Amino Acids (2003) 25: 107–110
DOI 10.1007/s00726-002-0351-3

Amino
Acids
Printed in Austria

Syntheses of two neuromedin U (NMU) analogues and their comparative reducing food intake effect in rats

Short Communication

T. Abiko and **Y. Takamura**

Research Laboratory, Global Shinwa Pharmaceutical Co. Ltd., Aoba-ku, Sendai, Japan

Received July 6, 2002
Accepted October 30, 2002
Published online January 20, 2003; Springer-Verlag 2003

Summary. To examine the roles of aromatic rings Tyr residues at positions 1 and 6 and Phe residues at positions 16, 17 and 19 of rat neuromedin U-23 (NMU-23) (Tyr-Lys-Val-Asn-Glu-Tyr-Gln-Gly-Pro-Val-Ala-Pro-Ser-Gly-Phe-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2) for reducing food intake activity in male Wistar rats, two NMU-23 analogues, [Phe(4F)^{16,17,19}]NMU-23 and [Tyr(Me)^{1,6}]NMU-23, were synthesized by Fmoc strategy of manual solid-phase method. The synthetic NMU-23 showed reducing effect on food intake in rats. [Phe(4F)^{16,17,19}]NMU-23 exhibited higher reducing food intake effect than that of NMU-23. On the contrary, [Tyr(Me)^{1,6}]NMU-23 showed no reducing effect on food intake in rats than that of NMU-23.

Abbreviations: NMU, neuromedin U; TLC, thin layer chromatography; 4F, 4-fluoro; TFA, trifluoroacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; NPY, neuropeptide Y; Obu^t, tert-butoxy; Trt, trityl; HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; AcOH, acetic acid; DCC, N,N′-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; Mts, mesitylenesulfonyl; Boc, tert-butoxycarbonyl; DMF, dimethyformamide; TMSOTf, trimethylsilyltrifrate; EtOH, ethanol.

Keywords: Neuromedin U – Solid-phase method – reducing food intake – Neuromedin U analogue – Fmoc strategy – Aromatic ring substitution

Introduction

Neuromedin U (NMU), possessing uterus contractile activity, was first isolated from porcine spinal cord as NMU-8 and NMU-25 (Colon et al., 1988; Minamino et al., 1988). Later, rat NMU was identified as a single entity, consisting of 23 amino acid residues (NMU-23) because of lack of the double basic site (Gly-Gly substitution). In addition to its role in smooth muscle contraction, NMU exerts some other biological activities such as increase of blood pressure, induction of ACTH release, etc. Recently, A.D. Howard et al. (2000) reported that NMU involved in the ventromedical hypothalamic regions and its level is reduced in rats fasted for 48 h.

When NMU is injected at a dose of 3 or 10 μg in rats, overnight food intake is significantly decreased. In the same experiments, water intake was found to be decreased by NMU. Taken together, NMU may be one of the physiological mediators of food intake and may be useful in clarifying the intricate mechanism of obesity and leanness.

In our preceding paper (Abiko and Nakatsubo, 2001), one of our synthetic cholecystokinin (CCK) fragment 26–33 analogues, [Phe(3F)^{33}]CCK 26–33 in which Phe^{33} of CCK 26–33 was replaced by Phe(4F) showed stronger reducing activity on food intake in rats than that of CCK 26–33. In addition to this result, further information on structure-food intake decreasing activity relationship has not been reported yet. These results prompted us to examine the roles of two different aromatic rings of NMU-23 using synthetic two aromatic ring substituted analogues, [Phe(4F)^{16,17,19}]NMU-23 and [Tyr(Me)^{1,6}]NMU-23.

In the present study, Phe residues at 16, 17 and 19 were replaced by Phe(4F) residues and Tyr residues at 1 and 6 were also replaced by Tyr(Me) residues simultaneously. Here, we demonstrate that Tyr^{1,6}

residues are dispensable and electronic density of Phe[16,17,19] residues influences reducing effect on food intake in rats. These results are the first report on structure-food intake decreasing activity relationship of NMU.

Materials and methods

Fmoc-Asn(Trt)-CLEAR-Amide-resin and Fmoc-amino acids, Fmoc-Lys(Boc)-OH, Fmoc-Val-OH, Fmoc-Glu(Obu^t)-OH, Fmoc-Gln(Trt)-OH, Fmoc-GlyOH, Fmoc-Pro-OH, Fmoc-Ala-OH, Fmoc-Ser(Bu^t)-OH, Fmoc-Phe-OH, Fmoc-Tyr(Bu^t)-OH, Fmoc-Leu-OH, Fmoc-Arg(Mts)-OH and FmocPhe(4F)-OH, were purchased from Sigma Chemical Co. (USA), BACHEM Feinchemikalien AG (Switzerland), Neosystem Laboratories (France), Protein Research Inc. (Mino, Osaka) and Kokusan Chemical Works Ltd. (Kyoto). Fmoc-Tyr(Me)-OH was synthesized from Tyr according to my preceding paper (Abiko, 1974). Other reagents and solvents were also purchased from Kokusan Chemical Works Ltd. (Kyoto) and Sigma Chemical Co. (USA). Solvents were freshly distilled before using. The amino acid compositions of the acid hydrolysates were determined with a Hitachi type 835–50 amino acid analyzer. HPLC was conducted with a Shimadzu LC-6A apparatus coupled to a Wakopak Wakosil-11 5C18AR column (6.0 × 150 mm). FAB-MS spectrum was obtained on a Auto Spec Q with an OPUS data processor. Purified peptides were chromatographed on silica-gel plates (Kieselgel G, Merck) and Rf^1 values refer to BuOH-AcOH-pyridine-H_2O (15:10:3:12 by volume) and Rf^2 values refer to BuOH-AcOH-pyridine-H_2O (5:5:1:4 by volume). Male Wistar rats were used for bioassay on reduction of food intake activity.

Solid-phase peptide synthesis

Solid-phase peptide syntheses of two NMU-23 analogues, [Phe(4F)[16,17,19]]NMU-23 and Tyr(Me)[1,6]]NMU-23, were carried out manually in a glass vessel by a stepwise strategy with Fmoc-Asn(Trt)-CLEAR-Amide-resin (0.5 mmol/g, 3 g), and the synthesis was continued by sequentially incorporating amino acid residues, one at a time, into the growing peptide chains according to the general principles of the solid-phase method. In all coupling cycles (120 min) three fold excess each of Fmoc-amino acid, HOBT and DCC were used. Double couplings were done when necessary as judged by the ninhydrin test.

The general procedure for each synthetic cycle was:

1. Three washings with dichloromethane.
2. Prewashings 20% piperidine in DMF.
3. Deprotecting for 30 min with 20% piperidine in DMF.
4. Three washings with dichloromethane.
5. Two washings with DMF.
6. Addition of 3 eq Fmoc-amino acid, HOBT and DCC in DMF-dichloromethane (1:1).
7. Reaction for 2 h.
8. Three washings each with dichloromethane, DMF, EtOH and dichloromethane. Double couplings were done when necessary as judged by the ninhydrin test.
9. 0.4 M acetylimidazole in DMF (1 X, 30 min).
10. Two washings with DMF.

Deprotection and purification

After deprotection of the last N^a-Fmoc group, the peptide-resin was washed with EtOH and dried in vacuo to yield the protected NMU-23 analogue-resin. The protected peptide-resin was treated with TFA/TFMSOTf-cresol/thioanisole (100:20:5:23:1.7; V/V) for 1 h at O°C and an additional 1 h at room temperature. The solution was filtered, washed with diethyl ether, and dried. The crude peptide was purified by PR-HPLC using a Shimadzu LC-6 apparatus. A sample (3 mg) was applied to a Nucleosil C18 column (250 × 10 mm), which was eluted with a gradient of acetonitrile (20→45%, 40 min) in 0.1% aqueous TFA at a flow rate of 3.0 ml/min. The eluates corresponding to the main peaks detected by ultraviolet absorption measurement at 260 nm were collected and the solvent was removed by lyophilization to give fluffy powders. The rest of the samples were similarly purified. Yields of the two synthetic analogues based on the C-terminal Asn loaded on the resin are shown in Table 1.

Homogeneity of the peptides was checked by TLC, analytical HPLC, FAB-MS, and amino acid analysis after 6 N HCl hydrolysis. The physicochemical data of the synthetic analogues are shown in Tables 1 and 2.

Reducing effect on food intake in rats by our centrally administered synthetic NMU-23 and its two analogues was examined. Comparative potency of NMU-23 and its two analogues was illustrated in Tables 3 and 4.

Table 1. Characterization of synthetic NMU-23 analogues

Peptide	Yield[a]	$[a]_D^{21}$	TLC[b]		HPLC[c]	FAB-MS[d] $(M + H)^+$
			Rf^1	Rf^2		
[Phe[16,17,19]]NMU-23	5.9	−79.8	0.13	0.18	17.6	2696.51 (2696.88)
[Tyr(Me)[1,6]]NMU-23	5.3	−82.6	0.15	0.20	18.3	2687.08 (2686.96)

[a] Final yield after deblocking and purification starting from Fmoc-Asn(Trt)-CLEAR-Amide-resin.
[b] See the Materials and Methods section.
[c] HPLC was performed on an analytical Wakopak Wakosil 11 5C18AR column (6.0 × 150 mm) by gradient elution with acetonitrile (20→45%) in 0.1% TFA at a flow rate of 1 ml/min and eluate was monitored at 260 nm.
[d] Found values were in agreement with calculated values. Values in parentheses are calculated values.

Table 2. Amino acid ratios in 6N HCl hydrolysates of NMU-23 analogues[a]

Amino acids	[Phe(4F)^{16,17,19}]NMU-23	[Tyr(Me)^{1,6}]NMU-23
Tyr	1.92	1.96
Val	2.02	1.97
Gly	3.01	3.02
Pro	2.89	2.87
Ala	0.97	1.02
Ser	0.92	0.93
Leu	1.00	1.00
Glu	1.95	1.98
Asp	1.93	1.96
Lys	1.02	0.99
Arg	1.91	1.90
Phe		2.94
Phe(4F)	2.90	
Average Recovery of Leu (%)	90.7	92.6

[a] Hydrolysis was carried out in 6N HCl in an evacuated sealed tube at 124°C for 26 h, and the results are expressed as ratios to the value for Leu, which was taken as the diagnostic amino acid in the acid hydrolysates.

Table 3. Reducing effect of food consumption by intercerebroventricular injection of NMU-23 and its analogues in freely fed rats[a]

Peptide	Dose (nmol/μg)	Food consumption (mg)
–	–	984 mg
NMU-23	3	564 mg*
NMU-23	30	396 mg*
[Phe(4F)^{16,17,19}]NMU-23	3	347 mg*
[Phe(4F)^{16,17,19}]NMU-23	30	220 mg*
[Tyr(Me)^{1,6}]NMU-23	3	968 mg
[Tyr(Me)^{1,6}]NMU-23	30	993 mg

[a] Designated amounts of synthetic NMU-23 and its two analogues were administered in a 5 μl bolus through a catcher placed in the left lateral ventricle in early light phase. Cultivated food consumption was measured in 4 h, after injection.
* Indicates significant difference from vehicle controls $p < 0.05$, n = 8–10, followed by Student-Newmann-Keuls test. Similar results were obtained in at least four independent sets of experiments.

Intracerebroventricular administration of synthetic NMU-23 and ITS two analogues

Male Wistar rats (180–200 g on arrival; Charles-River) were housed under controlled lighting (12 h light-dark cycle) and temperature (22°C) conditions. Food (standard chow pellets) and water were available ad libitum. Rats (200–220 g) were anesthetized with pentobarbital (50 mg/kg i.p.), positioned in a Koph Model 900 Stereotaxic frame, and implanted with a guide cannula into the left lateral ventricle under sterile conditions using a MEDIBIO Optical Brain Tracer (Muromachi Kikai) (Ikeda and Matsushita, 1980). The coordinates of the implants were; 6.1 mm midline, and −3.4 mm (guided by MEDIBIO) ventral to the skull surface, with the incisor bar set 3.3 mm below the interauricular line. Rats were then housed singly under the same conditions as above for a recovery period of at least 7 days, and body weights were monitored daily for the duration of the study. After recovery from surgery, rats were transferred to grid-floor cages and fed with powdered chow so that food intake measurements could be made. The rats were acclimated to the new environment at least for 1 day. The position of the cannula was verified by central administration of NPY (3 nmol in sterile water); for a positive test, at least 8 g of food was eaten over a 4 h period post-injection. Only positive testing animals (n = 8–10) were used. The studies were conducted according to a multidose, crossover design, with the order of dosing determined using the Latin square principle, leaving at least one rest day between administrations. All doses were delivered in a volume of 5 μl in sterile water over 30 s and the injector remained in position for a further 30 s to complete dispersal of the peptide. All intracerebroventricular administrations began at 2 h into light cycle, and food intake measured in 4 h. All peptides were dissolved in sterile water, initially at 6 mM, and diluted in water as needed. Water alone was used for the vehicle control.

Table 4. Relative potencies of synthetic NMU-23 and its two analogues on reducing food intake in rats

Peptide	Relative potency (molar basis)
NMU-23	1.00
[Phe(4F)^{16,17,19}]NMU-23	1.63
[Tyr(Me)^{1,6}]NMU-23	0.00

Results and discussion

The purpose of the present study was to examine the reducing effect of our synthetic two peptides, [Phe(4F)^{16,17,19}]NMU-23 and [Tyr(Me)^{1,6}]NMU-23, on the food intake activity in rats and compare the relative activity between the synthetic two analogues and NMU-23, since in our preceding papers (Abiko and Nakatsubo, 2001; Abiko and Fujimura, 2001), we reported that not only the synthetic [Phe(4F)^{97}]CART55–102 but also [Phe(4F)^{33}]CCK26–33 exhibited stronger reducing effect on food intake in rats than those of the synthetic parent peptides.

These results seem to suggest that changing electronic density of aromatic rings on the Phe residues of parent peptides influences biological activity. So, we decided to synthesize two analogues, [Phe(4F)^{16,17,19}]NMU-23 and [Tyr(Me)^{1,6}]NMU-23, by Fmoc-based solid-phase strategy (: fluorination at paraposition of an aromatic ring shows electron-withdrawing effect and CH_3O group at para-position of an aromatic ring shows electron-donating effect) and examine the reducing effect on food intake in rats whether changing electronic density on aromatic rings in NMU-23 influences biological activity or not ? The in vivo effect

of the synthetic analogues, [Phe(4F)^{16,17,19}]NMU-23, [Tyr(Me)^{1,6}]NMU-23 on food intake in rats is shown in Table 3. The synthetic NMU-23 exhibited reducing effect on food intake in rats at the concentration of 3 nmol/μg of NMU-23. The synthetic analogue in which Phe^{16,17,19} residues were replaced by Phe(4F) residues exhibited more potent reducing activity than that of NMU-23. On the contrary, the other synthetic analogue in which Tyr^{1,6} residues were replaced by Tyr(Me) residues exhibited no reducing activity.

These results seem to suggest that not only Phe residues at positions of 16, 17 and 19 of NMU-23 but also Tyr residues at positions of 1 and 6 of NMU-23 are key residues for regulation of food intake. Electronic density of these aromatic residues should be considered when we construct new NMU-23 analogues which have more food regulating activity.

Acknowledgements

We are grateful to Dr. Hiroshi Sekino of Kojinkai Central Hospital for giving us opportunities to do some experiments at Kidney Research Laboratory in Kojinkai Central Hospital in Sendai.

References

Abiko T (1974) Synthese of [Phe($4NO_2$)^5, Tyr(Me)^8]- and [Tyr(Me)^5, Phe(NO_2)^8]-bradykinin. Chem Pharm Bull 22: 2191–2196

Abiko T, Fujimura R (2001) Synthesis and biological effect of an anorectic peptide CART (cocaine- and amphetamine-regulated transcript) (human 55–102) analogue. Protein and Peptide Letters 8: 147–151

Abiko T, Nakatsubo S (2001) Syntheses of cholecystokinin (CCK) fragment analogues (26–33) and comparative effect on decreased feeding in rats by CCK fragment 26–33. Protein and Peptide Letters 8: 153–158

Colon JM, Domin J, Thim L, DiMarzo V, Morris HR, Bloom SR (1988) Primary structure of nueromedin U from the rat. J Neurochem 51: 988–991

Howard AD, Wang R, Pong SS, Mellin TN, Strack A, et al (2000) Identification of receptors for neuromedin U and its role in feeding. Nature 406: 70–74

Minamino N, Kangawa K, Honzawa M, Matsuo H (1988) Isolation and structure determination of rat neuromedin U. Biochem Biophys Res Commun 156: 355–360

Authors' address: Takashi Abiko, Ph. D., Research Laboratory, Global Shinwa Pharmaceutical Company, 4-17-29, Komatsushima, Aoba-ku, Sendai, 981-0905 Japan, E-mail: abiko@ma.mni.ne.jp