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Author(s)	Misu, Ryosuke; Noguchi, Taro; Ohno, Hiroaki; Oishi, Shinya; Fujii, Nobutaka
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Structure-activity Relationship Study of Tachykinin Peptides for the Development of Novel Neurokinin-3 Receptor Selective Agonists

Ryosuke Misu, Taro Noguchi, Hiroaki Ohno, Shinya Oishi*, and Nobutaka Fujii*

Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

*Corresponding Authors:

Shinya Oishi, Ph.D. and Nobutaka Fujii, Ph.D.

Graduate School of Pharmaceutical Sciences

Kyoto University

Sakyo-ku, Kyoto, 606-8501, Japan

Tel: +81-75-753-4551; Fax: +81-75-753-4570,

E-mail (S.O.): soishi@pharm.kyoto-u.ac.jp; E-mail (N.F.): nfujii@pharm.kyoto-u.ac.jp

ABSTRACT

Neurokinin B (NKB) is a potential regulator of pulsatile gonadotropin-releasing hormone (GnRH) secretion via activation of the neurokinin-3 receptor (NK3R). NKB with the consensus sequence of the tachykinin peptide family also binds to other tachykinin receptors [neurokinin-1 receptor (NK1R) and neurokinin-2 receptor (NK2R)] with low selectivity. In order to identify the structural requirements for the development of novel potent and selective NK3R agonists, a structure-activity relationship (SAR) study of [MePhe^7]-NKB and other naturally occurring tachykinin peptides was performed. The substitutions to naturally occurring tachykinins with Asp and MePhe improved the receptor binding and agonistic activity for NK3R. The corresponding substitutions to NKB provided an NK3R selective analog.

KEYWORDS

neurokinin B, [MePhe^7]-neurokinin B, NK3R, tachykinin, GnRH

Abbreviations: GnRH, gonadotropin-releasing hormone; ARC, arcuate nucleus; NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; NK3R, neurokinin-3 receptor; LH, Luteinizing hormone; SP, substance P; BH-SP, Bolton-Hunter labeled substance P; NKA, neurokinin A, NKB, neurokinin B; Dyn, dynorphin A; Nle, norleucine; MePhe, *N*-methylphenylalanine.

1. Introduction

Pulsatile release of gonadotropin-releasing hormone (GnRH) is a prerequisite for reproductive success in mammals.[1,2] Pulsatile GnRH secretion has been suggested to be regulated by potential "GnRH pulse generator(s)", which locate at the arcuate nucleus (ARC) in the hypothalamus;[3] however, the precise cellular and molecular mechanism to generate the rhythmic discharge of GnRH has not been revealed. One of the stimulatory regulators for the GnRH neuronal network is kisspeptin,[4] encoded by the *Kiss1* gene.[5] Kisspeptins and the related peptides promote GnRH secretion via activation of the corresponding receptor GPR54.[6,7] In ARC, kisspeptin neurons provide a tonic drive to GnRH neurons via transsynaptic inputs.[8] The administration of kisspeptins or related peptides[9,10] effectively induces ovulation by a transient increase in GnRH/LH secretion (a GnRH surge).[11,12] In addition, the peripheral administration of kisspeptin-10, which is a potent GPR54 agonist, elicits a robust LH release in goat and rat without any effect on the GnRH pulse.[13,14] These observations suggest that kisspeptins are unlikely to be involved in the regulation of pulsatile GnRH secretion.

Recently, it has been reported that kisspeptin-containing neurons coexpress neurokinin B (NKB) and dynorphin A (Dyn).[15-17] Central administration of NKB immediately induces pulsatile GnRH secretion, suggesting that NKB and its cognate receptor, the neurokinin-3 receptor (NK3R), are involved in generating GnRH pulses.[17] Indeed, it has been reported that a mutation in either the *Tac3* or *Tacr3* gene (which encodes NKB or NK3R, respectively) causes a severe gonadotropin deficiency in humans.[18] Thus, the NKB receptor(s) represents a potential pharmaceutical target to regulate pulsatile GnRH secretion.

NKB, an endogenous NK3R agonist, belongs to the tachykinin peptide family, which shares a common C-terminal sequence, -Phe-Xaa-Gly-Leu-Met-NH_2.[19-21] NKB acts as a neurotransmitter to regulate the release of GnRH and dopamine in CNS.[22-24] NKB also induces contraction of the portal vein, venoconstriction of the mesenteric bed and an increase in the heart rate in the periphery.[25-27]

Substance P (SP) and neurokinin A (NKA) are also known as mammalian tachykinins that show preferable binding to NK1R and NK2R, respectively. The receptor selectivity of these endogenous tachykinins is modest. There have also been several selective tachykinin receptor ligands reported, such as [Pro^9]-SP, [Pro^{10}]-SP and [Sar^9, Met(O_2)^{11}]-SP for NK1R[20,21] or [β-Ala^8]-NKA(4-10) and [Nle^{10}]-NKA(4-10) for NK2R.[28,29] [MePhe^7]-NKB (**1a**) and senktide, [succinyl-(Asp^6 MePhe^8)-SP(6-11)], were also reported to be NK3R-selective agonistic peptides.[30,31] Although these peptides were apparently designed to investigate the function of tachykinins and the cognate receptors,[28-31] the essential structural requirements for further potent and selective tachykinin receptor ligands have never been fully explored. In this article, we report a structure-activity relationship (SAR) of naturally occurring vertebrate/invertebrate tachykinin peptides and [MePhe^7]-NKB.

2. Results and discussion

2.1. Synthesis of tachykinin analog peptides

All peptide chains were constructed by standard Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) on Rink-amide resin. 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 more than 98% by analytical HPLC.

2.2. Structure-activity relationships of naturally occurring tachykinins and the derivatives

Initially, we investigated the biological activities of vertebrate and invertebrate tachykinins (Table 1). The biological activities were evaluated by competitive binding assays using [^{125}I]-BH-SP for NK1R, [^{125}I]-NKA for NK2R and ([^{125}I]His^3,MePhe^7)-NKB for NK3R. We also assessed the NK3R agonistic activity by monitoring the intracellular Ca^{2+} flux induced by NK3R activation.[32] All

naturally occurring tachykinins showed moderate to weak receptor binding to NK3R. Among the eight natural peptides **2a-9a**, PG-KII **2a** showed slightly higher NK3R agonistic activity [EC_{50}(**2a**) = 0.079 nM], whereas it bound to NK3R with more than 10-fold less potency when compared with that of [MePhe^7]-NKB **1a** [IC_{50}(**2a**) = 53 nM]. Uperolein **4a**, eledoisin **5a** and kassinin **6a** also exerted similarly potent NK3R agonistic effects [EC_{50}(**4a**) = 0.12 nM; EC_{50}(**5a**) = 0.22 nM; EC_{50}(**6a**) = 0.21 nM]. However, these peptides also showed potent binding to NK1R and NK2R at 10 μM.

It was reported that the substitution of the amino acid at position 7[33] in tachykinins [Phe^7 in NKB or Phe^8 in [pGlu^6]-SP(6-11)] with MePhe improved NK3R binding and selectivity.[30,31] At this position, naturally occurring tachykinin peptides contain various amino acids.[21] Alternatively, Severini *et al.* suggested that two acidic amino acids at positions 4 and 5 are responsible for the high NK3R selectivity.[21] An NK3R-selective agonist, senktide, also contains Asp at this position[31] On the basis of these previous investigations, we designed several analog peptides of vertebrate and invertebrate tachykinins with Asp and MePhe substitutions at positions 5 and 7, respectively.

All tachykinin derivatives **3b-9b** showed more potent binding affinity and agonistic activity to NK3R compared with the parent peptides **2a-9a** (Table 1). Peptide **3b** showed more potent NK3R binding affinity than PG-KII **2a** and PG-SP1 **3a** [IC_{50}(**3b**) = 19 nM], whereas the NK3R agonistic activity was similar to that of **2a** [EC_{50}(**3b**) = 0.083 nM]. Peptide **4b** also exhibited a similar NK3R agonistic activity of uperolein **4a** [EC_{50}(**4b**) = 0.13 nM]. Similarly potent bioactivities of an eledoisin derivative **5b** to those of **3b** and **4b** were observed [EC_{50}(**5b**) = 0.090 nM]. Peptide **6b** showed 3-times higher binding affinity and agonistic activity than kassinin **6a** [IC_{50}(**6b**) = 110 nM; EC_{50}(**6b**) = 0.085 nM] for NK3R. No binding of peptides **3b-7b** to NK1R and NK2R were observed, indicating that these peptides are NK3R-selective. Peptides **7b** and **8b** showed much more potent binding affinity and agonistic activity than the corresponding naturally occurring sialokinin II **7a** and physalaemin **8a** [IC_{50}(**7b**) = 81 nM; EC_{50}(**7b**) = 0.11 nM; IC_{50}(**8b**) = 54 nM; EC_{50}(**8b**) = 0.15 nM]. The agonistic activity of **7b** was comparable to [MePhe^7]-NKB **1a**. The NK3R binding of peptide **9b**

was significantly improved over bufokinin **9a** [IC_{50}(**9b**) = 31 nM], whereas a small improvement of the agonistic activity was observed [EC_{50}(**9b**) = 5.04 nM]. Peptides **8b** and **9b** remained moderate NK1R binders.[34] As such, Asp^5 and $MePhe^7$ are important for NK3R selective binding and agonistic activities. In addition, the receptor binding and agonistic activity of Asp^5/$MePhe^7$-substituted peptides towards NK3R is correlated. Although the contribution of these two residues and the C-terminal consensus sequence to the biological activities seems to be dominant, significantly different bioactivities among peptides **3b-9b** suggest that the N-terminal region is also playing a role in defining receptor binding and activation. For example, Lys^0 and Arg^2 in less potent peptides (**9b**) are unfavorable for NK3R agonistic activity.

To understand the optimization rationale by Asp and MePhe substitutions for receptor binding, selectivity and NK3R agonistic activity, we designed the analogs of eledoisin **5a** and kassinin **6a**, in which each residue at position 5 or 7 was substituted (Table 2). Asp substitution at position 5 in both peptides **5a** and **6a** led to slightly increased receptor binding to NK3R [IC_{50}(**5c**) = 35 nM; IC_{50}(**6c**) = 89 nM]. MePhe substitution at position 7 of **5a** and **6a** also improved binding affinity for NK3R [IC_{50}(**5d**) = 7.9 nM; IC_{50}(**6d**) = 13 nM]. Comparing these substitutions, MePhe at position 7 contributed more significantly to receptor binding. Dual substitutions led to the most potent binding to NK3R in eledoisin **5a**, whereas the single substitution with MePhe at position 7 resulted in highest binding of kassinin **6a**. In terms of NK3R agonistic activity, single Asp substitution in eledoisin **5a** or MePhe substitution in kassinin **6a** led to an increase in bioactivity [EC_{50}(**5c**) = 0.0054 nM; EC_{50}(**6d**) = 0.038 nM]. The potency of peptides **5c** and **6d** exceeded those of peptides **5b** and **6b**. Of note, NK1R and NK2R binding remained for peptides **5c,d** and **6c,d** with single Asp or MePhe substitutions at 10 μM, whereas peptides **5b** and **6b** did not bind to these receptors. Thus, dual Asp/MePhe substitutions provided the desired biological properties with high potency and selectivity for NK3R selective agonists.

2.3. Structure-activity relationship study of position 5 in [MePhe^7]-NKB

On the basis of the favorable effects of Asp/MePhe substitutions at positions 5 and 7 for selective NK3R agonists, we next designed [MePhe^7]-NKB analogs **1b-s** to optimize the Phe^5 position in [MePhe^7]-NKB **1a** (Table 3). Initially, we evaluated the binding affinity of the derivatives for NK3R. The substitution of Phe^5 with Arg, Tyr, Trp, Val, Ser or Met resulted in a slight increase in NK3R binding (**1d**, **1g**, **1h**, **1i**, **1o**, **1q**; IC_{50} = 1.5–3.6 nM), whereas the other derivatives showed lower binding to NK3R. For example, Asp- or Pro-substituted derivatives showed more than 10-times lower binding affinity than the parent [MePhe^7]-NKB **1a** [IC_{50}(**1b**) = 38 nM; IC_{50}(**1r**) = 45 nM]. The NK3R agonistic activity was largely similar among the derivatives except for the Trp-substituted peptide **1h** [EC_{50}(**1h**) = 0.28 nM]. Peptides **1f** and **1l** showed 4–5-times higher agonistic activities than that of [MePhe^7]-NKB **1a** [EC_{50}(**1f**) = 0.017 nM; EC_{50}(**1l**) = 0.012 nM]. Moderate correlations were observed between the binding affinity and agonistic activity for NK3R, suggesting that the amino acid at this position is not a critical factor in determining the biological activity for NK3R.

We also assessed the binding affinity of the peptides **1a-s** to NK1R and NK2R to evaluate the receptor selectivity. Peptides **1d**, **1e** and **1n** with a substitution with Arg, Lys and Gln, respectively, at position 5 exhibited moderate NK1R binding, whereas the other peptides did not bind to NK1R at 10 μM. In contrast, moderate to low NK2R binding was observed for peptides **1d-1s**, except for two peptides **1b** and **1c** that had substitutions with acidic amino acids (Asp or Glu). These observations suggest that the negative charge at this position is unfavorable for NK2R binding, and that the electrostatic property at this position is more important for an NK3R selective agonist. As such, the negatively charged amino acid at position 5 contributes to the receptor selectivity, rather than binding affinity and agonistic activity for NK3R.

3. Conclusions

In this study, we investigated the structure-activity relationships of naturally occurring tachykinins and an NK3R agonist, [MePhe^7]-NKB. Among the natural peptide derivatives evaluated, peptides **1b** and **1c** showed NK3R-selective agonistic activity. The SAR study of eledoisin and kassinin demonstrated that both Asp and MePhe at positions 5 and 7 contribute to the potent receptor binding to NK3R and high NK3R-selectivity. It was also revealed that in the optimization study of Phe^5 in [MePhe^7]-NKB, an acidic amino acid such as Asp and Glu improved the NK3R-selectivity. These structure-activity relationship data may facilitate the development of novel NK3R-selective peptide ligands as therapeutic agents that induce pulsatile GnRH secretion.

4. Experimental Section

4.1. Peptide synthesis

The protected linear peptides were constructed by Fmoc-based solid-phase synthesis on Rink-Amide resin (0.66 mmol/g, 45.5 mg, 0.025 mmol). Fmoc-protected amino acids (0.075 mmol) were coupled by using *N*,*N*′-diisopropylcarbodiimide (DIC, 11.6 μL, 0.075 mmol) and HOBt·H_2O (11.5 mg, 0.075 mmol) in DMF. Coupling of amino acid to *N*-methylamino acid was carried out with HATU (27.6 mg, 0.73 mmol) and (*i*-Pr)$_2$NEt (13.0 μL, 0.75 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 mL; 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 mL). The resulting powder was collected by centrifugation and then washed three times with ice-cold dry Et_2O (3 × 40 mL). The crude product was purified by HPLC on a Cosmosil 5C18-ARII preparative column (Nacalai Tesque, 20 × 250 mm). All peptides were characterized 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).

4.2. Evaluation of the binding affinity of tachykinin peptides to NK1R, NK2R and NK3R.

The experiment was performed using membranes from NK1R-, NK2R- or NK3R-expressing CHO cells. For ligand binding, membranes were incubated with 50 μL of increasing concentrations of the peptide, 25 μL of radio-active ligand [[^{125}I]-BH-SP for NK1R, [^{125}I]-NKA for NK2R, and ([^{125}I]His^{3}, MePhe^{7})-NKB for NK3R, respectively, 0.4 nM each, Perkin-Elmer Life Sciences] and 25 μL of the membrane solution in assay buffer [50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA]. The reaction mixtures were filtered through GF/B filters pretreated with 0.3% polyethyleneimine. The filters were washed with [50 mM HEPES (pH 7.4), 500 mM NaCl, 0.1% BSA], dried at 55 °C, and then subjected to γ-counting.

4.3. Evaluation of the NK3R agonistic activity

The NK3R agonistic activity of each peptide was evaluated by the $[Ca^{2+}]_i$ flux assay. NK3R expressing CHO-K1 cells (4.0×10^4 cells/50 μL/well) were inoculated in 10% FBS/Ham's F-12 onto a 96-well black clear-bottom plate (Greiner), followed by incubation at 37 °C overnight in 5% CO_2. After the medium was removed, 100 μL of the pigment mixture (Calcium 4 assay kit, Molecular Devices) was dispensed into each well of the plate, followed by incubation at 37 °C for 1 h. A total of 10 mM peptide in DMSO was diluted with HANKS/HEPES containing 2.5 mM probenecid and the dilution was transferred to a 96-well sample plate (V-Bottom plate, Coster). The cell and sample plates were set in a FlexStation (Molecular Devices) and 25 μL of the sample solution was automatically transferred to the cell plate.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.01.036.

References and footnotes

1. Knobil, E. *Recent Prog. Horm. Res.* **1980**, *36,* 53.
2. Karsch, F. J. In Reproduction in mammals, Hormonal control of reproduction, Ed 2; Austin, C. R.; Short, R. V., Eds.; Cambridge, UK: Cambridge UP. 1984; Vol . 3, pp 1-20.
3. Maeda, K.; Tsukamura, H.; Ohkura, S.; Kawakami, S.; Nagabukuro, H.; Yokoyama, A. *Neurosci. Biobehav. Rev.* **1995**, *19,* 427.
4. Gottsch, M. L.; Cunningham, M. J.; Smith, J. T.; Popa, S. M.; Acohido, B. V.; Crowley, W. F.; Seminara, S.; Clifton, D. K.; Steiner, R. A. *Endocrinology* **2004**, *145*, 4073.
5. Lee, J.; Miele, M. E.; Hicks, D. J.; Phillips, K. K.; Trent, J. M.; Weissman, B. E.; Welch, D. R. *J. Natl. Cancer Inst.* **1996**, *88,* 1731.
6. Ohtaki, T.; Shintani, Y.; Honda, S.; Matsumoto, H.; Hori, A.; Kanehashi, K.; Terao, Y.; Kumano, S. Takatsu, Y.; Masuda, Y.; Ishibashi, Y.; Watanabe, T.; Asada, M.; Yamada, T.; Suenaga, M.; Kitada, C.; Usuki, S.; Kurokawa, T.; Onda, H.; Nishimura, O.; Fujino, M. *Nature* **2001**, *411,* 613.
7. Kotani, M.; Detheux, M.; Vandenbogaerde, A.; Communi, D.; Vanderwinden, J.; Le Poul, E.; Brézillon, S.; Tyldesley, R.; Suarez-Huerta, N.; Vandeput, F.; Blanpain, C.; Schiffmann, S. N.; Vassart, G.; Parmentier, M. *J. Biol. Chem.* **2001**, *276,* 34631.
8. Irwig, M. S.; Fraley, G. S.; Smith, J. T.; Acohido, B. V.; Popa, S. M.; Cunningham, M. J.; Gottsch, M. L.; Clifton, D. K.; Steiner, R. A. *Neuroendocrinology* **2004**, *80*, 264.
9. Tomita, K.; Oishi, S.; Cluzeau, J.; Ohno, H.; Navenot, J. M.; Wang, Z. X.; Peiper, S. C.; Akamatsu, M.; Fujii, N. *J. Med. Chem.* **2007**, *50*, 3222.
10. Tomita, K.; Oishi, S.; Ohno, H.; Peiper, S. C.; Fujii, N. *J. Med. Chem.* **2008**, *51,* 7645.
11. Matsui, H.; Takatsu, Y.; Kumano, S.; Matsumoto, H.; Ohtaki, T. *Biochem. Biophys. Res. Commun.* **2004**, *320*, 383.

12. Inoue, N.; Sasagawa, K.; Ikai, K.; Sasaki, Y.; Tomikawa, J.; Oishi, S.; Fujii, N.; Uenoyama, Y.; Ohmori, Y.; Yamamoto, N.; Hondo, E.; Maeda, K. I.; Tsukamura, H. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108,* 17527.
13. Kinsey-Jones, J. S.; Li, X. F.; Luckman, S. M.; O'Byrne, K. T. *Endocrinology* **2008**, *149,* 1004.
14. Ohkura, S.; Takase, K.; Matsuyama, S.; Mogi, K.; Ichimaru, T.; Wakabayashi, Y.; Uenoyama, Y.; Mori, Y.; Steiner, R. A.; Tsukamura, H.; Maeda, K.-I.; Okamura, H. *J. Neuroendocrinol.* **2009**, *21,* 813.
15. Goodman, R. L.; Lehman, M. N.; Smith, J. T.; Coolen, L. M.; de Oliveira, C. V. R.; Jafarzadehshirazi, M. R.; Pereira, A.; Iqbal, J.; Caraty, A.; Ciofi, P.; Clarke, I. J. *Endocrinology* **2007**, *148,* 5752.
16. Navarro, V. M.; Gottsch, M. L.; Chavkin, C.; Okamura, H.; Clifton, D. K.; Steiner, R. A. *J. Neurosci.* **2009**, *29,* 11859.
17. Wakabayashi, Y.; Nakada, T.; Murata, K.; Ohkura, S.; Mogi, K.; Navarro, V. M.; Clifton, D. K.; Mori, Y.; Tsukamura, H.; Maeda, K.; Steiner, R. A.; Okamura, H. *J. Neurosci.* **2010**, *30,* 3124.
18. Topaloglu, A. K.; Reimann, F.; Guclu, M.; Yalin, A. S.; Kotan, L. D.; Porter, K. M.; Serin, A.; Mungan, N. O.; Cook, J. R.; Ozbek, M. N.; Imamoglu, S.; Akalin, N. S.; Yuksel, B.; O'Rahilly, S.; Semple, R. K. *Nat. Genet.* **2009**, *41,* 354.
19. Kangawa, K.; Minamino, N.; Fukuda, A.; Matsuo, H. *Biochem. Biophys. Res. Commun.* **1983**, *114,* 533.
20. Kurtz, M. M.; Wang, R.; Clements, M. K.; Cascieri, M. A.; Austin, C. P.; Cunningham, B. R.; Chicchi, G. G.; Liu, Q. *Gene* **2002**, *296,* 205.
21. For a review, see: Severini, C.; Improta, G.; Falconieri-Erspamer, G.; Salvadori, S.; Erspamer, V. *Pharmacol. Rev.* **2002**, *54,* 285.
22. Alonso, R. *Eur. J. Neurosci.* **1996**, *8,* 801.
23. Nalivaiko, E.; Michaud, J. -C.; Soubrié, P.; Le Fur, G.; Feltz, P. *Neuroscience* **1997**, *78,* 745.

24 Kinsey-Jones, J. S.; Grachev, P.; Li, X. F.; Lin, Y. S.; Milligan, S. R.; Lightman, S. L.; O'Byrne, K. T. *Endocrinology* **2012**, *153,* 307.

25. Mastrangelo, D.; Mathison, R.; Huggel, H. J. *Eur. J. Pharmacol.* **1987**, *134,* 321.

26. D'Orleans-Juste, P.; Claing, A.; Telemaque, S.; Warner, T. D.; Regoli, D. *Eur. J. Pharmacol.* **1991**, *204,* 329.

27. Thompson, G. W.; Hoover, D. B.; Ardell, J. L.; Armour, J. A. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **1998**, *275,* R1683.

28. Rovero, P.; Pestellini, V.; Patacchini, R.; Giuliani, S.; Santicioli, P.; Maggi, C. A.; Meli, A.; Giachetti, A. *Peptides* **1989**, *10,* 593.

29. Rovero, P.; Pestellini, V.; Rhaleb, N. -E.; Dion, S.; Rouissi, N.; Tousignant, C.; Telemaque, S.; Drapeau, G.; Regoli, D. *Neuropeptides* **1989**, *13,* 263.

30. Drapeau, G.; D'Orleans-Juste, P.; Dion, S. *Neuropeptides* **1987**, *10,* 43.

31. Wormser, U.; Laufer, R.; Hart, Y.; Chorev, M.; Gilon, C.; Selinger, Z. *EMBO J.* **1986**, *5,* 2805.

32. The NK3R activation in the Ca^{2+} flux assay was observed by significantly lower concentration (EC_{50}) of the peptides compared with that in the binding inhibition assay (IC_{50}). This was attributed to the relatively high receptor binding of radiolabeled ligand [([^{125}I]His^3, $MePhe^7$)-NKB] to NK3R in the binding inhibition assay.

33. In this article, the residue numbering is based on the human NKB sequence.

34. Of note, physalaemin **8a** was reported to originally show preferred NK1R binding: Buck, S. H.; Shatzer, S. A. *Life Sci.* **1988**, *42,* 2701.

Table 1. Structure-activity relationships of tachykinin peptides and their analogs.

Peptide	Sequence	IC_{50} (nM)[a]	EC_{50} (nM)[b]	Binding Inhibition (%)[c]	
				NK1R	NK2R
	-1 0 1 2 3 4 5 6 7 8 9 10				
[MePhe^7]-NKB (**1a**)	H-Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met-NH_2	3.0	0.11	< 10	59
PG-KII (**2a**)	pGlu-Pro-Asn-Pro-Asp-Glu-Phe- Val -Gly-Leu-Met-NH_2	53	0.079	94	92
PG-SP1 (**3a**)	pGlu-Pro-Asn-Pro-Asp-Glu-Phe- Tyr -Gly-Leu-Met-NH_2	710	1.38	98	< 10
3b	pGlu-Pro-Asn-Pro-Asp- **Asp**-Phe-**MePhe**-Gly-Leu-Met-NH_2	19	0.083	< 10	< 10
uperolein (**4a**)	pGlu-Pro-Ser-Pro-Asn-Ala-Phe- Tyr -Gly-Leu-Met-NH_2	82	0.12	100	60
4b	pGlu-Pro-Ser-Pro-Asn- **Asp**-Phe-**MePhe**-Gly-Leu-Met-NH_2	17	0.13	< 10	< 10
eledoisin (**5a**)	pGlu-Pro-Ser-Lys-Asp-Ala-Phe- Ile -Gly-Leu-Met-NH_2	180	0.22	100	95
5b	pGlu-Pro-Ser-Lys-Asp- **Asp**-Phe-**MePhe**-Gly-Leu-Met-NH_2	15	0.090	< 10	< 10
kassinin (**6a**)	H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe- Val -Gly-Leu-Met-NH_2	350	0.21	101	96
6b	H-Asp-Val-Pro-Lys-Ser-Asp- **Asp**-Phe-**MePhe**-Gly-Leu-Met-NH_2	110	0.085	< 10	< 10
sialokinin II (**7a**)	H-Asp-Thr-Gly-Asp-Lys-Phe- Tyr -Gly-Leu-Met-NH_2	1930	10.88	99	98
7b	H-Asp-Thr-Gly-Asp- **Asp**-Phe-**MePhe**-Gly-Leu-Met-NH_2	81	0.11	< 10	< 10
physalaemin (**8a**)	pGlu-Ala-Asp-Pro-Asn-Lys-Phe- Tyr -Gly-Leu-Met-NH_2	1550	3.36	101	68
8b	pGlu-Ala-Asp-Pro-Asn- **Asp**-Phe-**MePhe**-Gly-Leu-Met-NH_2	54	0.15	87	< 10
bufokinin (**9a**)	H-Lys-Pro-Arg-Pro-Asp-Gln-Phe- Tyr -Gly-Leu-Met-NH_2	2990	10.73	101	95
9b	H-Lys-Pro-Arg-Pro-Asp- **Asp**-Phe-**MePhe**-Gly-Leu-Met-NH_2	31	5.04	26	< 10

[a] IC_{50} values are the concentrations for 50% inhibition of 0.1 nM of ([^{125}I]His^3, MePhe^7)-NKB binding to NK3R ($n = 3$). The IC_{50} value of NKB was 76 nM in this assay. [b] EC_{50} values are the concentrations for 50% induction of Ca^{2+} influx in NK3R expressing CHO cell ($n = 3$). The EC_{50} value of NKB was 0.034 nM in this assay. [c] Binding inhibition (%) was calculated by the binding inhibition assay using a radioactive ligand (0.1 nM) and each peptide (10 μM). 100% binding inhibition was calculated based on the background signals, which were obtained by measurement of the wells without receptor membrane.

Table 2. Structure-activity relationships of eledoisin, kassinin and their analogs.

Peptide	Sequence	IC_{50} (nM)[a]	EC_{50} (nM)[b]	Binding Inhibition (%)[c]	
	-1 0 1 2 3 4 5 6 7 8 9 10			NK1R	NK2R
[$MePhe^7$]-NKB (**1a**)	H-Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met- NH_2	3.4	0.013	< 10	57
eledoisin (**5a**)	pGlu-Pro-Ser-Lys-Asp-Ala-Phe- Ile -Gly-Leu-Met- NH_2	96	0.24	100	99
5b	pGlu-Pro-Ser-Lys-Asp- **Asp**-Phe-**MePhe**-Gly-Leu-Met- NH_2	5.5	0.078	< 10	< 10
5c	pGlu-Pro-Ser-Lys-Asp- **Asp**-Phe- Ile -Gly-Leu-Met- NH_2	35	0.0054	91	56
5d	pGlu-Pro-Ser-Lys-Asp-Ala-Phe- **MePhe**-Gly-Leu-Met- NH_2	7.9	0.071	48	26
kassinin (**6a**)	H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe- Val -Gly-Leu-Met- NH_2	100	0.25	99	99
6b	H-Asp-Val-Pro-Lys-Ser-Asp- **Asp**-Phe-**MePhe**-Gly-Leu-Met- NH_2	27	0.15	< 10	< 10
6c	H-Asp-Val-Pro-Lys-Ser-Asp- **Asp**-Phe- Val -Gly-Leu-Met- NH_2	89	0.47	53	74
6d	H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe- **MePhe**-Gly-Leu-Met- NH_2	13	0.038	46	11

[a] IC_{50} values are the concentrations for 50% inhibition of ([^{125}I]His^3, $MePhe^7$)-NKB (0.1 nM) binding to NK3R ($n = 3$). [b] EC_{50} values are the concentrations for 50 induction of Ca^{2+} influx in NK3R expressing CHO cell ($n = 3$). [c] Binding inhibition (%) was calculated by the binding inhibition assay using a radioactive ligand (0.1 nM and each peptide (10 μM). 100% binding inhibition was calculated based on the background signals, which were obtained by measurement of the wells without recepto

Table 3. Structure-activity relationships of Phe[5]-substituted [MePhe[7]]-NKB analogs.

[MePhe[7]]-NKB `H-Asp-Met-His-Asp-`**`Xaa`**`-Phe-MePhe-Gly-Leu-Met-NH2`

Peptide	**Xaa**	IC_{50} (nM)[a]	EC_{50} (nM)[b]	Binding Inhibition (%)[c]	
				NK1R	NK2R
[MePhe[7]]-NKB[d] (**1a**)	Phe	3.8	0.070	< 10	57
1b	Asp	38	0.040	< 10	< 10
1c	Glu	7.5	0.024	< 10	< 10
1d	Arg	2.5	0.080	60	62
1e	Lys	11	0.026	40	35
1f	His	5.9	0.017	< 10	47
1g	Tyr	1.5	0.072	< 10	51
1h	Trp	1.8	0.28	< 10	79
1i	Val	1.5	0.065	< 10	29
1j	Leu	6.9	0.051	< 10	29
1k	Ile	4.0	0.060	< 10	35
1l	Ala	4.4	0.012	< 10	33
1m	Asn	18	0.036	< 10	10
1n	Gln	4.6	0.043	24	21
1o	Ser	3.6	0.040	< 10	31
1p	Thr	4.3	0.046	< 10	19
1q	Met	1.7	0.032	< 10	41
1r	Pro	45	0.041	< 10	38
1s	Gly	13	0.036	< 10	22

[a] IC_{50} values are the concentrations for 50% inhibition of ([^{125}I]His[3], MePhe[7])-NKB binding (0.1 nM) to NK3R (n = 3).

[b] EC_{50} values are the concentrations for 50% induction of Ca^{2+} influx in NK3R expressing CHO cells (n = 3).

[c] Binding inhibition (%) was calculated by the binding inhibition assay using a radioactive ligand (0.1 nM) and each peptide (10 μM). 100% binding inhibition was calculated based on the background signals, which were obtained by measurement of the wells without receptor membrane.

[d] [MePhe[7]]-NKB: H-Asp-His-Met-Asp-Xaa-Phe-MePhe-Gly-Leu-Met-NH_2.