*Supplementary Information*

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

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**Experimental procedures**

**Evaluation of binding affinity of tachykinin peptides to NK1R, NK2R and NK3R.** Binding inhibition assays were performed using membranes from NK1R-, NK2R- or NK3R-expressing CHO cells as described previously.[S1,S2] Briefly, membranes were incubated with 0.050 $cm^3$ of peptide, 0.025 $cm^3$ of radioactive 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, Perkin-Elmer Life Sciences], and 0.025 $cm^3$ of membrane solution in assay buffer [50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA]. Reaction mixtures were filtered through GF/B filters, pretreated with 0.3% polyethyleneimine. Filters were washed with [50 mM HEPES (pH 7.4), 500 mM NaCl, 0.1% BSA] and dried at 55 °C. Bound radioactivity was measured by TopCount (PerkinElmer Life Sciences) in the presence of MicroScint-O (0.030 $cm^3$) (PerkinElmer Life Sciences).

**Evaluation of NK3R agonist activity.** NK3R agonist activity of each peptide was evaluated by $[Ca^{2+}]_i$ flux assay as described previously.[S1,S2] NK3R expressing CHO cells ($4.0 \times 10^4$ cells 0.050 $cm^3$ $well^{-1}$) 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 medium removal, 0.1 $cm^3$ of pigment mixture (Calcium 4 assay kit, Molecular Devices) was dispensed into each well, followed by incubation at 37 °C for 1 h. 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 FlexStation (Molecular Devices) and 0.025 $cm^3$ sample solution automatically transferred to the cell plate.

**Preparation of animal sera.** Animal sera were prepared as described previously.[S1] Rat serum obtained from male Wistar rats (>5-month-old) was purchased from Shimuzu Animal Supplies Co., Ltd. Goat serum was obtained from an 14-month-old castrated male Shiba goat. Pig serum was obtained from 5-month-old female hybrid pigs. Cattle serum was obtained from a 7-year-old female Japanese Black cattle. After the collection, blood was incubated overnight at 4 °C, centrifuged (4 °C, 1,710 g, 15 min), and the supernatant were used as sera. The experiments were conducted in accordance with the guidelines of the Committee on the Care and Use of Experimental Animals at the Graduate School of Bioagricultural Sciences, Nagoya University.

**Evaluation of peptide stability in serum.** The stability of peptides in animal sera was evaluated as described previously.[S1] Briefly, 0.004 $cm^3$ of peptide solution (10 mM in DMSO containing 0.1% of *m*-cresol as an internal standard) was dissolved in rat, pig, goat, and cattle serum (0.196 $cm^3$). After incubation at 37 °C, a 0.030 $cm^3$ aliquot was sampled at intervals (indicated time). After addition of MeCN (0.090 $cm^3$), the sample solution was centrifuged (4 °C, 13000 g, 10 min), and then the

supernatant was analysed by HPLC with a linear gradient of $CH_3CN$ (5–50% over 45 min or 30–50% over 20 min; detection at 220 nm). The compound ratio was determined by the peak areas.

**Evaluation of peptide stability in the presence of NEP 24.11.** The stability of peptides in the presence of NEP 24.11 was evaluated as described previously.[S1] Briefly, 0.008 $cm^3$ of peptide solution (10 mM in DMSO containing 0.1% of *m*-cresol) was dissolved in Tris·HCl (pH 7.5) containing 0.05% of Brij-35 (WAKO) (0.192 $cm^3$). After addition of NEP 24.11 (R&D systems) [0.200 $cm^3$, 0.1 μg $cm^{-3}$ in Tris·HCl (pH 7.5)], solutions were incubated at 37 °C. After incubation, a 0.030 $cm^3$ aliquot was sampled at the indicated intervals, and quenched by addition of MeCN (0.030 $cm^3$). The sample solution was analysed by HPLC with a linear gradient of MeCN (5–50% over 45 min or 30–50% over 20 min; detection at 220 nm). The compound ratio was determined by the peak areas.

**Evaluation of effect of NK3R agonists on MUA volley in OVX goats.**[S1,S4,S5] All goat experiments were approved by the Committee on the Care and Use of Experimental Animals at the National Institute of Agrobiological Sciences (H23-002). OVX goats were implanted with an array of bilateral recording electrodes consisting of six Teflon-insulated platinum-iridium wires at the posterior region of the ARC as described previously.[S1,S4] After recovery, goats were kept in a condition-controlled room (12L/12D, 23 °C, and 50% relative humidity) and loosely held in an individual stanchion. Animals were maintained with a standard pellet diet and dry hay, and had free access to water and supplemental minerals. MUA was monitored in conscious goats. Signals were passed through a buffer amplifier and integrated circuit directly plugged into an electrode assembly. After additional amplification and amplitude discrimination, MUA signal was stored as counts per 20 s on a personal computer.

In OVX goats, the intervolley interval of spontaneously occurring MUA volleys differed slightly among individuals ranging from 20 to 35 min but was relatively constant within an individual, in which the interval variation was usually ± 2 min, allowing for a timed treatment between experiments. On each experimental day, the average value of 3 successive intervolley intervals (*T*) was calculated for each OVX goat and sample injections were made at 1/2 *T* after the preceding MUA volley.

## References


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S2 R. Misu, T. Noguchi, H. Ohno, S. Oishi and N. Fujii, *Bioorg. Med. Chem.,* 2013, **21**, 2413-2417.

S3 S. Ohkura, K. Takase, S. Matsuyama, K. Mogi, T. Ichimaru, Y. Wakabayashi, Y. Uenoyama, Y. Mori, R. A. Steiner, H. Tsukamura, K. Maeda and H. Okamura, *J. Neuroendocrinol.,* 2009, **21**, 813-821.

S4 Y. Wakabayashi, T. Nakada, K. Murata, S. Ohkura, K. Mogi, V. M. Navarro, D. K. Clifton, Y.

Mori, H. Tsukamura, K. Maeda, R. A. Steiner and H. Okamura, *J. Neurosci.,* 2010, **30**, 3124-3132.

**Table S1.** Analytical data of synthetic peptides[a]

| peptide | calcd ($MH^+$) | observed | peptide | calcd ($MH^+$) | observed |
|---|---|---|---|---|---|
| senktide | 794.37 | 794.13 | **3b** | 899.39 | 899.20 |
| **S1a**[b] | 904.39[c] | 904.74[c] | **3c** | 913.41 | 913.25 |
| **S1b** | 868.38 | 868.75 | **4a** | 899.39 | 899.20 |
| **S1c** | 842.34 | 842.18 | **4b** | 899.39 | 899.22 |
| **S1d**[b] | 848.78[c] | 848.33[c] | **4c** | 913.41 | 913.24 |
| **S1e** | 800.32 | 800.17 | **3d** | 913.41 | 913.34 |
| **S1f** | 840.36 | 840.80 | **4d** | 913.41 | 913.24 |
| **S1g** | 853.42 | 854.87 | **5b** | 842.37 | 842.21 |
| **S1h** | 867.44 | 867.94 | **5c** | 856.38 | 856.28 |
| **S1i** | 885.39 | 885.18 | **5d** | 856.38 | 856.26 |
| **S1j** | 881.42 | 881.26 | **5e** | 870.41 | 870.23 |
| **S1k** | 825.39 | 825.05 | **6a** | 814.34 | 814.15 |
| **S1l** | 867.44 | 867.18 | **6b** | 814.34 | 814.20 |
| **S1m** | 855.40 | 855.21 | **6c** | 828.35 | 828.12 |
| **S1n** | 895.43 | 895.80 | **6d** | 828.35 | 828.19 |
| **S1o** | 863.43 | 863.25 | **6e** | 842.37 | 842.07 |
| **S1p** | 858.36 | 858.25 | **7** | 785.94 | 785.76 |
| **S1q** | 881.38 | 881.28 | **8** | 801.94 | 801.82 |
| **S1r** | 832.37 | 832.22 | **9** | 800.38 | 800.81 |
| **1a** | 794.37 | 794.13 | **10**[b] | 850.94[c] | 850.55[c] |
| **1b** | 808.38 | 808.25 | **11** | 813.95 | 813.88 |
| **1c** | 858.36 | 858.15 | **12** | 821.99 | 821.83 |
| **1d** | 881.38 | 881.17 | **S2a** | 842.37 | 842.80 |
| **1e** | 808.38 | 808.22 | **S2b**[b] | 864.36[c] | 864.64[c] |
| **1f** | 810.33 | 810.09 | **S2c** | 856.39 | 856.84 |
| **1g** | 842.37 | 842.25 | **S3a** | 835.34 | 835.76 |
| **2a** | 857.38 | 857.24 | **S3b** | 835.34 | 835.61 |
| **3a** | 899.39 | 899.17 | **S3c** | 849.36 | 849.76 |

[a] Unless otherwise noted, the data were obtained by ESI-MS analysis. [b] The data were obtained by MALDI-TOF-MS. [c] [$MNa^+$].

**Table S2.** Structure-activity relationship of senktide derivatives with modification of tachykinin consensus sequence

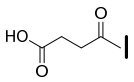
succinyl-Asp-**Xaa$^1$**-MePhe-**Xaa$^2$**-**Xaa$^3$**-**Xaa$^4$**-$NH_2$

| Peptide | **Xaa$^1$** | **Xaa$^2$** | **Xaa$^3$** | **Xaa$^4$** | $IC_{50}$ (nM)[a] |
|---|---|---|---|---|---|
| senktide | Phe | Gly | Leu | Met | 43 |
| **S1a** | Phe | Pro | Leu | Met | 7119 |
| **S1b** | Phe | Pro | Val | Met | >10000 |
| **S1c** | Phe | Ala | Val | Met | >10000 |
| **S1d** | Phe | Pro | Gly | Met | >10000 |
| **S1e** | Phe | Ala | Gly | Met | >10000 |
| **S1f** | Phe | Ala | Pro | Met | >10000 |
| **S1g** | Phe | Gly | Val | Arg | >10000 |
| **S1h** | Phe | Ala | Val | Arg | >10000 |
| **S1i** | Phe | Gly | Met | Arg | >10000 |
| **S1j** | Phe | Pro | Ser | Arg | >10000 |
| **S1k** | Phe | Gly | Ala | Arg | >10000 |
| **S1l** | Phe | Gly | Leu | Arg | >10000 |
| **S1m** | Phe | Gly | Thr | Arg | >10000 |
| **S1n** | Phe | Pro | Thr | Arg | >10000 |
| **S1o** | Phe | Pro | Pro | Lys | >10000 |
| **S1p** | Tyr | Gly | Leu | Met | 8480 |
| **S1q** | Trp | Gly | Leu | Met | >10000 |
| **S1r** | His | Gly | Leu | Met | >10000 |

[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).

**Table S3.** Structure-activity relationships of N-terminal methyloxalyl and aminosulfonyl 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 |  | L-Asp | 75 | 21 | >10000 | >10000 |
| **10** |  | L-Asp | 15 | 24 | >10000 | >10000 |
| **S2a** |  | L-Glu | 11 | 55 | >10000 | >10000 |
| **S2b** |  | D-Glu | 18 | 65 | >10000 | >10000 |
| **S2c** |  | L-Aad | 11 | 47 | >10000 | >10000 |
| **12** |  | L-Asp | 36 | 27 | >10000 | >10000 |
| **S3a** |  | L-Glu | 12 | 10 | >10000 | 8200 |
| **S3b** |  | D-Glu | 41 | 28 | >10000 | >10000 |
| **S3c** |  | L-Aad | 13 | 14 | >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.

**Figure S1.** Degradation profile of peptide **6c** and **6d** in animal-derived serum: **6c** (closed bar), **6d** (opened bar). Peptide **6c** and **6d** were incubated in rat, pig, goat and cattle serum at 37 °C for 24 h. The sample was analysed by HPLC (detection at 220 nm).

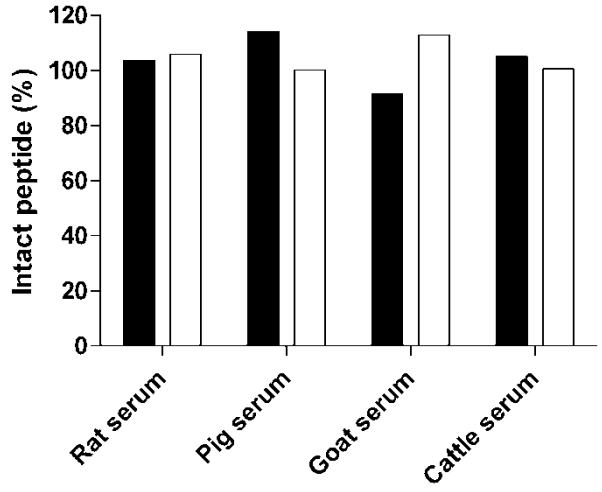


**Figure S2.** Effect of senktide and peptide **6d** on the induction of periodic burst of MUA volley in OVX goats. Representative profiles of MUA in an OVX goat that received intravenous administration of senktide (200 nmol, A) and peptide **6d** (200 nmol, B) are shown. The arrow indicates timing of injection of NK3R agonist. The open and closed triangles indicate spontaneous and ligand-induced MUA volleys, respectively.