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Structure-activity relationship study on senktide for development of novel potent neurokinin-3 receptor selective agonists

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ABSTRACT: Neurokinin B (NKB) regulates the secretion of gonadotropin-releasing hormone (GnRH) in the hypothalamus via activation of the cognate neurokinin-3 receptor (NK3R). The stimulatory effect of NKB and the derivatives in gonadotropin secretion can potentially be used for development of novel regulatory and therapeutic agents for reproductive dysfunctions. Here, we report a comprehensive structure-activity relationship study on the NK3R-selective agonist peptide, senktide. Substitution of the N-terminal succinyl-Asp substructure in senktide with oxalyl-Glu, oxalyl-D-Glu or oxalyl-L-2-aminoadipic acid (Aad) increased receptor binding and NK3R activation. Among these modifications, the oxalyl-D-Glu substructure prevented neutral endopeptidase (NEP) 24.11-mediated degradation, thus providing a novel NK3R agonist peptide with favourable biological and stability properties.

KEYWORDS: GnRH pulse, KNDy neuron, neurokinin B, NK3R, senktide

Abbreviations: Aad, L-2-aminoadipic acid; ARC, arcuate nucleus; BH-SP, Bolton-Hunter labelled substance P; DIC, *N*,*N*'-diisopropylcarbodiimide; DynA, dynorphin A; GnRH, gonadotropin-releasing hormone; HOAt, *N*-hydroxy-7-azabenzotriazole; LH, luteinising hormone; MUA, multiple-unit activity; NKA, neurokinin A; NKB, neurokinin B; NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; NK3R, neurokinin-3 receptor; MePhe, *N*-methylphenylalanine; OVX, ovariectomised; SP, substance P; SPPS, solid-phase peptide synthesis.

Introduction

Neurokinin-3 receptor (NK3R) belongs to the tachykinin receptor family, and is known as a cognate receptor of neurokinin B (NKB, Table 1). Recently, the hypothalamic regulation of reproduction by the NKB-NK3R axis has received considerable attention.[1,2] The genetic study of *TAC3* and *TACR3*, which encode NKB and NK3R, respectively, highlighted the prevalence of hypogonadotropic hypogonadism by inactivating mutations of these genes.[3,4] Exogenous administration of kisspeptin or gonadotropin-releasing hormone (GnRH) into patients with these genetic mutations restores the circulating level of luteinising hormone (LH),[5] suggesting that NKB positively regulates the reproductive hormone cascade via GnRH neuron activation.[6] The influence on LH secretion by central administration of an NK3R agonist into ovariectomised and estrogen-primed rats has also been reported previously.[7] Because LH secretion from the pituitary gland is implicated in a number of gonadal functions including testosterone production in males, maturation of preovulatory ovarian follicles, and ovulation in females,[8,9] selective NK3R modulators have been expected to be a novel class of therapeutic agents for gonadal dysfunctions as well as reproductive disorders. The NK3R modulators would also be applicable to the improvement of low reproductive efficiency of livestock such as cattle.[10]

In 2007, it was reported that NKB is colocalised with kisspeptin and dynorphin A (DynA) in a single subpopulation in the hypothalamic arcuate nucleus (ARC) in sheep.[11] These peptides cooperatively regulate the secretion of GnRH: NKB and kisspeptin mediate induction, while DynA mediates inhibition of GnRH secretion. Cells containing these three peptides are currently recognised as KNDy (kisspeptin, NKB and DynA) neurons.[12] Although NKB is expressed in many other brain regions, the colocalisation of these peptides is unique to ARC, which is conserved among mammalian species including human.[13-15]

Two NK3R-selective agonist peptides have been reported: [MePhe^7]-NKB[16] and senktide (Table 1).[17] [MePhe^7]-NKB was identified through a structure-activity relationship study on NKB (4-10), a

short NKB analogue, which was designed based on the sequence of the neurokinin-1 receptor (NK1R)/NK3R agonist peptide, DiMeC7.[16] Our previous structure-activity relationship study on naturally occurring tachykinin peptides revealed that substitution of Phe^5 with an acidic amino acid (Asp^5 or Glu^5) and of Val^7 with $MePhe^7$ in NKB are indispensable for potent activity and selectivity of [$MePhe^7$]-NKB for NK3R.[18] On the basis of these data, a novel potent NK3R agonist with higher NK3R selectivity compared with [$MePhe^7$]-NKB was identified.[18] Senktide is the most common NK3R agonist peptide, which was incidentally identified in an *N*-methylamino acid scanning study on substance P (SP)-related peptides for the development of NK1R-selective agonists.[17] It has been used in a number of *in vitro* and *in vivo* experiments to investigate NK3R functions.[6,7,19-25] For example, senktide-mediated NK3R stimulation evoked dopamine release from dopamine neurons *in vitro*.[19] In addition, the function of the KNDy neurons in the pulsatile secretion of GnRH was demonstrated by *in vivo* administration of senktide.[25] Although senktide is subjected to peptidase-mediated degradation, a novel peptidomimetic derivative of senktide exerts a prolonged effect on the GnRH pulse generator in ovariectomised (OVX) goats.[26]

In the current study, we report the structure-activity relationship of senktide derivatives with modification of component amino acids or N-terminal functional group for identifying a novel potent NK3R agonist. The stability under peptidase-mediated degradation conditions and the *in vivo* bioactivity of potent NK3R agonist derivatives are also discussed.

Results and Discussion

Synthesis of senktide analogue peptides

All peptide chains were constructed by standard Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) on Rink-amide resin. Succinylation, carbamoylation, or hydroxycarbamoylation of the peptide's N-terminus was carried out on resin as previously reported.[26-28] Modification with oxalyl, aminocarbamoyl, methyloxalyl, 2-amino-2-oxoacetyl, or sulfamoyl groups at the peptide N-terminus

was conducted using appropriate reagents as described in the experimental procedures. The final deprotection and cleavage from the resin with the cocktail [TFA/thioanisole/*m*-cresol/1,2-ethanedithiol/H_2O (80:5:5:5:5)], followed by RP-HPLC purification afforded the expected peptides as TFA salts. All peptides were identified with ESI-MS or MALDI-TOF-MS and the purity was determined to be more than 98% by analytical HPLC.

Structure-activity relationships of tachykinin consensus sequences in senktide

The C-terminal sequence, Phe-Xaa-Gly-Leu-Met-NH_2, is common among mammalian tachykinin peptides including SP, neurokinin A (NKA) and NKB, which are endogenous agonists of NK1R, neurokinin-2 receptor (NK2R) and NK3R, respectively.[29-31] In addition, tachykinin peptides share similar sequences among mammalian species, whereas the consensus sequence often contains several amino acid substitutions in other species. For example, insect-derived tachykinin-related peptides with the consensus Phe-Xaa-Gly/Ala-Yaa-Arg-NH_2 sequence[32] show potent agonistic activity towards the tachykinin peptide receptor, STKR, which has 41% homology with human NK3R. Octopus-derived tachykinin peptides have a variety of C-terminal sequences, including *oct*-TKRP I with Gly-Thr-Arg-NH_2 and *oct*-TKRP-V with Pro-Pro-Lys-NH_2.[33] These *oct*-TKRP peptides show potent agonistic activity towards the *oct*-TKRP receptor.[33] To optimise the amino acids of the consensus sequences in senktide for receptor binding and selectivity, we designed senktide derivatives based on these invertebrate tachykinin-related peptide sequences (see supplementary information). However, these modifications resulted in bioactivity loss, except for the peptide with Gly to Pro substitution, which exhibited weak NK3R binding. Substitution of Phe in the consensus sequence with an aromatic amino acid (Tyr, Trp or His) also led to significantly weak or no NK3R binding. These results suggest that the amino acids in the tachykinin consensus sequence, including the C-terminal Met-NH_2 are indispensable for the biological activity of senktide towards NK3R.

MePhe is a characteristic amino acid that contributes to the high NK3R selectivity of [MePhe^7]-NKB and senktide.[16-18] We next designed several senktide derivatives with substitution at the MePhe position with other *N*-methylamino acids (Table 2). Among derivatives **1a**–**c** with an aliphatic *N*-methylamino acid, the MeVal- (**1a**) and MeIle-derivatives (**1b**) showed slightly lower biological activity than senktide (**1a**: IC_{50} = 104 nM, EC_{50} = 64 pM; **1b**: IC_{50} = 91 nM, EC_{50} = 50 pM).[34-36] In contrast, substitution with MeLeu resulted in loss of binding to NK3R. The MeTyr- (**1d**) and MeTrp-substituted peptides (**1e**) also maintained the NK3R binding and activation (**1d**: IC_{50} = 28 nM, EC_{50} = 41 pM; **1e**: IC_{50} = 123 nM, EC_{50} = 33 pM). Substitution with D-MePhe resulted in loss of NK3R binding, suggesting that NK3R recognises the side chain orientation derived from the L-MePhe chiral centre in senktide. As the highly acidic succinyl-Asp substructure in senktide is likely to interact with the potential basic functional group(s) of NK3R, replacement of MePhe with MeAsp was also attempted; however, the MeAsp derivative (**1g**) showed no binding to NK3R. Taken together, some appropriate aliphatic or aromatic *N*-methylamino acids are acceptable at the MePhe position for potent NK3R agonists. Of note, the potent peptides **1a**, **1b**, **1d** and **1e** showed no binding affinity to NK1R and NK2R at 10 μM.

Structure-activity relationships of N-terminal substructures in senktide

Senktide possesses two anionic carboxyl groups at the N-terminus, which have been suggested to provide high NK3R selectivity.[18] We next investigated the modification of the senktide N-terminal substructure with a number of potential bioisosteres.

Initially we substituted the N-terminal succinyl group in senktide with L-Asp or D-Asp derivatives (Table 3). As the succinyl group in senktide is derived from the side chain of L-Asp^4 in NKB, regeneration of a chiral centre was expected to allow favourable orientation of negatively charged functional groups.[37] Introduction of unmodified L-Asp (**2a**) resulted in a slight decrease in receptor binding and agonistic activity (**2a**: IC_{50} = 175 nM, EC_{50} = 63 pM). The acetyl capping of the Asp

α-amino group (**3a**) restored the potent bioactivity (**3a**: IC_{50} = 68 nM, EC_{50} = 31 pM), suggesting that a basic functional group at the N-terminal region is unfavourable for NK3R binding and activation. Among derivatives **3a**–**d** with a modification at the second acidic amino acid, the L-Glu-substituted derivative **3c** showed 5-fold more potent receptor binding and 3-fold more potent agonist activity compared with those of senktide (**3c**: IC_{50} = 7.7 nM, EC_{50} = 8.1 pM). A similar structure-activity relationship was obtained among the **4a**–**d** series of peptides with an Ac-D-Asp modification at the N-terminus, in which the most potent L-Glu peptide **4c** exhibited 3-fold more potent receptor binding and 2-fold stronger agonist activity compared with those of senktide (**4c**: IC_{50} = 13 nM, EC_{50} = 12 pM). The advantage of L-Glu for NK3R activation is consistent with the potent biological activity of PG-KII with an L-Asp-L-Glu substructure at this position, which was derived from the Australian frog, *Pseudophryne guentheri*.[31]

Taking advantage of these key insights, further investigations were carried out for optimisation of the spatial arrangements of two N-terminal acidic functional groups in senktide (Table 4). Among the senktide derivatives in which L-Asp was substituted with other acidic amino acids including D-Asp, L-Glu, D-Glu or L-2-aminoadipic acid (L-Aad) (**5b**–**e**), substitution with D-Asp (**5b**), L-Glu (**5c**) or L-Aad (**5e**) resulted in 2- or 3-fold more potent NK3R agonist activity (**5b**: IC_{50} = 16 nM, EC_{50} = 29 pM; **5c**: IC_{50} = 6.1 nM, EC_{50} = 30 pM; **5e**: IC_{50} = 9.9 nM, EC_{50} = 19 pM) compared with that of senktide, whereas peptide **5d** with D-Glu showed slightly less potent agonist activity (**5d**: IC_{50} = 24 nM, EC_{50} = 84 pM). Interestingly, significant improvement in bioactivity was observed among the N-terminally oxalyl-modified derivatives, **6a**–**e**. The derivatives with the L-Glu (**6c**), D-Glu (**6d**) or L-Aad (**6e**) substitution exhibited 7- to 9-fold more potent biological activity (**6c**: IC_{50} = 0.43 nM, EC_{50} = 9.1 pM; **6d**: IC_{50} = 0.51 nM, EC_{50} = 14 pM; **6e**: IC_{50} = 1.4 nM, EC_{50} = 7.4 pM) compared with that of senktide. Although the peptides with the L-Asp (**6a**) or D-Asp (**6b**) substitution showed 10-fold more potent binding inhibition, significant improvement in NK3R activation was not observed (**6a**: IC_{50} = 3.6 nM, EC_{50} = 83 pM; **6b**: IC_{50} = 3.5 nM, EC_{50} = 22 pM). These results

suggest that a combination of carboxy functional groups with a short tether at the N-terminus and with a long side chain at the L-Asp position of senktide would be appropriate for potent agonist activity towards NK3R.

Further modifications of the senktide N-terminus with isosteric functional groups were performed in attempt to improve the biological activity (**7**–**12**) (Table 5). All the derivatives maintained the potent binding inhibition and agonist activity toward NK3R (IC_{50} = 4.1–39 nM; EC_{50} = 2.5–43 pM). Peptides **10** and **12**, with 2-methoxy-2-oxoacetyl and aminosulfonyl groups, respectively, showed slightly more potent biological activity compared with that of senktide [**10**: IC_{50} = 4.1 nM, EC_{50} = 2.9 pM; **12**: IC_{50} = 5.6 nM, EC_{50} = 2.5 pM], suggesting that a charged anionic functional group is not critical for the N-terminus of senktide. Using the potent peptides, **10** and **12**, we further optimised the L-Asp position using acidic amino acids (Table S3). However, the improvement in agonist activity towards NK3R by modifying the L-Asp position was limited, which was inconsistent with the case of the series of derivatives with an N-terminal oxalyl group. Altogether, we concluded that the oxalyl group is the best N-terminal functional group for NK3R agonists.

The highly potent senktide derivatives, **6c**–**e**, were evaluated for agonist activity towards NK3R derived from other mammalian species including rat, goat and cattle (Table 6). All the peptides showed more potent agonist activity towards mammalian NK3R compared with that of senktide. The most potent peptide, **6e**, with oxalyl-L-Aad at the N-terminus exhibited 3-, 2- and 4-fold higher potency than senktide in activation of rat, goat and cattle NK3R, respectively.[38]

Proteolytic degradation of potent NK3R agonist peptides in serum, and by endopeptidase treatment

NKB is degraded and inactivated by cleavage at the Gly-Leu peptide bond by membrane-anchored neutral endopeptidase 24.11 (NEP 24.11; also known as neprilysin or enkephalinase).[39] Senktide is also inactivated by NEP 24.11-mediated cleavage at the same site.[26] To

identify potent NK3R agonists with resistance to NEP 24.11-mediated degradation, we assessed the stability of the potent derivatives obtained in the above structure-activity relationship experiments.

Peptides **6c** and **6d** were stable in rat, pig, goat and cattle serum for 24 h similarly to senktide (Figure S1),[26,40] suggesting that the N-terminal oxalyl capping also prevents possible degradation by peptidases in the serum, which was observed in the case of [MePhe^7]-NKB.[26] Next, the resistance of peptides **6c**–**e** to NEP 24.11-mediated degradation was investigated (Figure 1). More than 70% of **6c** and **6e** were digested at the Gly-Leu dipeptide bond by 24 h of incubation with NEP 24.11. In contrast, little degradation of **6d** by NEP 24.11 was observed after 24 h. These results suggest that substitution of L-Asp in senktide with D-Glu is effective for resistance against NEP 24.11-mediated proteolytic degradation at the distal Gly-Leu cleavage site. Thus, peptide **6d** with oxalyl-D-Glu at the N-terminus is a potent NK3R agonist with high biological stability.

Effect of peripheral administration of peptide 6d on the induction of periodic burst of multiple-unit activity (MUA) volleys in goat

It has been reported that pulsatile GnRH secretion is synchronized with the induction of periodic burst of multiple-unit activity (MUA) volleys.[6,41] To assess the *in vivo* effect on pulsatile secretion of GnRH, we monitored the induction of MUA volleys after intravenous administration of **6d**. MUA volleys with interval less than 80% of the average spontaneous intervolley intervals (*T*) were defined as ligand-induced, whereas ones with intervals higher than 80% of *T* were spontaneous.[26] The action duration (*R*) was the period from the injection until the onset of the following spontaneous MUA volley, and the number of MUA volleys occurring in this period (*V*) was counted.[26] The peptide **6d** (200 nmol) immediately induced several MUA volleys. The *R* and *V* values of **6d** were similar to those of senktide (Table 7 and Figure S2).[42] This is contrary to the results in our recent study, in which the novel NK3R-selective peptidomimetic agonist **13** [succinyl-Asp-Phe-MePhe-Gly-ψ[(*E*)-CH=CH]-Leu-Met-NH_2] showed prolonged *in vivo* bioactivity

in goat compared with that of senktide.[26] These results may suggest that the clearance of the peptide from the circulation should be further optimised to develop NK3R agonists with prolonged in vivo bioactivity.

Conclusions

In this study, we investigated the structure-activity relationships of senktide derivatives in relation to agonist activity towards NK3R. The consensus sequence of tachykinin peptides in senktide is important for NK3R binding and activation, while MePhe can be substituted with several hydrophobic *N*-methylamino acids. Optimisation of the N-terminal substructures resulted in identification of novel potent NK3R agonists (**6c**–**e**) with an oxalyl group at the peptide N-terminus. The investigation of the peptide resistance to NEP 24.11-mediated degradation revealed that peptide **6d** with D-Glu at the L-Asp position in senktide was highly stable under *in vitro* conditions. Although further improvement for preventing the possible rapid clearance of senktide and the derivatives is needed, peptide **6d** with its enhanced metabolic stability could be an appropriate lead peptide for designing novel NK3R agonists with prolonged *in vivo* bioactivity.

Experimental procedures

General method for peptide synthesis

The protected linear peptides were constructed by Fmoc-based solid-phase synthesis on Rink-Amide resin (0.66 mmol g^{-1}, 45.5 mg, 0.025 mmol). Fmoc-protected amino acids (0.075 mmol) were coupled by *N*,*N*′-diisopropylcarbodiimide (DIC, 0.0116 cm^3, 0.075 mmol) and HOBt·H_2O (11.5 mg, 0.075 mmol) in DMF. Coupling of amino acid (0.075 mmol) to *N*-methylamino acid was carried out with HATU (28.5 mg, 0.075 mmol) and (iPr)$_2$NEt (DIPEA, 0.013 cm^3, 0.075 mmol). Completion of each coupling reaction was ascertained using the Kaiser ninhydrin test. The Fmoc-protecting group was removed by treating the resin with 20% piperidine in DMF. The resulting protected peptide resin was treated with TFA/thioanisole/*m*-cresol/1,2-ethanedithiol/H_2O (5 cm^3; 80:5:5:5:5) at room temperature for 2 h. After removal of the resin by filtration, the filtrate was poured into ice-cold dry Et_2O (40 cm^3). The resulting powder was collected by centrifugation and then washed three times with ice-cold dry Et_2O (3 × 40 cm^3). The crude product was purified by HPLC on a Cosmosil 5C18-ARII preparative column (Nacalai Tesque, 20 × 250 mm). All peptides were characterised by ESI-MS or MALDI-TOF-MS and the purity was calculated as >98% by HPLC on a Cosmosil 5C18-ARII analytical column (Nacalai Tesque, 4.6 × 250 mm).

General procedures for modification of the peptide N-terminus

Acetylation: The peptide resin (0.025 mmol) was treated with Ac_2O (0.024 cm^3, 0.25 mmol) and pyridine (0.020 cm^3, 0.25 mmol) in DMF (1.0 cm^3) for 1.5 h to produce acetyl-modified peptide resin for peptides **3a**–**d** and **4a**–**d**.

Succinylation: The peptide resin (0.025 mmol) was treated with succinic anhydride (12.5 mg, 0.125 mmol) and DIPEA (0.0436 cm^3, 0.125 mmol) in DMF (1.0 cm^3) for 1.5 h, to produce succinyl-modified peptide resin for peptides **1a**–**g**, **5b**–**e** and **S1a**–**r**.

Oxalylation: The peptide resin (0.025 mmol) was treated with *tert*-butyl chloro(oxo)acetate[43] (20.6 mg, 0.125 mmol) and DIPEA (0.0436 cm^3, 0.25 mmol) in CH_2Cl_2 (1.0 cm^3) for 1.5 h, to produce oxalyl-modified peptide resin for peptides **6a**–**e**.

Carbamoylation: The peptide resin (0.025 mmol) was treated with chlorosulfonyl isocyanate (0.0109 cm^3, 0.125 mmol) and pyridine (0.0202 cm^3, 0.25 mmol) in CH_2Cl_2 (1.0 cm^3) for 16 h. Subsequently, the resin was treated with H_2O (0.050 cm^3) for 48 h, to produce carbamoyl-modified peptide resin for peptide **7**.

Hydroxycarbamoylation: The peptide resin (0.025 mmol) was treated with *p*-nitrophenyl chloroformate (186.4 mg, 0.925 mmol) and DIPEA (0.161 cm^3, 0.925 mmol) in THF/CH_2Cl_2 (1:1, 1.0 cm^3) for 30 min. After removal of the above reagents, a solution of hydroxylamine hydrochloride (64.3 mg, 0.925 mmol) and DIPEA (0.322 cm^3, 1.85 mmol) in DMF (1.0 cm^3) was added. The suspension was stirred for 15 min, to produce hydroxycarbamoyl-modified peptide resin for peptide **8**.

Aminocarbamoylation: A solution of *p*-nitrophenyl chloroformate (100.8 mg, 0.5 mmol) in dry THF (1.0 cm^3) was added to a solution of *tert*-butyl carbazate (66.1 mg, 0.5 mmol) and *N*-methylmorpholine (0.055 cm^3, 0.5 mmol) in dry THF (4.0 cm^3). After stirring for 40 h, the reaction mixture was concentrated to give Boc-NHNH-$CO_2C_6H_4$(4-NO_2). This reagent in dry THF (1.0 cm^3) and TEA (0.069 cm^3, 0.5 mmol) was added to the peptide resin (0.025 mmol). The suspension was agitated for 42 h to produce aminocarbamoyl-modified peptide resin for peptide **9**.

2-Methyl-2-oxoacetylation: The peptide resin (0.025 mmol) was treated with methyl chloroglyoxalate (0.0114 cm^3, 0.125 mmol) and pyridine (0.0201 cm^3, 0.25 mmol) in CH_2Cl_2 (1.0 cm^3) for 2 h, to produce methyloxalyl-modified peptide resin for peptides **10** and **S2a**–**c**.

2-Amino-2-oxoacetylation: The peptide resin (0.025 mmol) was treated with oxamic acid (11.1 mg, 0.125 mmol), DIC (19.4 μL, 0.125 mmol), and HOBt·H_2O (19.1 mg, 0.125 mmol) in CH_2Cl_2 (1.0 cm^3) for 3 h, to produce 2-amino-2-oxoacetyl-modified peptide resin for peptide **11**.

Sulfamoylation: *tert*-butyl alcohol (0.0237 cm^3, 0.25 mmol) in dry THF (0.1 cm^3) was added to chlorosulfonyl isocyanate (21.8 cm^3, 0.25 mmol) in dry THF (0.15 cm^3) and the mixture was stirred for 15 min at 0 °C. The reaction mixture and TEA (0.0693 cm^3, 0.5 mmol) in dry CH_2Cl_2 (0.25 cm^3) were added to the peptide resin (0.025 mmol), and the suspension was agitated for 3 h, to produce sulfamoyl-modified peptide resin for peptides **12** and **S3a**–**c**.

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Supplementary information

Electronic supplementary information (ESI) available. See DOI: ##########

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towards cattle NK3R or low expression level of cattle NK3R on the cell membrane. This is supported by the less binding of radiolabelled [MePhe^7]-NKB towards cattle NK3R (data not shown).

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Figure 1. Proteolytic degradation profile of senktide derivatives by NEP 24.11. The peptides were incubated in NEP 24.11 solution for 24 h at 37 °C and were analysed by HPLC (detection at 220 nm). Data represent the mean ± SD (n = 3).

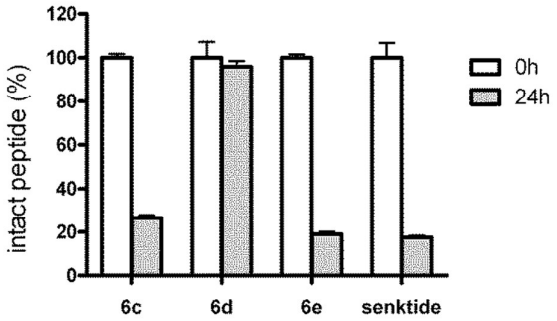


Table 1. Sequences of NKB and senktide.

Peptide	sequence
neurokinin B (NKB)	H-Asp-Met-His-Asp-Phe-Phe- Val -Gly-Leu-Met-NH_2
[MePhe^7]-NKB	H-Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met-NH_2
Senktide	succinyl-Asp-Phe-MePhe-Gly-Leu-Met-NH_2

Table 2. Structure–activity relationship of senktide derivatives with a modification at the MePhe position.

succinyl-Asp-Phe-**Xaa**-Gly-Leu-Met-NH_2

Peptide	**Xaa**	NK3R		NK1R	NK2R
		IC_{50} (nM)[a]	EC_{50} (pM)[b]	IC_{50} (nM)[a]	IC_{50} (nM)[a]
senktide	MePhe	45	17	>10000	>10000
1a	MeVal	104	64	>10000	>10000
1b	MeIle	91	50	>10000	>10000
1c	MeLeu	>10000	-[c]	-[c]	-[c]
1d	MeTyr	28	41	>10000	>10000
1e	MeTrp	123	33	>10000	>10000
1f	D-MePhe	>10000	-[c]	-[c]	-[c]
1g	MeAsp	>10000	-[c]	-[c]	-[c]

[a] IC_{50} values indicate the concentration needed for 50% inhibition of receptor binding of ([^{125}I]His^3, $MePhe^7$)-NKB to NK3R, [^{125}I]-BH-SP to NK1R, and [^{125}I]-NKA to NK2R. [b] EC_{50} values are the concentration needed for 50% of the full agonist activity induced by 100 nM senktide. [c] Not evaluated.

Table 3. Structure–activity relationship of senktide derivatives with a modification of the N-terminal acetyl-Asp substructure.

R-Xaa-Phe-MePhe-Gly-Leu-Met-NH_2

Peptide	**R**	**Xaa**	NK3R		NK1R	NK2R
			IC_{50} (nM)[a]	EC_{50} (pM)[b]	IC_{50} (nM)[a]	IC_{50} (nM)[a]
senktide	succinyl	L-Asp	40	23	>10000	>10000
2a	H-L-Asp	L-Asp	175	63	>10000	>10000
3a	Ac-L-Asp	L-Asp	68	31	>10000	>10000
3b	Ac-L-Asp	D-Asp	83	24	>10000	>10000
3c	Ac-L-Asp	L-Glu	7.7	8.1	>10000	>10000
3d	Ac-L-Asp	D-Glu	37	18	>10000	>10000
4a	Ac-D-Asp	L-Asp	73	18	>10000	>10000
4b	Ac-D-Asp	D-Asp	55	17	>10000	>10000
4c	Ac-D-Asp	L-Glu	13	12	>10000	>10000
4d	Ac-D-Asp	D-Glu	68	17	>10000	>10000

[a] IC_{50} values indicate the concentration needed for 50% inhibition of receptor binding of ([^{125}I]His^{3}, MePhe^{7})-NKB to NK3R, [^{125}I]-BH-SP to NK1R, and [^{125}I]-NKA to NK2R. [b] EC_{50} values are the concentration needed for 50% of the full agonist activity induced by 100 nM senktide.

Table 4. Structure–activity relationship of N-terminal succinyl and oxalyl senktide derivatives.

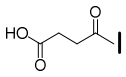
R-Xaa-Phe-MePhe-Gly-Leu-Met-NH_2

Peptide	**R**	**Xaa**	NK3R		NK1R	NK2R
			IC_{50} (nM)[a]	EC_{50} (pM)[b]	IC_{50} (nM)[a]	IC_{50} (nM)[a]
senktide	succinyl	L-Asp	43	63	>10000	>10000
5b	succinyl	D-Asp	16	29	>10000	>10000
5c	succinyl	L-Glu	6.1	30	>10000	>10000
5d	succinyl	D-Glu	24	84	>10000	>10000
5e	succinyl	L-Aad	9.9	19	>10000	>10000
6a	oxalyl	L-Asp	3.6	83	>10000	>10000
6b	oxalyl	D-Asp	3.5	22	>10000	>10000
6c	oxalyl	L-Glu	0.43	9.1	>10000	>10000
6d	oxalyl	D-Glu	0.51	14	>10000	>10000
6e	oxalyl	L-Aad	1.4	7.4	>10000	>10000

[a] IC_{50} values indicate the concentration needed for 50% inhibition of receptor binding of ([^{125}I]His^3, $MePhe^7$)-NKB to NK3R, [^{125}I]-BH-SP to NK1R, and [^{125}I]-NKA to NK2R. [b] EC_{50} values are the concentration needed for 50% of the full agonist activity induced by 100 nM senktide.

Table 5. Optimisation of the N-terminal accessory substructure.

R-Asp-Phe-MePhe-Gly-Leu-Met-NH_2

Peptide	**R**	NK3R		NK1R	NK2R
		IC_{50} (nM)[a]	EC_{50} (pM)[b]	IC_{50} (nM)[a]	IC_{50} (nM)[a]
senktide		29	3.4	>10000	>10000
7		39	13	>10000	>10000
8		10	43	>10000	>10000
9		34	6.0	>10000	>10000
10		4.1	2.9	>10000	>10000
11		7.9	11	>10000	>10000
12		5.6	2.5	>10000	>10000

[a] IC_{50} values indicate the concentration needed for 50% inhibition of receptor binding of ([^{125}I]His^{3}, MePhe^{7})-NKB to NK3R, [^{125}I]-BH-SP to NK1R, and [^{125}I]-NKA to NK2R. [b] EC_{50} values are the concentration needed for 50% of the full agonist activity induced by 100 nM senktide.

Table 6. Biological activity of potent peptides towards rat, goat and cattle NK3R.

Peptide	EC_{50} (pM)[a]			
	human	rat	goat	cattle
senktide	63	23	19	610
6c	9.1	13	12	169
6d	14	9.9	9.4	152
6e	7.4	7.2	9.3	150

[a] EC_{50} values are the concentration needed for 50% of the full agonist activity induced by 100 nM senktide.

Table 7. *In vivo* bioactivity of senktide and peptide **6d**.[a]

	R (min)[b]	*V*[c]
senktide	47.2 ± 14.2	2.6 ± 1.1
6d	50.4 ± 23.8	2.7 ± 1.5

[a] MUA volley induction in OVX goats by intravenous injection of NK3R ligands (200 nmol, $n \geq 3$). Values are represented as the mean ± SD. [b] *R* values indicate the duration of the effect of NK3R ligands. [c] *V* values indicate the number of ligand-induced MUA volleys.