[Chem. Pharm. Bull.]
35(8)3442—3446(1987)

Synthesis of Neurokinin B Analogs and Their Activities as Agonists and Antagonists

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(Received December 22, 1986)

Nine neurokinin B (NKB) related octapeptide analogs, substituted with Arg for His³, or Gly, D-Trp, D-Ala or D-Phe for Phe⁶, or Gly for Val⁷, or D-Trp or D-Ala for Gly⁸ in the original sequence, were synthesized by the solid-phase method. The biological activity of the synthetic analogs was assayed on isolated guinea-pig ileum and rat duodenum. None of the analogs possessed agonistic activity. In the guinea-pig ileum assay, [Arg³, Gly⁶, D-Ala⁸]- and [Arg³, Gly⁶, D-Trp⁸]-NKB (3—10) were found to act as fairly potent antagonists against NKB, but showed no antagonistic effect against substance P (SP) or neurokinin A (NKA). [Arg³, D-Ala⁶, D-Trp⁸]- and [Arg³, D-Trp^{6,8}, Gly⁷]-NKB (3—10) showed antagonistic activities against NKB and SP, and NKB, SP and NKA, respectively, in the ileum assay, while the latter acted as an antagonist against NKB, but not against SP or NKA in the rat duodenum assay.

Keywords—neurokinin B; neurokinin B octapeptide analog; solid-phase synthesis; isolated guinea-pig ileum; isolated rat duodenum; agonistic activity; relative activity; antagonistic activity; nonspecific antagonist; specific antagonist

Neurokinin B^{1,2)} (NKB) is a decapeptide amide isolated from porcine spinal cord, and has close structural homologies with tachykinins such as neurokinin A (NKA), substance P (SP), kassinin and physalaemin (Fig. 1), particularly in the C-terminal regions.

In a study³⁾ of the relationship between chain length and activity, it was found that the contractile activity of NKB on the guinea-pig ileum, rat *vas deferens* and rat duodenum remains nearly intact after removal of the N-terminal tripeptide portion from the native peptide.

Our study⁴⁾ on the structure-activity relationship of NKB (3—10) provided a clear indication of the location of the active site, that is, the replacement of Phe⁶ or Val⁷ with Gly brings about a drastic decrease of the contractile activities on isolated guinea-pig ileum and rat *vas deferens*, while the substitution of Gly for Phe⁵ enhances the activities. In other words, NKB related octapeptide analogs [Gly⁶]-NKB (3—10) **1** and [Gly⁷]-NKB (3—10) **2** have no intrinsic activity on isolated guinea-pig ileum and rat *vas deferens*. In a recent study⁵⁾ of NKB related peptides, it has been found that the substitution of an unusual amino acid such as Sar, D-Trp, D-Ala, D-Phe, D-Arg, D-Pro, D- α -phenylglycine, D-homoglutamine,⁶⁾ or D-homo-

	1	10
neurokinin B (NKB)	H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH ₂	
neurokinin A (NKA)	H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH ₂	
substance P (SP)	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH ₂	
kassinin	H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH ₂	
physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH ₂	

Fig. 1. Amino Acid Sequences of Tachykinin Peptides

glutamic acid⁷⁾ for Phe⁶ significantly reduces the contractile activity on isolated guinea-pig ileum, and the replacement of Phe⁶ with Gly or D-Ala changes the pharmacological spectrum of NKB from that of an agonist to that of an antagonist. Octapeptide analogs [Gly⁶]-NKB (3—10) **1** and [Arg³, D-Ala⁶]-NKB (3—10) **3** have been found to act as fairly potent antagonists of NKB.

Based on these observations, a new series of octapeptide analogs of NKB (3—10) (Fig. 2) was designed in order to obtain a specific and potent antagonist of NKB. Analogs **1** and **2** were modified by replacements with Arg at position 3, Gly, D-Ala, D-Trp and D-Phe at position 6, Gly at position 7, and D-Ala and D-Trp at position 8 of NKB. It has been indicated that the antagonistic activity⁸⁾ of SP related peptide analogs against SP can be increased with double or triple substitutions of D-amino acids such as D-Trp and D-Ala for natural residues, mainly at the C-terminal region of SP. The N-terminal Arg³ moiety was incorporated to improve the solubility of the peptides in water, as in SP antagonists⁸⁾ derived from the C-terminal hexapeptide sequence of SP.

The new NKB analogs were synthesized in the same way as described for the preparation of NKB analogs⁴⁾ on an automated peptide synthesizer by the standard solid-phase method⁹⁾ starting from *tert*-butoxycarbonyl(Boc)-Met-benzhydrylamine-resin. Boc protection was used

	3	6	7	8	10
1 [Gly ⁶]-NKB (3—10)	H-His-Asp-Phe—Gly-Val—Gly-Leu-Met-NH ₂				
2 [Gly ⁷]-NKB (3—10)	H-His-Asp-Phe—Phe-Gly—Gly-Leu-Met-NH ₂				
3 [Arg ³ , D-Ala ⁶]-NKB (3—10)	H-Arg-Asp-Phe—D-Ala-Val—Gly-Leu-Met-NH ₂				
4 [Arg ³ , Gly ⁶ , D-Ala ⁸]-NKB (3—10)	H-Arg-Asp-Phe—Gly-Val—D-Ala-Leu-Met-NH ₂				
5 [Arg ³ , Gly ⁶ , D-Trp ⁸]-NKB (3—10)	H-Arg-Asp-Phe—Gly-Val—D-Trp-Leu-Met-NH ₂				
6 [Arg ³ , D-Ala ⁶ , D-Trp ⁸]-NKB (3—10)	H-Arg-Asp-Phe—D-Ala-Val—D-Trp-Leu-Met-NH ₂				
7 [Arg ³ , D-Trp ^{6,8}]-NKB (3—10)	H-Arg-Asp-Phe—D-Trp-Val—D-Trp-Leu-Met-NH ₂				
8 [Arg ³ , D-Phe ⁶ , Gly ⁷ , D-Ala ⁸]-NKB (3—10)	H-Arg-Asp-Phe—D-Phe-Gly—D-Ala-Leu-Met-NH ₂				
9 [Arg ³ , D-Phe ⁶ , Gly ⁷ , D-Trp ⁸]-NKB (3—10)	H-Arg-Asp-Phe—D-Phe-Gly—D-Trp-Leu-Met-NH ₂				
10 [Arg ³ , D-Ala ⁶ , Gly ⁷ , D-Trp ⁸]-NKB (3—10)	H-Arg-Asp-Phe—D-Ala-Gly—D-Trp-Leu-Met-NH ₂				
11 [Arg ³ , D-Trp ⁶ , Gly ⁷ , D-Ala ⁸]-NKB (3—10)	H-Arg-Asp-Phe—D-Trp-Gly—D-Ala-Leu-Met-NH ₂				
12 [Arg ³ , D-Trp ^{6,8} , Gly ⁷]-NKB (3—10)	H-Arg-Asp-Phe—D-Trp-Gly—D-Trp-Leu-Met-NH ₂				

Fig. 2. NKB Related Octapeptide Analogs

TABLE I. Physical Properties and Yields of the Synthetic Analogs

Analog	$[\alpha]_D^{25}$ (°) (c=0.1, DMF)	Retention time ^{a)} (min)	R _f ¹	R _f ²	Yield (%)
4	−23.0	17.1	0.07	0.64	31.1
5	−36.0	21.6	0.09	0.67	18.4
6	−32.0	21.8	0.10	0.68	29.2
7	−20.0	26.3	0.16	0.70	15.8
8	−10.0 ^{b)}	17.4	0.07	0.65	14.9
9	+0.3 ^{b)}	23.5	0.13	0.68	14.2
10	−26.0 ^{b)}	18.3	0.06	0.64	11.5
11	+3.0 ^{b)}	18.4	0.07	0.64	29.1
12	−17.0	23.0	0.11	0.67	12.3

a) Conditions of analytical HPLC: column, Chemcosorb ODS (3.9×300 mm); flow rate, 1 ml/min; detection, 210 nm; eluent system, linear gradient from 14% to 35% MeCN (15 min) in 20 mM phosphate buffer (pH 3.0). b) $[\alpha]_D^{25}$ (°).

TABLE II. Amino Acid Analyses of the Synthetic Analogs

Analog	Found (Calcd)									
	Asp	Gly	Ala	Val	Met	Leu	Phe	Trp	Arg	NH ₃
4	1.00 (1)	1.01 (1)	1.03 (1)	1.05 (1)	0.99 (1)	1.03 (1)	1.01 (1)	—	0.88 (1)	1.10 (1)
5	1.03 (1)	0.99 (1)	—	0.94 (1)	0.91 (1)	1.01 (1)	0.97 (1)	1.05 (1)	1.09 (1)	1.12 (1)
6	1.04 (1)	—	1.02 (1)	0.93 (1)	0.94 (1)	1.03 (1)	1.00 (1)	1.05 (1)	0.98 (1)	1.14 (1)
7	1.06 (1)	—	—	0.94 (1)	0.92 (1)	1.03 (1)	0.98 (1)	2.00 (2)	1.07 (1)	1.31 (1)
8	1.00 (1)	0.98 (1)	0.99 (1)	—	0.99 (1)	1.01 (1)	1.98 (2)	—	1.06 (1)	1.34 (1)
9	1.00 (1)	1.00 (1)	—	—	0.97 (1)	1.02 (1)	1.96 (2)	1.07 (1)	0.99 (1)	1.41 (1)
10	0.99 (1)	1.02 (1)	0.98 (1)	—	0.98 (1)	1.01 (1)	0.98 (1)	0.91 (1)	1.14 (1)	1.42 (1)
11	0.97 (1)	1.05 (1)	0.97 (1)	—	1.01 (1)	1.02 (1)	0.99 (1)	1.04 (1)	0.95 (1)	1.23 (1)
12	1.02 (1)	0.98 (1)	—	—	0.91 (1)	0.99 (1)	0.96 (1)	2.10 (2)	1.05 (1)	1.26 (1)

TABLE III. Biological Activities of the Synthetic Analogs on Isolated Guinea-Pig Ileum and Rat Duodenum

Analog	Guinea-pig ileum				Rat duodenum			
	RA ^{a)}	pA ₂ /NKB	pA ₂ /SP	pA ₂ /NKA	RA	pA ₂ /NKB	pA ₂ /SP	pA ₂ /NKA
1	0 ^{b)}	5.82 ^{c)}	—	—	0	—	—	—
2	0 ^{b)}	— ^{c)}	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
3	0 ^{c)}	5.69 ^{c)}	—	—	0	—	—	—
4	0	5.11	—	—	0	—	—	—
5	0	6.30	—	—	0	—	—	—
6	0	5.61	4.32	—	0	—	—	—
7	0	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
8	<0.01	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
9	0	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
10	<0.01	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
11	0	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
12	0	6.70	5.94	5.85	0	5.70	—	—

a) Relative activity based on NKB as 1. b) Data from ref 4). c) Data from ref 5). —, no antagonistic activity. N.T., not tested.

for the α -amino function. The side-chain protective groups used were cyclohexyl for Asp and *p*-toluenesulfonyl for Arg. The synthetic products were purified by preparative reverse-phase high-performance liquid chromatography (HPLC) using 0.1% TFA in MeCN as the eluent. Highly purified peptides (Tables I and II) were obtained after gel filtration on a Sephadex LH-20 column. Homogeneity of the peptides was demonstrated by analytical HPLC and thin-layer chromatography (TLC). When a single peak and a single spot were observed for a peptide in all chromatographic systems, the peptide was considered to be sufficiently pure for bioassay. The chromatographic data were supported by the amino acid analytical data.

The biological properties of the synthetic peptides were examined on isolated guinea-pig ileum and rat duodenum. The agonistic effects of analogs 4–12 were compared with that of our synthetic NKB in the ileum assay. Analogs 4–12 were tested for their antagonistic effects against NKB, and analogs 1, 3–6, and 12 were examined against SP and NKA in the ileum assay. The agonistic and antagonistic activities of analogs 1, 3–6, and 12 were further assayed in the rat duodenum preparation. The antagonistic activities of the analogs were evaluated in terms of pA₂ (negative concentration of antagonist that reduces the contractile response to a double dose of agonist in the presence of the antagonist to that obtained with a

single dose without the antagonist), according to Schild.¹⁰⁾ In the presence of analogs at concentrations of 8×10^{-6} M for SP and 2×10^{-6} M for NKB and NKA, the maximal responses induced by NKB, SP and NKA were almost the same as those without analogs. The results are presented in Table III.

Analog 4—12, and 1, 3—6, and 12 showed no agonistic activities in the ileum and duodenum assays, respectively. Though NKB at the concentration of 10^{-7} M induced maximal response in both assays, the analogs did not induce a contractile response when applied at up to 10^{-6} M. Analog 4—6 and 12 were found to act as antagonists of NKB, while analogs 7—11 exerted no antagonistic effect against NKB in the ileum assay. Analog 12 possessed the highest antagonistic activity against NKB among analogs 1, 3—6, and 12. The six NKB antagonists were tested for their antagonistic effects against SP and NKA. Analog 6 was a weak antagonist against SP, but not against NKA. The most potent antagonist of NKB, analog 12, also showed fairly potent antagonism to SP and NKA. None of analogs 1 and 3—5 was found to act as an antagonist of SP or NKA. The analogs 1, 3—6, and 12, which possessed antagonistic activities in the ileum assay, were tested for their agonistic activities in the duodenum assay. Analog 1 and 3—6 were found to exert no antagonistic effect against NKB, SP, or NKA, while analog 12, a nonspecific antagonist in the ileum assay, showed antagonism to NKB, but not to SP or NKA. The data indicate that analogs 12, and 1, 3—5 are specific and fairly potent antagonists against NKB in the duodenum and ileum assays, respectively, under the conditions of this investigation. The results show that a double substitution in positions 6 and 8 of NKB did not increase the activity of the octapeptide analog as an antagonist. A triple substitution in positions 6, 7, and 8 appears to be unfavorable for a specific NKB antagonist.

Experimental

Amino acid derivatives were purchased from the Peptide Institute, Inc., Osaka, Japan, and benzhydrylamine (BHA) resin (available amine of the resin: 0.6 mmol/g of support) from Beckman Inc., Palo Alto, Calif. Optical rotations were measured in a Nippon Bunkoh DIP-4 polarimeter. Amino acid analyses on samples previously hydrolyzed with 6 N HCl (110 °C, 24 h) or on analogs 5—7 and 9—12 previously hydrolyzed with 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (115 °C, 24 h) were performed on a Beckman System 7300 amino acid analyzer. TLC was carried out on silica-gel plates (Merck). The following solvent systems were used, and allowed to ascend for 10 cm: R_f^1 , *n*-BuOH–AcOH–H₂O (4:1:5, upper phase); R_f^2 , *n*-BuOH–pyridine–AcOH–H₂O (30:20:6:24). Analytical HPLC was done with the following systems: column, Chemcosorb ODS (Chemco, 3.9 × 300 mm); flow rate, 1 ml/min; detection, 210 nm; eluent system, linear gradient for 15 min from 14% to 35% MeCN in 20 mM phosphate buffer (pH 3.0).

General Procedure for the Preparation of Analogs—The solid-phase synthesis was carried out using a Beckman System 990C peptide synthesizer as described previously.⁴⁾ BHA-resin hydrochloride (0.5 g for each analog) served as the solid support. A 2.5 fold excess of the amino acid derivative was used for all couplings. The coupling was effected with dicyclohexylcarbodiimide–1-hydroxybenzotriazole. The Boc group on Trp-peptide resin was deblocked by the use of 25% TFA in dichloromethane containing 5% 1,2-ethanedithiol. The protected peptide resin was treated with anhydrous liquid HF¹¹⁾ containing 10% anisole. After evaporation of the HF *in vacuo* under ice-cooling, the residue was washed with AcOEt and the peptide was extracted with 50% AcOH.

Purification of the Peptides—The crude peptide was subjected to HPLC as reported previously.⁴⁾ The apparatus was composed of a model 590 pump and a U6K injector (Waters) connected to a column of Chemcosorb ODS (Chemco, 20 × 300 mm) or μ -Bondasphere C₁₈ (Waters, 19 × 150 mm). The eluates were monitored with a UV detector (S-310A model-II, Soma) at 210 nm. The MeCN–0.1% TFA solvent system was used for elution at a flow rate of 10 or 7 ml/min. Each peptide emerged at 40–60 min on isocratic elution with 21–32% MeCN in the solvent system. The desired fraction was passed through a Sephadex LH-20 column (16 × 500 mm) eluted with aqueous DMF (90%) containing 0.02% 1,2-ethanedithiol. Homogeneity of the peptides was checked by analytical HPLC and TLC.

Bioassay—The agonistic activity of the synthetic analogs was measured on isolated guinea-pig ileum and rat duodenum, as described before.⁴⁾ The contraction was recorded by means of an isotonic transducer (Nippon Kohden, TD-111T) with a load of 2 g on a Servocorder (Watanabe Instruments, SR6204). Concentration–response curves were obtained by using a cumulative dose assay, and the time between measurements of two consecutive dose–response curves was longer than 10 min. Though NKB at the concentration of 10^{-7} M induced maximal response, all

the analogs were applied up to 10^{-6} M. In the tests for antagonistic activity in the guinea-pig ileum and rat duodenum systems, the analog was added 10 min before NKB, SP or NKA. The cumulative dose-response curve of NKB, SP or NKA in the presence or absence of the analog was obtained. In the presence of analogs at concentrations of 8×10^{-6} M for SP and 2×10^{-6} M for NKB and NKA, the maximal response induced by NKB, SP and NKA was almost the same as the response in the absence of the analogs.

References and Notes

- 1) S. Kimura, M. Okada, Y. Sugita, I. Kanazawa, and E. Munekata, *Proc. Jpn. Acad.*, **59B**, 101 (1983); H. Kangawa, N. Minamino, A. Fukuda, and H. Matsuo, *Biochem. Biophys. Res. Commun.*, **114**, 533 (1983).
- 2) Amino acids, peptides and their derivatives mentioned in this paper are of L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature given in *Eur. J. Biochem.*, **138**, 9 (1984). Other abbreviations used: DMF, dimethylformamide; TFA, trifluoroacetic acid; AcOH, acetic acid; BuOH, butyl alcohol; MeCN, acetonitrile; AcOEt, ethyl acetate.
- 3) F. Osakada, K. Kubo, K. Goto, I. Kanazawa, and E. Munekata, *Eur. J. Pharmacol.*, **120**, 201 (1986).
- 4) T. Hashimoto, T. Moro, and Y. Uchida, *Bull. Chem. Soc. Jpn.*, **59**, 4006 (1986).
- 5) Y. Uchida, K. Okimura, K. Kurosawa, N. Sakura, K. Hirose, and T. Hashimoto, *Bull. Chem. Soc. Jpn.*, **60**, 1561 (1987).
- 6) T. Hashimoto, Y. Uchida, M. Nishijima, T. Moro, N. Sakura, and K. Hirose, *Bull. Chem. Soc. Jpn.*, **59**, 4009 (1986).
- 7) N. Sakura, K. Hirose, and T. Hashimoto, *Chem. Pharm. Bull.*, **34**, 3506 (1986).
- 8) D. Regoli, E. Escher, and J. Mizrahi, *Pharmacology*, **28**, 301 (1984).
- 9) R. B. Merrifield, *Adv. Enzymol.*, **32**, 221 (1969).
- 10) H. O. Schild, *Br. J. Pharmacol.*, **4**, 277 (1949).
- 11) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).