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Naltriben Analogues as Peptide Anticancer Drugs

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Abstract Apoptosis inducers for cancer therapy have been studied. Among hundreds of inducers, peptide anticancer drugs have many advantages such as being not harmful to humans, high selectivity, and dependence on their structures. Naltriben (NTB) is an octapeptide consisting of DPhe-Cys-Tyr-DTrp-Orn-Thr-Pen-Thr-NH_2. Several NTB analogues are known. In this experiment, apoptotic activities of NTB analogues with 8 amino acids were tested using flow cytometry. The conformational study of NTB was carried out using NMR spectroscopy and molecular modeling. Here, the relationships between conformations of NTB analogues and their apoptotic effects are reported.

Key words: Peptide, anticancer, drug, NTB, naltriben, apoptosis

Most anticancer drugs based on apoptosis do not induce apoptosis directly but rather induce apoptosis indirectly by cell proliferation or cell survival signals [3, 9]. Inhibition of cell proliferation and survival signals result in apoptosis. Several cancer drugs that have effect by the proliferative or survival pathways have been developed, which include inhibitors of cell cycle regulation kinases and monoclonal antibodies [1]. Instead of small molecules, recently, peptide anticancer drugs (PAD) have been introduced. Among their several advantages, such as being not harmful to humans and high selectivity, the structure dependence of their activity can rise to introduce structure-based drug design easily. We tested the apoptotic activities of four naltriben (NTB) analogues and tried to find the relationships between conformations of NTB analogues and their activities.

NTB is an octapeptide consisting of DPhe-Cys-Tyr-DTrp-Orn-Thr-Pen-Thr-NH_2 [4], and differentiates subtypes of δ opioid receptors. Here, Orn and Pen denote ornithine and pennicillamine, respectively. Several NTB analogues are known. In this experiment, NTB analogues composed of 8 amino acids, μ opioid receptor antagonist CTAP (DPhe-Cys-Tyr-DTrp-Arg-Thr-Pen-Thr-NH_2) [2], growth hormone release inhibitor RC-160 (DPhe-Cys-Tyr-DTrp-Lys-Val-Cys-Trp-NH_2) [10], and μ opioid receptor antagonist, Octreotide (DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr-ol) were studied [7].

For analysis of their apoptotic effects, the human cervical adenocarcinoma cell line, HeLa, was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured in MEM containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cell density in culture did not exceed 1×10^6 cells/ml. HeLa cells ($1-2\times10^6$ cells /ml) were incubated in MEM medium with 5% FBS for 48 h after treatment with 20 μM, 20 μM, 0.01 μM, and 0.01 μM of NTB, CTAP, RC-160, and Octreotide, respectively. The cells were then washed twice with ice-cold PBS, harvested, fixed with ice-cold PBS in 70% ethanol, and stored at 4°C. For flow cytometric analysis, cells were incubated with 0.1 mg/ml RNase A at 37°C for 30 min, stained with 50 μg/ml propidium iodide for 30 min on ice, and then measured using a FASTAR flow cytometer (Becton Dickinson, San Diego, U.S.A.) with Cell Quest software [5].

Since the conformations of three peptides, CTAP, RC-160, and Octreotide were determined already, in this experiment only conformational study of NTB was performed using NMR spectroscopy and molecular modeling. All NMR measurements were performed on a Bruker Avance 600 spectrometer system (14.1 T, Karlsruhe, Germany) with a cryoprobe at 298 K. The NMR spectra of ^{1}H-NMR, Correlated spectroscopy (COSY), Total correlated spectroscopy (TOCSY), Rotating frame Overhauser enhancement spectroscopy (ROESY), and Nuclear Overhauser

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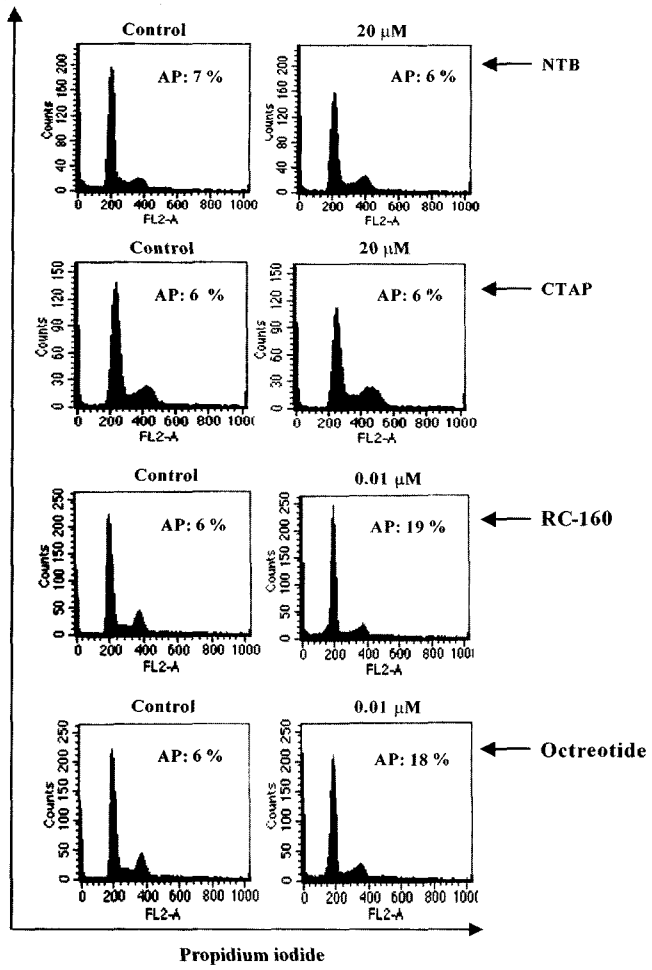

Fig. 1. Quantification of apoptosis by flow cytometry.
HeLa cells were treated with 20 μM, 20 μM, 0.01 μM, and 0.01 μM of NTB, CTAP, RC-160, and Octreotide, respectively. Cells were then stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry using Cell Quest software. A total of 10,000 nuclei were analyzed from each sample.

and exchange spectroscopy (NOESY) were collected in 90%H_2O/10%D_2O [6, 8]. The concentration of the samples was approximately 1 mM. For ^{1}H-NMR analysis, 128 transients were acquired with a 1 sec relaxation delay using 32 K data points. The 90° pulse was 9.7 μsec with a spectral width of 4,000 Hz. Two-dimensional spectra were acquired with 2,048 data points for t2 and 256 for t1 using time proportional phase increments except COSY experiments where magnitude mode was applied. NOESY experiments of allatostains were performed at the various mixing times between 50 msec and 1,500 msec. The mixing time for TOCSY with MLEV17 spin-lock pulse program was 80 msec. The mixing time for ROESY was 200 msec. In all experiments, water peaks were suppressed by presaturation. Prior to Fourier transformation, zero filling of 2 K and sine-squared bell window function were applied using XWIN-NMR (Bruker, Karlsruhe, Germany). Data analysis was carried out using Sparky. The molecular modeling calculations were carried out according to the method previously published [11].

As shown in Fig. 1, the flow cytometric analysis revealed an appreciable arrest of the cells in the cell cycle after treatment with 20 μM, 20 μM, 0.01 μM, and 0.01 μM of NTB, CTAP, RC-160, and Octreotide, respectively. While the cases of NTB and CTAP did not show any increment of the HeLa cell population, the cases of RC-160 and Octreotide showed an increment at a much lower concentration.

The NMR data of NTB obtained in 90%H_2O/10%D_2O was assigned based on the standard procedures. Even though all of the backbone amide cross peaks were not observed in COSY and TOCSY, the chemical shifts of the residues could be assigned from the interpretation of the cross peaks between amide protons and alpha protons. Assignments of NMR data of NTB are listed in Table 1. NTB did not show meaningful NOE peaks. Therefore, the ROESY experiment was performed and several meaningful ROE cross peaks were observed (Table 2). Based on RoE cross peaks observed in NTB, constraints were given on the primary sequence of NTB using the InsightII/Discover program (Accelrys, San Diego, U.S.A.). Simulated annealing was applied to obtain the refined structure of NTB using InsightII software. The best 20 conformers were collected

Table 1. Assignments of NMR data of NTB.

	Chemical shifts of ^{1}H/ppm			
Amino acid	NH	CαH	CβH	CγH and Others
dPhe[1]	-	3.925	3.00	C_δH 7.184; C_εH 7.28; C_ζH 7.235
Cys[2]	8.353	4.527	2.771	-
Tyr[3]	7.736	4.567	2.786	C_δH 6.917; C_εH 6.631
dTrp[4]	8.609	4.30	2.977, 2.849	C_δH 7.073; C_εH 7.485, 10.070 C_ζH 7.079; 7.388 C_ηH 7.145
Orn[5]	8.208	3.856	1.10, 1.523	0.5625, 0.728; C_δH 2.52
Thr[6]	8.063	4.232	4.137	1.033
Pen[7]	8.208	3.856	-	1.19, 1.267
Thr[8]	8.316	4.251	4.137	1.122
NH_2	7.55, 7.079	-	-	-

Table 2. ROE cross peaks observed in the ROESY spectrum of NTB.

DPhe^1:C_δH	Pen^7:C_γH
DPhe^1:C_εH	Pen^7:C_γH
Cys^2:C_βH	Tyr^3:C_δH
Tyr^3:NH	Pen^7:C_γH
DTrp^4:C_αH	Tyr^3:C_δH
DTrp^4:C_αH	DPhe^1:C_δH
DTrp^4:C_ηH	Orn^5:C_δH
DTrp^4:C_ηH	Orn^5:C_δH
Orn^5:C_γH	DTrp^4:C_δH
Orn^5:C_γH	DTrp^4:C_ζH
Orn^5:C_αH	DTrp^4:C_ζH
Thr^6:C_γH	Tyr^3:C_δH
Thr^6:C_αH	Tyr^3:NH
Thr^8:C_γH	Tyr^3:C_εH
Thr^8:C_βH	NH
Thr^8:C_αH	NH

and superimposed as shown in Fig. 2. The root mean-square deviation (RMSD) of 20 conformers was 0.60 Å. The conformer with the lowest total energy was validated using PROCHECK. The statistical analysis of Ramachandran plot showed that 33.3% of the residues are in the most favored regions, 50.0% in additional allowed regions, 16.7% in generously allowed regions, and 0% in disallowed regions.

As shown in Fig. 1, while NTB and CTAP do not show an apoptotic activity, RC-160 and Octreotide have an activity. RC-160 and Octreotide have a disulfide bond so that they are cyclic octapeptides. The distinguishable structural

Fig. 3. Superimposed image of NTB (thick line) and RC-160 (thin line).

difference is a cyclization. Even though NTB and CTAP do not have flexible structures, they are different to those of RC-160 and Octreotide. When NTB and RC-160 are superimposed, their RMSD value is 3.21 Å. As shown in Fig. 3, in the case of RC-160, the distance between Cys^2

Fig. 2. Superimposed image of the best 20 refined structures of NTB collected from simulated annealing results.

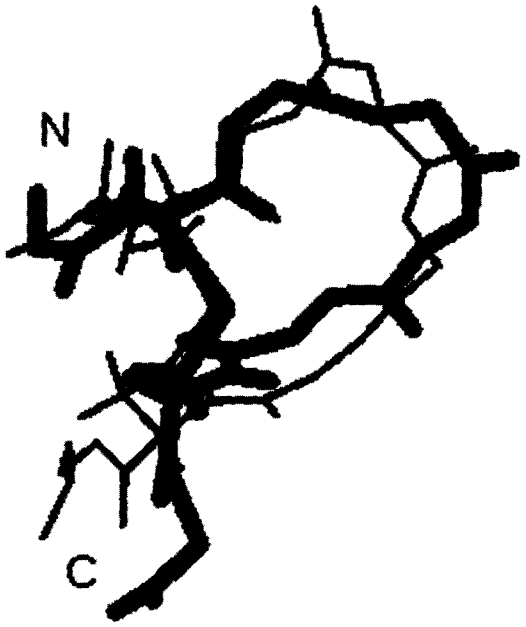

Fig. 4. Ssuperimposed image of Octreotide (thick line) and RC-160 (thin line).

and Val^6 is 1.97 Å, and in NTB the distance between Cys^2 and Thr^6 is 6.79 Å. The cyclization by a disulfide bond makes a big difference between NTB and RC-160. While the C-terminal of RC-160 is amidated, that of Octreotide is hydroxylated. However, their apoptotic activities do not show any difference. Though three residues of RC-160 and Octreotide are not the same, their superposition gives the RMSD value of 1.97 Å (Fig. 4) which is smaller than that of the case shown in Fig. 3. As a result, it can be considered that the apoptotic activity of NTB analogues depends on a cyclization of peptides instead of their residues.

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