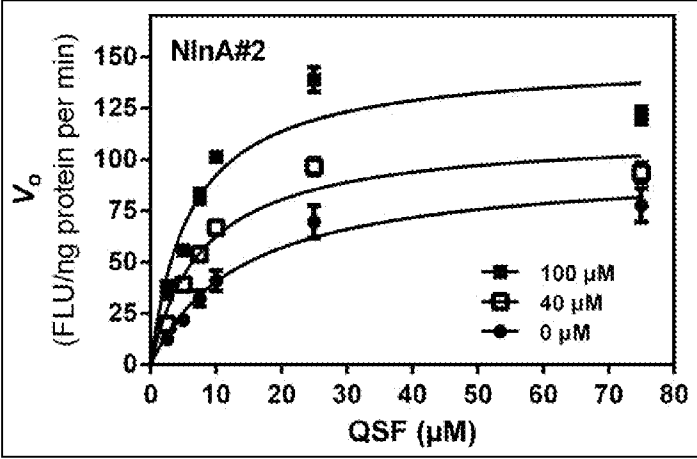


FIG. 6A

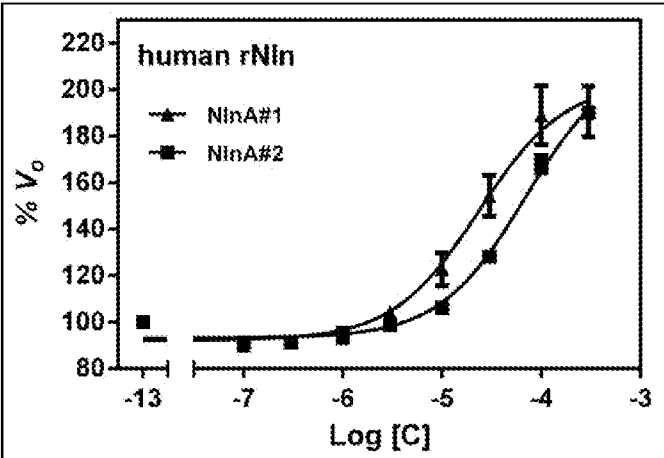


FIG. 6B

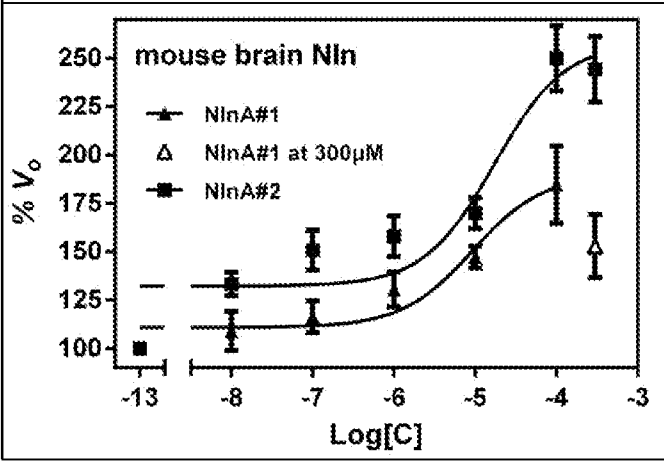


FIG. 7A

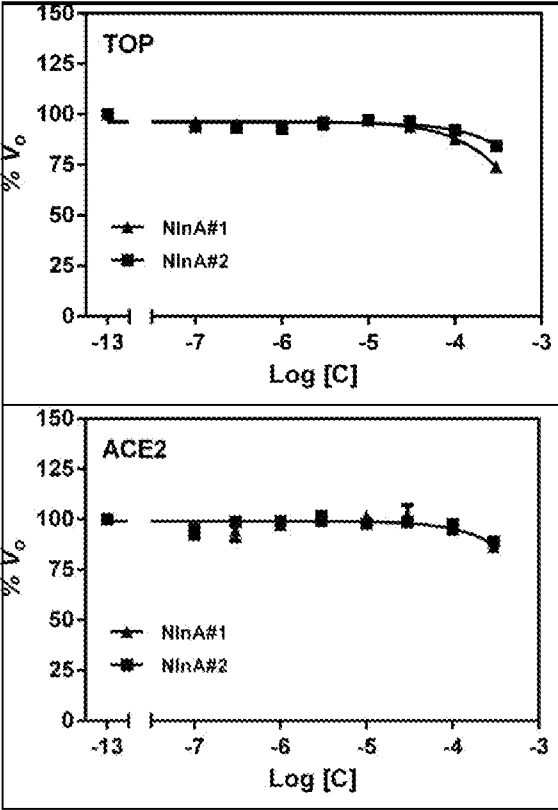


FIG. 7B

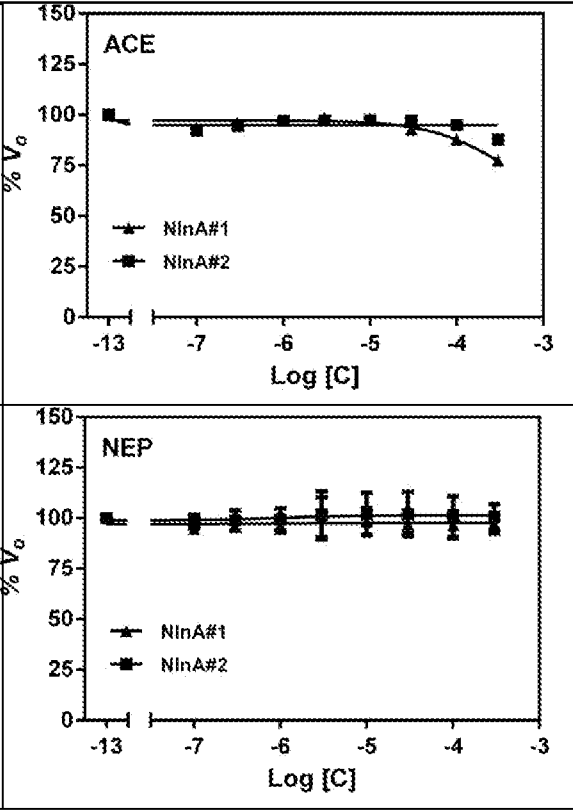


FIG. 7C

FIG. 7D

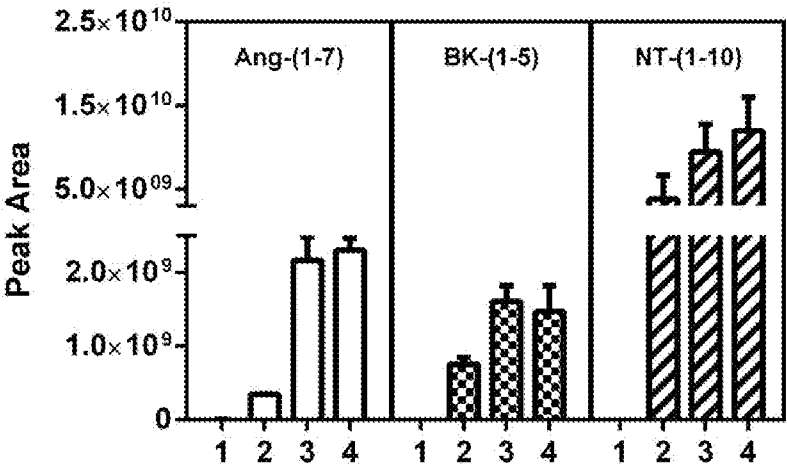


FIG. 8

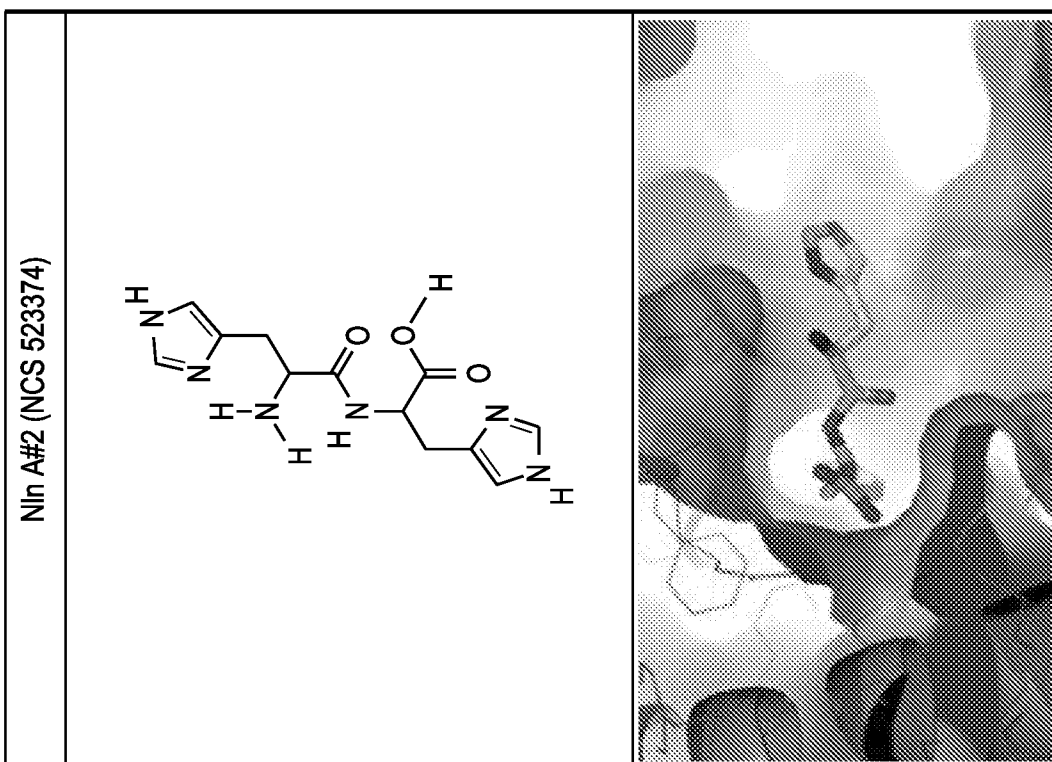


FIG. 9B

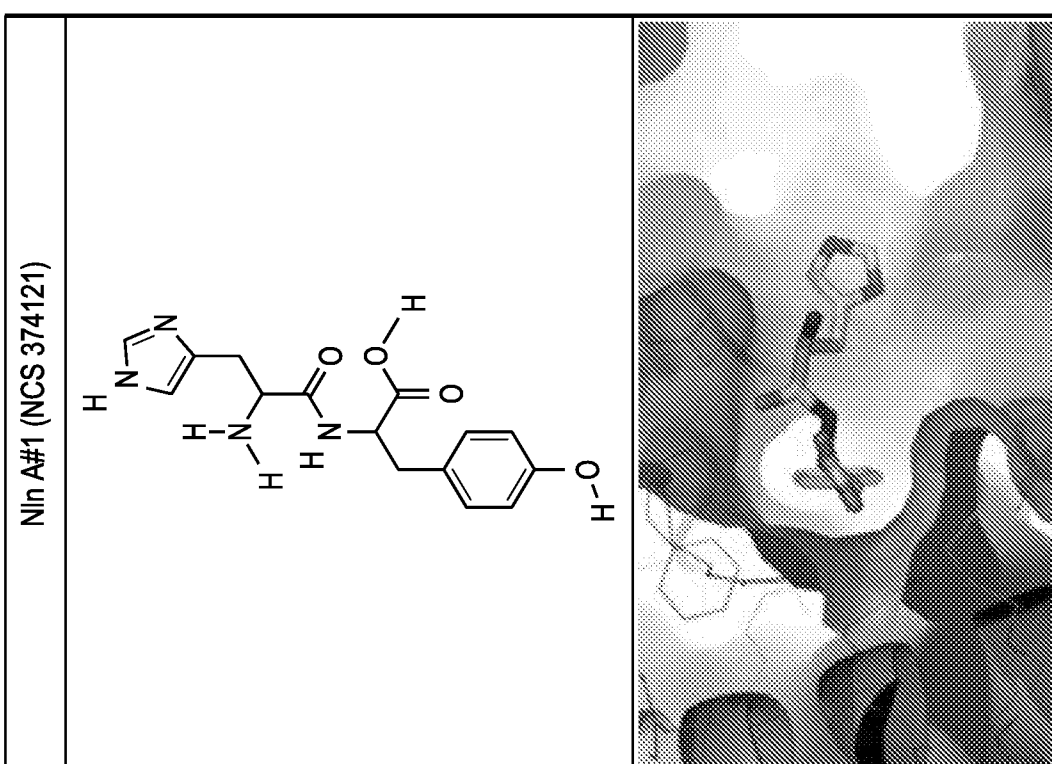


FIG. 9A

FIG. 10A

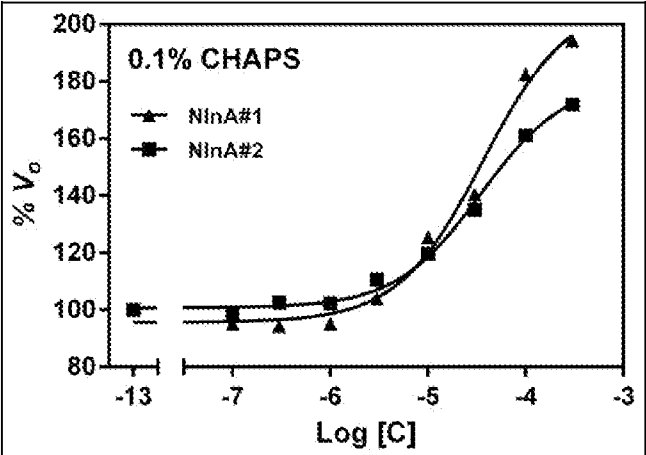


FIG. 10B

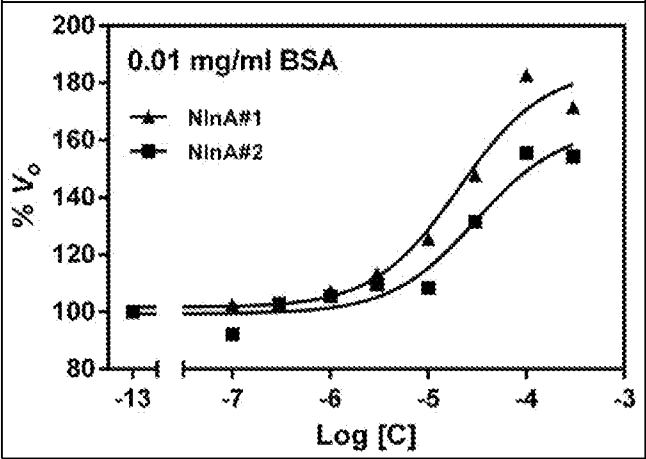


FIG. 11A

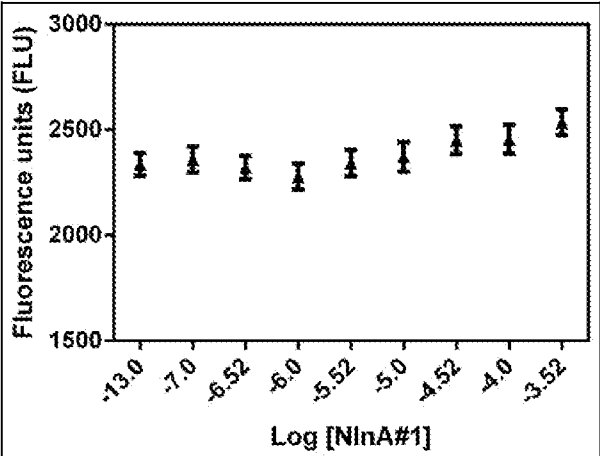
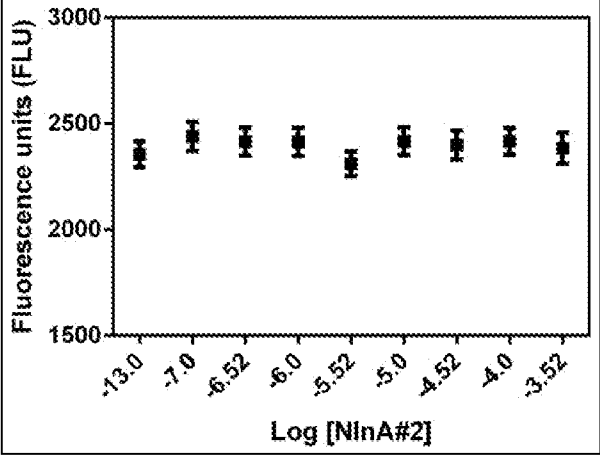
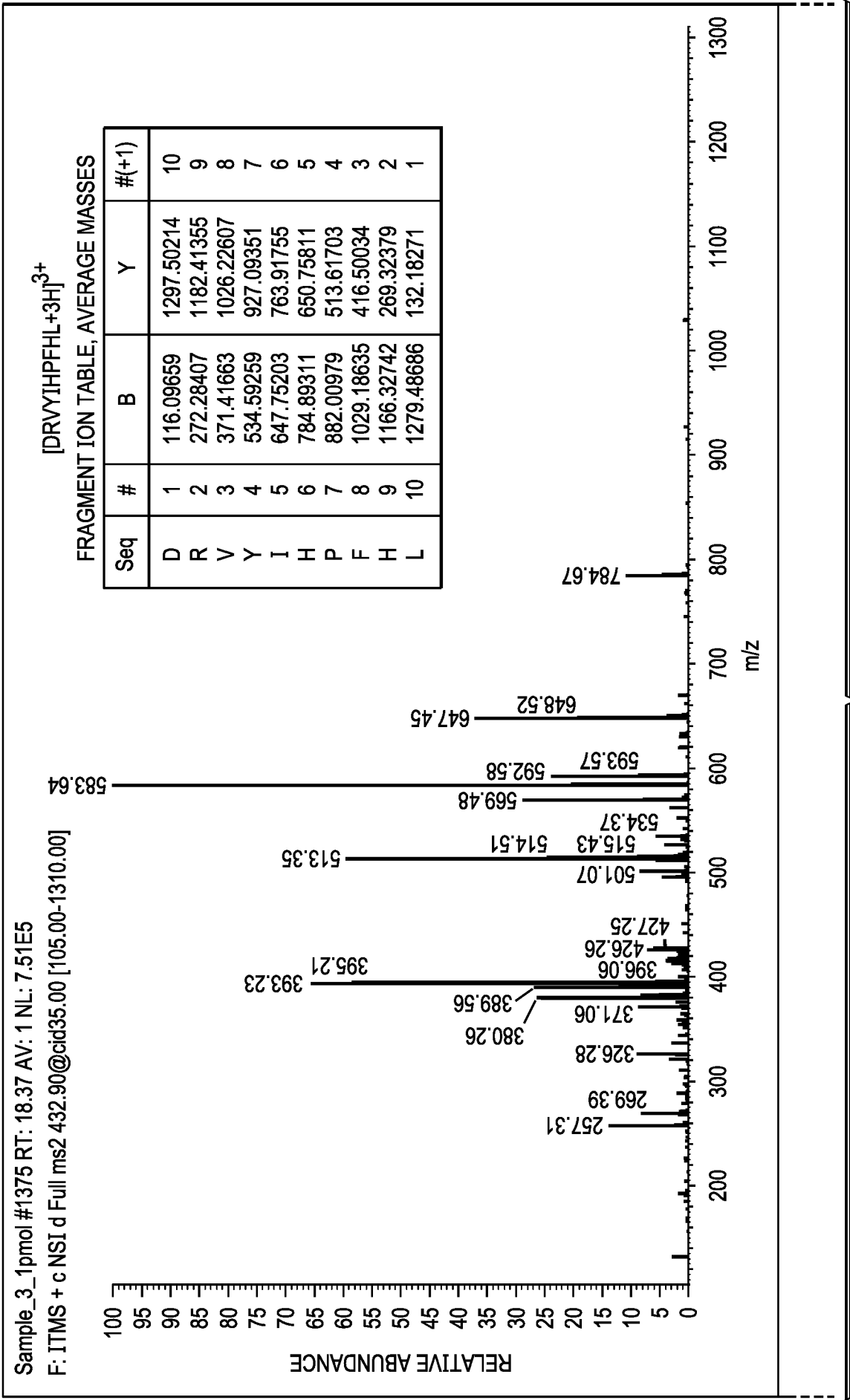


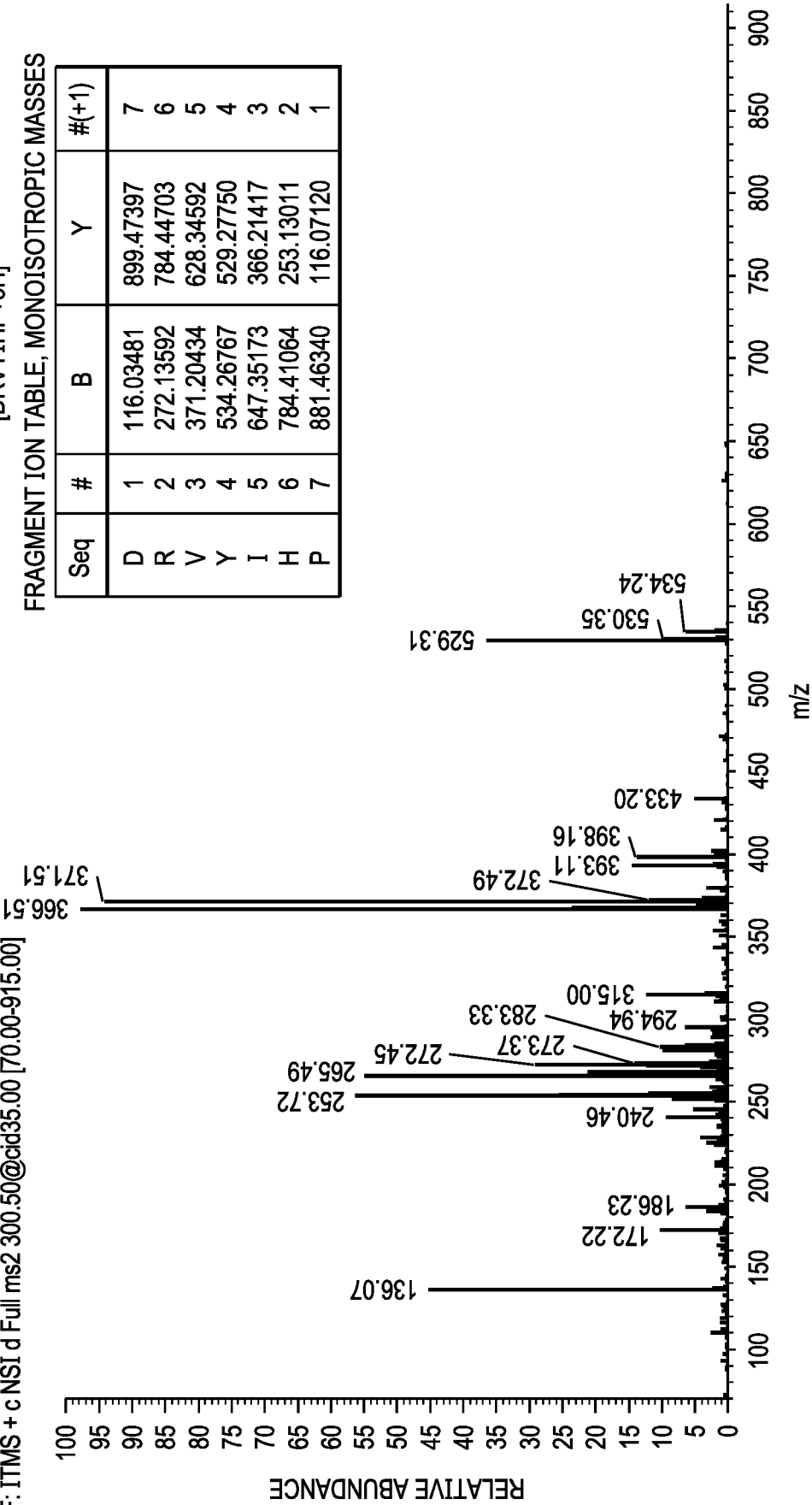
FIG. 11B





FROM FIG. 12

Sample_3_1pmol #608 RT: 16.02 AV: 1 NL: 4.05E4
F: ITMS + c NSI d Full ms2 300.50@cid35.00 [70.00-915.00]



[DRVYIHP+3H]³⁺

FRAGMENT ION TABLE, MONOISOTROPIC MASSES

Seq	#	B	Y	#(+1)
D	1	116.03481	899.47397	7
R	2	272.13592	784.44703	6
V	3	371.20434	628.34592	5
Y	4	534.26767	529.27750	4
I	5	647.35173	366.21417	3
H	6	784.41064	253.13011	2
P	7	881.46340	116.07120	1

FIG. 12 (CONTINUED) TO FIG. 12 (CONTINUED)

FROM FIG. 12 (CONTINUED)

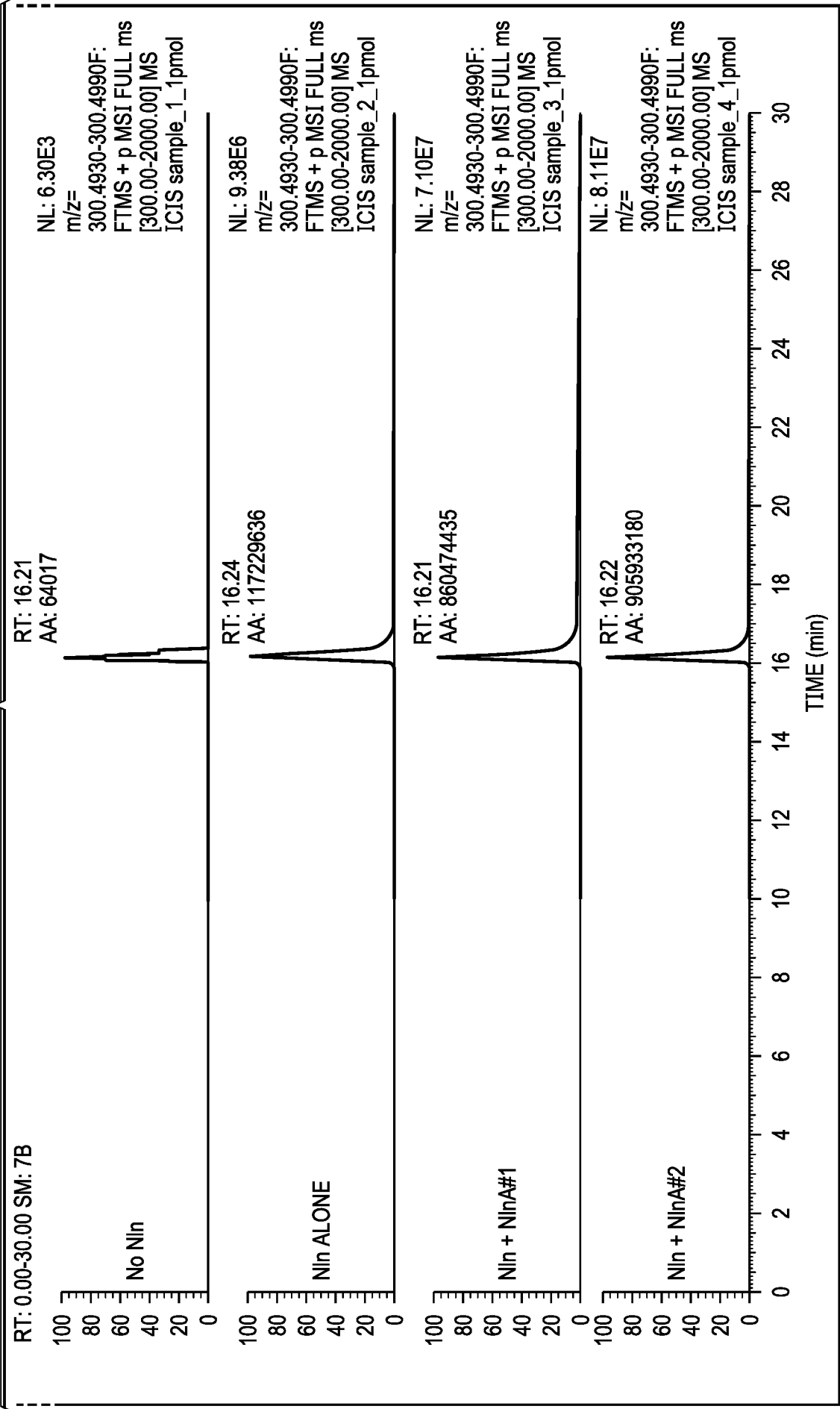


FIG. 12 (CONTINUED)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/048702

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 1/22; C07K 14/47; C07K 17/14; C07K 19/00; C12N 9/16; C12P 21/02 (2019.01)

CPC - C07K 1/22; C07K 14/4702; C07K 23/19/20; C07K 23/19/92; C12N 9/16; C12P 21/02 (2019.08)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

USPC - 435/196; 435/325; 435/320.1; 506/9; 536/23.2 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 8,933,056 B2 (BACHOVCHIN et al) 13 January 2015 (13.01.2015) entire document	1-9, 11-24
A	US 2017/0196908 A1 (CASE WESTERN RESERVE UNIVERSITY) 13 July 2017 (13.07.2017) entire document	1-9, 11-24
A	US 4,611,076 A (DONG et al) 09 September 1986 (09.09.1986) entire document	1-9, 11-24
A	JAYARAMAN et al. "Allosteric Potentiation of Peptidase Neurolysin by Small Molecules," Biophysical Journal, 16 February 2016 (16.02.2016), Vol. 110, Iss. 3, Suppl. 1, Pgs. 396A-397A. entire document	1-9, 11-24
A	US 7,863,272 B2 (OKSENBERG et al) 04 January 2011 (04.01.2011) entire document	1-9, 11-24

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 December 2019

Date of mailing of the international search report

10 JAN 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/048702

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NO:1 was searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/048702

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See extra sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-9 and 11-24 to the extent that they read on dipeptide His-Tyr.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/048702

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-9 and 11-26 are drawn to histidine-containing dipeptides, and methods comprising the same.

The first invention of Group I+ is restricted to a histidine-containing dipeptide, and methods comprising the same, wherein the histidine-containing dipeptide is selected to be His-Tyr. It is believed that claims 1-9 and 11-24 read on this first named invention and thus these claims will be searched without fee to the extent that they read on dipeptide His-Tyr.

Applicant is invited to elect additional histidine-containing dipeptides, each with specified chemical structure, to be searched in a specific combination by paying an additional fee for each set of election. An exemplary election would be a histidine-containing dipeptide, and methods comprising the same, wherein the histidine-containing dipeptide is selected to be His-Phe. Additional histidine-containing dipeptides will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element responsible for allosterically potentiating neurolysin, requiring the selection of alternatives for the chemical structure of the histidine-containing dipeptides, where "the histidine-containing dipeptide is selected from... (see Table instant Claim 4) ...derivatives, salts, or enantiomers thereof".

Additionally, even if Groups I+ were considered to share the technical features of a method of allosterically potentiating the activity of neurolysin comprising: contacting the neurolysin with an amount of a small molecule that allosterically increases the activity of neurolysin, wherein the small molecule is a histidine-containing dipeptide; an allosteric activator of neurolysin; a method of treating the symptoms of peripheral inflammatory disorder comprising: identifying a subject in need of treatment for ischemic stroke; and providing the subject with an amount of an allosteric potentiator of neurolysin sufficient to increase the activity of neurolysin; these shared technical features do not represent a contribution over the prior art.

Specifically, "Allosteric Potentiation of Peptidase Neurolysin by Small Molecules" to Jayaraman et al. discloses a method of allosterically potentiating the activity of neurolysin (allosteric potentiation of peptidase neurolysin by small molecules, Pg. 396a, right-hand column, bottom of page; we describe discovery of two small molecules which specifically enhance catalytic efficiency of peptidase neurolysin, Pg. 397a, left-hand column, first paragraph) comprising: contacting the neurolysin with an amount of a small molecule that allosterically increases the activity of neurolysin (we describe discovery of two small molecules which specifically enhance catalytic efficiency of peptidase neurolysin, Pg. 397a, left-hand column, first paragraph; Both modulators reduced Km and increased Vmax values for hydrolysis of the synthetic substrate by neurolysin in a concentration-dependent manner, Pg. 397a, left-hand column, first paragraph), an amount of an allosteric potentiator of enzyme sufficient to increase the activity of enzyme (we describe discovery of two small molecules which specifically enhance catalytic efficiency of peptidase neurolysin, Pg. 397a, left-hand column, first paragraph; Both modulators reduced Km and increased Vmax values for hydrolysis of the synthetic substrate by neurolysin in a concentration-dependent manner, Pg. 397a, left-hand column, first paragraph).

US 4,611,076 A to Dong et al. discloses a histidine containing dipeptide ((L-phenylalanyl-L-histidine) dipeptide, Col. 2, Ln. 16).

US 7,863,272 B2 to Oskenberg et al. discloses a method of treating the symptoms of peripheral inflammatory disorder (The invention discloses methods and compositions useful for facilitating neuronal regeneration and functional recovery in neurodegenerative diseases, Abstract; that result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, Col. 6, Lns. 49-51) comprising: identifying a subject in need of treatment for ischemic stroke (are used as neuronal regeneration agents for the treatment of neurodegenerative diseases such as those resulting from ischemic strokes, Col. 3, Lns. 55-58; the subject invention is directed to a method for treating or preventing neurodegenerative disease in a subject in need thereof, Col. 3, Lns. 60-62); and providing the subject with an enzyme activator (the topical composition may further comprise one or more additional ingredients, Para. [0119]; the additional ingredient may be... enzyme activators, Para. [0124]; the administration of an agent to a subject, Para. [0175]).

US 2017/0196908 A1 to Case Western Reserve University discloses a compound of instant Formula (3), where n = 0 (2-amino-3-(1H-imidazol-4-yl)propanamide, Para. [0136]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.