TITLE OF THE INVENTION

OPIORPHIN PEPTIDE DERIVATIVES AS POTENT INHIBITORS OF ENKEPHALIN-DEGRADING ECTOPEPTIDASES

[0001] The instant application claims the benefit of US provisional application Serial Number 61/042,922 which was filed on April 7, 2008, and which is incorporated by reference.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0002] The present invention relates to modified opiorphin peptides as new inhibitors of metallo-ectopeptidases.

DESCRIPTION OF THE RELATED ART

[0003] Zinc metal ectopeptidases control the receptor-dependent activity of neural and hormonal mediators involved in the regulation of important physiological functions in mammals. They are located at the surface of cells in nervous and systemic tissues and catalyze postsecretory processing or metabolism of neuropeptides and regulatory peptides (Roques, BP, Noble, F, Dauge, V, Fournie-Zaluski, MC & Beaumont, A. (1993) *Pharmacol Rev* **45**, 87–146. Turner, AJ, Isaac, RE & Coates, D. (2001) *BioEssays* **23**, 261–269).

[0004] Prominent among these neuronal and/or hormonal peptide signals are substance P (SP) and enkephalins, which are implicated in the receptor-dependent modulation of behavioral adaptive responses to stressful or threatening environmental stimuli. They notably regulate spinal processing of nociceptive information and analgesic mechanisms, emotional and/or motivational responses, anxiety, aggression, and neuroimmune inflammatory phenomena (Dickenson, AH. (1995) *Br J Anaesth* **75**, 193–200. Sora, I, Takahashi, N, Funada, M, Ujike, H, Revay, RS, Donovan, DM, Miner, LL & Uhl, GR. (1997) *Proc Natl Acad Sci USA* **94**, 1544–1549; Konig, M, Zimmer, AM, Steiner, H, Holmes, PV, Crawley, JN, Brownstein, MJ & Zimmer, A. (1996) *Nature* **383**, 535–538; Filliol, D, Ghozland, S, Chluba, J, Martin, M, Matthes, HW, Simonin, F, Befort, K, Gaveriaux-Ruff, C, Dierich, A & LeMeur, M, *et al.* (2000) *Nat Genet* **25**, 195–200).

[0005] Because of the physiological importance and the critical role of zinc ectopeptidases in modulating the functional potency of downstream neuronal and hormonal signals, it is essential to focus on what controls their activity and, as a consequence, the overall regulatory cascade. The discovery of upstream regulators of ectopeptidase activity also is

exciting from physiopathological and therapeutic points of view because of the potential for developing new candidate drugs.

[0006] A brain-specific heptapeptide named spinorphin was isolated and characterized from bovine spinal cord based on its inhibitory activity toward enkephalin-degrading ectoenzymes, such as neutral endopeptidase (NEP; EC 3.4.24.11 [EC]) and aminopeptidase N (AP-N; EC 3.4.11.2 [EC]) (Nishimura, K & Hazato, T. (1993) *Biochem Biophys Res Commun* **194**, 713–719; Yamamoto, Y, Ono, H, Ueda, A, Shimamura, M, Nishimura, K & Hazato, T. (2002) *Curr Protein Pept Sci* **3**, 587–599). In addition, we characterized rat sialorphin, a peptide mediator involved in adaptation to environmental changes in rat. Rat sialorphin is an endocrine peptide signal whose expression is activated by androgen regulation and whose secretion is stimulated under adrenergic-mediated response to environmental stress in male rats. It is a physiological inhibitor of the membrane-anchored rat NEP activity and is a powerful inhibitor of pain sensation in rats (Rougeot, C, Rosinski-Chupin, I, Njamkepo, E & Rougeon, F. (1994) *Eur J Biochem* **219**, 765–773; Rougeot, C, Vienet, R, Cardona, A, Le Doledec, L, Grognet, JM & Rougeon, F. (1997) *Am J Physiol* **273**, R1309–R1320.; Rougeot, C, Rosinski-Chupin, I & Rougeon, F. (1998) in Biomedical Reviews eds. Chaldakov, GN & Mathison, R. (Bulgarian-American Center, Varna, Bulgaria,) **Vol 9**, pp. 17–32; Rosinski-Chupin, I, Huaulme, JF, Rougeot, C & Rougeon, F. (2001) *Endocrinology* **142**, 4550–4559; Rougeot, C, Messaoudi, M, Hermitte, V, Rigault, AG, Blisnick, T, Dugave, C, Desor, D & Rougeon, F. (2003) *Proc Natl Acad Sci USA* **100**, 8549–8554).

[0007] Previously we demonstrated that human Opiorphin native peptide (QRFSR-peptide), the first characterized in human to date, is an efficient dual inhibitor of two enkephalin-inactivating ectopeptidases, neutral endopeptidase NEP (EC 3.4.24.11) and aminopeptidase AP-N (EC 3.4.11.2) (Wisner et al. *Proc Natl Acad Sci USA*, Nov. 2006, 103(47): 17979-84).

[0008] Opiorphin peptide derivatives have been described previously, see, e.g., Wisner et al. *Proc Natl Acad Sci USA*, Nov. 2006, 103(47): 17979-84 and has the basic peptide sequence as QRFSR.

[0009] Thus, this sequence and the sequence defined by the following formula may be modified: X1-X2-Arg-Phe-Ser-Arg, wherein X1 represents H atom or a Tyr amino acid, X2 represents Gln or Glp when X1 is H, or X2 represents Gln when X1 is Tyr or Cys. Preferred is QRFSR, YQRFSR, and/or CQRFSR with QRFSR most preferred. It is understood that Glp is pyroglutamate, Tyr or Y is Tyrosine, Gln or Q is glutamine, Arg or

R is Arginine, Phe or F is Phenylalanine, Ser or S is Serine, and Cys or C for Cysteine. These peptides have been described in the international patent application published as WO2005090386.

SUMMARY OF THE INVENTION

[0010] The invention provides new Opiorphin derivatives and in vitro functional characterization by using highly selective biochemical assays.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIGS. 1-4 The kinetic of hydrolysis of corresponding FRET-peptide substrates by recombinant hNEP or hAP-N in the presence of vehicle or in the presence of 50 μM QRFSR-peptide analogs. Each point represents the intensity of the signal expressed in RFU (Relative Fluorescence Unit), which was proportional to the quantity of metabolites formed, as function of reaction time (min).

[0012] FIG. 5: Inhibition by QRFSR-peptide analogs of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or AP-N. Each bar represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of 50 μM of QRFSR-peptide analogs.

[0013] FIG. 6: substrates by pure recombinant human hNEP or AP-N. Each bar represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of 50 μM of QRFSR-peptide analogs.

[0014] FIGS. 7-8: Inhibition by QRFSR-peptide analogs of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or AP-N. Each bar represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of 50 μM of QRFSR-peptide analogs.

[0015] FIG. 9: Concentration-dependent Inhibition by CQRFSR peptide of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or AP-N. Each point represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of various concentrations of CRFSR-peptide plotted in μM (log-scale).

[0016] FIG. 10: Concentration-dependent Inhibition by QRFS[O-octanoyl]R peptide of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or

AP-N. Each point represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of various concentrations of QRFS[O-octanoyl]R -peptide plotted in μM (log-scale).

[0017] FIG. 11: Concentration-dependent Inhibition by Y(PE12)QRFSR (i.e. Y-[NH-$(CH2)_{12}$-CO]-QRFSR) peptide of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or AP-N. Each point represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of various concentrations of Y(PE12)QRFSR -peptide plotted in Log μM.

[0018] FIGS 12-17: Kinetics of hydrolysis of corresponding FRET-peptide substrates by recombinant hNEP or hAP-N in the presence of vehicle or in the presence of 1 to 50 μM of CQRFSR or CQRFS[O-octanoyl]R Opiorphin-peptide analogs. Each point represents the intensity of the signal expressed in RFU (Relative Fluorescence Unit), which was proportional to the quantity of metabolites formed, as function of reaction time (min).

[0019] FIG.18: Concentration-dependent Inhibition by CQRFSR peptide of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or AP-N. Each point represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of various concentrations of CRFSR-peptide plotted in μM (log-scale).

[0020] FIG.19: Concentration-dependent Inhibition by Y[PE12]QRFSR-COOH peptide (i.e. Y-(-HN-$(CH_2)_{12}$-CO-)-QRFSR) of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or AP-N. Each point represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of various concentrations of Y(PE12)QRFSR-COOH peptide plotted in μM (log scale).

[0021] FIG.20: Concentration-dependent Inhibition by C[PE6]QRFSR-COOH peptide (i.e. C-(-HN-$(CH_2)_6$-CO-)-QRFSR) of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or AP-N. Each point represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in

the presence of various concentrations of C[PE6]QRFSR-COOH peptide plotted in μM (log scale).

[0022] FIG.21: Concentration-dependent Inhibition by C(PE12)QRFSR-COOH peptide (i.e. C-(-HN-$(CH_2)_{12}$-CO-)-QRFSR) of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or AP-N. Each point represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of various concentrations of C(PE12)QRFSR-COOH peptide plotted in μM (log scale).

[0023] FIG. 22: Concentration-dependent Inhibition by C[PE6]QRF[S-O-Octanoyl]R peptide (i.e. C-(-HN-$(CH_2)_6$-CO-)-QRF-S(O-octanoyl)-R) of hydrolysis of corresponding FRET-peptide substrates by pure recombinant human hNEP or AP-N. Each point represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of various concentrations of C[PE6]QRF[S-O-Octanoyl]R -peptide plotted in μM (log scale).

[0024] FIG. 23: Concentration-dependent Inhibition by C[PE6]QRF[S-O-Octanoyl]R peptide (i.e. C-(-HN-$(CH_2)_6$-CO-)-QRF-S(O-octanoyl)-R) of hydrolysis of the substance P physiological NEP-substrate by membrane-bound human NEP expressed by LNCaP epithelial cells in culture. Each point represents the percentage of intact substrate recovered and calculated as: percentage of velocity without inhibitor - velocity in presence of inhibitor / velocity without inhibitor, which was measured in the absence or in the presence of various concentrations of C[PE6]QRF[S-O-Octanoyl]R -peptide plotted in nM (log scale).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0025] The invention here provides modified versions of these peptides where each or combinations of the amino acid residues that compose the opiorphin peptide are modified with one or more functional groups.

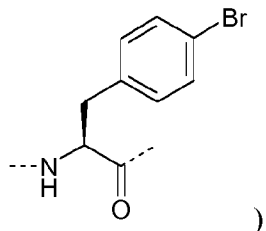
[0026] Non-limiting examples of such modified opiorphin peptides include:

NH2-QRFSR-CONH2;

NH2-QRGPR-COOH;

NH2-QHNPR-COOH;

NH2-QR(4BromoF)SR-COOH (i.e. NH2-QR-F[4Br]-SR-COOH, wherein -F[4Br]- is a phenylalanine, the phenyl group of which is substituted in the para position by a bromo atom so that -F[4Br]- has the following formula:



);

N-(Acetyl)QRFSR-COOH;

N-(C8-polyethylene)QRFSR-COOH;

N-(biotine-C6)QRFSR-COOH;

NH2-dRdSdFdRdQ-COOH (retroinversion D-enantiomere);

NH2-YQRFSR-COOH;

NH2-Y-(C6-polyethylene)QRFSR-COOH;

NH2-Y-(C12-polyethylene)QRFSR-COOH;

NH2-QRF[S-O-C8-polyethylene]R-COOH;

NH2-CQRFSR-COOH;

NH2-CQRF[S-O-C8-polyethylene]R-COOH;

NH2-CQRF[S-O-C12-polyethylene]R-COOH;

NH2-C-(C8-polyethylene)QRFSR-COOH;

NH2-C-(C12-polyethylene)QRFSR-COOH;

NH2-C-(C8-polyethylene)QRF[S-O-C8-polyethylene]R-COOH;

NH2-[Cß2]QRF[S-O-C8-polyethylene]R-COOH;

NH2-C-(C8-polyethylene)QRFS[ß3R]-COOH;

NH2-C[dQ]RF[S-O-C8-polyethylene][dR]-COOH;

NH2-C-(C8-polyethylene)QRFS[dR];

NH2-[dC]QRF[S-O-C8-polyethylene][dR]-COOH;

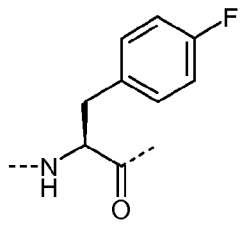
NH2-[Cß2]QRF[S-O-C8-polyethylene][ß3R]-COOH;

[CQRFSR]2 as cystine-dipeptide through disulfide linkage;

Glp-RFSR-COOH;

NH2-QRYSR-COOH;

NH2-QRF[4F]SR-COOH, wherein -F[4F]- is a phenylalanine, the phenyl group of which is substituted in the para position by a fluoro atom so that -F[4F]- has the following formula:



;

NH2-QKFSR-COOH;

NH2-QRFSK-COOH;

NH2-C-(C6-polyethylene)QRFSR-COOH;

NH2-C-(C6-polyethylene)-QRFS[S-O-C8-polyethylene]R-COOH;

NH2-C-(C12-polyethylene)QRFS[S-O-C8-polyethylene]R-COOH;

NH2-C[PE12]QRFS-dR-COOH;

NH2-C-(C12-polyethylene)-QRFS[S-O-C8-polyethylene]-β3R-COOH;

NH2-C-(C8-polyethylene)-QRFS[S-O-C8-polyethylene]-β3R-COOH,

wherein

Cß2 replaces natural Cysteine residue by ß2-cysteine incorporating methylene residue adjacent to Cα carbon near the α carbonyl group of Cysteine ($H_2N(-CH_2-SH)-CH_2-CO-$);

ß3R replaces natural Arginine residue by ß3-Arginine incorporating methylene residue adjacent to Cα carbon near the α amine group of Arginine ($-NH-CH_2-C[-(CH_2)_3-NH-C(NH)(NH_2)]-COOH$);

[S-O-C8-polyethylene] and [S-O-octanoyl] mean a serine, the hydroxyl group of which is substituted by an octanoyl group;

[S-O-C12-polyethylene] means a serine, the hydroxyl group of which is substituted by a dodecanoyl group;

C6, C8 or C12 –polyethylene correspond to spacer of 6, 8 or 12 ethylene carbons ($(-HN-(CH_2)_6-CO-$, $-HN-(CH_2)_8-CO-$ and $(-HN-(CH_2)_{12}-CO-$, respectively).

[0027] In the above peptides, NH2- represents the terminal amine of the peptide and COOH the terminal carboxylic acid (-CO-NH2 when the terminal function is an amide). The list above can also be read as:

$QRFSR-NH_2$;

QRGPR;

QHNPR;

(Acetyl)QRFSR;

$C-(-HN-(CH_2)_8-CO-)-QRFSR$;

$biotine-(-HN-(CH_2)_6-CO-)-QRFSR$;

dR-dS-dF-dR-dQ;

YQRFSR;

Y-(-HN-$(CH_2)_6$-CO-)-QRFSR;

Y-(-HN-$(CH_2)_{12}$-CO-)-QRFSR;

QRF-S(O-octanoyl)-R;

CQRFSR;

CQRF-S(O-octanoyl)-R;

CQRF-S(O-dodecanoyl)-R;

C-(-HN-$(CH_2)_8$-CO-)-QRFSR;

C-(-HN-$(CH_2)_{12}$-CO-)-QRFSR;

C-(-HN-$(CH_2)_8$-CO-)-QRF-S(O-octanoyl)-R;

[Cß2]QRF-S(O-octanoyl)-R;

C-(-HN-$(CH_2)_8$-CO-)-QRFS-[ß3R];

C-[dQ]-RF-S(O-octanoyl)-[dR];

C-(-HN-$(CH_2)_8$-CO-)-QRFS-[dR];

[dC]-QRF-S(O-octanoyl)-[dR];

[Cß2]-QRF-S(O-octanoyl)-[ß3R];

$[CQRFSR]_2$;

Glp-RFSR;

QRYSR;

QR-F[4F]-SR, wherein -F[4F]- is a phenylalanine, the phenyl group of which is substituted in the para position by a fluoro atom;

QR-F[4Br]-SR, wherein -F[4Br]- is a phenylalanine, the phenyl group of which is substituted in the para position by a bromo atom;

QKFSR;

QRFSK;

C-(-HN-$(CH_2)_6$-CO-)-QRFSR;

C-(-HN-$(CH_2)_6$-CO-)-QRF-S(O-octanoyl)-R;

C(-HN-$(CH_2)_{12}$-CO-)QRF-S(O-octanoyl)-R;

C-(-HN-$(CH_2)_{12}$-CO-)-QRFS-dR;

C-(-HN-$(CH_2)_{12}$-CO-)-QRF-S(O-octanoyl)-β3R;

C-(-HN-$(CH_2)_8$-CO-)-QRF-S(O-octanoyl)β3R,

wherein :

Cß2 is $H_2N(-CH_2-SH)-CH_2-CO-$;

ß3R is $-NH-CH_2-C[-(CH_2)_3-NH-C(NH)(NH_2)]-COOH$;

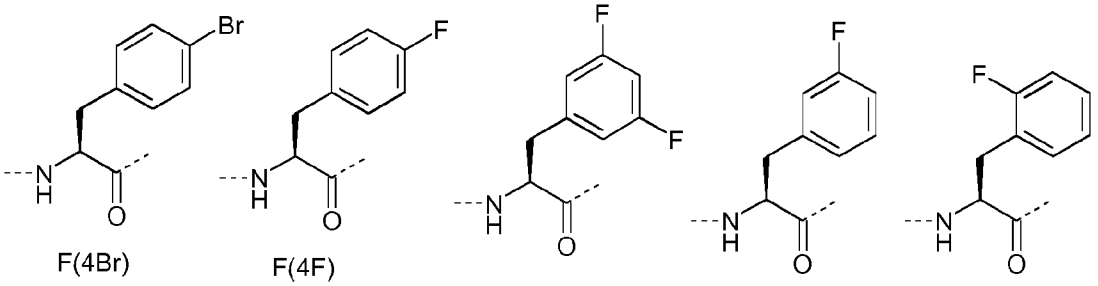
-S(-O-octanoyl) means a serine, the hydroxyl group of which is substituted by an octanoyl group;

[0028] -S(-O-dodecanoyl) means a serine, the hydroxyl group of which is substituted by a dodecanoyl group.The following definitions are used throughout the instant application.

[0029] A "peptide" is a molecule comprised of a linear array of amino acid residues connected to each other in the linear array by peptide bonds. Such linear array may optionally be cyclic, *i.e.*, the ends of the linear peptide or the side chains of amino acids within the peptide may be joined, *e.g.*, by a chemical bond. Such peptides may further include secondary, tertiary or quaternary structures, as well as intermolecular associations with other peptides or other non-peptide molecules. Such intermolecular associations may be through, without limitation, covalent binding (*e.g.*, through disulfide linkages), or through chelation, electrostatic interactions, hydrophobic interactions, hydrogen bonding, ion-dipole interactions, dipole-dipole interactions, or any combination of the above.

[0030] Any aminoacid of the following peptides may be in L-configuration or D-configuration. In particular, the entire peptide may be either in L or D configuration. The hydrocarbon chain can optionally contain a further methylene group compared to the natural aminoacid, so that the aminoacids can also be β aminoacids, more precisely β2 or β3 aminoacids. For example, R may represent one of the following aminoacid: lR (i.e. L-Arg), dR (i.e. D-Arg), β3R or β2R. Any aminoacid of the following peptides may also be an aza-aminoacid or a β–aza-aminoacid.

[0031] "F(X)" means a phenylalanine, the phenyl group of which is substituted by one or more halogen atoms, preferably fluor, the preferred F(X) having one the following formulae:


Br
F
F
F

octanoyl or a dodecanoyl group. In the present application, S(O-C8-polyethylene) or S(O-C8) means a serine, the hydroxyl group of which is substituted by an octanoyl group.

[0033] "C-[linker]-" means Cys-[NH-$(CH_2)_n$-CO]-, wherein n is an integer between 1 and 20, preferably between 4 and 15, 6, 8 or 12 being particularly preferred. In the present application, "C-(C6-polyethylene)" or "C[PE6]" means Cys-[NH-$(CH_2)_6$-CO]-; "C-(C8-polyethylene)" or "C[PE8]" means Cys-[NH-$(CH_2)_8$-CO]-; and "C-(C12-polyethylene)" or "C[PE12]" means Cys-[NH-$(CH_2)_{12}$-CO]-.

[0034] "Y-[linker]-" means Tyr-[NH-$(CH_2)_{n'}$-CO]-, wherein n' is an integer between 1 and 20, preferably between 4 and 15, 6, 8 or 12 being particularly preferred. In the present application, "Y-(C6-polyethylene)" or "Y[PE6]" means Tyr-[NH-$(CH_2)_6$-CO]-; "Y-(C8-polyethylene)" or "Y[PE8]" means Tyr-[NH-$(CH_2)_8$-CO]-; and "Y-(C12-polyethylene)" or "Y[PE12]" means Tyr-[NH-$(CH_2)_{12}$-CO]-.

[0035] "Inhibitory potency" of a peptide derivative of the invention towards a particular peptidase can readily be assessed by the one skilled in the art, e.g. either by measuring Ki values or IC_{50} values. In particular, where IC_{50} values are determined, the relative inhibitory potencies of two modified opiorphin peptides can be compared by measuring the IC_{50} value of each modified opiorphin peptide towards a given peptidase, in given experimental conditions (same buffer, same concentrations of peptidase and substrate), and by comparing said IC_{50} values of the modified opiorphin peptides with the IC_{50} value of opiorphin in the same experimental conditions.

[0036] The invention concerns a peptide derivative of formula (I):

$$\zeta\text{-}\ AA_1\text{-}AA_2\text{-}AA_3\text{-}AA_4\text{-}AA_5\text{-}OH \quad (I),$$

wherein:

- ζ is hydrogen atom, tyrosine, Y-[linker]- or a Zn chelating group, such as cysteine, C-[linker]-, N-acetyl-cysteine, , N-mercaptoacetyl (HS-CH_2-CO-), hydroxamic acid (HO-NH-CO-) or an optionally substituted hydroxyquinoline,
- AA_1 is Q or Glp,
- AA_2 is K, R or H, preferably R,
- AA_3 is Y, G, N, F or F(X), preferably F or F(X),
- AA_4 is P, S or S(OAlk), preferably S or S(OAlk),
- AA_5 is K or R, preferably R,
- C-[linker]- meaning Cys-[NH-$(CH_2)_n$-CO]-, wherein n is an integer between 1 and 20, n being preferably 6, 8 or 12,

- Y-[linker]- meaning Tyr-[NH-$(CH_2)_{n'}$-CO]-, wherein n' is an integer between 1 and 20, n' being preferably 6, 8 or 12,
- F(X) meaning a phenylalanine, the phenyl group of which is substituted by one or more halogen atoms,
- S(OAlk) meaning a serine, the hydroxyl group of which is substituted by a linear or branched alkyl group having from 1 to 20 carbon atoms,
- said AA_1, AA_2, AA_3, AA_4, and AA_5 may be independently either in the L-configuration or D-configuration, and any one of AA_1, AA_2, AA_3, AA_4, and AA_5 may be optionally a β aminoacid, an aza-aminoacid or a β–aza-aminoacid;

wherein if the peptide derivative comprises a cysteine, said peptide derivative is optionally a dimer,with the proviso that the peptide is not QRFSR, QHNPR, QRGPR, YQRFSR or GlpRFSR.

[0037] Generally, in the peptide derivatives according to the invention, AA_1, AA_2, AA_3, AA_4, and AA_5 are independently either in the L-configuration or D-configuration ,and any one of AA_1, AA_2, AA_3, AA_4, and AA_5 is optionally a β aminoacid, an aza-aminoacid or a β–aza-aminoacid.

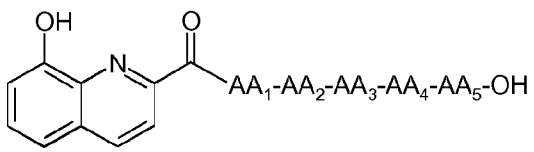
[0038] In an embodiment, at least one of AA_1, AA_2, AA_3, AA_4, and AA_5 is a β aminoacid. In another embodiment, all of AA_1, AA_2, AA_3, AA_4, and AA_5 are β aminoacids.

[0039] In an embodiment, at least one of AA_1, AA_2, AA_3, AA_4, and AA_5 is an aza-aminoacid. In another embodiment, all of AA_1, AA_2, AA_3, AA_4, and AA_5 are an aza-aminoacids.

[0040] In an embodiment, at least one of AA_1, AA_2, AA_3, AA_4, and AA_5 is a β–aza-aminoacid. In another embodiment, all of AA_1, AA_2, AA_3, AA_4, and AA_5 are β–aza-aminoacid.

[0041] In one embodiment, the peptide derivative comprises a cysteine and is a dimer wherein two sulfur atoms of two cysteines are bound with a disulfur bond. For example, $[CQRFSR]_2$ is the dimer of the peptide derivative CQRFSR, the cysteine aminoacids of which are linked with a disulfur bond.

[0042] In a preferred embodiment, when ζ is a hydroxyquinoline, the peptide derivative has the following formula:



[0043] Peptide derivatives of formula (I) are modified opiorphin peptides which advantageously have inhibitory potency against neutral endopeptidase NEP and/or aminopeptidase AP-N.

[0044] Preferred peptide derivatives of formula (I) are:

CQRFSR,

QRF(X)SR,

QRGPR,

QHNPR,

QRFPR,

QRFS(OAlk)R, preferably QRFS(O-octanoyl)R,

C-[linker]-QRFSR, preferably C-[NH-$(CH_2)_6$-CO]-QRFSR, C-[NH-$(CH_2)_8$-CO]-QRFSR, or C-[NH-$(CH_2)_{12}$-CO]-QRFSR,

QRYSR,

QKFSR,

QRFSK,

C-[linker]-QRFSR, preferably C-[NH-$(CH_2)_6$-CO]-QRFSR, C-[NH-$(CH_2)_8$-CO]-QRFSR, or C-[NH-$(CH_2)_{12}$-CO]-QRFSR,

$[CQRFSR]_2$,

C-[NH-$(CH_2)_6$-CO]-QRFS(OAlk)R, preferably C-[NH-$(CH_2)_6$-CO]-QRFS(O-octanoyl)R and

Y-[NH-$(CH_2)_{12}$-CO]-QRFSR.

[0045] In an embodiment, the invention is related to a peptide derivative of formula (II):

$$\zeta^a - Q\text{-}AA^a{}_2\text{-}AA^a{}_3\text{-}P\text{-}R\text{-}OH \quad (II),$$

wherein:

- ζ^a is a hydrogen atomor a Zn chelating group, such as cysteine, C-[linker]-, N-acetyl-cysteine, N-mercaptoacetyl (HS-CH_2-CO-), hydroxamic acid (HO-NH-CO-) or an optionally substituted hydroxyquinoline,
- $AA^a{}_2$ is R or H, preferably R,
- $AA^a{}_3$ is G, N, F or F(X), preferably F,
- said Q, AA_2, AA_3, P, and R may be independently either in the L-configuration or D-configuration ,and any one of Q, AA_2, AA_3, P, and R may be optionally a β aminoacid, an aza-aminoacid or a β–aza-aminoacid;

wherein if the peptide derivative comprises a cysteine, said peptide derivative is optionally a dimer,

with the proviso that the peptide is not QHNPR or QRGPR.

[0046] Said peptide derivative of formula (II) is a modified opiorphin peptide and is advantageously a human AP-N inhibitor. In particular it may have an inhibitory potency towards AP-N higher (preferably at least 6 fold, 8 fold, or 10 fold) than its inhibitory potency towards NEP endopeptidase and/or carboxydipeptidase activity(ies).

[0047] Preferred peptide derivatives of formula (II) are:

QRGPR,

QHNPR, and

QRFPR.

[0048] In an embodiment, the invention is related to a peptide derivative of formula (III):

$$\zeta^n - AA_1\text{-}AA^n{}_2\text{-}AA^n{}_3\text{-}\ AA^n{}_4\text{-}AA^n{}_5\text{-}OH \quad (III),$$

wherein:

- ζ^n is a hydrogen atom, tyrosine or Y-[linker]-,
- AA_1 is Q or Glp, preferably Q,
- $AA^n{}_2$ is K or R, preferably R,
- $AA^n{}_3$ is Y, F or F(X), preferably F or F(X),
- $AA^a{}_4$ is S or S(OAlk),
- $AA^n{}_5$ is K or R, preferably R,
- said AA_1, AA_2, AA_3, AA_4, and AA_5 may be independently either in the L-configuration or D-configuration ,and any one of AA_1, AA_2, AA_3, AA_4, and AA_5 may be optionally a β aminoacid, an aza-aminoacid or a β–aza-aminoacid;

with the proviso that the peptide is not QRFSR, QHNPR, YQRFSR or GlpRFSR.

[0049] Said peptide derivative of formula (III) is a modified opiorphin peptide and is advantageously a human NEP inhibitor. In particular, it may have an inhibitory potency towards NEP endopeptidase and/or carboxydipeptidase activity(ies) higher (preferably at least 6 fold, 8 fold, or 10 fold than its inhibitory potency towards AP-N.

[0050] In one embodiment, the invention is related to a peptide derivative of formula (IIIa):

$$AA_1\text{-}R\text{-}AA^n{}_3\text{-}S(OAlk)\text{-}AA^n{}_5\text{-}OH \quad (IIIa),$$

wherein:

- AA_1 is Q or Glp,

- AA^n_3 is F or F(X),
- AA^n_5 is K or R, preferably R,

with the proviso that the peptide is not QRFSR, QHNPR, YQRFSR or GlpRFSR.

[0051] Preferred peptide derivatives of formula (III) or (IIIa) are:

QRYSR,

QRF(X)SR,

QKFSR,

QRFSK,

QRFS(OAlk)R, preferably QRFS(O-octanoyl)R and

Y-[NH-$(CH_2)_{12}$-CO]-QRFSR.

[0052] In an embodiment, the invention is related to a peptide derivative of formula (IV):

$$\zeta^m\text{-Q-R-}AA^m_3\text{-}AA^m_4\text{-R-OH} \quad (IV),$$

wherein:

- ζ^m is a hydrogen atom, or a Zn chelating group, such as cysteine, C-[linker]-, N-acetyl-cysteine, N-mercaptoacetyl (HS-CH_2-CO-), hydroxamic acid (HO-NH-CO-) or an optionally substituted hydroxyquinoline,
- AA^n_3 is F or F(X), preferably F(X),
- AA^m_4 is S or S(OAlk), preferably S or S(OAlk),
- said Q, R, AA_3, AA_4, and R may be independently either in the L-configuration or D-configuration ,and any one of Q, R, AA_3, AA_4, and R may be optionally a β aminoacid, an aza-aminoacid or a β–aza-aminoacid;

wherein if the peptide derivative comprises a cysteine, said peptide derivative is optionally a dimer,

with the proviso that the peptide is not QRFSR or YQRFSR.

[0053] Peptide derivatives of formula (IV) are modified opiorphin peptides which are advantageously dual human NEP and human AP-N inhibitors, as they display significative inhibitory potency towards both human NEP and human AP-N.

[0054] Preferred peptide derivatives of formula (IV) are:

CQRFSR,

$[CQRFSR]_2$,

C-(-HN-$(CH_2)_{12}$-CO-)-QRFSR;

C-(-HN-$(CH_2)_6$-CO-)-QRFSR;

C-[linker]-QRF-S(O-octanoyl)-R, preferably C-(-HN-$(CH_2)_6$-CO-)-QRF-S(O-octanoyl)-R, C-(-HN-$(CH_2)_8$-CO-)-QRF-S(O-octanoyl)-R or C-(-HN-$(CH_2)_{12}$-CO-)-QRF-S(O-octanoyl)-R;

QRF(X)SR and

QRFS(OAlk)R, preferably QRFS(O- octanoyl)R.

[0055] Preferably, the peptide derivatives of the invention here are modified opiorphin peptides and have inhibitory potency against neutral endopeptidase NEP and/or aminopeptidase AP-N. Most preferably, the modified opiorphin peptides of the invention have inhibitory potency against endopeptidase NEP and aminopeptidase AP-N.

[0056] The peptide derivatives according to the present invention may be prepared in a conventional manner by peptide synthesis in liquid or solid phase by successive couplings of the different amino acid residues to be incorporated (from the N-terminal end to the C-terminal end in liquid phase, or from the C-terminal end to the N-terminal end in solid phase) wherein the N-terminal ends and the reactive side chains are previously blocked by conventional groups.

[0057] For solid phase synthesis the technique described by Merrifield may be used in particular. Alternatively, the technique described by Houbenweyl in 1974 may also be used.

[0058] The peptide derivatives according to the present invention may also be obtained using genetic engineering methods, provided they consist of natural aminoacids.

[0059] Modifications to the opiorphin peptides include chemical (e.g., containing additional chemical moieties, such as methyl, polyethylene C_2, C_4, C_6, C_8, C_{10}, C_{12} and polyethyleneglycol thereof, and/or glycosylated forms, and peptidomimetics (e.g., a low molecular weight compound that mimics a peptide in structure and/or function (see, e.g., Abell, *Advances in Amino Acid Mimetics and Peptidomimetics*, London: JAI Press (1997); Gante, *Peptidmimetica – massgeschneiderte Enzyminhibitoren Angew. Chem.* 106: 1780-1802 (1994); and Olson et al., *J. Med. Chem.* 36: 3039-3049 (1993)).

[0060] Other modifications which may be introduced in the peptide derivatives of the invention include using unnatural amino acids, D amino acid , β_2 amino acid, β_3 amino acid, Aza amino acid or β-Aza amino acid instead of L amino acid, conformational restraints, isosteric replacement, cyclization, or other modifications. Other examples are where one or more amide bond is replaced by a non-amide bond, and/or one or more amino acid side chain is replaced by a different chemical moiety, or one or more of the N-terminus, the C-terminus or one or more side chain is protected by a protecting group, and/or double bonds

and/or cyclization and/or stereospecificity is introduced into the amino acid chain to increase rigidity and/or binding affinity.

[0061] Based on the crystal structure of the binding domain of the metallo-ectopeptidase targeted by the peptide derivative of the invention, mimetics can also be obtained by means of computer-assisted drug design development (Oefner et al. J. Mol. Biol. (2000) 296(2):341-9; Gomeni et al. Eur. J. Pharm. Sci. (2001) 13(3):261-70; Kan, Curr Top Med Chem (2002), 2(3):247-69).

[0062] Still other modifications include:

[0063] - protecting the NH_2 and COOH hydrophilic groups by esterification (COOH) with lipophilic alcohols or by amidation (COOH) and/or by acetylation (NH_2) or added carboxyalkyl or aromatic hydrophobic chain at the NH_2 terminus ;

[0064] - retroinversion isomers of the CO-NH amide bonds or methylation (or ketomethylene, methyleneoxy, hydroxyethylene) of the amide groups ;

[0065] - insertion of $-[HN-(CH_2)_n-CO-]-$ moieties between two aminoacids, n being an integer from 1 to 20, preferably 6, 8 or 12.

[0066] The nucleic acids, also named polynucleotides, such as DNA or RNA molecules, that encode the peptides, including peptide derivatives containing natural amino acids, defined above are also part of the invention, while taking into account the degeneration of the genetic code. Preferably, the nucleic acid comprises a sequence incoding a peptide derivative which consists of natural aminoacids.

[0067] The nucleic acids of the invention include sequences that are hybridizable to any of the above sequences or their complementary sequences under high stringency hybridization conditions which refers to hybridization and/or washing conditions at 68°C in 0.2 X SSC, at 42°C in 50 % formamide, 4 X SSC, or under conditions that afford levels of hybridization equivalent to those observed under either of these two conditions.

[0068] The present invention further relates to vectors for cloning and/or expression comprising a nucleic acid sequence of the invention and to host cell comprising the nucleic acid or said vector, *i.e.* a host cell wherein at least one of these vectors was transferred. The expression vector according to the invention comprises a nucleic acid sequence encoding a peptide, including a peptide derivative, or protein of the invention, said nucleic acid sequence being operably linked to elements allowing its expression. The vector advantageously contains a promoter sequence, signals for initiation and termination of translation, as well as appropriate regions for regulation of translation. Its insertion into the

host cell may be transient or stable. The vector may also contain specific signals for secretion of the translated protein.

[0069] Host cells may be prokaryotic or eukaryotic, including but not limited to bacteria, yeasts, plant cells, insect cells, mammalian cells, including cell lines which are commercially available. Preferred examples for host cells are COS-1, HEK cells, 293 cells, or CHO cells.

[0070] The present invention further provides antibodies, monoclonal or polyclonal, or fragments thereof specifically directed against (*i.e.* that specifically recognizes) the modified opiorphin peptides described herein. Such antibodies may be useful for instance for studying pharmacokinetic properties of the peptide derivatives of the invention.

[0071] The term "antibody" in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, Fab', F(ab')2 and F(v).

[0072] Procedures for raising polyclonal antibodies are also well known. Typically, such antibodies can be raised by administering the peptide derivative, including conjugate peptide derivative, of the present invention subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 50 µl per site at ten different sites or at least five different sites. The rabbits are then bled five weeks after the first injection and periodically boosted with the same antigen administered subcutaneously at five fold lower concentration than the primary injection at maximum depending on quality of the immune response three times every six weeks. A sample of serum is then collected every 10 days after each boost. Polyclonal antibodies are then recovered from the serum by affinity chromatography using the corresponding antigen to capture the antibody. This and other procedures for raising polyclonal antibodies are disclosed in E. Harlow, et. al., editors, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988).

[0073] A "monoclonal antibody" in its various grammatical forms refers to a population of antibody molecules that contain only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody thus typically displays a single binding affinity for any epitope with which it immunoreacts. A monoclonal antibody

may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different epitope, e.g. a bispecific monoclonal antibody.

[0074] Laboratory methods for preparing monoclonal antibodies are well known in the art (see, for example, Harlow et al., supra). Monoclonal antibodies (Mabs) may be prepared by immunizing a mammal, e.g. a mouse, rat, rabbit, goat, human and the like, against the peptide derivatives of the present invention, including conjugated peptide derivatives. The antibody-producing cells in the immunized mammal are isolated and fused with myeloma or heteromyeloma cells to produce hybrid cells (hybridoma). The hybridoma cells producing the monoclonal antibodies are used as a source of the desired monoclonal antibody.

[0075] While Mabs can be produced by hybridoma culture, the invention is not to be so limited. Also contemplated is the use of Mabs produced by an expressing nucleic acid cloned from a hybridoma. That is, the nucleic acid expressing the molecules secreted by a hybridoma can be transferred into another cell line to produce a transformant. The transformant is genotypically distinct from the original hybridoma but is also capable of producing antibody molecules of this invention, including immunologically active fragments of whole antibody molecules, corresponding to those secreted by the hybridoma. In addition, the literature provides methods for forming chimeric antibodies, humanized antibodies, single chain antibodies and the like variations on a basic immunoreactive antibody fragment. All of these are considered within the scope of the invention insofar as a class and specificity of antibody is disclosed and claimed, regardless of the precise variant structure that one skilled in the art may construct.

[0076] The modified opiorphin peptides can be formulated in pharmaceutical compositions in association with a pharmaceutically acceptable carrier. For instance, the pharmaceutical compositions are suitable for a topical, oral, sublingual, parenteral, intranasal, intravenous, intramuscular, subcutaneous, transcutaneous or intraocular administration and others.

[0077] The modified opiorphin peptides described above are useful in the treatment of diseases or disorders, wherein a modulation of the activity of a membrane metallo-ectopeptidase is sought, more particularly a membrane-zinc metallopeptidase, such as NEP and AP-N.

[0078] Natural NEP substrates are mainly the peptide hormones : Enkephalins, Substance P, Bradykinin, Angiotensin II and Atrial Natriuretic Peptide which play key role in the control of central and peripheral pain perception, inflammatory phenomena, mineral exchange and/or arterial tone (Roques et al., Pharmacol Rev. 1993;45(1):87-146).

[0079] More particularly, neutral endopeptidase, NEP 24-11, is distributed both in nervous and peripheral tissues of mammals, and in the periphery it is particularly abundant in the kidney and placenta. In these tissues the cell-surface metallopeptidase NEP participates in the postsecretory processing and metabolism of neuropeptides, systemic immunoregulatory peptides and peptide-hormones. By controlling the active levels of circulating or secreted regulatory peptides, NEP modulates their physiological receptor-mediated action. Hence, the membrane-anchored NEP is involved in regulating the activity of : potent vasoactive peptides such as Substance P, Bradykinin (BK), Atrial Natriuretic peptide (ANP), and Angiotensin II (AII) ; potent inflammatory/immunoregulatory peptides such as Substance P and BK and fMet-Leu-Phe (fMLP) ; potent opioid neuropeptides such as Met and Leu-Enkephalins (Enk) and potent mineral exchange and fluid homeostasis regulatory peptides such as ANP, C-type Natriuretic Peptide (CNP) and B-type Natriuretic Peptide (BNP). However the levels of these peptides are changed through the NEP-induced formation/degradation only in regions where they are tonically released or where their release is triggered by a stimulus.

[0080] From an integrative point of view, the NEP biological activity is to control the active levels of peptidergic signals involved in arterial tension regulation, in inflammatory phenomena, emotional states and in water-mineral homeostasis, as well as, in the control of pain processing. From a clinical point of view, this substantiates the fact that NEP is an important drug target in various disease states. For example, by inhibiting NEP and APN, thereby increasing the levels and duration of action of central or peripheral endogenous opioids, an analgesic effect or an anti-depressant or psychostimulant effect could be obtained. The main advantage of modifying the concentrations of endogenous regulatory peptides by use of NEP and/or AP-N inhibitors is that the pharmacological effects are induced only at receptor sites activated by the natural ligands, and are critically dependent on their tonic or stimulus-evoked release happening upon specific environmental, behavioral and physiopathological stressful situations (Roques et al, 1993).

[0081] Examples of mammalian membrane metallopeptidases besides NEP are NEP2, ECE (Endothelin-Converting Enzymes), in particular ECE1 and ECE2, the erythrocyte cell-surface antigen KELL and the product of PEX gene associated with X-linked hypophosphatemic rickets and AP-N (Aminopeptidase N).

[0082] NEP2 is distributed specifically in the nervous system and genital tissues.

[0083] AP-N is a ubiquitous enzyme present in a wide variety of human organs, tissues and cell types (endothelial, epithelial, fibroblast, leukocyte) and is in particular abundant in

the kidneys and central nervous system. Identified substrates include Angiotensin III (Ang III); neuropeptides, including enkephalins and endorphins; and hormones, including kallidan and somatostatin. AP-N is a multifunctional enzyme, related with tumorigenesis, immune system, pain, regulation of arterial blood pressure etc. AP-N is also involved in the trimming of antigen and the process of antigen presentation. These functions facilitate the modulation of bioactive peptide responses (pain management, vasopressin release) and influence immune functions and major biological events (cell proliferation, secretion, invasion, angiogenesis) thereby providing treatment options for many kinds of diseases.

[0084] Inhibition of ECE has a significant application in the treatment of hypertension and the prevention and treatment of atherosclerosis.

[0085] Inhibition of AP-N in conjunction with NEP has significant application in the treatment of pain and depression, anxiety, sedation and socio-sexual emotional disorders.

[0086] Inhibition of related membrane metallopeptidases has therapeutic effects in the treatment of tumors, namely ovarian, colorectal, brain, lung, pancreas, gastric and melanoma cancers, and reducing the incidence of metastasis, atherosclerosis and/or hypertension.

[0087] Inhibition of related membrane metallopeptidases has effects in pain relief. Such antinociceptive effects on acute pain are analgesic effects but also effects on chronic inflammatory pain such as arthritis or inflammatory bowel disease, post-operative injury and neuropathic severe pain associated or not with cancer and/or cancer therapy.

[0088] Furthermore, inhibition of bacterial or viral metallopeptidase is expected to have anti-infection effects.

[0089] Metallopeptidases playing an important role in pathogen host tissue invasion and immunological and inflammatory processes, for example those of *Streptococcus pyogenes, Pseudomonas aeruginosa, Porphyromonas gingivalis and Legionella pneumophila.*

[0090] Furthermore, bacterial metallopeptidases, especially zinc-metallopeptidases play an important role in the diseases caused by proteolytic toxins, such as the toxins of *B. anthracis* (Anthrax Lethal factor) and the neurotoxins of *C. tetanum* and *botulinum.*

[0091] Other metallopeptidases play an important role in various infections such as infections caused by HIV (FR 2 707 169).

[0092] The importance of proteinase inhibitors for the treatment of bacterial or viral diseases may be found in J. Potempa and J. Travis.

[0093] The different roles of metallopeptidases are disclosed in Turner et al, 2001; Kenny et al, 1977 ; Kenny et al, 1987 ; Beaumont et al, 1996.

[0094] One object of the present invention is providing an analgesia or anti-depressant therapy with the modified opiorphin peptides which acts by inhibiting NEP and AP-N at peripheral, spinal and/or supraspinal levels and thereby increasing the levels and duration of action of central or peripheral endogenous opioids, including enkephalins.

[0095] The treatment of pain, especially acute and chronic pain, visceral inflammatory and neuropathic pain, is contemplated.

[0096] The treatment of any hydro-mineral imbalance is also an aim of the invention. Among target disorders one may cite bone, teeth, kidney, parathyroid, pancreas, intestine, stomach mucosa, prostate, and salivary gland disorders that are caused by hydro-mineral imbalance.

[0097] In particular, the disorder may be selected from hyper or hypo-parathyroidism, osteoporosis, pancreatitis, submandibular gland lithiasis, nephrolithiasis and osteodystrophy.

[0098] The treatment of impaired interpersonal and behavioural disorders is of further interest. Various mental disorders are described in WO 02/051434.

[0099] In particular the invention is drawn at any disorder selected from the group consisting of avoidance disorder, decreased awareness disorder, autistic disorder, attention deficit hyperactivity disorder, arousal disorder, hospitalism, impaired interpersonal functioning and relationship to the external world, schizoid personality disorder, schizophrenia, depressive disorder, decreased interest in environment, impaired social activity linked to sexuality, and impaired sexual behaviour, including untimely ejaculation, hyperactive sexuality and erectile dysfunction.

[00100] The invention also relates to the use of the peptides or peptide derivatives according to the invention as psychostimulating agents. Accordingly the prevention or treatment of a narcolepsy, hypersomnia, obsessional compulsive troubles, mood disorders such as depressive disorders or major depressive disorder, either major depressive disorder single episode or major depressive disorder recurrent, type I or II bipolar disorder, dysthymic disorder and cyclothymic disorder.

[00101] Diseases wherein a modulation of a membrane metallopeptidase is sought also include hypertension, aterosclerosis, tumor, inflammatory arthritis and bowel disease.

[00102] Treatment of infections is also encompassed. Especially, the importance of proteinase inhibitors for the treatment of bacterial or viral diseases may be found in J. Potempa and Travis.

[00103] The modified opiorphin peptides described above are also useful for controlling immuno-inflammatory responses.

[00104] The modified opiorphin peptides as defined above are also useful as a natriuretic agent or a diuretic agent.

[00105] Thus, the present invention advantageously provides peptide derivatives as defined above for use in the treatment of a disease or disorder selected from the group consisting of pain, depressive disorders, impaired social activity linked to sexuality, and impaired sexual behaviour.

[00106] Another object of the present invention is the use of the above described peptide derivatives or nucleic acids as a substitute in the treatment of drug abuse, notably morphine drug abuse.

[00107] Indeed, studies have suggested that the vulnerability to drug abuse and the development of reward and drug dependence is at least in part, a result of pre-existent or induced modifications and/or defect of the endogenous opioid system. In this regard, using modified opiorphin peptides or nucleic acid to potentiate the effects of endogenous enkephalins will reduce the various side-effects (somatic signs of withdrawal) produced by interruption of chronic morphine or heroin administration.

[00108] Preferred diseases or disorders which may be treated with the peptide derivatives of the invention include pain, depressive disorders, impaired social activity linked to sexuality, and impaired sexual behaviour.

[00109] The invention further relates to the use of an agent that modulates the interaction between endogenous BPLP protein or maturation product, *e.g.* natural opiorphin QRFSR, and a membrane metallopeptidase for the preparation of a therapeutic composition for preventing or treating diseases wherein a modulation of the activity of said membrane metallopeptidase is sought.

[00110] The invention provides an *in vitro* method for screening compounds for their ability to bind to the NEP and/or APN binding site for the BPLP protein or a the QRFSR peptide. Details of this procedure are described, e.g., in U.S. application serial no. 10/593,071 filed on September 15, 2006, also published as WO 2005/090386, as well as in the international patent application PCT/EP2009/050567, the contents of which are incorporated herein by reference.

[00111] Another object of the present invention is a process for determining the relative affinity of the modified opiorphin peptides that specifically bind to the NEP and/or to the AP-N binding sites for the BPLP protein, or maturation products, (or the peptide that

retains the binding specificity or the physiological activity of the BPLP protein or of its matured products) as described in e.g., in U.S. application serial no. 10/593,071 filed on September 15, 2006, the contents of which are incorporated herein by reference.

[00112] EXAMPLES

[00113] Example 1: Methods for Biochemical Assays

[00114] Formal kinetic analysis was performed for each assay using real-time fluorescence monitoring of specific substrate hydrolysis. For each 96-well adapted fluorimetric model, all parameters allowing the analysis of human NEP and human AP-N enzyme activity were defined under conditions of initial velocity measurement.

[00115] 1- *Sources of the human ectopeptidases, hNEP and hAP-N*

[00116] Recombinant human NEP and recombinant human AP-N (devoid of their respective N-terminal cytosol and transmembrane segment) that were purchased from R&D Systems, were used as pure source of peptidase.

[00117] 2- *Substrates and synthetic inhibitors*

[00118] *In vitro*, Amino-, CarboxyDi- and Endo- Peptidase activities were assayed by measuring the breakdown of the following synthetic selective substrates:

[00119] - Abz-dR-G-L-EDDnp FRET-peptide that is an internally quenched fluorescent substrate specific for NEP-EndoPeptidase activity, was synthesized by Thermo-Fisher Scientific (Germany).

[00120] - Abz-R-G-F-K-DnpOH FRET-peptide that is an internally quenched fluorescent substrate specific for NEP-CarboxyDiPeptidase activity, was synthesized by Thermo-Fisher Scientific (Germany)

[00121] - Mca-R-P-P-G-F-S-A-F-K-(Dnp)-OH FRET-peptide (Mca-BK2) that is an intramolecularly quenched fluorogenic peptide structurally related to bradykinin, which is a selective substrate for measuring NEP and ECE activity, was purchased from R&D Systems.

[00122] FRET is the distance-dependant transfer of energy from a donor fluorophore (Abz=ortho-aminobenzoyl or Mca=7-methoxycoumarin-4-yl-acetyl) to an acceptor fluorophore (DnpOH=2,4-dinitrophenyl or EDDnp=2,4-dinitrophenyl ethylenediamine).

[00123] - L-alanine-Mca, Ala-Mca, a fluorogenic substrate for measuring aminopeptidase activity was purchased from Sigma.

[00124] Measuring the hydrolysis rate of these substrates by soluble ectopeptidases in the presence and absence of different available selective synthetic peptidase inhibitors assessed the specificity of each enzyme assay: – Thiorphan (NEP inhibitor) (Bachem), – Bestatin (AP inhibitor) (Calbiochem).

[00125] *3- Measurement of peptidase Activities using 96-well adapted fluorimetric assays*

[00126] According to conditions of initial velocity measurement: the time, pH and temperature of incubation as well as enzyme and substrate concentrations were defined for each assay. Hydrolysis of substrates was measured by real-time monitoring their metabolism rate by the two peptidases in the presence and absence of tested inhibitory compound (concentrations ranging from 1 to 50 µM). These were added to the preincubation medium. The background rate of substrate autolysis representing the fluorescent signal obtained in the absence of enzyme was subtracted to calculate the initial velocities in RFU (Relative Fluorescent Unit)/min.

[00127] *Measurement of NEP-endopeptidase activity using FRET specific peptide-substrate, Abz-dR-G-L-EDDnp.* Using black half-area 96 well microplate, the standard reaction consisted of enzyme (12.5 ng) in 100 mM Tris-HCl pH 7 containing 200 mM NaCl (100 µl final volume). The substrate (15 µM final concentration) was added after preincubation for 10 min at 28°C and the kinetics of appearance of the fluorescent signal (RFU) was directly analyzed for 40 min at 28 °C (2.3 min-interval successive measures) by using a fluorimeter microplate reader (monochromator Infinite 200-Tecan) at 320 nm and 420 nm excitation and emission wavelengths, respectively.

[00128] Under conditions of initial velocity measurement, the intensity of the signal was directly proportional to the quantity of metabolites formed during the 20-40 min time-period of the reaction. Thus, in absence of inhibitor, the initial velocity of rhNEP-mediated specific endoproteolysis of Abz-dR-G-L-EDDnp, was calculated from the linear regression (slope = NEP activity in presence of vehicle / incubation time) as 8218 ± 2878 RFU/min/µg rhNEP, n=3 independent determinations.

[00129] *Measurement of NEP-CarboxyDiPeptidase activity using FRET specific peptide-substrate Abz-R-G-F-K-DnpOH.* Using black half-area 96 well microplate, the standard reaction consisted of enzyme (2.5 ng) in 100 mM Tris-HCl pH 6.5 containing 50 mM NaCl (100 µl final volume). The substrate (4 µM final concentration) was added after

preincubation for 10 min and the kinetics of appearance of the fluorescent signal (RFU) was directly analyzed for 40 min at 28 °C (2.3 min-interval successive measures) by using the fluorimeter reader at 320 nm excitation and 420 nm emission wavelengths. Under these conditions of initial velocity measurement, human NEP-mediated specific hydrolysis of Abz-R-G-F-K-DnpOH was evaluated at 59796 ± 18685 RFU/min/µg rhNEP, n= 4 independent determinations. NEP-CarboxyDiPeptidase activity assayed with the substrate Abz-R-G-F-K-DnpOH is referred to as "NEP-CDP1 activity" in Figures 5-8.

[00130] In addition, the intramolecularly quenched fluorogenic peptide, Mca-BK2 (10 µM), was submitted to hydrolysis by 5 ng rhNEP under the same experimental conditions as those described behind. Under these conditions the hNEP-enzyme acted upon Mca-R-P-P-G-F-S-A-F-K-(Dnp)-OH mainly as a CarboxyDiPeptidase preferentially cleaving A-F bond but also as an EndoPeptidase cleaving the G-F bond. Under conditions of initial velocity measurement, human NEP-mediated specific hydrolysis of Mca-BK2 was evaluated at 121910 ± 24755 RFU/min/µg rhNEP, n= 3 independent determinations. NEP-CarboxyDiPeptidase activity assayed with the substrate Abz-R-G-F-K-DnpOH is referred to as "NEP-CDP2 activity" in Figures 5-8.

[00131] *Measurement of AP-N-ectopeptidase activity using Ala-Mca substrate.* Using black half-area 96 well microplate the standard reaction consisted of enzyme (4 ng) in 100mM Tris-HCl pH 7.0 (100 µl final volume). The Ala-Mca substrate (25 µM final concentration) was added after preincubation for 10 min at 28°C and the kinetics of appearance of the signal was monitored for 40 min at 28°C by using the fluorimeter reader at 380 nm excitation and 460 nm emission wavelengths. The intensity of the signal was directly proportional to the quantity of metabolites formed during the 10-40 min time-period of the reaction. Under these conditions of initial velocity measurement, the human AP-N-mediated aminoproteolysis of Ala-Mca was directly calculated (from the slope: AP-N activity in absence of inhibitor in function of incubation time) as 147042 ± 44657 RFU/min/µg rhAP-N, n = 3 independent determinations.

Other FRET-peptide substrates for assaying NEP carboxydipeptidase activity have been described in *Barros et al.* Biol. Chem., 2007, 388:447-455.

[00132] **Example 2: Results of Biochemical Assays**

[00133] *1- Screening of Opiorphin peptide-derivatives on hNEP and hAP-N*

[00134] Compounds were tested in triplicate at 50 µM final concentration.

[00135] Figures 1 to 9 display the results of biochemical assays on NEP endopeptidase and carboxydipeptidase activities and AP-N activity with various peptide derivatives of the invention.

[00136] Various Opiorphin peptido-analogs were tested for their inhibitory potency towards the two membrane-anchored ectoenzymes, NEP (specific endopeptidase and carboxydipeptidase activities) and APN by using selective fluorescence-based enzyme assays : the FRET-based Enzyme in Vitro Models previously developed and validated in the laboratory (PCT application PCT/ filed January 18, 2009, incorporated herein by reference).

[00137] The following compounds were analyzed. In the compounds below, NH2- represents the terminal amine of the peptide and COOH the terminal carboxylic acid of the peptide.

[00138] - NH2-QRFSR-CONH2 (Opiorphin); NH2-QRGPR-COOH; NH2-QHNPR-COOH;
NH2-QR(4BromoF)SR-COOH (i.e. QRF(4Br)SR); NH2-QRFPR-COOH: they displayed inhibitory preference for human AP-N and a weak inhibitory potency towards human NEP

[00139] - N-(Acetyl)QRFSR-COOH; N-(C8-polyethylene)QRFSR-COOH; N-(biotine-C6-polyethylene)QRFSR-COOH : they displayed weak inhibitory potency towards human AP-N and NEP with a preference for NEP endopeptidase activity

[00140] - NH2-dRdSdFdRdQ-COOH (retroinversion D-enantiomere): It did not display significant inhibitory activity towards human AP-N and NEP with a preference for AP-N activity

[00141] - NH2-YQRFSR-COOH; NH2-Y-(C6-polyethylene)QRFSR-COOH; NH2-Y-(C12-polyethylene)QRFSR-COOH: they displayed inhibitory preference for human NEP endopeptidase activity in particular Y-(C12-polyethylene)QRFSR

[00142] - NH2-QRF[S-O-C8-polyethylene]R-COOH; NH2-CQRFSR-COOH: They were potent dual inhibitors of human NEP (specific endopeptidase and carboxydipeptidase activities) and AP-N.

[00143] Other modified opiorphin peptides are:

[00144] NH2-CQRF[S-O-C8-polyethylene]R-COOH;

[00145] NH2-CQRF[S-O-C12-polyethylene]R-COOH

[00146] NH2-C-(C8-polyethylene)QRFSR-COOH;

[00147] NH2-C-(C12-polyethylene)QRFSR-COOH

[00148] NH2-C-(C8-polyethylene)QRF[S-O-C8-polyethylene]R-COOH

[00149] NH2-[Cß2]QRF[S-O-C8-polyethylene]R-COOH

[00150] NH2-C-(C8-polyethylene)QRFS[ß3R]-COOH

[00151] NH2-C[dQ]RF[S-O-C8-polyethylene][dR]-COOH

[00152] NH2-C-(C8-polyethylene)QRFS[dR]

[00153] NH2-[dC]QRF[S-O-C8-polyethylene][dR]-COOH

[00154] NH2-[Cß2]QRF[S-O-C8-polyethylene][ß3R]-COOH

[00155] [CQRFSR]2 as cystine-dipeptide through disulfide linkage .

[00156] *2- Concentration-dependent Inhibition of selected Opiorphin peptide-derivatives on hNEP and hAP-N*

[00157] **CQRFSR-COOH peptide** (Figures 9 and 18)

[00158] The CQRFSR-COOH peptide prevented in a concentration dependent manner the Abz-dR-G-L-EDDnp cleavage mediated by the rhNEP-Endopeptidase activity: $r^2 = 0.97$, n = 18 determination points, with a IC50 at 7 ± 3 µM.

[00159] CQRFSR-COOH peptide prevented in a concentration dependent manner the Abz-R-G-F-K-DnpOH cleavage mediated by the rhNEP activity: $r^2 = 0.99$, n = 18 determination points, with a IC50 at 6 ± 1 µM.

[00160] CQRFSR-COOH peptide prevented in a concentration dependent manner the Ala-AMC cleavage mediated by the rhAP-N activity: $r^2 = 0.99$, n = 30 determination points, with a IC50 at 0.8 ± 0.1 µM.

[00161] CQRFSR-COOH peptide prevented in a concentration dependent manner the Mca-R-P-P-G-F-S-A-F-K-(Dnp)-OH FRET-peptide (Mca-BK2) cleavage mediated by the rhNEP activity: $r^2 = 0.99$, n = 16 determination points, with a IC50 at 17 ± 2 µM.

[00162] Therefore, modification of the N-terminal position of the NH2-QRFSR peptide by formation of amide link with a Cysteine amino acid (the thiol functional group is a strong Zn-chelating group) led to a compound (CQRFSR peptide) displaying reinforced inhibitory potency towards hAP-N (10-fold compared to QRFSR native peptide) and towards hNEP (5-fold).

[00163] **QRF[S-O-Octanoyl]R peptide** (Figure 10)

[00164] QRF[S-O-C8]R peptide prevented in a concentration dependent manner the Abz-dR-G-L-EDDnp cleavage mediated by the rhNEP-Endopeptidase activity: $r^2 = 0.98$, $n = 18$ determination points, with a IC50 at 2.8 ± 0.2 µM.

[00165] QRF[S-O-C8]R peptide prevented in a concentration dependent manner the Abz-R-G-F-K-DnpOH cleavage mediated by the rhNEP activity: $r^2 = 0.99$, $n = 18$ determination points, with a IC50 at 12 ± 5 µM.

[00166] QRF[S-O-C8]R peptide prevented in a concentration dependent manner the Ala-AMC cleavage mediated by the rhAP-N activity: $r^2 = 0.99$, $n = 20$ determination points, with a IC50 at 10 ± 1 µM.

[00167] QRF[S-O-C8]R peptide prevented in a concentration dependent manner the Mca-R-P-P-G-F-S-A-F-K-(Dnp)-OH FRET-peptide (Mca-BK2) cleavage mediated by the rhNEP activity: $r^2 = 0.99$, $n = 18$ determination points, with a IC50 at 14 ± 4 µM.

[00168] **Y-[amino-dodecanoic-acid spacer]-QRFSR peptide** (Figures 11 and 19)

[00169] Y(PE12)QRFSR-COOH peptide prevented in a concentration dependent manner the Abz-dR-G-L-EDDnp cleavage mediated by the rhNEP-Endopeptidase activity: $r^2 = 0.98$, $n = 18$ determination points, with a IC50 at 10 ± 1 µM.

[00170] Y(PE12)QRFSR-COOH peptide prevented in a concentration dependent manner the Abz-R-G-F-K-DnpOH cleavage mediated by the rhNEP activity: $r^2 = 0.99$, $n = 18$ determination points, with a IC50 at 24 ± 2 µM.

[00171] Y(PE12)QRFSR-COOH peptide prevented in a concentration dependent manner the Ala-AMC cleavage mediated by the rhAP-N activity: $r^2 = 0.99$, $n = 20$ determination points, with a IC50 at 122 ± 20 µM.

[00172] Y(PE12)QRFSR-COOH peptide prevented in a concentration dependent manner the Mca-R-P-P-G-F-S-A-F-K-(Dnp)-OH FRET-peptide (Mca-BK2) cleavage mediated by the rhNEP activity: $r^2 = 0.99$, $n = 18$ determination points, with a IC50 at 31 ± 2 µM.

Thus the inhibitory preference for human NEP endopeptidase activity of Y-(C12-polyethylene)QRFSR was confirmed

[00173] **C[PE6]QRFSR peptide** (PE6=amino-hexanoic-acid) (Figure 20)

[00174] C[PE6]QRFSR-COOH peptide prevented in a concentration dependent manner the Abz-dR-G-L-EDDnp cleavage mediated by the rhNEP-Endopeptidase activity: $r^2 = 0.98$, $n = 18$ determination points, with a IC50 at 40 ± 5 µM.

[00175] C[PE6]QRFSR-COOH peptide prevented in a concentration dependent manner the Abz-R-G-F-K-DnpOH cleavage mediated by the rhNEP activity: $r^2 = 0.99$, $n = 18$ determination points, with a IC50 at about 217.

[00176] C[PE6]QRFSR-COOH peptide prevented in a concentration dependent manner the Ala-AMC cleavage mediated by the rhAP-N activity: $r^2 = 0.99$, $n = 20$ determination points with a IC50 at 0.8 ± 0.1 μM.

[00177] C[PE6]QRFSR-COOH peptide prevented in a concentration dependent manner the Mca-R-P-P-G-F-S-A-F-K-(Dnp)-OH FRET-peptide (Mca-BK2) cleavage mediated by the rhNEP activity: $r^2 = 0.99$, $n = 18$ determination points, with a IC50 at about 227 μM.

[00178] **C-[amino-dodecanoic-acid-spacer]-QRFSR peptide** (Figure 21)

[00179] C[PE12]QRFSR-COOH peptide prevented in a concentration dependent manner the Abz-dR-G-L-EDDnp cleavage mediated by the rhNEP-Endopeptidase activity: $r^2 = 0.96$, $n = 24$ determination points, with a IC50 at 5.7 ± 0.5 μM.

[00180] C[PE12]QRFSR-COOH peptide prevented in a concentration dependent manner the Abz-R-G-F-K-DnpOH cleavage mediated by the rhNEP activity: $r^2 = 0.97$, $n = 30$ determination points, with a IC50 at 19 ± 2 μM.

[00181] C[PE12]QRFSR-COOH peptide prevented in a concentration dependent manner the Ala-AMC cleavage mediated by the rhAP-N activity: $r^2 = 0.98$, $n = 27$ determination points, with a IC50 at 0.8 ± 0.1 μM.

[00182] **C-[amino-hexanoic-acid spacer]-QRFS[O-Octanoyl]R peptide** (Figure 22)

[00183] C[PE6]QRF[S-O-Octanoyl]R peptide prevented in a concentration dependent manner the Abz-dR-G-L-EDDnp cleavage mediated by the rhNEP-Endopeptidase activity: $r^2 = 0.99$, $n = 27$ determination points, with a IC50 at 759 ± 56 nM.

[00184] C[PE6]QRF[S-O-Octanoyl]R peptide prevented in a concentration dependent manner the Abz-R-G-F-K-DnpOH cleavage mediated by the rhNEP activity: $r^2 = 0.99$, $n = 27$ determination points, with a IC50 at 848 ± 58 nM.

[00185] C[PE6]QRF[S-O-Octanoyl]R peptide prevented in a concentration dependent manner the Ala-AMC cleavage mediated by the rhAP-N activity: $r^2 = 0.99$, $n = 24$ determination points, with a IC50 at 1.7 ± 0.1 μM.

[00186] In a biologically relevant in vitro assay, using the Substance P physiological NEP-substrate and human cell membranes as source of hNEP, the C[PE6]QRF[S-O-Octanoyl]R derivative peptide prevented in a concentration dependent manner the Substance P cleavage mediated by the membrane-bound hNEP-Endopeptidase activity: r2 = 0.95, n = 13 determination points, with a IC50 at 1.6 ± 0.4 μM (at least 5-fold increase inhibitory potency compared to Opiorphin native peptide for the same test) (figure 23).

[00187] [CQRFSR] dipeptide ([CQRFSR]$_2$)

[00188] [CQRFSR] dipeptide prevented in a concentration dependent manner the Abz-dR-G-L-EDDnp cleavage mediated by the rhNEP-Endopeptidase activity: $r^2 = 0.87$, $n = 24$ determination points, with a IC50 at 1.4 ± 0.5 µM.

[00189] [CQRFSR] dipeptide prevented in a concentration dependent manner the Abz-R-G-F-K-DnpOH cleavage mediated by the rhNEP activity: $r^2 = 0.99$, $n = 27$ determination points, with a IC50 at 3.3 ± 0.2 µM.

[00190] [CQRFSR] dipeptide prevented in a concentration dependent manner the Ala-AMC cleavage mediated by the rhAP-N activity: $r^2 = 0.99$, $n = 24$ determination points, with a IC50 at 0.8 ± 0.1 µM.

[00191] Table 1: Summary of IC50 values of various peptides and peptide derivatives on NEP and AP-N activities

Opiorphin derivatives	IC50, µM towards hAP-N	IC50, µM towards hNEP-Endopeptidase	IC50, µM towards hNEP-CarboxyDiPeptidase
Y-[PE12]QRFSR	122 ± 20	10 ± 1	24 ± 2 / 31 ± 2
QRFS[O-C8]R	10 ± 1	2.8 ± 0.2	12 ± 5 / 14 ± 4
CQRFSR	0.8 ± 0.1	7 ± 3	6 ± 1 / 17 ± 2
C-[PE6]-QRFSR	0.8 ± 0.1	40 ± 5	~220
C-[PE12]-QRFSR	0.8 ± 0.1	5.7 ± 0.5	19 ± 2
C-[PE6]-QRFS[O-C8]R	1.7 ± 0.1	0.76 ± 0.06	0.85 ± 0.06
[CQRFSR]2	0.8 ± 0.1	1.4 ± 0.5	3.3 ± 0.2

[00192] All together data showed the importance of the N-terminal α amine-group of the NH2-QRFSR peptide for the inhibitory potency of Opiorphin towards human AP-N.

[00193] Indeed, as compared to Opiorphin-QRFSR native peptide, its acetylation (Ac-QRFSR) or cyclisation (pGlu-RFSR), its octanoylation (C8-QRFSR) or biotinylation (biotine-C6)QRFSR, led to compounds displaying a weak inhibitory potency towards hAP-N.

[00194] However, compared to Opiorphin-QRFSR native peptide, the two compounds: pGlu-RFSR and C8-QRFSR displayed at least equivalent if not better inhibitory potency for human hNEP.

[00195] These data further demonstrate the importance of the free carboxyl terminus of the QRFSR-COOH peptide for its inhibitory potency towards hNEP, especially towards the CarboxyDiPeptidase activity of NEP as compared to Opiorphin (QRFSR) native peptide, amidation (QRFSR-CONH2) led to a compound displaying a weak inhibitory potency towards hNEP activity.

[00196] The Phe residue of the QRFSR peptide is important for the inhibitory potency of Opiorphin towards hNEP and hAP-N peptidase activities. Indeed, as compared to Opiorphin (QRFSR) native peptide,substitution of Tyr residue for Phe (QRYSR) led to a compound displaying a 6-fold decrease in inhibitory potency for hAP-N and a light reduction of inhibitory potency towards human NEP.

[00197] However, interestingly substitution of 4-Fluoro-Phe residue for Phe (QR[4F]FSR) led to a compound displaying only a 2-fold decrease in inhibitory potency towards hNEP and a equivalent inhibitory potency towards hAP-N compared to Opiorphin-QRFSR native peptide. Conversely, substitution of (4Bromo-Phe) residue or Phe (QR[4Br]FSR) led to a compound displaying a weak inhibitory potency towards human NEP.

[00198] Modification of the RFS central residues of the QRFSR peptide affects the inhibitory potency of Opiorphin towards hNEP, since, as compared to Opiorphin-QRFSR native peptide, the compounds QRGPR, QHNPR, and QRFPR displayed equivalent inhibitory potency for human AP-N and a weak inhibitory potency towards human NEP.

[00199] The results further demonstrate the importance of the guanidium ion of the Arg residue at position 2 of the QRFSR peptide for the inhibitory potency of Opiorphin towards hAPN. As compared to Opiorphin-QRFSR native peptide, substitution of a ε-amine of Lys residue (QKFSR) for Arg2 led to a compound displaying more than a 10-fold decrease in inhibitory potency towards hAP-N and a light reduction in inhibitory potency towards human NEP.

[00200] Similarly the guanidium ion of the Arg residue at position 5 of the QRFSR peptide is important for the inhibitory potency of Opiorphin towards hAPN, as compared to

Opiorphin-QRFSR native peptide, substitution of a ε-amine of Lys residue for Arg5 (QRFSK) led to a compound displaying a 10-fold decrease in inhibitory potency for hAP-N and an equivalent inhibitory potency for human NEP.

[00201] Interestingly, esterification of the hydroxyl group of the serine residue of the QRFSR peptide by octanoic acid (QRF[octanoyl-Serine]R) led to a compound displaying equivalent inhibitory potency towards hAP-N compared to Opiorphin-QRFSR native peptide and reinforced inhibitory potency towards hNEP-Endo and CarboxyDi-peptidase activities (at least 10-fold more inhibitory potency than Opiorphin native peptide) (see Figure 19).

[00202] **Example 3: Identification of in vitro potent bioactive peptido-mimetics of Opiorphin that would potentially displaying in vivo bioavailability properties superior to the native peptide**

[00203] *1. Potential gain in biological adsorption: trans-membrane transport (epithelial and endo-epithelial cell membrane passage)*

[00204] Modifications to Opiorphin-peptide by addition of chemical hydrophobic moieties such as polyethylene C6, C8, C10 or C12 spacers would allow to increase its biological in vivo adsorption potency towards membranes. Among all compounds tested, NH2-QRF[S-O-Octanoyl]R-COOH – NH2-Y(PE12)QRFSR-COOH (PE= [CH2]n; PE12=amino-dodecanoic-acid) were shown to be potent dual inhibitors of human NEP (specific Endopeptidase and CarboxyDiDeptidase activities) and AP-N activities

[00205] *2- Potential gain in metabolic stability: Cystine-dipeptide: $[CQRFSR]_2$*

[00206] [CQRFR] di-peptide displayed at least 2-fold increase inhibitory potency towards hNEP-Endo and CarboxyDi-peptidase activities compared to CQRFSR monomer peptide. The di-peptide sequence should allow to protect the derivative compound against degradation by circulating aminopeptidase.

[00207] Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

CLAIMS:

1. A peptide derivative of formula (I):

$$\zeta\text{-}AA_1\text{-}AA_2\text{-}AA_3\text{-}AA_4\text{-}AA_5\text{-}OH \quad (I),$$

wherein:

- ζ is hydrogen atom, tyrosine, Y-[linker]- or a Zn chelating group, such as cysteine, C-[linker]-, N-acetyl-cysteine, N-mercaptoacetyl ($HS\text{-}CH_2\text{-}CO$-), hydroxamic acid (HO-NH-CO-) or an optionally substituted hydroxyquinoline,
- AA_1 is Q or Glp,
- AA_2 is K, R or H, preferably R,
- AA_3 is Y, G, N, F or F(X), preferably F or F(X),
- AA_4 is P, S or S(OAlk), preferably S or S(OAlk),
- AA_5 is K or R, preferably R,
- C-[linker]- meaning Cys-[NH-(CH_2)n-CO]-, wherein n is an integer between 1 and 20,
- Y-[linker]- meaning Tyr-[NH-$(CH_2)_{n'}$-CO]-, wherein n' is an integer between 1 and 20,
- F(X) meaning a phenylalanine, the phenyl group of which is substituted by one or more halogen atoms,
- S(OAlk) meaning a serine, the hydroxyl group of which is substituted by a linear or branched alkyl group having from 1 to 20 carbon atoms,
- said AA_1, AA_2, AA_3, AA_4, and AA_5 may be independently either in the L-configuration or D-configuration, and any one of AA_1, AA_2, AA_3, AA_4, and AA_5 may be optionally a β aminoacid, an aza-aminoacid or a β–aza-aminoacid;

wherein if the peptide derivative comprises a cysteine, said peptide derivative is optionally a dimer,

with the proviso that the peptide is not QRFSR, QHNPR, QRGPR, YQRFSR or GlpRFSR.

2. The peptide derivative according to claim 1, of formula (II) :

$$\zeta^a\text{-}Q\text{-}AA^a{}_2\text{-}AA^a{}_3\text{-}P\text{-}R\text{-}OH \quad (II),$$

wherein:

- ζ^a is a hydrogen atom, or a Zn chelating group, such as cysteine, C-[linker]-, N-acetyl-cysteine, N-mercaptoacetyl (HS-CH_2-CO-), hydroxamic acid (HO-NH-CO-) or an optionally substituted hydroxyquinoline,
- AA^a_2 is R or H, preferably R,
- AA^a_3 is G, N, F or F(X), preferably F,
- C-[linker]-, F(X) and S(OAlk) being as defined in claim 1,
- said Q, AA_2, AA_3, P, and R may be independently either in the L-configuration or D-configuration ,and any one of Q, AA_2, AA_3, P, and R may be optionally a β aminoacid, an aza-aminoacid or a β–aza-aminoacid;

wherein if the peptide derivative comprises a cysteine, said peptide derivative is optionally a dimer,

with the proviso that the peptide is not QHNPR or QRGPR.

3. The peptide derivative according to claim 1, of formula (III):

$$\zeta^n - AA_1\text{-}AA^n_2\text{-}AA^n_3\text{-}AA^n_4\text{-}AA^n_5\text{-}OH \quad (III),$$

wherein:

- ζ^n is a hydrogen atom, tyrosine or Y-[linker]-,
- A A_1 is Q or Glp, preferably Q,
- AA^n_2 is K or R, preferably R,
- AA^n_3 is Y, F or F(X), preferably F or F(X),
- AA^a_4 is S or S(OAlk),
- AA^n_5 is K or R, preferably R,
- Y-[linker]-, F(X) and S(OAlk) being as defined in claim 1,
- said AA_1, AA_2, AA_3, AA_4, and AA_5 may be independently either in the L-configuration or D-configuration ,and any one of AA_1, AA_2, AA_3, AA_4, and AA_5 may be optionally a β aminoacid, an aza-aminoacid or a β–aza-aminoacid;

with the proviso that the peptide is not QRFSR, QHNPR, YQRFSR or GlpRFSR.

4. The peptide derivative according to claim 1, of formula (IV):

$$\zeta^m\text{-}Q\text{-}R\text{-}AA^m_3\text{-}AA^m_4\text{-}R\text{-}OH \quad (IV),$$

wherein:

- ζ^m is a hydrogen atom, or a Zn chelating group, such as cysteine, C-[linker]-, N-acetyl-cysteine, N-mercaptoacetyl (HS-CH_2-CO-), hydroxamic acid (HO-NH-CO-) or an optionally substituted hydroxyquinoline,
- AA^n_3 is F or F(X), preferably F(X),
- AA^m_4 is S or S(OAlk), preferably S or S(OAlk),
- C-[linker]-, F(X) and S(OAlk) being as defined in claim 1,
- said Q, R, AA_3, AA_4, and R may be independently either in the L-configuration or D-configuration ,and any one of Q, R, AA_3, AA_4, and R may be optionally a β aminoacid, an aza-aminoacid or a β–aza-aminoacid;

wherein if the peptide derivative comprises a cysteine, said peptide derivative is optionally a dimer,

with the proviso that the peptide is not QRFSR or YQRFSR.

5. The peptide derivative of claim 1, which is

QRFSR-NH_2;

QR-F[4Br]-SR, wherein -F[4Br]- is a phenylalanine, the phenyl group of which is substituted in the para position by a bromo atom;

QRFPR;

(Acetyl)-QRFSR;

C-(-HN-$(CH_2)_8$-CO-)-QRFSR;

biotine-(-HN-$(CH_2)_6$-CO-)-QRFSR;

dR-dS-dF-dR-dQ;

Y-(-HN-$(CH_2)_6$-CO-)-QRFSR;

Y-(-HN-$(CH_2)_{12}$-CO-)-QRFSR;

QRF-S(O-octanoyl)-R;

CQRFSR;

CQRF-S(O-octanoyl)-R;

CQRF-S(O-dodecanoyl)-R;

C-(-HN-$(CH_2)_8$-CO-)-QRFSR;

C-(-HN-$(CH_2)_{12}$-CO-)-QRFSR;

C-(-HN-$(CH_2)_8$-CO-)-QRF-S(O-octanoyl)-R;

[Cß2]QRF-S(O-octanoyl)-R;

C-(-HN-$(CH_2)_8$-CO-)-QRFS-[ß3R];

C-[dQ]-RF-S(O-octanoyl)-[dR];

C-(-HN-$(CH_2)_8$-CO-)-QRFS-[dR];

[dC]-QRF-S(O-octanoyl)-[dR];

[Cß2]-QRF-S(O-octanoyl)-[ß3R];

$[CQRFSR]_2$;

QRYSR;

QR-F[4F]-SR, wherein -F[4F]- is a phenylalanine, the phenyl group of which is substituted in the para position by a fluoro atom;

QR-F[4Br]-SR, wherein -F[4Br]- is a phenylalanine, the phenyl group of which is substituted in the para position by a bromo atom;

QKFSR;

QRFSK;

C-(-HN-$(CH_2)_6$-CO-)-QRFSR;

C-(-HN-$(CH_2)_6$-CO-)-QRF-S(O-octanoyl)-R;

C(-HN-$(CH_2)_{12}$-CO-)QRF-S(O-octanoyl)-R;

C-(-HN-$(CH_2)_{12}$-CO-)-QRFS-dR;

C-(-HN-$(CH_2)_{12}$-CO-)-QRF-S(O-octanoyl)-β3R;

C-(-HN-$(CH_2)_8$-CO-)-QRF-S(O-octanoyl)β3R;

wherein :

Cß2 is $H_2N(-CH_2-SH)-CH_2-CO-$;

ß3R is $-NH-CH_2-C[-(CH_2)_3-NH-C(NH)(NH_2)]-COOH$;

-S(-O-octanoyl) means a serine, the hydroxyl group of which is substituted by an octanoyl group,

-S(-O-dodecanoyl) means a serine, the hydroxyl group of which is substituted by a dodecanoyl group.

6. The peptide derivative according to anyone of claims 1 to 5, which has inhibitory potency against neutral endopeptidase NEP and/or aminopeptidase AP-N.

7. The peptide derivative according to claim 2, which has inhibitory potency against human AP-N.

8. The peptide derivative according to claim 3, which has inhibitory potency against human NEP.

9. The peptide derivative according to claim 4, which is a dual neutral endopeptidase NEP and aminopeptidase AP-N inhibitor.

10. A composition comprising at least one peptide derivative according to any one of claims 1 to 9, and a pharmaceutically acceptable carrier.

11. A method of treatment of diseases or disorders in a subject in need of such treatment, wherein a modulation of the activity of a membrane metallo-ectopeptidase is sought, the method comprising administering one or more of the peptide derivative (s) of any one of claims 1 to 9 to the subject in an amount to treat the disease or disorder.

12. The method according to claim 11, wherein the membrane metallo-ectopeptidase is a membrane-zinc metallopeptidase.

13. The method according to claim 11 or 12, wherein the membrane metallo-ectopeptidase is NEP and/or AP-N.

14. The method according to any one of claims 11 to 13, for the treatment of a disease or disorder selected from the group consisting of pain, depressive disorders, impaired social activity linked to sexuality, and impaired sexual behaviour.

15. A peptide derivative as defined in any one of claims 1 to 9, for use in the treatment of diseases or disorders wherein a modulation of the activity of a membrane metallo-ectopeptidase is sought.

16. The peptide derivative according to claim 15, wherein the membrane metallo-ectopeptidase is a membrane-zinc metallopeptidase.

17. The peptide derivative according to claim 15 or 16, wherein the membrane metallo-ectopeptidase is NEP and/or AP-N.

18. The peptide derivative according to any one of claims 15 to 17, for use in the treatment of a disease or disorder selected from the group consisting of pain, depressive disorders, impaired social activity linked to sexuality, and impaired sexual behaviour.

19. A nucleic acid which comprises a sequence encoding a peptide derivative according to anyone of claims 1 to 9, wherein said peptide derivative consists of natural aminoacids.

20. An antibody directed against a peptide derivative as defined in any one of claims 1 to 9.