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		pKi		
		MOR	KOR	DOR
SEQ ID NO: 3	Dyn A	8.1	10.8–8.3	7.4
SEQ ID NO: 4	Dyn A {1–13}	8.3	10.7–9.3	7.8
SEQ ID NO: 5	Dyn A {1–8}	8.4	9.9–8.0	8.4
SEQ ID NO: 6	Dyn B	8.5	9.9–8.1	7.8
SEQ ID NO: 1	Leu-Enk	8.1	6.0	8.7–8.4
SEQ ID NO: 2	Met-Enk	8.2	6.0	7.4

K receptor
selectivity decrease

FIG. 1

(57) Abstract: Opioid receptor ligands (ORLs) that are multifunctional having agonist activity at mu opioid receptor (MOR), agonist activity at delta opioid receptor (DOR), and antagonist (or partial agonist) activity at kappa opioid receptor (KOR). The ORLs comprise peptide portions that are analogs derived from enkephalins, EM-1, or DALDA, as well as tail portions that comprise a lipophilic molecule such as a 4-anilidopiperidine moiety.

MULTIFUNCTIONAL OPIOID RECEPTOR LIGANDS AND METHODS OF TREATING PAIN

CROSS REFERENCE

[0001] This application claims priority to U.S. Patent Application No. 62/165,063 filed May 21, 2015, the specification(s) of which is/are incorporated herein in their entirety by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to ligands for mu, delta, and kappa opioid receptors, more particularly to multifunctional opioid peptides that function as mu opioid receptor (MOR), delta opioid receptor (DOR) agonists, and kappa opioid receptor (KOR) antagonists (or partial agonists). The present invention also relates to treating pain or other conditions using the multifunctional opioid peptides herein.

BACKGROUND OF THE INVENTION

[0003] Opioids are commonly used in the treatment of severe pain. Opioids have analgesic activity through their interaction with the opioid receptors (e.g., mu (μ) opioid receptor (MOR), delta (δ) opioid receptor (DOR), kappa (κ) opioid receptor (KOR)), mostly with MOR. However, the clinical use of opioids is limited by associated side effects such as respiratory depression, constipation, development of tolerance, and addiction. Indeed, chronic pain and subsequent chronic administration of a MOR agonist can lead to KOR activation, which results in undesirable adverse and addictive behaviors. For this reason, a KOR antagonist (or partial agonist) could be used to reduce such undesirable effects of chronic MOR activation.

[0004] Inventors have surprisingly discovered opioid peptides, e.g., opioid receptor ligands (ORLs) that are multifunctional, e.g., acting as MOR agonists, DOR agonists, and KOR antagonists (or partial agonists). In some embodiments, the multifunctional ORLs may comprise peptide analogs derived from enkephalins. Enkephalins are pentapeptides (peptides containing 5 amino acids) that are endogenous ligands of the opioid receptors (e.g., MOR, and DOR). There are two known forms of enkephalins: leucine-containing enkephalin (Leu-Enk, or YGGFL (SEQ ID NO: 1)) and methionine-containing enkephalin (Met-Enk, or YGGFM (SEQ ID NO: 2)). In

some embodiments, the ORLs comprise a 4-anilidopiperidine moiety, e.g., fentanyl analog, etc., (or a moiety that is an analog of a 4-anilidopiperidine).

SUMMARY OF THE INVENTION

[0005] The present invention features multifunctional opioid receptor ligands (ORLs) and methods of use of said multifunctional ORLs.

[0006] In some embodiments, the ORLs are mu opioid receptor (MOR) agonists and delta opioid receptor (DOR) agonists. In some embodiments, the ORLs are also kappa opioid receptor (KOR) antagonists (or partial agonists), and methods of use of said multifunctional ORLs. In some embodiments, the ORL comprises a peptide portion and a tail portion linked to a C-terminus of the peptide portion, wherein the ORL has a formula according to Formula 1: Aaa-DBbb-Ccc-Ddd(X)-Eee. In some embodiments, Aaa is selected from 2'-6'-dimethyltyrosine (Dmt) and Tyrosine (Tyr); D-Bbb is selected from D-Alanine (D-Ala), D-Norleucine (D-Nle), Proline (Pro), and D-Arginine (D-Arg); Ccc is selected from Gly, Phenylalanine(X) (Phe(X)), and naphthylalanine (Nal) or is absent; Ddd(X) is Gly, Phe(X), or Lys; Eee is the tail portion, the tail portion is lipophilic; and X is selected from H, F, Cl, and Br. In some embodiments, Eee is selected from -NH₂ and a 4-anilidopiperidine moiety. In some embodiments, the 4-anilidopiperidine moiety comprises N-phenyl-N-piperidin-4-ylpropionamide (Ppp).

[0007] In some embodiments, the peptide portion comprises 3 or 4 amino acids or derivatives thereof. The present invention is not limited to peptide portions comprising 3 or 4 amino acids (e.g., in some embodiments, the peptide portion comprises 5 amino acids, 6 amino acids, or more than 6 amino acids, etc.). In some embodiments, the peptide portion comprises a peptide having a formula according to Formula 2: Dmt-DXxx-Gly-Phe(X), wherein Dmt is selected from 2'-6'-dimethyltyrosine (Dmt), Tyrosine (Tyr), Phenylalanine (Phe), Tmt, Dmp, or Mdp, DXxx is selected from DAlanine (DAla), DNorleucine (DNle), or D-tetrahydroisoquinoline-3-carboxylic acid (DTic), Gly is either Glycine (Gly) or no residue, Phe is Phenylalanine (Phe), and X is either H, Cl, or F. In some embodiments, the tail portion comprises a lipophilic molecule. In some embodiments, the lipophilic molecule comprises a 4-anilidopiperidine moiety. In some embodiments, the 4-anilidopiperidine moiety comprises N-phenyl-N-piperidin-

4-ylpropionamide (Ppp).

[0008] The present invention also features methods of treating or reducing pain. In some embodiments, the method comprises identifying a subject in need of a kappa opioid receptor (KOR) antagonist (or partial agonists) and introducing to the subject a multifunctional ORL according to the present invention, wherein the ORL is effective for reducing pain.

[0009] The present invention also features methods of blocking kappa opioid receptor. In some embodiments, the method comprises introducing to the KOR a multifunctional ORL according to the present invention.

[0010] The present invention also features methods of blocking KOR, activating MOR, and activating DOR in a subject. In some embodiments, the method comprises introducing to the subject a multifunctional ORL according to the present invention.

[0011] Any feature or combination of features described herein are included within the scope of the present invention provided that the features included in any such combination are not mutually inconsistent as will be apparent from the context, this specification, and the knowledge of one of ordinary skill in the art. Additional advantages and aspects of the present invention are apparent in the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1 shows the well-known structure-activity relationship (SAR) results of Dynorphin A (Dyn A (SEQ ID NO: 3)) and Enkephalins for opioid activities. Peptides tested include Dyn A (SEQ ID NO: 3), an endogenous KOR ligand, a peptide containing the first 13 amino acids of Dyn A (Dyn A 1-13 (SEQ ID NO: 4)), a peptide containing the first 8 amino acids of Dyn A (Dyn A 1-8 (SEQ ID NO: 5)), Dyn B (SEQ ID NO: 6), Leu-Enk (SEQ ID NO: 1), and Met-Enk (SEQ ID NO: 2).

[0013] FIG. 2 shows a schematic representation of an ORL of the present invention. For reference, Dmt refers to 2'-6'-dimethyltyrosine, DXxx refers to a D-amino acid, and Phe(X) refers to a halogenated or methylated Phe residue. N-phenyl-N-piperidin-4-ylpropionamide (Ppp) is shown as the tail.

[0014] FIG. 2A shows a formula representing various ORLs of the present

invention. In some embodiments, Eee comprises Ppp or NH₂. In some embodiments, Aaa comprises Tyr or Dmt. In some embodiments, Bbb comprises Nle, Pro, or DArg. In some embodiments, Ccc comprises Gly, Phe(X), or Nal. In some embodiments, Ddd comprises Gly, Phe(X), or Lys. In some embodiments, X is H, F, Cl, or Br. The present invention is not limited to the formula or substitutions shown in FIG. 1A.

[0015] FIG. 3 shows non-limiting examples of anilidopiperidine analogs as tails of the ORLs of the present invention.

[0016] FIG. 4 shows GTPyS activity of LYS739 (SEQ ID NO: 10), U50,488, and Naloxone at KOR. U50,488 is known to have agonist activity at KOR. Naloxone is known to have antagonist activity at KOR. LYS739 (SEQ ID NO: 10) appears to have partial agonist/antagonist activities at KOR.

[0017] FIG. 5A and FIG. 5B show von Frey tests and IR tests of LYS739 (SEQ ID NO: 10). Reversal of thermal hyperalgesia and tactile allodynia is observed with LYS739 (SEQ ID NO: 10) at 10 µg/5µl in L5/L6 SNL-operated male SD rats.

[0018] FIG. 6A shows [³⁵S]GTPyS assays: MOR (left) and DOR (right) antagonist modes. LYS739 (SEQ ID NO: 10), LYS744 (SEQ ID NO: 15), and MR115 (SEQ ID NO: 28) do not possess antagonist activity at MOR and DOR.

[0019] FIG. 6B shows [³⁵S]GTPyS assays: KOR agonist (left) and antagonist (right) modes. LYS54 (SEQ ID NO: 9), LYS644 (SEQ ID NO: 14), and MR121 (SEQ ID NO: 126) are partial agonist/antagonist at KOR. CYF132 (SEQ ID NO: 13) is observed as a partial agonist at KOR.

[0020] FIG. 7 shows examples of ORL design for MOR/DOR agonist and KOR antagonist activities.

[0021] FIG. 8 shows examples of ORL design for MOR agonist and KOR antagonist activities.

[0022] FIG. 9 shows a HPLC profile showing the stability of LYS739 (SEQ ID NO: 10) in human plasma. LYS739 (SEQ ID NO: 10) is stable in human plasma.

[0023] FIG. 10 shows the effects of fentanyl analogs, LYS436, LYS739 and LYS416 and biphalin on H/A and reoxygenation challenge. For both the graphs; '*' compared to no drug treated group; '#' compared to biphalin treated group; *p<0.05, ***p<0.001, ****p<0.0001; #p<0.05, ##p<0.01; data from 3 to 4 independent primary neuron isolations with 2-3 replicates treatment per isolation. Compared to normoxic and 0.1% tritonX, all experimental groups were significantly different (p<0.0001). A) MTT assay: Effect of fentanyl analogs LYS436, LYS739 and LYS416 and biphalin on

3 hr H/A ad 24 hr reoxygenation. Compared to no drug treated group, LYS436 ($p<0.0001$), LYS739 ($p<0.0001$), LYS416 ($p<0.0001$), biphalin ($p<0.001$) and fentanyl ($p<0.05$) significantly increased neuronal survival. Again, compared to biphalin, LYS739 ($p<0.01$) and LYS416 ($p<0.05$) showed better neuroprotection in terms of neuronal cell survival. LYS436 ($p<0.05$), LYS739 ($p<0.0001$) and LYS416 ($p<0.001$) demonstrated better neuronal survival compared to fentanyl alone. NTX reversed the effect of LYS436, LYS739, LYS416 and biphalin. B) LDH assay: Relative neuronal death in terms of LDH production was assessed upon 3 hr H/A and 24 hr reoxygenation. Fentanyl analogs LYS436 ($p<0.001$), LYS739 ($p<0.0001$) and LYS416 ($p<0.0001$) and biphalin ($p<0.001$) and fentanyl ($p<0.05$) significantly decreased neuronal cell death compared to no drug treated group. LYS739 ($p<0.05$) significantly decreased neuronal cell death in comparison to biphalin. LYS739 ($p<0.001$) and LYS416 ($p<0.01$) showed better neuroprotection compared to fentanyl alone. NTX reversed the effect of LYS436, LYS739, LYS416 and biphalin.

[0024] FIG. 11 shows the effects of fentanyl analogs, LYS436, LYS739 and LYS416 and biphalin on NMDA challenge. For both the graphs; '*' compared to no drug treated group; '#' compared to biphalin treated group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; # $p<0.05$, ## $p<0.01$; data from 3 to 4 independent primary neuron isolations with 2-3 replicates treatment per isolation. All experimental groups were significantly different ($p<0.0001$) compared to normoxia and 0.1% tritonX. A) MTT assay: effects of fentanyl analogs and biphalin (10 nM) on primary cortical neuron with NMDA (50 μ M) exposure for 3 hr assessed by relative neuronal survival. LYS436 ($p<0.0001$), LYS739 ($p<0.0001$), LYS416 ($p<0.001$), biphalin ($p<0.01$) and fentanyl ($p<0.05$) significantly improved relative neuronal survival compared to no drug treated group. Effect of LYS739 ($p<0.01$) and LYS436 ($p<0.05$) were significantly better than biphalin. LYS436 ($p<0.01$) and LYS739 ($p<0.001$) also increased neuronal survival when compared to fentanyl alone. NTX reversed the effect of LYS436, LYS739, LYS416 and biphalin. B) LDH assay: effects of fentanyl analogs and biphalin (10 nM) on primary cortical neuron with NMDA (50 μ M) exposure for 3 hr assessed by relative neuronal death. In comparison to no drug treated group, LYS436 ($p<0.0001$), LYS739 ($p<0.0001$), LYS416 ($p<0.01$), biphalin ($p<0.0001$) and fentanyl ($p<0.05$) significantly decreased relative neuronal death. LYS739 ($p<0.05$) and LYS436 ($p<0.05$) showed better neuroprotection compared to biphalin. Compared to fentanyl alone, LYS436 ($p<0.0001$) and LYS739 ($p<0.0001$)

displayed better neuroprotection in terms of LDH production. NTX reversed the effect of LYS436, LYS739, LYS416, biphalin and fentanyl.

[0025] FIG. 12 shows the effects of fentanyl analogs and biphalin on primary cortical neuronal ROS production upon exposure to 3 hr H/A and 24 hr reoxygenation. ('*' compared to no drug treated group; '#' compared to biphalin treated group; * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, # $p < 0.05$; data from 3 to 4 independent primary neuron isolations with 2-3 replicates treatment per isolation). All experimental groups were significantly different compared to normoxia ($p < 0.0001$) and H₂O₂ ($p < 0.001$). LYS436 ($p < 0.001$), LYS739 ($p < 0.001$), LYS416 ($p < 0.01$) and biphalin ($p < 0.05$) significantly decreased ROS production compared to no drug treated group. LYS739 ($p < 0.05$) showed better neuroprotection compared to biphalin in terms of ROS production. In comparison to fentanyl alone, LYS436 ($p < 0.001$) and LYS739 ($p < 0.001$) significantly reduced ROS production. NTX reversed the effect of biphalin, LYS436, LYS739 and LYS416.

[0026] FIGs. 13A, B, and C show the effects of fentanyl analog LYS739 and biphalin (5 mg/kg, I.P. administration, 10 min after reperfusion), fentanyl (0.2 mg/kg, I.P. administration, 10 min after reperfusion) and non-selective OR antagonist NTX (1 mg/kg, I.P. administration, 10 min before surgery) or vehicle (0.9% saline) on edema and infarct formation in transient MCAO (60 min occlusion and 24 hr reperfusion). A) Representative TTC staining of brain slices from vehicle and drug treated mice. B) Brain edema ratio of brain in vehicle and drug treated groups. Fentanyl analog LYS739 ($p < 0.05$) and biphalin ($p < 0.05$) significantly decreased edema formation compared to vehicle treated group. In comparison to fentanyl alone, both LYS739 ($p < 0.05$) and biphalin ($p < 0.05$) significantly reduced edema formation. NTX reversed the effect of both biphalin ($p < 0.05$) and LYS739 ($p < 0.05$). NTX and FENT alone did not show any significant effect compared to vehicle treated group. C) Brain infarct ration in vehicle and drug treated mice. In comparison to vehicle treated group, fentanyl analog LYS739 ($p < 0.0001$) and biphalin ($p < 0.0001$) significantly reduced infarct formation in mice. Fentanyl and NTX alone did not show any improvement compared to saline treated group. Both biphalin ($p < 0.0001$) and LYS739 ($p < 0.0001$) decreased infarct formation compared to fentanyl alone. NTX reversed the effect of biphalin ($p < 0.0001$) and LYS739 ($p < 0.0001$). ('*' compared to vehicle treated group; * $p < 0.05$; **** $p < 0.0001$; numbers indicated in the parenthesis in the figure columns denote to the number of experimental animals per group).

[0027] FIG. 14 shows the neurological score evaluation of mice 24 hr after ischemia and drug treatment. Both biphalin ($p<0.05$) and LSY739 ($p<0.05$) improved neurological behavior compared to vehicle treated group whereas FENT and NTX alone did not improve any neurological score compared to vehicle treated group. NTX reversed the effect of biphalin ($p<0.05$) and fentanyl analog LYS739 ($p<0.05$). “*” compared to vehicle treated group; * $p<0.05$; numbers indicated in the parenthesis in the figure columns denote to the number of experimental animals per group).

DESCRIPTION OF PREFERRED EMBODIMENTS

[0028] Referring now to FIG. 1-14, the present invention features multifunctional opioid receptor ligands (ORLs), acting as MOR agonists, DOR agonists, and KOR antagonists (or partial agonists). The present invention also features methods of use of said multifunctional ORLs, e.g., methods of treating pain or other conditions using peptides of the present invention.

[0029] FIG. 1 shows the well-known structure-activity relationship (SAR) results of Dynorphin A (Dyn A) and enkephalins for opioid activities. Enkephalins shown are Leu-Enk (YGGFL, SEQ ID NO: 1) and Met-Enk (YGGFM, SEQ ID NO: 2). Dyn A is an endogenous kappa opioid receptor (KOR) ligand. The sequence for Dyn A is YGGFLRRIRPKLKWDNQ (SEQ ID NO: 3). (Note that the first five amino acids of Dyn A is Leu-Enk). Other peptides tested include a peptide containing the first 13 amino acids of Dyn A (Dyn A 1-13, YGGFLRRIRPKLK (SEQ ID NO: 4)), a peptide containing the first 8 amino acids of Dyn A (Dyn A 1-8, YGGFLRRI (SEQ ID NO: 5)), and Dyn B (YGGFLRRNFLVVT (SEQ ID NO: 6)). Without wishing to limit the present invention to any theory or mechanism, it appears that KOR selectivity decreases as the C-terminal residues of Dyn A are removed (e.g., Dyn A is more selective for KOR than is Leu-Enk). Without wishing to limit the present invention to any theory or mechanism, it is thought that residues following the first four amino acids of enkephalin, e.g., the residue(s) following the Phe/F residue of the enkephalin (or derivative) may be a region that helps make the ORL active for KOR, e.g., the residues following the first four amino acids of the enkephalin (or derivative thereof) may provide specificity for KOR.

[0030] The ORLs of the present invention comprise a peptide portion, e.g., a peptide analog derived from enkephalins (e.g., Leu-Enk (YGGFL, SEQ ID NO: 1) or

Met-Enk (YGGFM, SEQ ID NO: 2)) and a tail portion linked to the C-terminus of the peptide portion. In some embodiments, the peptide portion comprises four residues (e.g., amino acids, analogs or derivatives thereof), occupying position 1, 2, 3, and 4. In some embodiments, the peptide portion comprises three residues (e.g., amino acids, analogs or derivatives thereof), occupying position 1, 2, and 4. The peptide portion may be based on the enkephalin sequence e.g., Leu-Enk (YGGFL, SEQ ID NO: 1) or Met-Enk (YGGFM, SEQ ID NO: 2).

[0031] In some embodiments, the tail portion comprises a lipophilic molecule (e.g., a 4-anilidopiperidine moiety), e.g., the tail portion may comprise a residue or compound that increases the lipophilicity of the peptide portion. In some embodiments, the tail comprises a N-phenyl-N-piperidin-4-ylpropionamide (Ppp) moiety. In some embodiments, the tail comprises -NH₂. Other non-limiting examples of tail portion molecules (tail compounds) are shown in FIG. 3.

[0032] FIG. 2 and FIG. 2A show examples of schematic representations of ORLs of the present invention. For reference in FIG. 2, Dmt refers to 2'-6'-dimethyltyrosine, DXxx refers to a D amino acid, and X refers to a halogen or other appropriate compound, e.g., H, Cl, F, or a methyl group. N-phenyl-N-piperidin-4-ylpropionamide (Ppp) is shown as the tail. Referring to FIG. 2 and FIG. 2A, in some embodiments, residue 1 (Dmt, as shown in FIG. 2 or Aaa in FIG. 2A) comprises Dmt or Tyr. In some embodiments, residue 2 (DXxx, as shown in FIG. 2 or DBbb in FIG. 2A) comprises DAla, DNle (D-norleucine), Pro, or DArg. In some embodiments, residue 3 (Gly, as shown in FIG. 2 or Ccc in FIG. 2A) comprises Gly, Phe, Phe(X), or Nal, wherein X may refer to H, Cl, F, methyl group, or any other appropriate modification of Phe. In some embodiments, residue 3 is absent. In some embodiments, residue 4 (Phe(X) as shown in FIG. 2 or Ddd in FIG. 2A) comprises Gly, Phe, Phe(X), wherein X may refer to H, Cl, F, methyl group, or any other appropriate modification of Phe. In some embodiments, the tail (e.g., shown as Eee in FIG. 2A) comprises Ppp or NH₂. The present invention is not limited to the formula or substitutions shown in FIG. 2 or FIG. 2A. For reference, DTic refers to D-tetrahydroisoquinoline-3-carboxylic acid.

[0033] Table 1 below shows non-limiting examples of ORLs of the present invention. Note that the Phe residues in SEQ ID NO: 10, SEQ ID NO: 11, and SEQ

ID NO: 12 are halogenated with F, and the Phe residue in SEQ ID NO: 15 is halogenated with Cl.

Table 1. Examples of ORLs

SEQ ID NO:	Compound	Residue (Position from N-terminus to C-terminus)			
		1	2	3	4
7	LYS729	Tyr	DAla	Gly	Phe-NH ₂
8	LYS544	Tyr	DAla	Gly	Phe-Ppp
9	LYS540	Dmt	DAla	Gly	Phe-Ppp
10	LYS739	Dmt	DNle	Gly	Phe(F)-Ppp
11	MR106	Tyr	DNle	Gly	Phe(4-F)-NH ₂
12	MR107	Dmt	DNle	Gly	Phe(F)-NH ₂
13	CYF132	Dmt	DNle	Gly	Phe-NH ₂
14	LYS644	Dmt	DNle	Gly	Phe-Ppp
15	LYS744	Dmt	DNle	Gly	Phe(4-Cl)-Ppp
16	LYS702	Dmt	DTic		Phe-Ppp
17	CYF136	Dmt	DNle	Gly	Phe(4-Cl)-NH ₂
18	MR119	Dmt	DNle	Gly	Phe(Br)-Ppp
19	MR111* (*see note below)	Dmt	DNle	Homocys	Phe(4-F)-Ppp
20	MR112* (*see note below)	Dmt	Homocys	Gly	Phe(4-F)-Ppp
21	MR124	Dmt	DArg	Phe	Gly-Ppp
22	MR125	Dmt	DArg	1Nal	Gly-Ppp
23	MR110	Dmt	DArg	Phe	Lys-Ppp
24	MR120	Dmt	DArg	1Nal	Lys-Ppp
25	MR122	Dmt	DArg		1Nal-Ppp
26	MR121	Dmt	DArg		Phe(4-Cl)-Ppp
27	MR114	Dmt	Pro	Trp	Phe(4-Cl)-Ppp
28	MR115	Dmt	Pro	Phe	Phe(4-Cl)-Ppp
29	MR116	Dmt	Pro	Phe(4-Cl)	Phe(4-Cl)-Ppp
30	MR123	Dmt	Pro	Gly	Phe(4-Cl)-Ppp

[0034] Note MR111 comprises two units of SEQ ID NO: 19, e.g., MR111 comprises (Dmt-DNle-Homocys-Phe(4-F)-Ppp)₂. MR112 comprises two units of SEQ ID NO: 20, e.g., MR112 comprises (Dmt-Homocys-Gly-Phe(4-F)-Ppp)₂.

[0035] The ORLs of the present invention may be synthesized as appropriate (see, for example, Lee et al., 2011, J. Med. Chem. 54:882-886). For example, the ORLs of the present invention may be synthesized by a protocol for liquid phase peptide synthesis (LPPS), e.g., using Boc-chemistry in high yields. In some embodiments, halogen modification on the aromatic ring is on the para position, e.g., to help avoid unfavorable steric hindrance.

[0036] Table 2 shows analytical data of various multifunctional ORLs of the present invention with a Ppp group at the C-terminus. ^aFAB-MS (JEOL HX110 sector instrument) or MALDI-TOF. ^bPerformed on a Hewlett Packard 1100 [C-18, Vydac, 4.6 mm x 250 mm, 5 μm, 10-100% of acetonitrile containing 0.1% TFA within 45 min, 1 mL/min]. ^c<http://www.vcclab.org/lab/alogps/>. ^dLow resolution-Mass. ^eRetention time. n.d. not determined.

Table 2

ORL/ SEQ ID NO:	Structure	Molecular Formula	HR MS ^a (M-TFA + H) ⁺		HPLC ^b % ACN	ALOGPs ^c
			observed	calculated		
LYS729/ 7	Tyr-DAla-Gly-Phe-NH ₂	C ₂₃ H ₂₈ N ₂ O ₅	456.2239	456.2246	14.0 ^e	0.32
MR106/ 11	Tyr-DNle-Gly-Phe(4-F)-NH ₂	C ₂₆ H ₂₄ FN ₂ O ₅	n.d.	515.2544	39.64	1.36
MR107/ 12	Dmt-DNle-Gly-Phe(4-F)-NH ₂	C ₂₈ H ₂₀ FN ₂ O ₅	n.d.	543.2857	41.39	1.75
CYF136/ 17	Dmt-DNle-Gly-Phe(4-Cl)-NH ₂	C ₂₈ H ₂₆ ClN ₂ O ₅	560.2653	560.2640	16.0 ^e	2.36
CYF132/ 13	Dmt-DNle-Gly-Phe-NH ₂	C ₂₃ H ₂₈ N ₂ O ₅	526.3035	526.3030	16.5	1.69
LYS544/ 8	Tyr-DAla-Gly-Phe-Ppp	C ₂₇ H ₃₈ N ₄ O ₆	671.3579	671.3557	19.1 ^e	2.80
LYS540/ 9	Dmt-DAla-Gly-Phe-Ppp	C ₂₉ H ₃₀ N ₄ O ₆	699.3852	699.3870	20.1 ^e	2.96
LYS644/ 14	Dmt-DNle-Gly-Phe-Ppp	C ₄₂ H ₃₂ N ₆ O ₆	741.4325	741.4340	19.3 ^e	3.66
LYS739/ 10	Dmt-DNle-Gly-Phe(F)-Ppp	C ₄₂ H ₂₉ FN ₆ O ₆	759.4247	759.4245	20.0 ^e	3.74
LYS744/ 15	Dmt-DNle-Gly-Phe(4-Cl)-Ppp	C ₄₂ H ₂₈ ClN ₆ O ₆	775.3995	774.3950	53.52	4.18
LYS702/ 16	Dmt-DTic-Phe-Ppp	C ₄₄ H ₃₀ ClN ₆ O ₅	764.3632	765.3658	23.0 ^e	4.91
MR111/ 19	(Dmt-DNle-Homocys-Phe(4-F)-Ppp) ₂	C ₂₈ H ₁₁₆ F ₂ N ₁₂ O ₁₂ S ₂	1635.5 ^d	1635.8324	62.24	5.58
MR112/ 20	(Dmt-Homocys-Gly-Phe(4-F)-Ppp) ₂	C ₂₈ H ₁₀₆ F ₂ N ₁₂ O ₁₂ S ₂	1524.4 ^d	1523.7072	56.70	4.89

MR121/ 26	Dmt-DArg-Phe(4-Cl)-Ppp	C ₃₀ H ₃₅ ClN ₆ O ₅	761.3 ^d	761.3906	50.56	3.00
MR114/ 27	Dmt-Pro-Trp-Phe(4-Cl)-Ppp	C ₃₀ H ₃₅ ClN ₇ O ₅	888.6 ^d	888.4216	62.00	5.20
MR116/ 29	Dmt-Pro-Phe(4-Cl)-Phe(4-Cl)-Ppp	C ₃₅ H ₃₈ ClN ₈ O ₅	883.37	883.3717	57.83	5.23

[0037] As shown in Table 3, Table 4, and FIG. 4 it was surprisingly discovered that the ORL LYS739 (SEQ ID NO: 10, Dmt-DNle-Gly-Phe(*p*-F)-Ppp) interacts with KOR ($K_i = 0.70$ nM) as well as MOR ($K_i = 0.02$ nM) and DOR ($K_i = 0.40$ nM). Considering well known structure-activity relationships (SAR) of enkephalin analogues, the sub nanomolar range of binding affinity of LYS739 (SEQ ID NO: 10) at the KOR was unexpected and could not be predicted. LYS739 (SEQ ID NO: 10) turned out to be the first potent MOR/DOR agonist (IC_{50} : 0.26 nM, and 0.37 nM in GPI, and MVD, respectively) and KOR partial agonist/antagonist among ORLs. In GTP- γ -assay, LYS739 (SEQ ID NO: 10) showed mixed partial agonist ($EC_{50} = 21$ nM, $E_{max} = 39\%$) / antagonist activity ($EC_{50} = 60$ nM, $E_{max} = 65\%$) for KOR. Note that in Table 4, potency and efficacy reported as mean $\pm$ SEM from each experiment (n=3) independent experiments for both modes; curves use the mean value of each point from each experiment combined together; n.d. is not determined.

Table 3. Binding Affinities of Enkephalin Analogs at MOR, DOR, and KOR

SEQ ID NO:	Compound	K _i (nM)		
		MOR [³ H]DAMGO	DOR [³ H]DPDPE	KOR [³ H]Nor-BNI
7	LYS729	2.8	300	220
8	LYS544	26	5.2	190
9	LYS540	0.38	0.36	n/d
10	LYS739	0.02	0.4	0.7
11	MR106	n.d	n.d	210
12	MR107	n.d	n.d	0.11
13	CYF132	n.d	n.d	
14	LYS644	0.39	0.18	n.d
15	LYS744	0.1	0.08	n.d
16	LYS702	0.45	0.76	n.d

Table 4. Functional Activities of LYS739 (SEQ ID NO: 10) at MOR, DOR, and KOR						
[³⁵ S]GTP-γ-S binding assay				IC ₅₀ (nM)		
Agonist EC ₅₀ (nM) (E _{max} %)			Antagonist IC ₅₀ (nM) (I _{max} %)			
hDOR [³ H]DAMGO	rMOR [³ H]DPDPE	hKOR [³ H]U50,488	hKOR [³ H]naloxone	GPI (μ)	MVD (δ)	GPI (κ)
0.07 (48%)	0.29 (98%)	21 (39%)	60 (65%)	0.26	0.37	n.d.

[0038] Preliminary *in vivo* studies of LYS739 (SEQ ID NO: 10) showed that intrathecal (i.th.) administration of LYS739 (SEQ ID NO: 10) at 10 μg/5μl in L₅/L₆ SNL-operated male SD rats can reverse thermal hyperalgesia in nerve injured animals (FIG. 5A, left) and reverse tactile allodynia (FIG. 5A, right). In FIG. 6A, statistical significance was determined by 95% confidence interval (*P < 0.05 compared with pre-dose SNL baseline vehicle; #p < 0.05 compared with the vehicle at the same time point; n>6). Vehicle was DMSO/Tween 80/Saline (1:1:8). FIG. 5B shows that intravenous (i.v.) administration of LYS739 (SEQ ID NO: 10) (3 mg/mL/Kg) in L₅/L₆ SNL-operated male SD rats shows reversal of thermal hyperalgesia. This represents high potency of analgesic effects through MOR (and DOR).

[0039] For reference, Table 5 lists examples of ORLs with various tail portions (e.g., NH₂ and Tail Compounds 1-5). Structures of the Tails (e.g., anilidopiperidine moieties) can be found in FIG. 3. Table 5 also shows lipophilicity values and MOR/DOR agonist activities of the ORLs. Note that SEQ ID NO: 8 refers to both LYS544 and LYS436.

Table 5. MOR/DOR agonist activities of C-terminal modified lipophilic enkephalin analogues			
ORL	Tail	aLogP	K _i (nM) MOR/DOR
LYS729 Tyr-DAla-Gly-Phe-NH ₂ (SEQ ID NO: 7)	-NH ₂	0.32	2.8/300
LYS416 Tyr-DAla-Gly-Phe-Tail (SEQ ID NO:31)	4-Anilidopiperidine analogue 1	2.93	14/14
LYS620 Tyr-DAla-Gly-Phe-Tail (SEQ ID NO:32)	4-Anilidopiperidine analogue 2	2.63	1.2/3.7

LYS429 Tyr-DAla-Gly-Phe-Tail (SEQ ID NO:33)	4-Anilidopiperidine analogue 3	4.04	1.1/6.1
LYS544 (or LYS436) Tyr-DAla-Gly-Phe-Ppp (SEQ ID NO: 8)	4-Anilidopiperidine analogue 4 (Ppp)	2.80	23/0.69
LYS437 Tyr-DAla-Gly-Phe-Tail (SEQ ID NO:34)	4-Anilidopiperidine analogue 5	2.13	5.7/3.2

[0040] The present invention also features ORLs that are derived from LYS739 (SEQ ID NO: 10), e.g., LYS739 analogs. In some embodiments, the ORLs are obtained by modifying LYS739 (SEQ ID NO: 10) by substitution, dimerization, and/or cyclization. Modifications may involve the incorporation of an unnatural amino acid and/or constrained amino acids. For example, in some embodiments, Dmt is substituted with trimethyltyrosine (Tmt). In some embodiments, the ORL comprises 2-methyl-3-(2',6'-dimethyl-4'-hydroxyphenyl)-propionic acid (Mdp).

[0041] In some embodiments, the ORL comprises a bivalent ligand. In some embodiments, a disulfide bond is used to link two monomeric pharmacophores. For example, a disulfide bond may be used through a homocysteine residue at position 2 (or 3). In some embodiments, ORLs comprise cyclic structures, e.g., the ORLs are cyclic and retain the pharmacophoric structure for the receptors within a constrained structure, e.g., since linear peptide ligands can be flexible even with multiple modifications due to high flexibility of enkephalins. Cyclization may be through the formation of various bonds such as a disulfide and a lactam, but is not limited to these mechanisms. See FIG. 7 for examples of ORL design.

[0042] In some embodiments, the ORLs are bifunctional ligands. In some embodiments, the ORLs are trifunctional ligands. In some embodiments, ORLs are constructed using endomorphin-1 (EM-1) and/or DALDA (D-Arg², Lys⁴]dermorphin. FIG. 8 shows examples of ORL design using EM-1 (Tyr-Pro-Trp-Phe-NH₂, SEQ ID NO: 35) and DALDA (Tyr-DArg-Phe-Lys-NH₂, SEQ ID NO: 36).

[0043] Various ORLs (e.g., analogs of LYS739 (SEQ ID NO: 10)) were tested for their binding affinities at MOR, DOR, and KOR using [³H]-Diprenorphine in the membranes of Chinese Hamster Ovary (CHO) cells expressing the relevant human opioid receptor. Analogues with particular binding affinity (K_i < 10 nM for MOR and

DOR; $K_i < 30$ nM for KOR) as well as others were tested for receptor functional activity in the [^{35} S]-GTP γ S assay. In this assay, antagonist activity at all three receptors expressed in CHO cells were determined by the inhibition of stimulation caused by 100 nM of control agonist (DAMGO for MOR, SNC80 for DOR, U50,488 for KOR) in a 96-well plate. Table 6 summarized in vitro biological activities of multifunctional ligands at MOR, DOR, and KOR with a Ppp group at the C-terminus. (Note: ^a=Competition analyses were carried out using membrane preparations from transfected HNB9.10 cells that constitutively expressed the respective receptor types; ^b= [^{3} H]DAMGO, $K_d = 0.85$ nM; ^c= [^{3} H]DPDPE, $K_d = 0.50$ nM; ^d= [^{3} H]U69,593, $K_d = 5.3$ nM; ^e=Expressed in CHO cells; ^f=Mean $\pm$ SEM of the % relative to 10 μ M U50,488 stimulation; ^g=Mean $\pm$ SEM of the % relative to 10 μ M naloxone inhibition of 100 nM U50,488; ^h=at 10 μ M.

TABLE 6

SEQ ID NO:	ligand	K_i , nM ^a			KOR ^e [^{35} S]GTP γ S-binding				KOR function
		MOR ^a	DOR ^a	KOR ^d	EC ₅₀ , nM	E _{max} , % ^f	IC ₅₀ , nM	I _{max} , % ^g	
7	LYS729	2.8	300	2000	-	< 30 ^h	-	< 10 ^h	no function
11	MR106	2.3	0.44	7.8	-	70 ^h			weak agonist
12	MR107	4.5	0.99	604	10	62	250	37	partial agonist/antagonist
17	CYF136	2.9	0.61	156	7.0	18	66	60	partial agonist/antagonist
13	CYF132	1.1	0.25	3.4	84	59	n.c. ^a	-	partial agonist
8	LYS544	23	0.69	2000	-	< 10 ^h	-	< 10 ^h	no function
9	LYS540	0.38	0.36	21	540	40	630	49	partial agonist/antagonist
14	LYS644	0.39	0.18	77	260	53	290 ^a	70	partial agonist/antagonist
10	LYS739	0.02	0.40	0.70	21	39	60	65	partial agonist/antagonist
15	LYS744	0.10	0.08	1.4	-	< 10% ^h	52	122	antagonist
16	LYS702	0.45	0.76	2000	-	< 10% ^h			no function
19	MR111	0.02	2.6	220					MOR selective
20	MR112	n.c.	9.9	n.c.					DOR selective
26	MR121	1100	960	62	470	32	450 ^a	38	partial agonist/antagonist
27	MR114	4700	68	200					DOR selective
29	MR116	990	30	n.c.					DOR selective

[0044] Analogues were tested for their activity at KOR, and GTPyS assays were performed at the MOR and DOR for LYS739 (SEQ ID NO: 10) and LYS744 (SEQ ID NO: 15) (see FIG. 6A). The assay results shows that two ligands are pure agonists for the MOR and DOR. GTPyS assays showed that LYS540 (SEQ ID NO: 9) and LYS644 (SEQ ID NO:14) are partial agonist/antagonist for the KOR, which has a potential to reduce KOR related side effects (see FIG. 6B). MR107 (SEQ ID NO: 12) and CYF136 (SEQ ID NO: 17) also revealed partial agonist/antagonist activities.

[0045] The present invention also features ORLs having half lives longer than 4 , etc. For example, in some embodiments, the ORL has a half life longer than 1 hour. In some embodiments, the ORL has a half life longer than 2 hours. In some embodiments, the ORL has a half life longer than 3 hours. In some embodiments, the ORL has a half life longer than 4 hours. In some embodiments, the ORL has a half life longer than 5 hours. In some embodiments, the ORL has a half life longer than 10 hours. In some embodiments, the ORL has a half life longer greater than 24 hours. For example, FIG. 9 shows LYS739 (SEQ ID NO: 10) is stable in a metabolic condition, and there was no trace of degradation observed after a 4 day incubation.

[0046] In some embodiments, the ORL is 4 amino acids in length. In some embodiments, the ORL is 5 amino acids in length. In some embodiments, the ORL is 6 amino acids in length. In some embodiments, the ORL is 7 amino acids in length. In some embodiments, the ORL is 8 amino acids in length. In some embodiments, the ORL is 9 amino acids in length. In some embodiments, the ORL is 10 amino acids in length. In some embodiments, the ORL is more than 10 amino acids in length.

[0047] In some embodiments, the ORL is between 4 to 6 amino acids in length. In some embodiments, the ORL is between 4 to 7 amino acids in length. In some embodiments, the ORL is between 4 to 8 amino acids in length. In some embodiments, the ORL is between 4 to 9 amino acids in length. In some embodiments, the ORL is between 4 to 10 amino acids in length. In some embodiments, the ORL is between 4 to 20 amino acids in length. In some embodiments, the ORL is between 4 to 30 amino acids in length. In some embodiments, the ORL is between 4 to 40 amino acids in length. In some embodiments, the ORL is between 4 to 50 amino acids in length.

[0048] Example 1

[0049] Example 1 describes non-limiting approaches to designing ORLs.

[0050] Step 1: Discover pharmacophoric structures of EM-1 and DALDA for MOR agonist/KOR antagonist activities. The C-terminus of EM-1 and DALDA may be modified with Ppp(R) (the R group may be decided by SAR results). This modification may improve their lipophilicities (aLogP increase > 2) and metabolic stabilities, and thus afford high potential of BBB penetration. This modification may cause a biological profile change. The Ppp(R) group may be kept at the C-terminus, and the other positions may be modified. Substitution of Tyr with 2',6'-dimethyltyrosine (Dmt) in opioid peptides can increase opioid activities dramatically, thus a Tyr¹ residue may be replaced in both ligands with a Dmt residue or a β -methyl-2,6-dimethyltyrosine (Tmt) residue, which is more sterically hindered due to an extra methyl group. EM-1 and DALDA have distinct primary structures in positions 2, 3, and 4 but a Phe residue in common. The Phe residue in both ligands may be substituted with Phe(p-X) for altering receptor selectivity and inducing KOR interactions. A Phe³ residue in DALDA may also be substituted with a Phe(p-X) residue to observe SAR. However, to conserve its MOR selectivity over DOR, positions 2 and 4 of DALDA may be limited to basic amino acid residues. Likewise, position 2 of EM-1 may be limited to turn making amino acid residue. A Trp³ residue in EM-1 may be modified with other aromatic amino acid residues.

[0051] Step 2: Build dimerized ligands of MOR agonist/KOR antagonist using pharmacophores discovered in the first step. Position 2 and 4 of EM-1 and DALDA, respectively, may be consumed. Two homo pharmacophores may be linked through a disulfide bond of homocysteine residue. Cyclic bifunctional ligands may be designed. Insertion (l, m, and/or n= 1) or deletion (l, m, and/or n= 0) of Bbb, Ccc, and Ddd may optimize the distance between two aromatic rings, which may be the most important factor of high potency and selectivity.

[0052] Example 2 – Multifunctional ORLs as neuroprotectants for ischemic stroke treatment

[0053] Ischemic stroke is one of the leading causes of mortality and morbidity in the world. Example 2 describes the evaluation of multifunctional ORLs, e.g., LYS436 (SEQ ID NO: 8), LYS739 (SEQ ID NO: 10) and LYS416 (YGGF-Ppp, SEQ ID NO: 37), for their neuroprotective potential using *in vitro* and *in vivo* ischemic models. *In*

vitro, neuronal death and total reactive oxygen species level, upon exposure to hypoxia- aglycemia followed by reoxygenation or challenged with NMDA was significantly decreased when treated with non-selective opioid agonists compared to no drug treatment group. Fluorinated enkephalin- fentanyl conjugate, LYS739 (SEQ ID NO: 10) showed better neuroprotection in all *in vitro* ischemic models compared to biphalin. An *in vivo* mouse middle cerebral artery occlusion (MCAO) stroke model was utilized to screen biphalin and LYS739 (SEQ ID NO: 10). Both agonists significantly decreased brain infarct ratio and edema ration measured with TTC staining compared to saline treated group. Neuronal deficit was improved in terms of neurological score and locomotor activity with LYS739 (SEQ ID NO: 10) and biphalin treatment. All enkephalin fentanyl conjugates and biphalin demonstrated better neuroprotection compared to fentanyl treated groups. Neuroprotective effects of biphalin and multivalent analogs were reversed, in most cases, by naltrexone, a non-selective opioid antagonist. This suggests that LYS739 (SEQ ID NO: 10) is a potential neuroprotective agent for ischemic stroke.

[0054] Primary cortical neuron survival upon exposure to 3 hr H/A and 24 hr reperfusion in presence or absence of fentanyl analogs and biphalin (10 nM) was determined using MTT (see FIG. 10A) and LDH (see FIG. 10B) assays. In MTT assay, fentanyl analogs, LYS436 (57.9% more neuronal survival, $p < 0.0001$), LYS739 (68.1% more neuronal survival, $p < 0.0001$) and LYS416 (66.4% more neuronal survival, $p < 0.0001$) and biphalin (42.6% more neuronal survival, $p < 0.001$) and fentanyl (28.7% more neuronal survival, $p < 0.05$) reproducibly improved neuronal survival compared to no drug treatment group. The protective effect of fentanyl analogs, LYS436 ($p > 0.05$), LYS739 ($p < 0.01$) and LYS416 ($p < 0.05$) were significantly better than that of biphalin. They also showed better neuroprotection (LYS436: $p < 0.05$, LYS739: $p < 0.0001$ and LYS416: $p < 0.001$) compared to fentanyl itself. Among the analogs, LYS739 showed the most significant activity in terms of neuronal survival. Likewise, LDH assay showed reproducible, statistically significant neuroprotection upon treatment with biphalin (30.5% less LDH release, $p < 0.001$), LYS436 (29.37% less LDH release, $p < 0.001$), LYS739 (45.7% less LDH release, $p < 0.0001$), LYS416 (41.28% less LDH release, $p < 0.0001$) and FENT (21.59% less LDH release, $p < 0.05$) compared to no drug treated group. In comparison to biphalin, LYS739 ($p < 0.05$) showed less neuronal death upon H/A and reoxygenation

exposure. The fentanyl analogs showed less neuronal death compared to fentanyl itself. Notably, non-selective OR antagonist NTX reversed the effect of biphalin and fentanyl analogs in both assays. No statistical significant difference was found for NTX treated group compared to no drug treated group in both assays.

[0055] The effect of three fentanyl analogs, LYS436, LYS739 and LYS416 and biphalin and fentanyl (10 nM) were evaluated in primary cortical neurons exposed to 50 μ M NMDA for 3 hours followed by 24 hours normal condition media exposure. Relative neuronal survival and cytotoxicity were quantified using MTT (see FIG. 11A) and LDH (see FIG. 11B) assay, respectively. MTT assay showed that LYS436 (52.1% more neuronal survival, $p < 0.0001$), LYS739 (54.7% more neuronal survival, $p < 0.0001$), LYS416 (43.4% more neuronal survival, $p < 0.001$), biphalin (28.7% more neuronal survival, $p < 0.01$) and fentanyl (22.6% more neuronal survival, $p < 0.05$), which was statistically significant when compared to no drug treated group. Compared to biphalin, fentanyl analog LYS436 ($p < 0.05$) and LYS739 ($p < 0.01$) showed better neuroprotection in terms of neuronal survival quantified with MTT assay kit. These two analogs, LYS436 ($p < 0.01$) and LYS739 ($p < 0.001$) also increased neuronal survival when compared to fentanyl itself. Similar reproducible results were observed when the neuroprotective effect was evaluated with an LDH assay kit. With LDH assay, LYS436 (27.5% less LDH release, $p < 0.0001$), LYS739 (28.6% less LDH release, $p < 0.0001$), LYS416 (12.9% less LDH release, $p < 0.01$), biphalin (18.4% less LDH release, $p < 0.0001$) and fentanyl (10.2% less LDH release, $p < 0.05$) showed statistically significantly increased neuroprotection compared to no drug treated group. Compared to biphalin, LYS436 ($p < 0.05$) and LYS739 ($p < 0.05$) treated neurons released less LDH denoting a more potent effect than biphalin. LYS436 ($p < 0.0001$) and LYS739 ($p < 0.0001$) also showed better neuroprotection compared to fentanyl. In both assay, non-selective OR antagonist, NTX reversed the effect of most analogs and NTX did not show any significant effect compared to non-treated group.

[0056] Generation of total ROS in primary cortical neuron exposed to 3 hr H/A and 24 hr reoxygenation in presence or absence of OR agonist fentanyl analogs and biphalin (10 nM) was assessed in this experiment (see FIG. 12). Total ROS generation was statistically significantly reduced when neuron were treated with LYS436 (52.2% less ROS production, $p < 0.001$), LYS739 (54.4% less ROS

production, $p < 0.001$), LYS416 (35.0% less ROS production, $p < 0.01$) and biphalin (29.1% less ROS production, $p < 0.05$) compared to no drug treated group. The effect of LYS739 was significantly better ($p < 0.05$) than that of biphalin. Both LYS436 ($p < 0.001$) and LYS739 ($p < 0.001$) significantly decreased ROS production compared to fentanyl. Non-selective OR antagonist NTX did not show significant decrease in ROS production compared to no drug treated group but it reversed the effect of OR agonists (except for fentanyl) used in this experiments.

[0057] The effect of lead fentanyl analog LYS739, biphalin and fentanyl on brain edema formation (see FIG. 13B) and infarct volume (FIG. 13C) after focal brain ischemia induced by 1 hr occlusion followed by 24 hr reperfusion. Compared to the vehicle treated group LYS739 produced a 59.45% reduction in edema formation, $p < 0.05$ and biphalin produced a 56.17% reduction in edema formation, $p < 0.05$ that was statistically significant when administered 10min after reperfusion at a dose of 5 mg/kg in saline (i.p.). Fentanyl (0.2 mg/kg, 10 minute post reperfusion) and/or antagonist NTX (1 mg/kg, 10 min before stroke) did not show any significant reduction in edema formation compared to saline treated group. LYS739 produced a 67.7-% reduction in infarct ratio, $p < 0.0001$) and biphalin produced a 67.0% reduction in infarct ratio, $p < 0.0001$ that were statistically significant compared to saline treated group. Again, fentanyl and NTX did not show any improvement in terms of infarct ratio. For both edema formation and infarction volume, NTX reversed the effect of both LYS739 and biphalin. Mean cerebral blood flow reduction $\pm$ SEM in ischemic brain for saline group $80.7 \pm 1.2\%$, BIP $81.1 \pm 1.3\%$, BIP+NTX $79.7 \pm 2.0\%$, LYS739 $82.1 \pm 1.2\%$, LYS739+NTX $80.6 \pm 2.2\%$, FENT $80.4 \pm 1.6\%$, NTX $76.9 \pm 2.0\%$.

[0058] Twenty-four hours after the reperfusion neurological score was evaluated in the experimental groups (see FIG. 14). LYS739 (30.4% improvement, $p < 0.05$) and biphalin (25.5% improvement, $p < 0.05$) significantly improved the neurological score compared to saline treated control group. Fentanyl or OR antagonist NTX did not improve any neurological score under same experimental conditions. NTX reversed the effect of both LYS739 and biphalin but the effects were not statistically significant.

[0059] Locomotor activity (horizontal activity, vertical activity, total distance, rest time, stereotype counts and number of movements) was evaluated 24 hr after

reperfusion in experimental animals (Table 7). Before the start of surgery all animals went through locomotor evaluation to get the baseline. Both LYS739 and biphalin (5 mg/kg, 10 min post reperfusion, i.p.) statistically significantly improved all the locomotor parameters compared to saline treated control animals. When compared the effect of LYS739 to that of biphalin most of the parameter were improved although they were not statistically significant except for vertical activity ($p < 0.05$). But, in comparison to fentanyl treated group, both LYS739 and biphalin showed better locomotor activity and the effects were statistically significant. Non-selective OR antagonist NTX did not improve any locomotor parameters.

[0060] Table 7 shows measurement of locomotor activity 24 h after stroke and drug treatments. Data represent the mean $\pm$ S.E.M. of 4-5 independent determinations; numbers indicated in parenthesis in the line of the table columns donate to the number of experimental animals per group. '*' Compared to Saline treated group - * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; '#' Compared to biphalin treated group - # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$; #### $p < 0.0001$; 'φ' Compared to fentanyl treated group - φ $p < 0.05$; φφ $p < 0.01$; φφφ $p < 0.001$; φφφφ $p < 0.0001$

TABLE 7

Parameters	Sham (N = 7)	0.9% Saline (N = 7)	BIP (N = 7)	BIP + NTX (N = 5)	LYS739 (N = 4)	LYS739 + NTX (N = 4)	FENT (N = 4)	NTX (N = 4)
Horizontal Activity	1300 $\pm$ 230 **** #	150 $\pm$ 34 ##	760 $\pm$ 75 ** φ	390 $\pm$ 130	1000 $\pm$ 160 *** φφφ	120 $\pm$ 9 ##	140 $\pm$ 50 #	160 $\pm$ 36 #
Vertical Activity	36 $\pm$ 8 **	0 ## φ	28 $\pm$ 3 **	20 $\pm$ 2	50 $\pm$ 13 **** # φφφφ	0 ##	3 $\pm$ 3 #	1.8 $\pm$ 1 ##
Total Distance (CM)	1100 $\pm$ 280 ****	17 $\pm$ 5 #	650 $\pm$ 81 ** φφ	86 $\pm$ 64 #	680 $\pm$ 110 ** φφ	8 $\pm$ 2 ##	15 $\pm$ 6 ##	45 $\pm$ 16 #
No. of Movements	75 $\pm$ 16 ***** #	10 $\pm$ 2 #	42 $\pm$ 6 * φ	19 $\pm$ 6	61 $\pm$ 7 ** φφ	6 $\pm$ 2 #	6 $\pm$ 1 #	11 $\pm$ 3
Stereotypy	970 $\pm$	62 $\pm$ 12	390 $\pm$ 46	230 $\pm$ 71	340 $\pm$ 76	39 $\pm$ 2	83 $\pm$ 35	77 $\pm$ 13

	110							
Counts	**** ####	##	** Φ		*	##	#	#
Rest Time (Seconds)	220 ± 19 ****	300 ± 1 ##	240 ± 7 ** ΦΦΦ	270 ± 7	220 ± 14 **** ΦΦΦΦ	300 ± 27 ###	300 ± 1 ###	300 ± 2 ##

[0061] Various modifications of the invention, in addition to those described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Each reference cited in the present application is incorporated herein by reference in its entirety.

[0062] Although there has been shown and described the preferred embodiment of the present invention, it will be readily apparent to those skilled in the art that modifications may be made thereto which do not exceed the scope of the appended claims. Therefore, the scope of the invention is only to be limited by the following claims. Reference numbers recited in the claims are exemplary and for ease of review by the patent office only, and are not limiting in any way. In some embodiments, the figures presented in this patent application are drawn to scale, including the angles, ratios of dimensions, etc. In some embodiments, the figures are representative only and the claims are not limited by the dimensions of the figures. In some embodiments, descriptions of the inventions described herein using the phrase "comprising" includes embodiments that could be described as "consisting of", and as such the written description requirement for claiming one or more embodiments of the present invention using the phrase "consisting of" is met.

[0063] The reference numbers recited in the below claims are solely for ease of examination of this patent application, and are exemplary, and are not intended in any way to limit the scope of the claims to the particular features having the corresponding reference numbers in the drawings.

WHAT IS CLAIMED IS:

1. A multifunctional opioid receptor ligand (ORL), wherein the multifunctional ORL has agonist activity at mu opioid receptor (MOR), agonist activity at delta opioid receptor (DOR), and antagonist activity at kappa opioid receptor (KOR), the ORL comprises LYS739 (SEQ ID NO: 10).
2. A multifunctional opioid receptor ligand (ORL), wherein the multifunctional ORL has agonist activity at mu opioid receptor (MOR), agonist activity at delta opioid receptor (DOR), and antagonist activity at kappa opioid receptor (KOR), the ORL comprises a peptide portion and a tail portion linked to a C-terminus of the peptide portion, the ORL has a formula according to Formula 1: $Aaa-DBbb-Ccc-Ddd(X)-Eee$ wherein
 - Aaa is selected from 2'-6'-dimethyltyrosine (Dmt) and Tyrosine (Tyr);
 - D-Bbb is selected from D-Alanine (D-Ala), D-Norleucine (D-Nle), Proline (Pro), and D-Arginine (D-Arg);
 - Ccc is selected from Gly, Phenylalanine(X) (Phe(X)), and naphthylalanine (Nal) or is absent;
 - Ddd(X) is Gly, Phe(X), or Lys;
 - Eee is the tail portion, the tail portion is lipophilic; and
 - X is selected from H, F, Cl, and Br.
3. The multifunctional ORL of claim 2, wherein Eee is selected from $-NH_2$ and a 4-anilidopiperidine moiety.
4. The multifunctional ORL of claim 2, wherein the 4-anilidopiperidine moiety comprises N-phenyl-N-piperidin-4-ylpropionamide (Ppp).
5. A method of reducing pain, said method comprising
 - a. identifying a subject in need of a kappa opioid receptor (KOR) antagonist or partial agonist; and
 - b. introducing to the subject a multifunctional ORL having agonist activity at mu opioid receptor (MOR), agonist activity at delta opioid receptor (DOR), and antagonist activity at kappa opioid receptor (KOR), the ORL comprises a peptide portion and a tail portion linked to a C-

terminus of the peptide portion, the ORL has a formula according to Formula 1: Aaa-DBbb-Ccc-Ddd(X)-Eee wherein

Aaa is selected from 2'-6'-dimethyltyrosine (Dmt) and Tyrosine (Tyr);

D-Bbb is selected from D-Alanine (D-Ala), D-Norleucine (D-Nle), Proline (Pro), and D-Arginine (D-Arg);

Ccc is selected from Gly, Phenylalanine(X) (Phe(X)), and naphthylalanine (Nal) or is absent;

Ddd(X) is Gly, Phe(X), or Lys;

Eee is the tail portion, the tail portion is lipophilic; and

X is selected from H, F, Cl, and Br;

wherein the ORL is effective for reducing pain.

6. The method of claim 5, wherein Eee is selected from $-NH_2$ and a 4-anilidopiperidine moiety.
7. The method of claim 6, wherein the 4-anilidopiperidine moiety comprises N-phenyl-N-piperidin-4-ylpropionamide (Ppp).

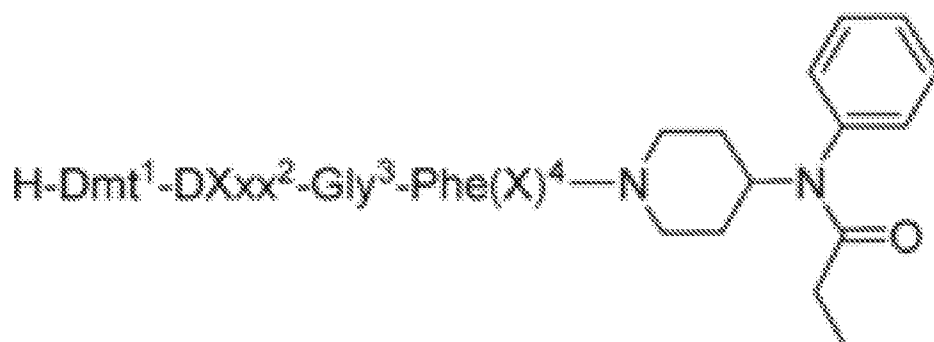
1/14

		pKi		
		MOR	KOR	DOR
SEQ ID NO: 3	Dyn A	8.1	10.8-8.3	7.4
SEQ ID NO: 4	Dyn A (1-13)	8.3	10.7-9.3	7.8
SEQ ID NO: 5	Dyn A (1-8)	8.4	9.9-8.0	8.4
SEQ ID NO: 6	Dyn B	8.5	9.9-8.1	7.8
SEQ ID NO: 1	Leu-Enk	8.1	6.0	8.7-8.4
SEQ ID NO: 2	Met-Enk	9.2	6.0	7.4

↓
K receptor
selectivity decrease

FIG. 1

2/14

*(Formula 2 + Ppp)***FIG. 2**H-Aaa-DBbb-Ccc-Ddd(X)-Eee *(Formula 1)*

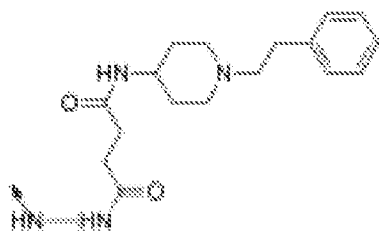
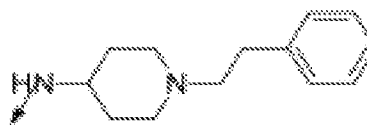
Eee: Ppp, NH ₂ Aaa: Tyr, Dmt Bbb: Nle, Pro, DArg Ccc: Gly, Phe(X), Nal, Ddd: Gly, Phe(X), Lys X: H, F, Cl, Br

FIG. 2A

3/14

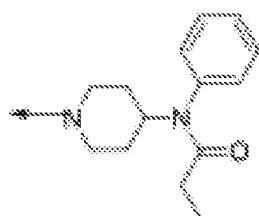
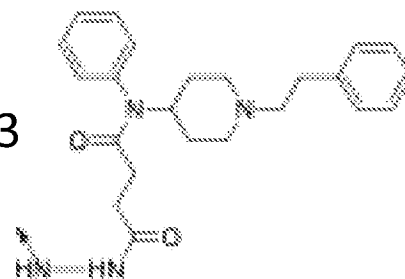
 NH_2 

4-Anilidopiperidine Analogue 1

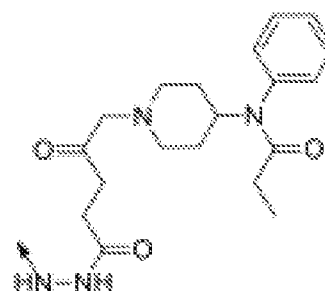


4-Anilidopiperidine Analogue 2

4-Anilidopiperidine Analogue 3

4-Anilidopiperidine Analogue 4
(Ppp)

4-Anilidopiperidine Analogue 5

**FIG. 3**

GTP γ S Activity at KOR

4/14

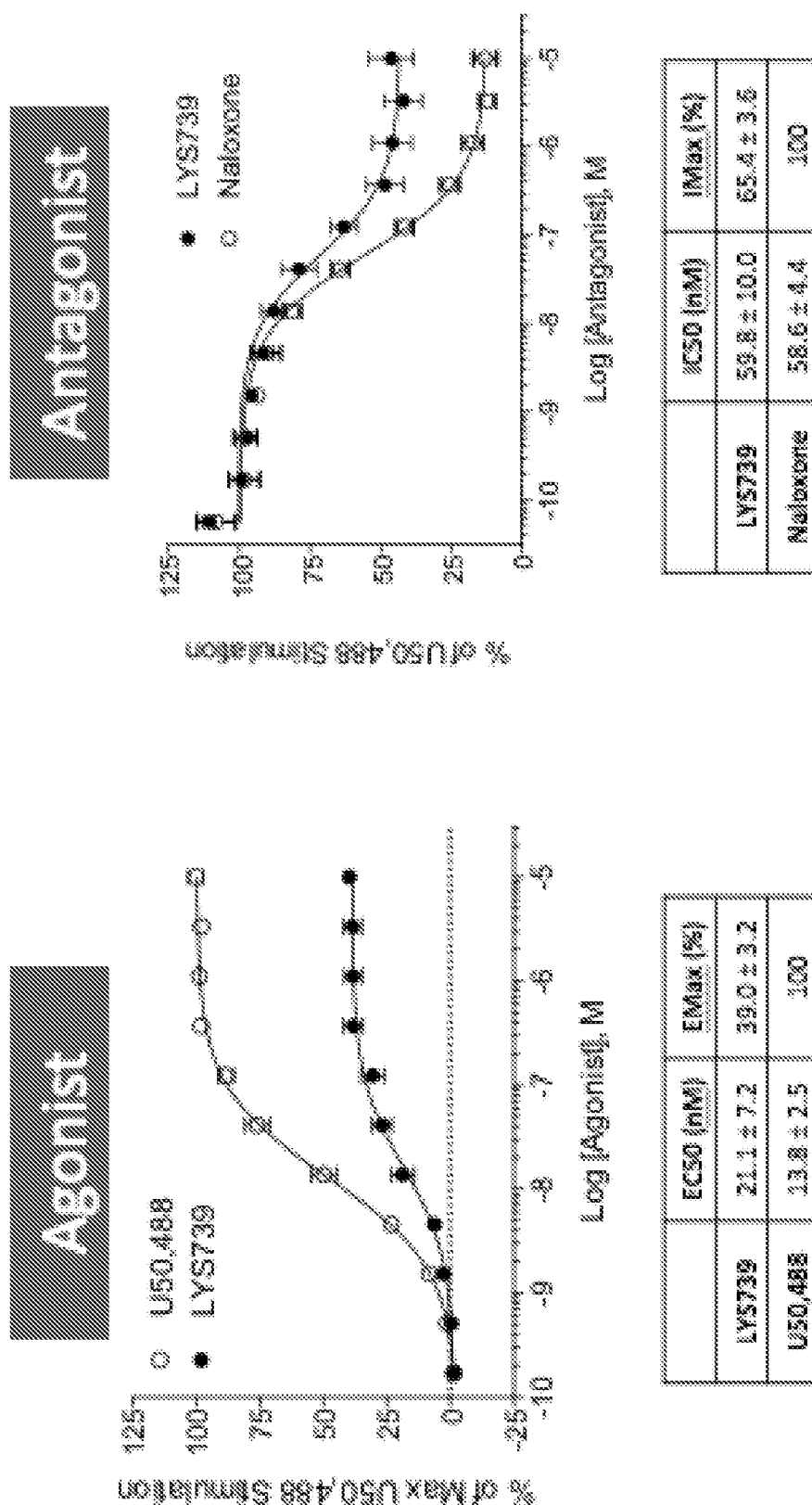
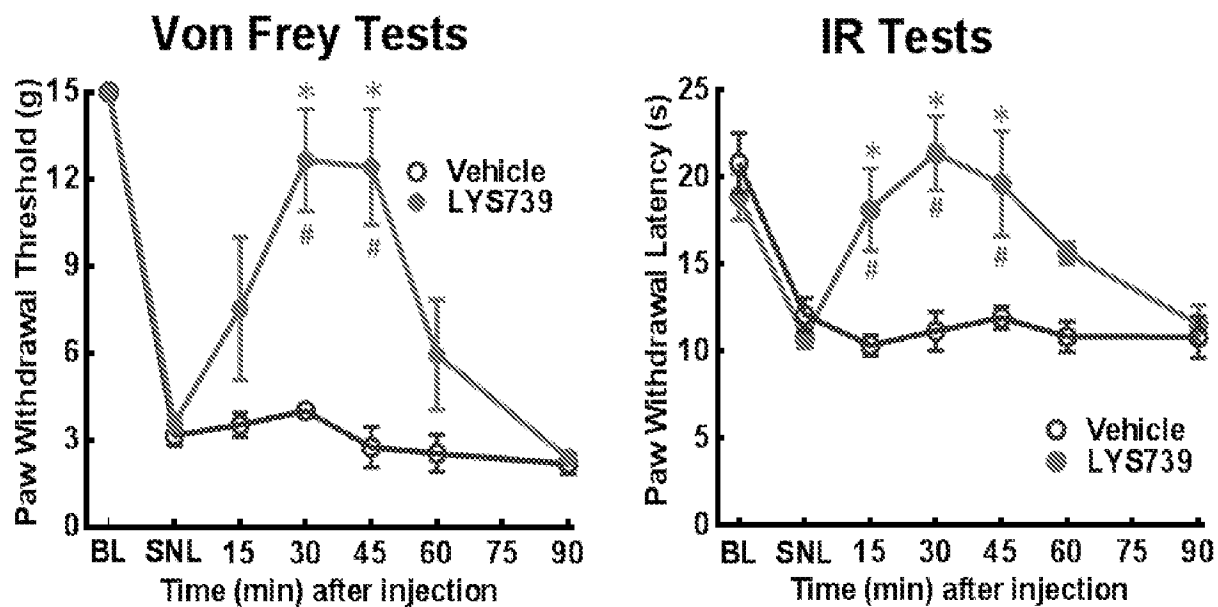
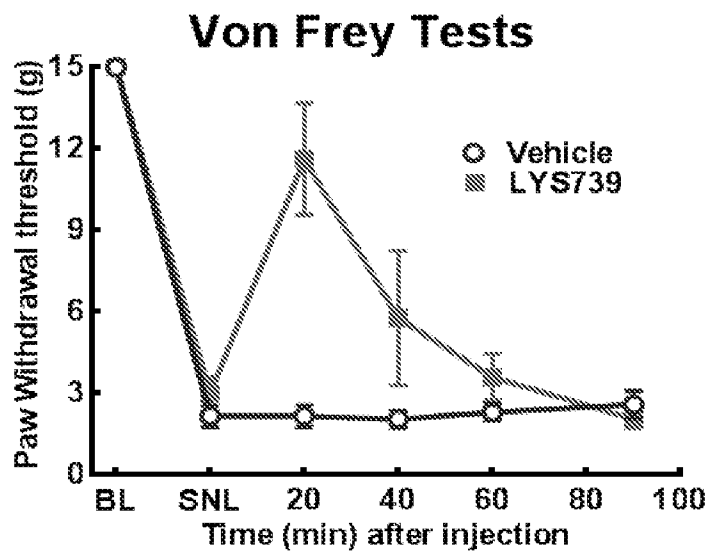


FIG. 4

5/14

**FIG. 5A****FIG. 5B**

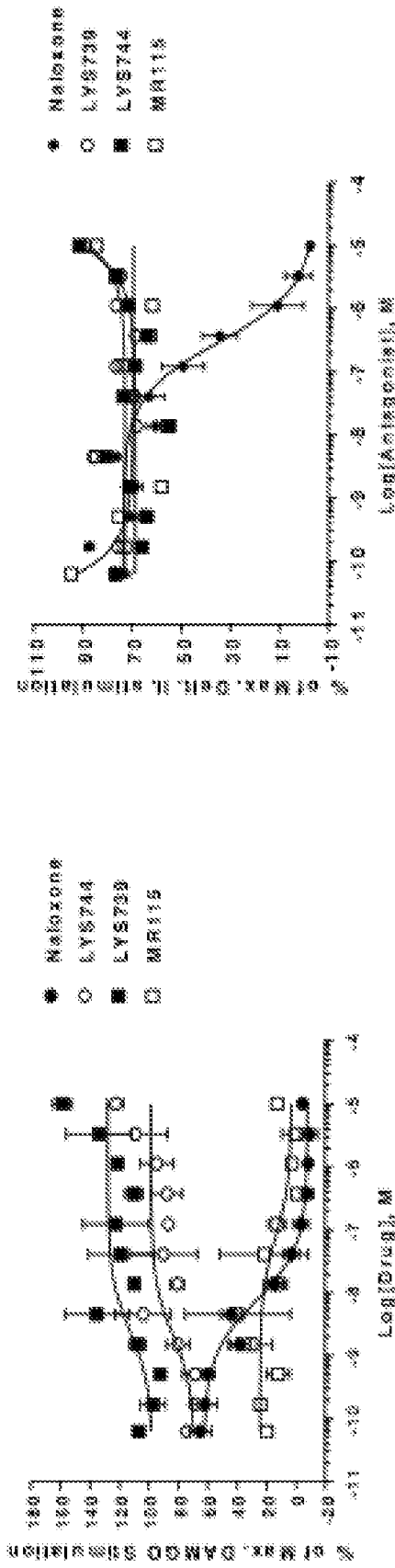


FIG. 6A

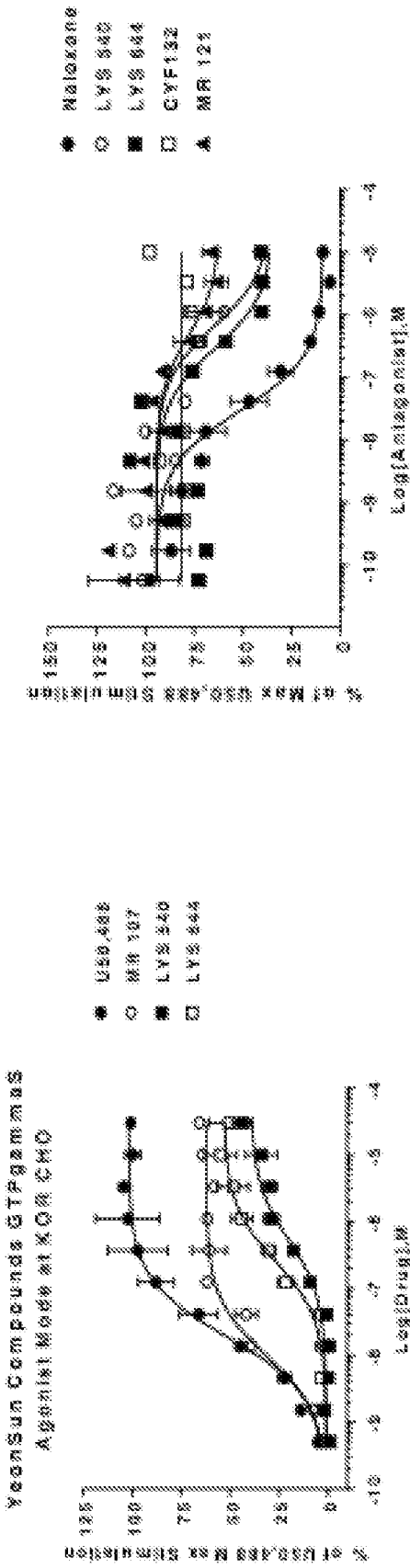
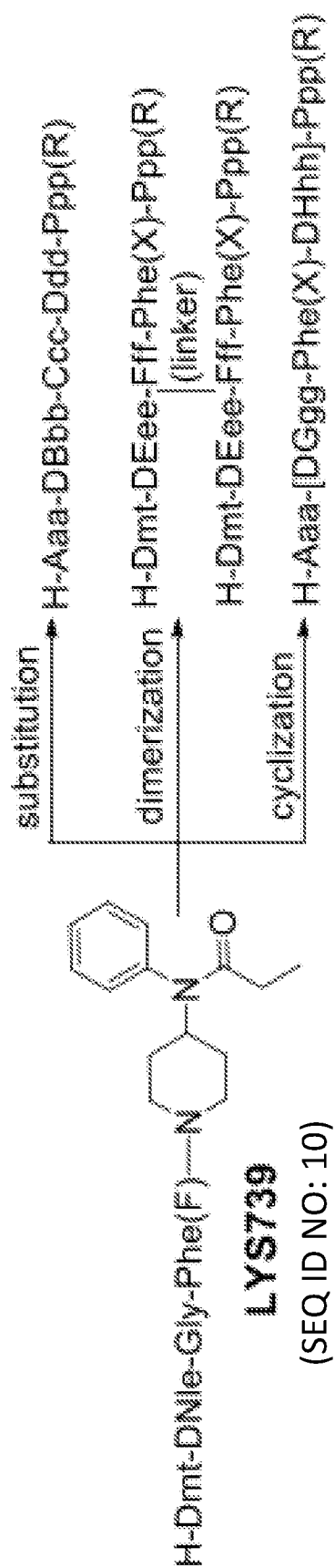


FIG. 6B

7/14



Ppp: N-phenyl-N-piperidin-4-yl-propionamide

Aaa: Tmt, Dmt, Tyr, Phe, Dmp, Mdp, etc

Bbb: Nle, Pro, Arg, Ala, etc

Ccc: Gly, Phe, Trp, etc

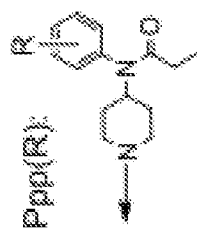
Ddd: Phe(X), Trp, Lys, Sar, etc

Eee / Fff: Bbb / Ccc or homocysteine

Fff / Ggg: Lys, Orn, Pen, Cys, etc

X: H, F, Cl, Br, etc

R: 3-Cl, 4-Cl, 3-F, 4-f, 2,4-diCl, etc

**FIG. 7**

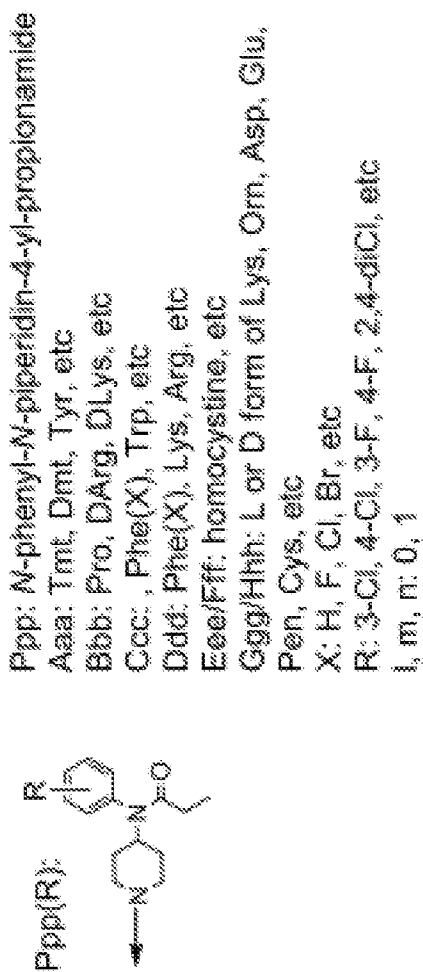
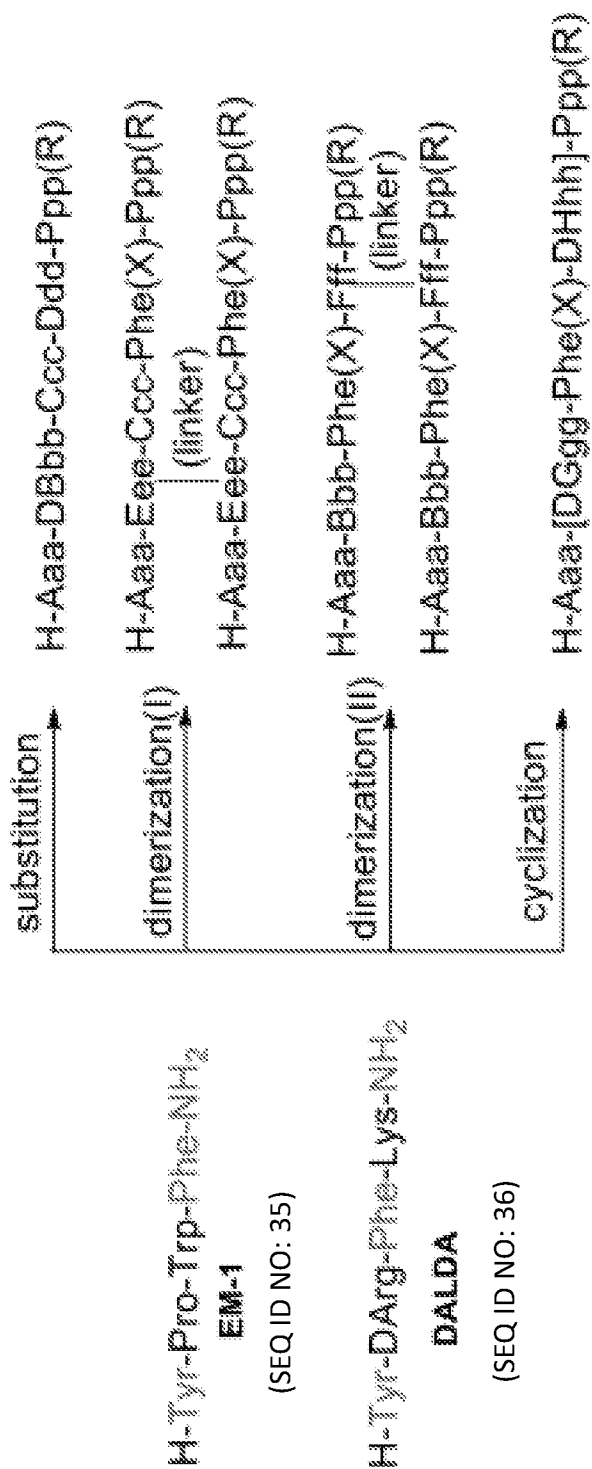
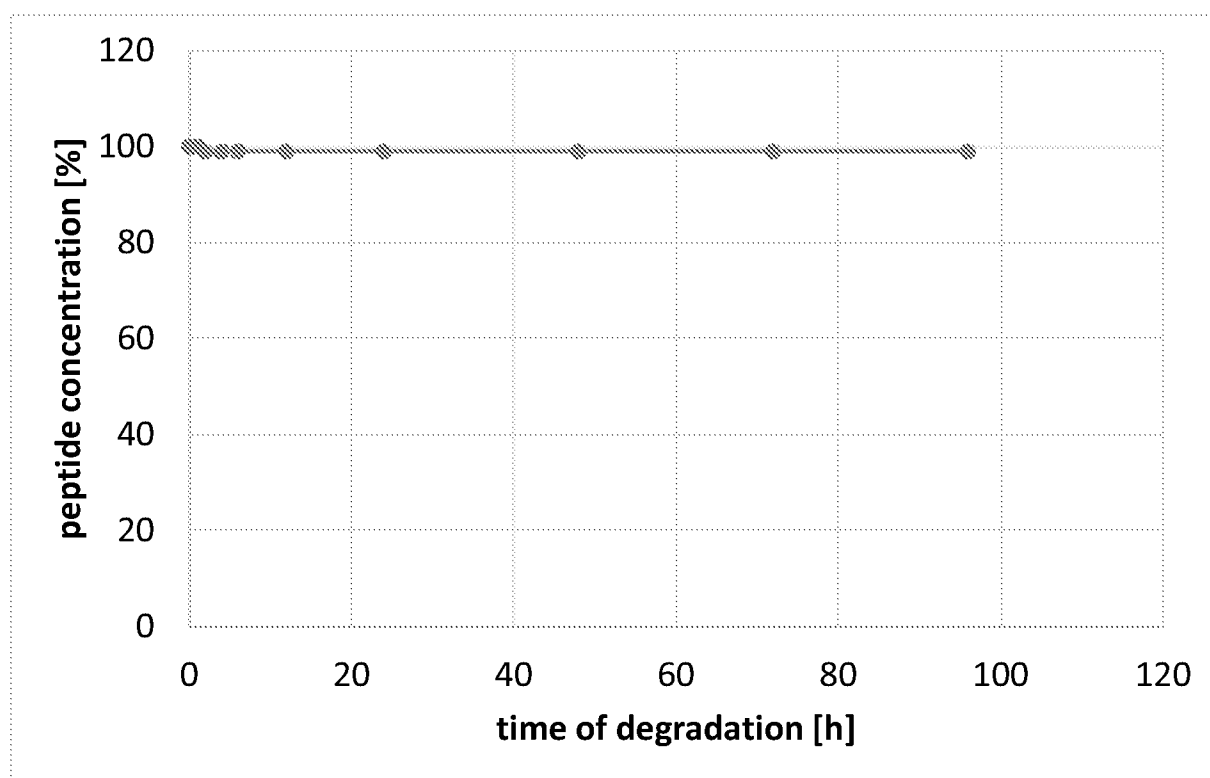


FIG. 8

9/14

**FIG. 9**

10/14

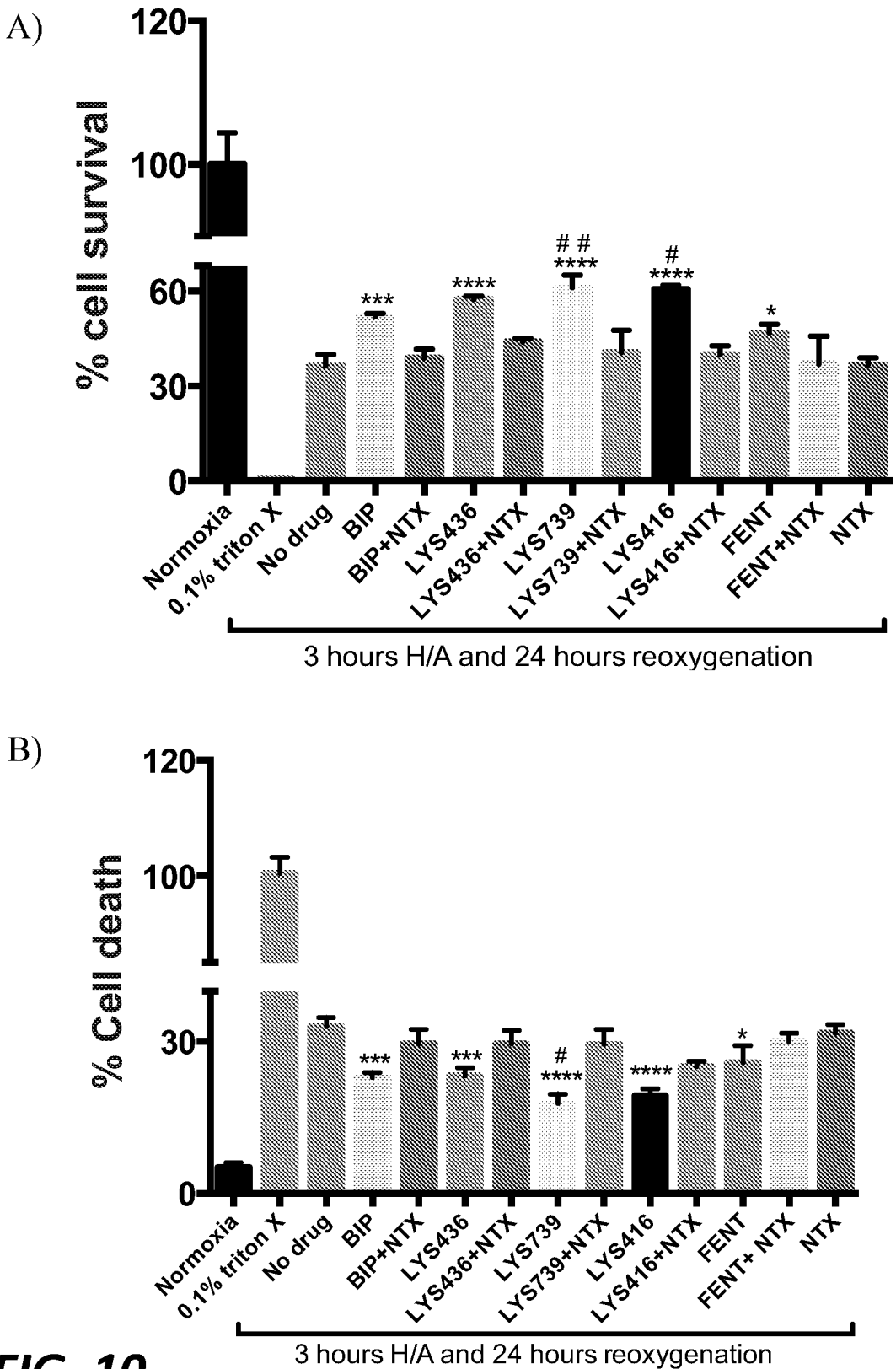
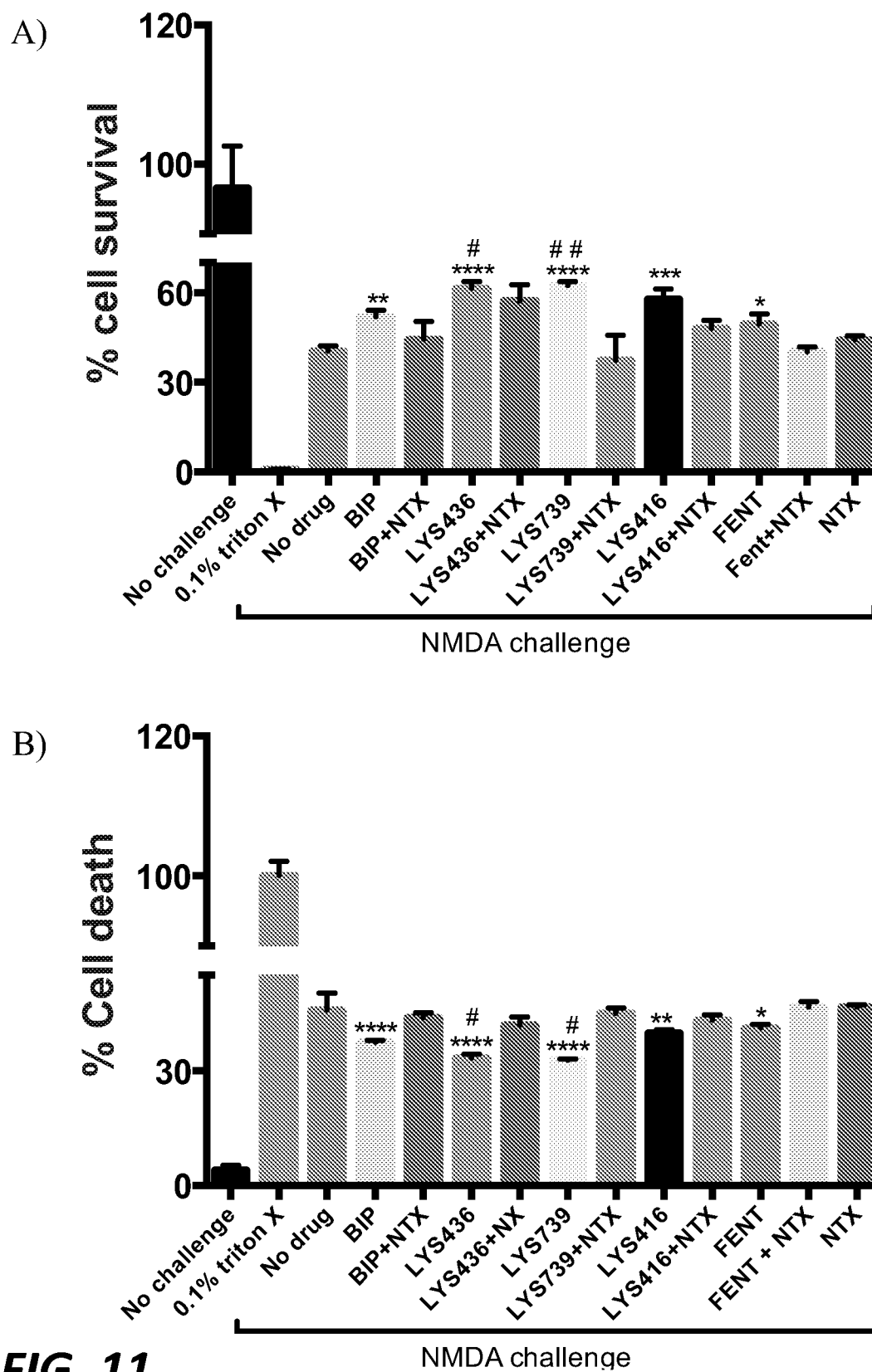


FIG. 10

11/14

**FIG. 11**

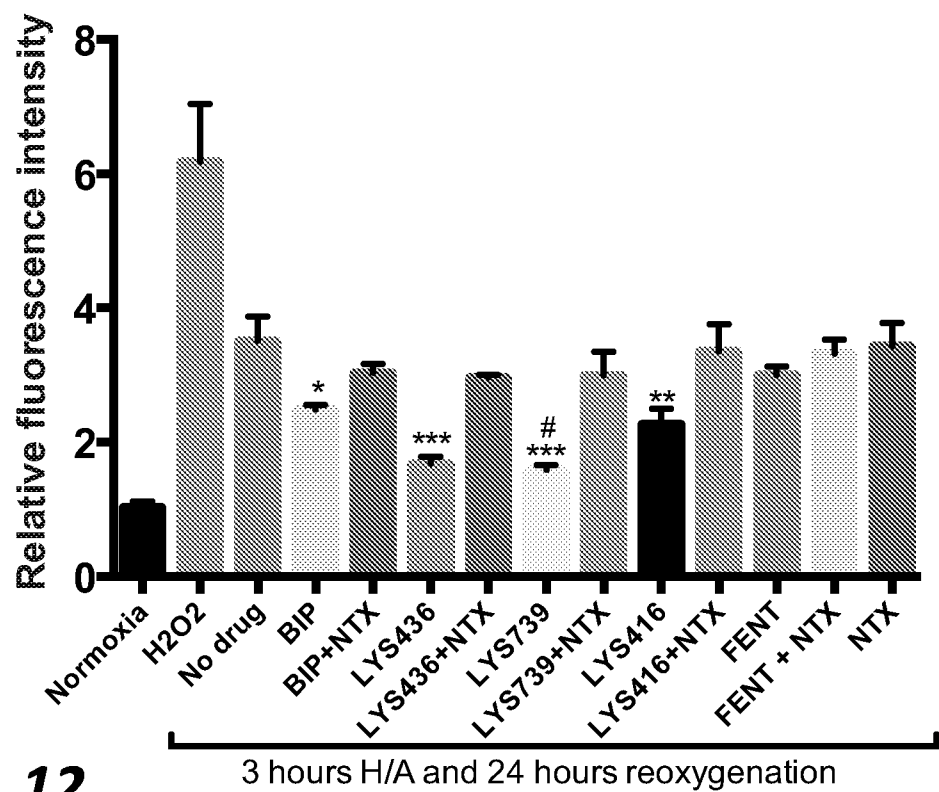


FIG. 12

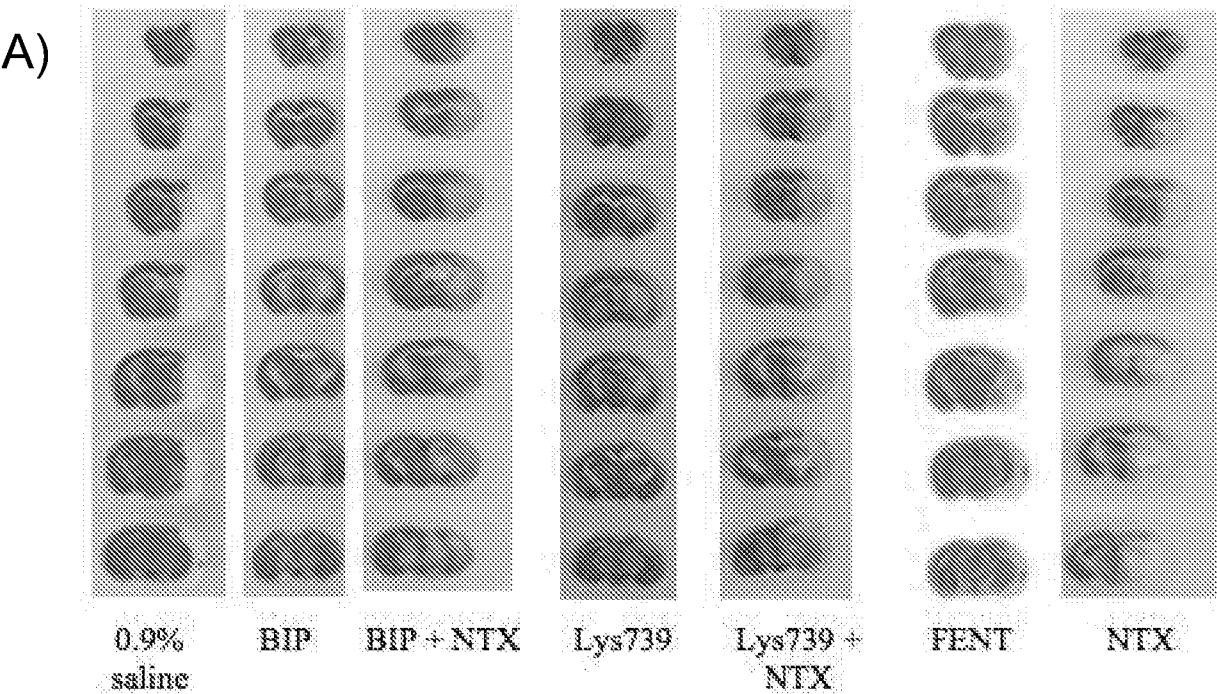
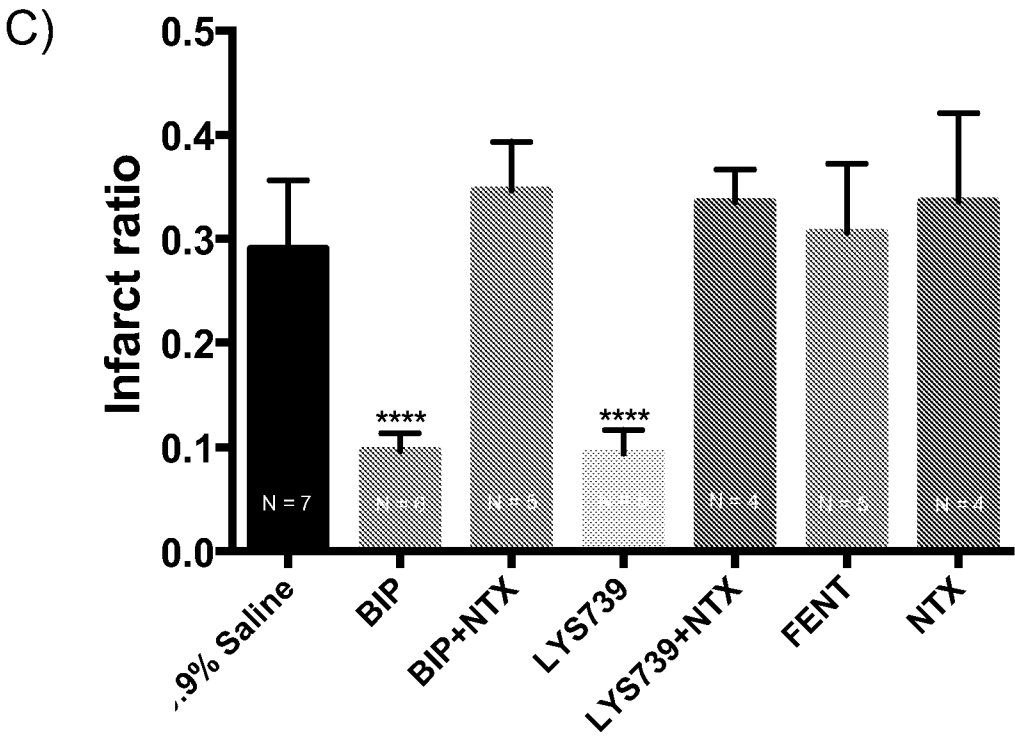
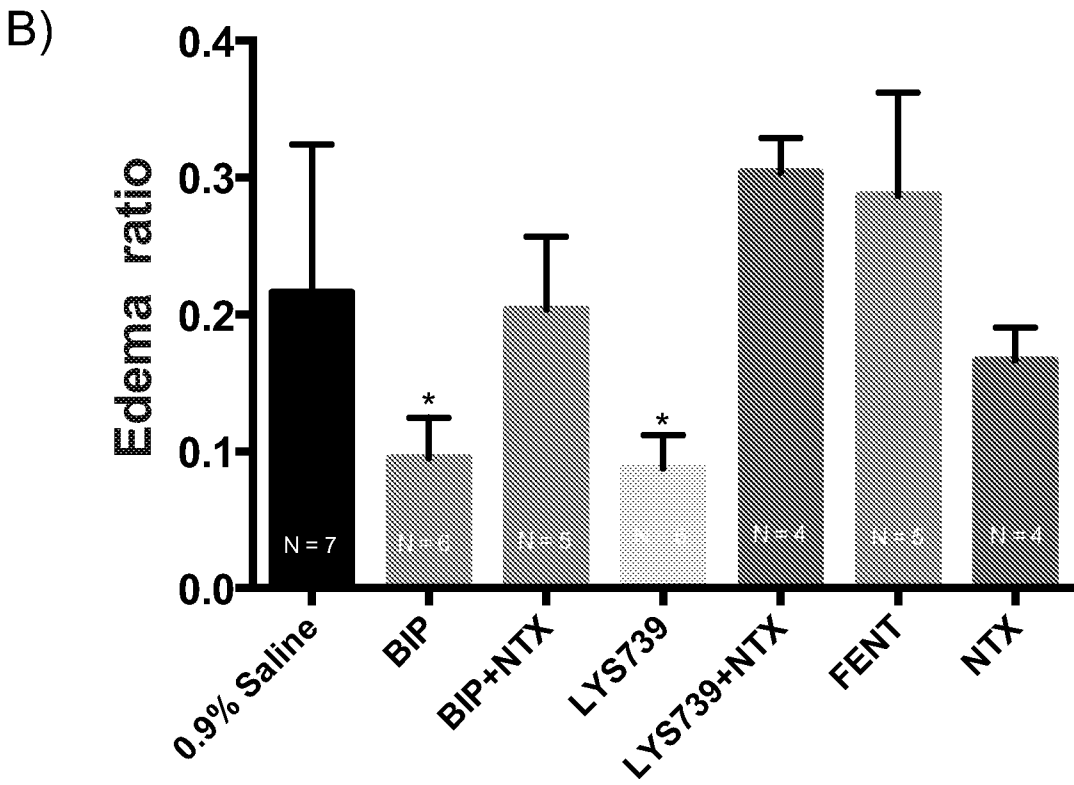
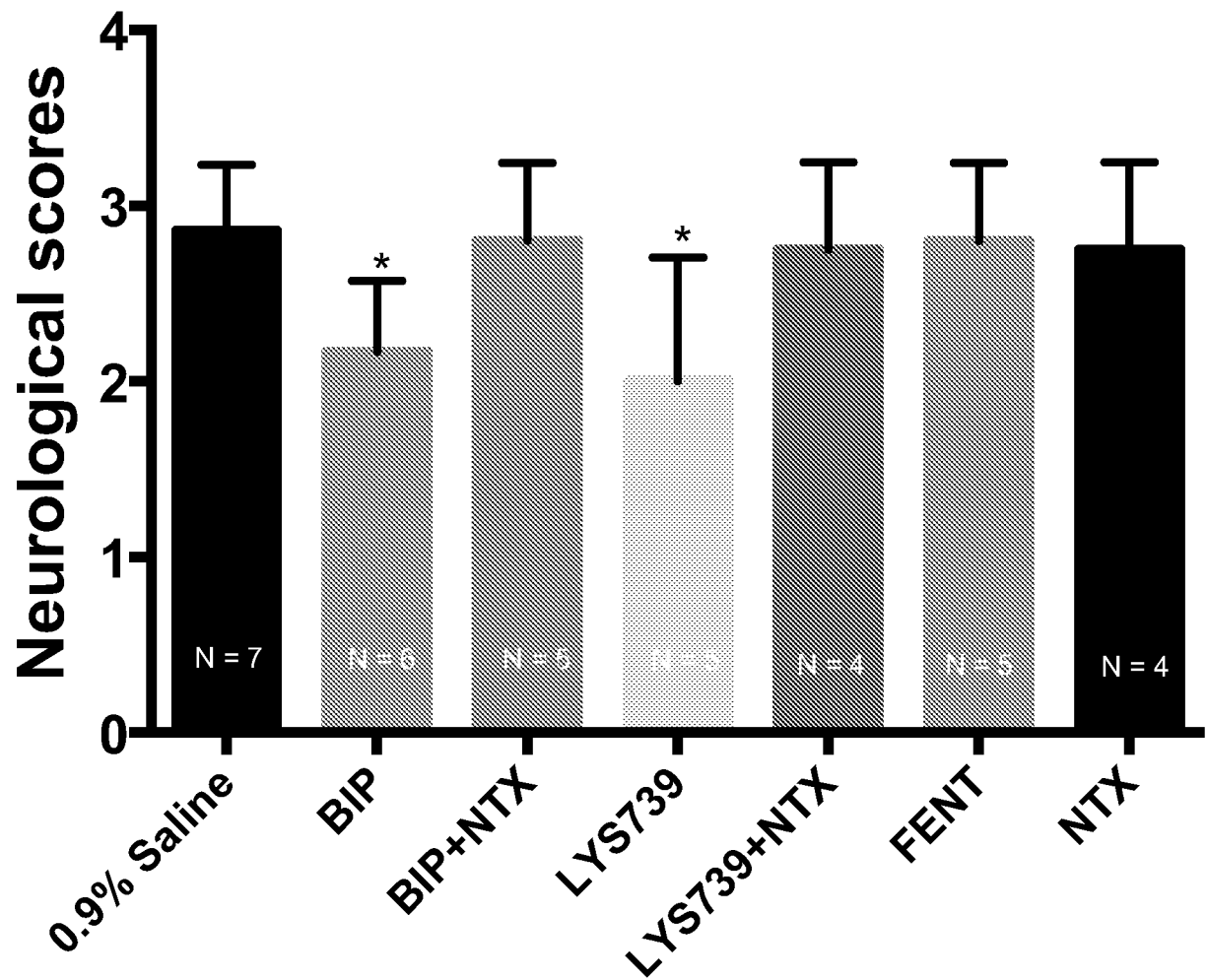


FIG. 13



14/14

**FIG. 14**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/33529

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/07, A61K 38/08, A61K 38/17 (2016.01)

CPC - A61K 38/177, C07K 7/22, C07K 19/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 38/07, A61K 38/08, A61K 38/17 (2016.01)

CPC: A61K 38/177, C07K 7/22, C07K 19/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/18.3, 530/330Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Cooglc Scholar, Google Web, search terms: multifunctional opioid receptor ligand, agonist, antagonist, Kd, inhibit, mu opioid receptor, delta opioid receptor, kappa opioid receptor, MOR, KOR, DOR, KOP, DOP, MOP, 4-anilidopiperidine, N-phenyl-N-piperidin-4-ylpropionamide, D-Norleucine, tail portion, lipophilic, reducing pain

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Lee et al. Development of Novel Enkephalin Analogues that Have Enhanced Opioid Activities at Both mu and delta Opioid Receptors. J. Med. Chem. (1 November 2007), vol 50, No. 22 pp 5528-5532, abstract, pg 5531 co 1, para 1, Table 1, structure 16	2-7 ----- 1
Y	Mehr-un-Nisa et al. Design, synthesis, and biological evaluation of a series of bifunctional ligands of opioids/SSRIs. Bioorganic and Medicinal Chemistry (15 March 2015) vol 23, no 6, pp 1251-1259 pg 1252, col 1 para 2 pg 1252, col 2, para 4 - pg 1253, col 1, para 1, pg1253, Table 2, peptide structure 7	1
A	US 2008/0039404 A1 (HRUBY et al.) 14 February 2008 (14.02.2016) abstract	1-7

☐ Further documents are listed in the continuation of Box C.

* 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

14 July 2016 (14.07.2016)

Date of mailing of the international search report

17 AUG 2016

Name and mailing address of the ISA/US

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