Design, Syntheses, and Conformational Study of Angiogenesis Inhibitors

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Since anti-angiogenesis could lead to the suppression of tumor growth, angiogenesis inhibitors have received particular attention for their therapeutic potential. In this study, two angiogenic inhibitors using the bioactive sequence from the kringle 5, AK1(KLYDY), AK2(KLWDF) were designed and synthesized. We have investigated their solution structures using NMR spectroscopy and their activities as angiogenesis inhibitors. AK2 has an intramolecular hydrogen bon d between the side chain amino proton of Lys1 and the carboxyl oxygen of Asp4 with a N···O distance of 3.27 Å, while AK1 shows more flexible structures than AK2. Indole ring in Trp is much bigger than the phenyl ring in Tyr and may have good face-to-edge interaction enforcing more rigid and constrained conformational features of AK2. Because of this relatively stable structure, Trp3 in AK2 may have better hydrophobic interaction with Phe5 than Tyr3 in AK1 if two adjacent aromatic groups are located in hydrophobic pocket of receptor. Since AK2 shows the similar anti-angiogenic activities to AK1, we are also able to confirm that the activity of AK1 is irrelevant to the Tyr phosphorylation. More rigid drug with higher activities can be provided by the mimetic approaches. For the further development of the angiogenesis inhibitors, these conformational studies on our lead peptides will be helpful in design of peptidomimetics.

Keywords : Angiogenesis inhibitor, Peptide, NMR spectroscopy, CAM assay, Peptidomimetics.

Introduction

Angiogenesis is a complex process of new blood vessel formation, which is essential for cell reproduction, development, and wound repair under normal condition. This process involves endothelial cell proliferation, migration, and membrane degradation. Since Folkman suggested angiogenesis is important for the growth and metastasis of tumors, many studies have been focused on the understanding of angiogenesis.[1,2] Now one of the main strategy of cancer treatment is developing molecules of anti-angiogenic activity.[3-6]

Angiostatin,[7,8] a proteolytic fragment of human plasminogen, is known to have profound inhibitory activity of angiogenesis by blocking the proliferation and migration of endothelial cell. This protein consists of homologous four kringle domains (kringle 1-kringle 4), each containing about 80 amino acids with conserved three disulfide linkages. Kringle 5 domain of plasminogen, which shares high sequence homology with other four kringles of angiostatin, was shown to antagonize endothelial cell growth and known to have better anti-proliferative activity than angiostatin.[9,10] From the sequence homology of kringle 1 through kringle 5, KRYDY in kringle 1 is similar to KLYDY in kringle 5. From the fact that among angiostatin fragments kringle 1 domain has the best anti-proliferating activity, KLYDY peptide in kringle 5 may play an important role on the activity of kringle 5. According to the crystal structure of kringle 5,[11] it has been reported that KLYDY (the region from residue 70 to residue 74 in kringle 5) is involved in lysine binding although the lys binding capability is not responsible for its bioactivity.[12] Even though Abbott has patented KL(R)YD peptides as anti-angiogenesis agents,[13] there has been no report regarding conformational study of this peptide.

Peptides are extremely important molecules in many biochemical functions. Thesedays, there is increasing research activity on the use of biologically active peptides as drugs or lead compounds for the development of peptidomimetic drugs. NMR-derived structures in the drug design process using peptides can help to design new pharmaceutical agents. In this study, two angiogenic inhibitors using the bioactive sequence from the kringle 5, AK1(KLYDY), AK2(KLWDF) were designed and synthesized. We have investigated their solution structures using NMR spectroscopy and their activities as angiogenesis inhibitors, and have tried to understand the relationships between the structures and their activities.

Experimental Section

Peptide synthesis. Peptide syntheses were performed on a manual synthesizer equiped with Glas-Col WS180 shaker. Peptide sequence elongation was accomplished using stepwise solid-phase synthesis. All couplings were carried out in DMF using 3-fold excess (based on resin loading) of protected amino acids, BOP, HOBt, 4-methylmorpholine (or DIPEA). Each coupling was monitored using the Kaiser test for free amine. N^{α}-Fmoc protection was removed 20%(v/v) piperidine in DMF (2×5 min). The peptide-resin was washed very throughly with DMF after each coupling and deprotection step. Peptides were removed from the resin

with simultaneous side-chain deprotection using TFA containing 2.5%(v/v) water and 2.5%(v/v) TIS for 2×2 hr at room temperature, then washed with 50%(v/v) TFA, 50%(v/v) DCM (2×30 min). Crude peptides were precipitated with cold ether and washed throughly with ether, then separated with prep-RP-HPLC, and lyophilized Separation was performed using a linear gradient of water (0.1%(v/v) TFA) and MeCN (0.1%(v/v) TFA) at a flow rate 3 mL/min. Mass spectra were obtained on a doble focusing mass spectrometer (JEOL, JMS-AX505WA) using FAB ionization.

Chorioallantoic membrane (CAM) assay. The fertilized chicken eggs were kept in a humidified incubator at 37 °C. After 3 days incubation, about 2 mL of albumin was aspirated from the eggs, and the shell covering the air sac was punched out and removed by forceps. 25 ug of AK1 and AK2 were applied to sterile Thermanox discs separately and allowed to dry under laminar flow conditions. The loaded discs were inverted and applied to the CAM surface of 5-day-old embryos through the windows. PBS (Phosphate buffered saline) was used as a control. The air sac ends of the embryo with shell were sealed with tape. Two days later, an appropriate volume of a 10% fat emulsion was injected into the 7-day embryo chorioallantois. The CAM was examined under a microscope and photographed. The anti-angiogenic effect was expressed as the percentage of the number of eggs which showed an avascular zone of the CAM under the disc.[14,15] At least twenty eggs were used for each dose of agent.

NMR experiments. In order to investigate the conformation of AK1 and AK2, 1mg of peptides was dissolved in 0.4 mL of DMSO-d_6 solvent for NMR experiment. All of the phase sensitive two-dimensional experiments, such as DQF-COSY,[16] TOCSY,[17] NOESY,[18] and ROESY[19] experiments were performed using TPPI method, *i.e.*, time-proportional phase incrementation.[20] For these experiments, 512 transients with 2 K complex data points were collected for each increment, with a relaxation delay of 1.2 sec between successive transients, and the data along the t_1 dimension were zerofilled to 1 K before 2D-Fourier transformation. TOCSY experiments were performed using 20 msec and 50 msec MLEV-17 spin-lock mixing pulses. Mixing times of 250 and 400 msec were used for ROESY and NOESY experiments. The $^3J_{HN\alpha}$ coupling constants were measured from the DQF-COSY spectrum. In order to calculate temperature coefficients, chemical shifts were measured from 298 K to 328 K at every 5°. Spectra were recorded at 298 K on an Avance-400 spectrometer at Konkuk University. All NMR spectra were processed off-line using the FELIX software package on the SGI workstation in our laboratory (Molecular Simulations Inc., San Diego, CA, USA).

Structure calculation. Distance constraints were extracted from the NOESY spectra with mixing times of 250 msec. The volumes of the NOEs between the two beta protons of the Tyr and Trp residues were used as references. All other volumes were converted into the distances by assuming a simple $1/r^6$ distance dependence. All of the NOE intensities are divided into three classes (strong, medium, and weak) with the distance ranges of 1.8-2.7, 1.8-3.3, and 1.8-5.0 Å, respectively.[21,22] Structure calculations were carried out using X-PLOR version 3.851 with the topology and parameter sets topallhdg and parallhdg, respectively.[23] Standard pseudo-atom corrections were applied to the non-stereospecifically assigned restraints, and an additional 0.5 Å was added to the upper bounds for the NOEs involving methyl protons.[24,25] A hybrid distance geometry-dynamical simulated annealing protocol was employed to generate the structures.[26,27] A total of 50 structures was generated, and the 20 structures with the lowest energies were selected for further analysis.

Results and Discussion

CAM assay. To determine the anti-angiogenic effects of AK1 and AK2, CAM assays were used. Each of AK1 and AK2 was applied to the CAMs of 5-day-old chicken embryos and the anti-angiogenic activity was evaluated by measuring the frequency of avascular zone which looked

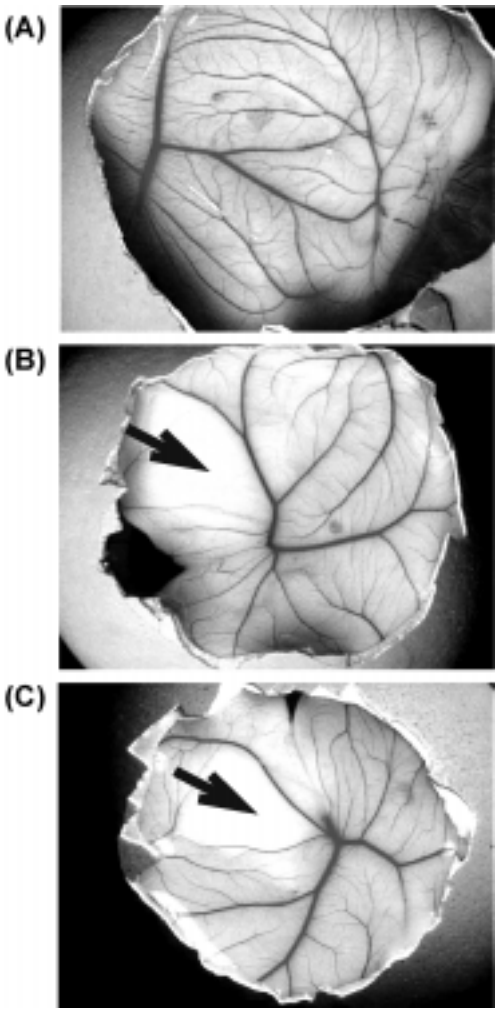

Figure 1. Anti-angiogenic activity of AK1 and AK2 in the chicken CAM assay. (a) Control CAM treated with PBS. CAMs implanted with thermanox discs loaded with (b) AK1 and (c) AK2. Arrows indicates the avascular zone due to inhibition of new vessel formation.

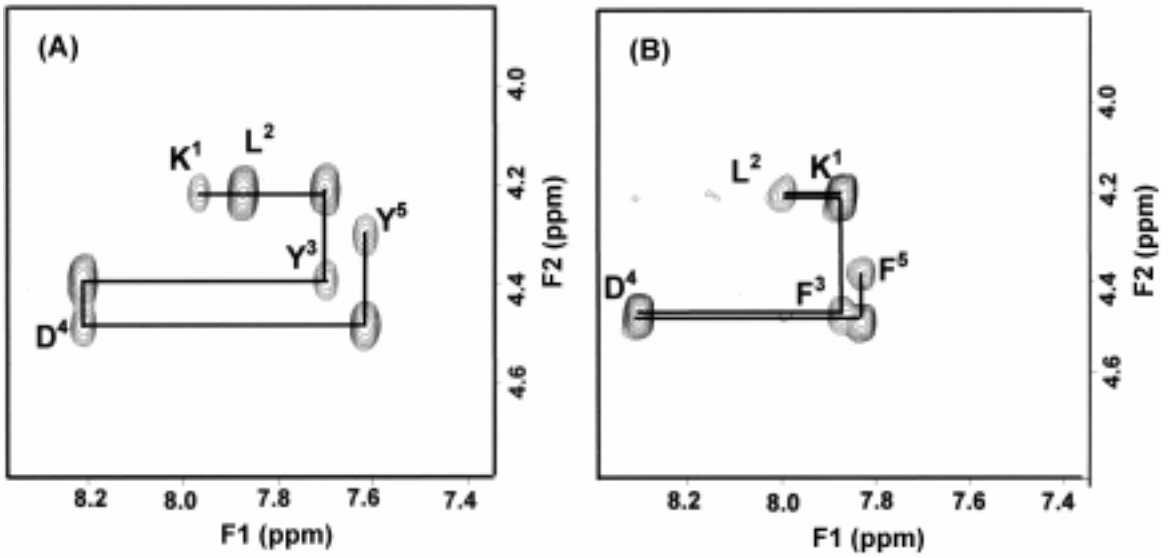

Figure 2. NH-CαH region of a 250 msec mixing time ROESY spectrum of (a) AK1 and (b) AK2. For the sake of clarity, only the intraresidual NH-CαH cross peaks are labeled.

white 2 days after implantation of sample. Control CAM treated with PBS in Figure 1A showed no disturbance of angiogenesis. CAMs implanted with thermanox discs loaded with AK1 and AK2 showed white avascular zones indicated by arrows due to inhibition of new vessel formation as shown in Figure 1B and 1C, respectively. The percentage of angiogenesis inhibition of AK1 and AK2 were 89% and 88%, respectively, whereas control PBS had no inhibitory effect. Therefore, AK1 and AK2 showed similar anti-angiogenic activity in the chicken CAM.

Resonance assignments and the temperature coefficients. Sequence specific resonance assignments were performed using mainly the DQF-COSY, TOCSY, and ROESY data.[28] Figure 2 shows the ROESY spectra with the sequential assignments of AK1 and AK2 in the NH-C$_\alpha$H region. ROESY and TOCSY experiments at 298 K allowed to complete the assignment. Chemical shifts of AK1 and AK2 in DMSO at 298 K, referenced to TMS, are listed in Table 1. The sequential NOE connectivities are illustrated in Figure 3. Since these peptides are short, there are not many NOE connectivities. In case of AK1, there are connectivities between Lys1 and Tyr3. In case of AK2, there are connectivities between Lys1 and Trp3 and connectivities between Trp3 and Tyr5. The observed values of the $^3J_{HN\alpha}$ coupling constants for these peptides are generally about 8 Hz.

Table 1 also lists the temperature coefficients of the backbone amide protons and side chain amino protons of Lys1. A reduction in temperature susceptibility (ppb/deg)

Table 1. Chemical shifts(ppm) referenced to TMS, coupling constants, $^3J_{HN\alpha}$ (Hz) and temperature coefficients[a] κ (-10^{-3} ppm/deg) of AK1 and AK2 in DMSO-d_6

Residue	Chemical shift of AK1				$^3J_{HN\alpha}$	Temperature coefficient[a]
	NH	αH	βH	others		
Lys[1]	8.03	4.22	1.60, 1.61	γ: 1.47 δ: 1.57 ε_1 : 2.76 ε_2 : 7.66	7.4	8.5 (NH_3) 2.0
Leu[2]	7.92	4.22	1.49, 1.50	γ: 1.37 δ: 0.82	7.4	8.5
Tyr[3]	7.77	4.38	2.68, 2.87	2,6H : 7.00 3,5H : 6.63	7.4	7.0
Asp[4]	8.30	4.48	2.47, 2.66		7.5	15.0
Tyr[5]	7.67	4.38	2.74, 2.88	2,6H : 6.97 3,5H : 6.61	7.4	8.5
Residue	Chemical shift of AK2				$^3J_{HN\alpha}$	Temperature coefficient[a]
	NH	αH	βH	others		
Lys[1]	7.99	4.20	1.58	γ: 1.27, δ: 1.47 ε_1 : 2.72, ε_2 : 7.62	7.5	8.5 (NH_3) 1.5
Leu[2]	7.89	4.23	1.37, 1.50	γ: 1.37, δ: 0.772, 0.82	8.6	8.0
Trp[3]	7.88	4.48	2.94, 3.05	2H : 7.04, 4H : 7.57, 5H : 6.97, 6H : 7.11, 7H : 7.29	7.6	8.0
Asp[4]	8.24	4.49	2.41, 2.62		8.6	11.0
Phe[5]	7.77	4.37	2.84, 3.01	2,6H : 7.16, 3,5H : 7.29, 4H :7 .22	7.5	9.0

[a]Calculated slope from the linear least squares analysis of seven data points from 298 K to 328 K.

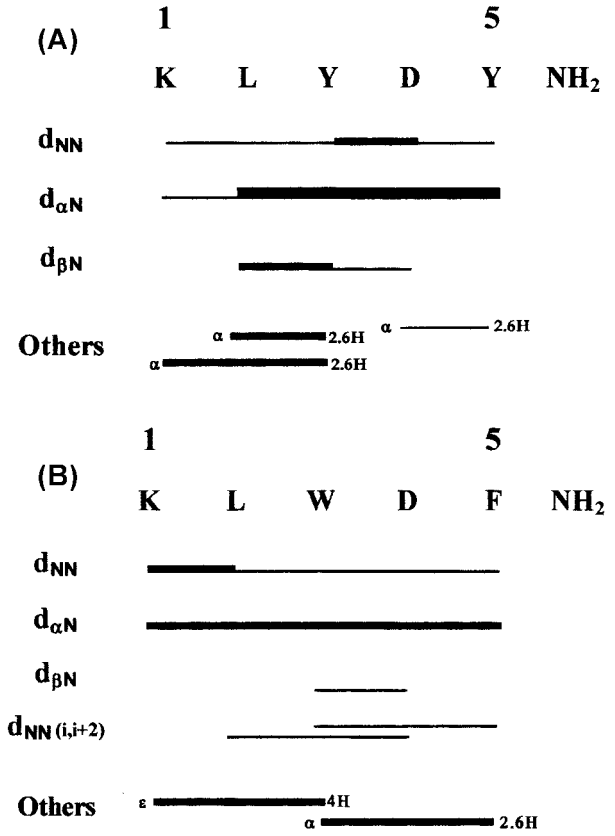

Figure 3. Summary of the NOE connectivities for (A) AK1 and (B) AK2. The line thickness for the NOEs reflects the intensity of the NOE connectivities.

has been commonly accepted as an indicator of reduced interaction with solvent, due to the intramolecular hydrogen bonding.[29,30] Very small temperature coefficients were observed for the amino protons in the side chain of Lys1 in both AK1 and AK2, compared to those of the backbone amide protons. Specially, chemical shift of amino protons in the side chain of Lys1 in AK2 retained in the almost same positions with the change of temperature and this results in a very small temperature coefficient of 1.5 ppb/deg. This should correspond to a transfer of electron density from the protons in the amino group in Lys1 as a result of hydrogen bonding with the other atoms as electron accepters.

Structure-activity relationships. Figure 4A shows the ribbon diagram of the crystal structure of kringle 5 showing the region from residue 70 to residue 74 (KLYDY). Figure 4B and 4C show the ribbon diagrams of the lowest energy structures of AK1 and AK2 with all heavy atoms, respectively. As expected from the NOE connectivities, AK2 has a simple turn structure. According to the analysis using PROCHECK, this turn structure is characterized as type IV β-turn. As shown in Figure 4A, in the crystal structure of kringle 5, the active sequence K70-L71-Y72-D73-Y74 has an intramolecular hydrogen bond between the side chain amino protons of Lys70 and the carboxyl oxygen of Asp73 and it results in a stable structure. Distance between the carboxyl oxygen in Asp73 and the side chain amino nitrogen

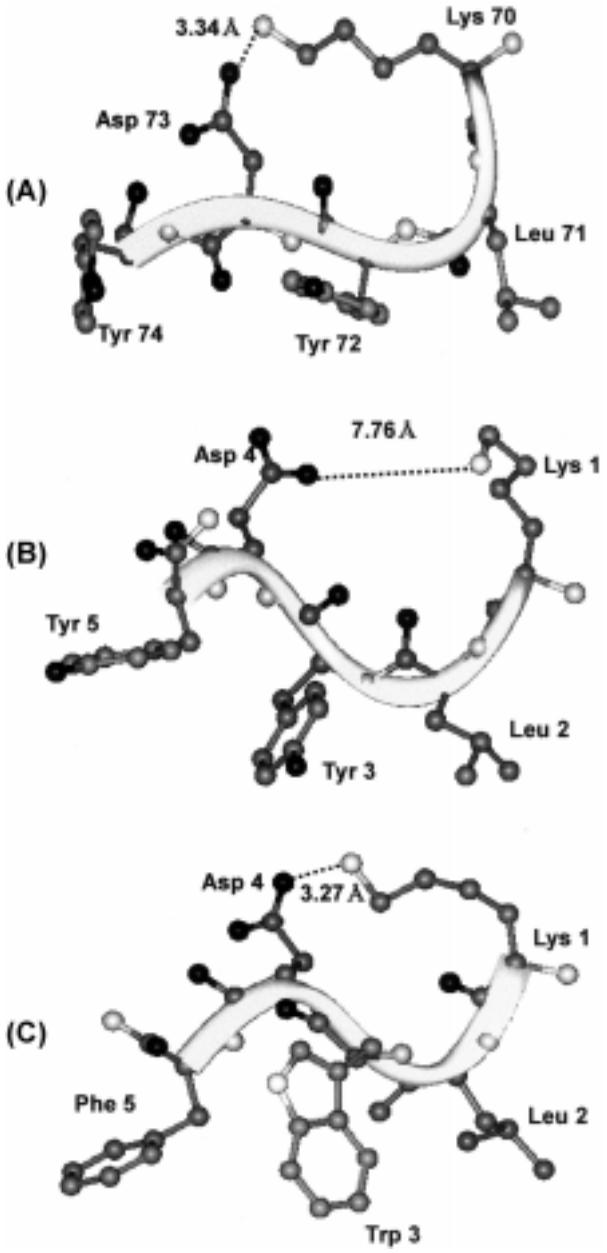

Figure 4. (A) Ribbon diagram of the crystal structure of kringle 5 showing the region from residue 70 to residue 74 (KLYDY).[11] Figure 4B and 4C shows the ribbon diagrams of the lowest energy structures of AK1 and AK2 with all heavy atoms, respectively. Carbon atoms are dictated with gray and oxygen atoms are dictated with black, and nitrogen atoms are dictated with light gray.

in Lys70 is 3.34 Å in the crystal structure. Even though AK1 has the same amino acid sequence as that of active fragment of kringle 5, AK1 has more extended solution structure than the crystal structure of kringle 5 as shown in Figure 4B. AK2 also has an intramolecular hydrogen bond between the side chain amino proton of Lys1 and the carboxyl oxygen of Asp4 with the N···O distance of 3.27 Å as shown in Figure 4C. This agrees well with the result of temperature coefficient data. Unfortunately, no NOEs were found between the amino protons in the side chains of Lys1 and the other protons in AK2.

Peptide with five residues can not adopt single predominant conformation in solution. AK2 has two sets of structures while AK1 shows more diverse structures in solution than AK2. AK2 has two sets of structures, one with a turn structure which has an intramolecular hydrogen bond and the other with a extended structure. AK2 has more stable solution structure than AK1 because of an intramolecular hydrogen bond. Indole ring in Trp is much bigger than the phenyl ring in Tyr and may have better face-to-edge interaction enforcing more rigid and constrained conformational features of AK2. Therefore, Trp3 in AK2 may have better hydrophobic interactions with Phe5 than Tyr3 in AK1 if two adjacent aromatic groups are located in hydrophobic pocket of receptor.

Conclusion

Angiogenesis inhibitors have received particular attention because of their therapeutic potential. In this study, two angiogenic inhibitors, AK1 (KLYDY) and AK2 (KLWDF) were designed based on the bioactive sequence from the kringle 5 and synthesized. These peptides showed similar angiogenic inhibition activities. The solution structures determined by NMR spectroscopy show that AK2 (KYWDF) has an intramolecular hydrogen bond and has relatively stable structures in solution compared to AK1. Indole ring in Trp may provide better face-to-edge interaction enforcing more rigid and constrained conformational feature in AK2 than Tyr in AK1. However, AK2 also has sets of structures in solution. The mechanism of action of angiogenesis inhibitors on endothelial cells and their receptors is so far unknown. Conformationally constrained structure can offer a chance to overcome the problems of flexibilities of peptides, and can be helpful to elucidate the relationship between the structures of peptides and biological activities. In order to provide the higher activities and better match with the receptor bound conformations, a peptide should have more rigid structure. More rigid drugs with higher activities can be provided by the mimetic approaches. For the further development of the angiogenesis inhibitors, these conformational studies on our lead peptides will be helpful in design of peptidomimetics.

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