Angiogenesis Inhibitor Derived from Angiostatin Active Sites

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Angiogenesis is essential for the growth and persistence of solid tumors. Their metastases, anti-angiogenesis could lead to the suppression of tumor growth. One of the main strategies of cancer treatment is developing molecules of anti-angiogenic activity. In this study, two angiogenic inhibitors, Ang3 (KLFDF) and Ang4 (XLFDF) derived from KLYDY, which is the sequence of angiostatin active sites kringle 5, were designed and synthesized. Previously we reported the activities and structures of two inhibitors, Ang1 (KLYDY) and Ang2 (KLWDF). In order to investigate the effect of Phe substitution, Ang3 was designed with a sequence of KLFDF. In order to reduce conformational flexibility of side chain in Lys, Ang4 was designed with a sequence of XLFDF, where X has amino substituted phenyl ring. Solution structures of those inhibitors were investigated using NMR spectroscopy and their activities as angiogenesis inhibitors were studied. Ang1 and Ang2 show angiogenic activities, while Ang3 and Ang4 have no activities and have extended structures compared to Ang1 and Ang2. Therefore, Phe rings do not have effective hydrophobic interactions with other aromatic residues in Ang3 and Ang4. The representative structure of Ang2 has a stable intramolecular hydrogen bond. Therefore, intramolecular hydrogen bonding might be more important in stabilizing the structure than the hydrophobic interactions in these inhibitors. More rigid structure, which can be expected to have higher activities and better match with the receptor bound conformations, can be obtained with a constrained cyclic structure. Further peptidomimetic approaches should be tried to develop angiogenesis inhibitors.

Key Words : Angiogenesis inhibitor, Kringle, CAM assay, Peptidomimetics, Structure

Introduction

The formation of new blood vessels is crucial for tissue regeneration and for efficient tumor growth, and requires the concerted action of various angiogenic factors and inhibitors. 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 angiogenesis is important for the growth and metastasis of tumors, many studies are being focused on the understanding of angiogenesis and inhibitors have received particular attention because of their therapeutic potential.[1,2] Now one of the main strategies of cancer treatment is developing molecules with anti-angiogenic activity.[3-6]

Angiostatin, a proteolytic fragment of human plasminogen, is known to have profound inhibitory activity of angiogenesis by blocking the proliferation and migration of endothelial cell.[7,8] 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] It has been reported that KLYDY peptide in kringle 5 may play an important role on the activity of kringle 5.[11] We have reported the activities and the structures of two angiogenic inhibitors, Ang1 (KLYDY) and Ang2 (KLWDF).[12] NMR-derived structures in the drug design process using peptides can help to design new pharmaceutical agents. In this study, two angiogenic inhibitors derived from angiostatin active sites, kringle 5, Ang3 and Ang4 were designed as listed in Table 1 and synthesized. We have investigated their solution structures using NMR spectroscopy and their activities as angiogenesis inhibitors, and tried to understand the relationships between the structures and their activities.

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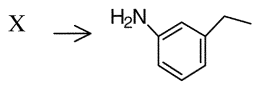
Table 1. Amino acid sequences of Ang1, Ang2, Ang3, and Ang4

Peptide	Sequence
Ang1	KLYDY-NH_2
Ang2	KLWDF-NH_2
Ang3	KLFDF-NH_2
Ang4	XLFDF-NH_2 

Materials and Method

Peptide Synthesis. Peptide syntheses were performed on a manual synthesizer equipped with Glas-Col WS180 shaker. Peptide sequence elongation was accomplished using stepwise solid-phase synthesis as reported previously.[12] Crude peptides were precipitated with cold ether and washed thoroughly 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 double 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 μg of Ang3, and Ang4 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.[13,14] At least twenty eggs were used for each dose of agent.

NMR Experiments and Structure Calculation. 1 mg of peptide was dissolved in 0.45 mL of DMSO-d_6 solvent for NMR experiment. All of the phase sensitive two-dimensional experiments, such as DQF-COSY, TOCSY, NOESY, and ROESY experiments were performed using TPPI method, i.e., time-proportional phase incrementation.[15-19] 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 study the intramolecular hydrogen bonding, temperature coefficients were calculated using chemical shifts measured from 298 K to 328 K at every 5°. Spectra were recorded on a Bruker Avance-400 spectrometer at Konkuk University and at 500 MHz Varian instrument at KBSI. 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).

NOESY spectra with mixing times of 250 msec were used to get distance constraints. The volumes of the NOEs between the two beta protons of the Tyr or Phe residues were used as references. 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.[20,21] Nonnatural amino acid X in Ang4 was built using the InsightII and Discover program (Molecular Simulations Inc., San Diego, CA, USA). Structure calculations were carried out using X-PLOR version 3.851 with the topology and parameter sets topallhdg and parallhdg, respectively.[22] A hybrid distance geometry-dynamical simulated annealing protocol was employed to generate the structures.[23-26] A total of 50 structures were generated, and the 20 structures with the lowest energies were selected for further analysis.

Results and Discussion

CAM Assay. CAM assays were used to determine the anti-angiogenic effects of Ang3 and Ang4. Each of peptides 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 looks white 2 days after implantation of sample. Control CAM treated with PBS in Figure 1A showed no disturbance of angiogenesis. For comparison, CAMs implanted with thermanox discs loaded with Ang3 and Ang4 are shown in Figure 1B and 1C. We have reported that Ang1 and Ang2 showed white avascular zones due to inhibition of new vessel formation. However, Ang3 and Ang4 did not show any disturbance of angiogenesis at all as shown in Figure 1B and 1C.

Resonance Assignments and the Temperature Coefficients. Sequence specific resonance assignments were performed using mainly the DQF-COSY, TOCSY, and ROESY data.[27] Chemical shifts of Ang3 and Ang4 in DMSO at 298 K, referenced to TMS, are listed in Table 2.

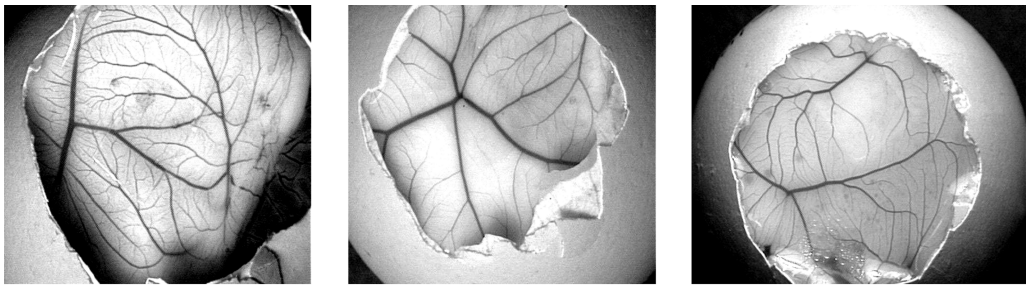


Figure 1. Anti-angiogenic activity of (B) Ang3 and (C) Ang4 in the chicken CAM (Chorioallantoic membrane) assay. (A) Control CAM treated with PBS (Phosphate buffered saline).

Table 2. ^{1}H chemical shifts (ppm) for (A) Ang3 and (B) Ang4 in DMSO at 298 K

(A)

Residue	Chemical shift (ppm)[a]				$J_{HN\alpha H}$	Temperature coefficient
	NH	αH	βH	Others		
K[1]	7.99	4.20	1.56*	γ1.26; δ1.45; ε* 2.74; ε NH_3^+ 7.65	8	9.0 (NH3: 7.0)
L[2]	7.88	4.22	1.47*	γ 1.34; δ0.77, 0.83	8	8.5
F[3]	7.87	4.47	2.75, 2.94	2,6H 7.15; 3,5H 7.31; 4H 7.21	8	8.0
D[4]	8.31	4.49	2.48, 2.65		7	8.5
F[5]	7.83	4.38	2.85, 3.02	2,6H 7.17; 3,5H 7.27; 4H 7.21	8	10.5

(B)

Residue	Chemical shift (ppm)[a]				$J_{HN\alpha H}$	Temperature coefficient
	NH	αH	βH	Others		
X[1]			3.51, 3.57	2,6H 7.01; 3,5H 7.23		8.5
L[2]	8.21	4.18	1.41*	γ1.30; δ0.752, 0.677	8	8.5
F[3]	7.96	4.39	2.66, 2.96	2,6H 7.11; 3,5H 7.21; 4H 7.14	8	9.0
D[4]	8.20	4.40	2.25, 2.42		7	10.0
F[5]	7.79	4.31	2.77, 2.86	2,6H 7.09; 3,5H 7.21; 4H 7.14	8	8.5

[a]chemical shifts are relative to TMS (0 ppm)

Since these peptides are short, there are not many NOE connectivities except sequential ones. Table 2 lists the temperature coefficients of the backbone amide protons and side chain amino protons of Lys1. A reduction in temperature susceptibility (ppb/deg) has been commonly accepted as an indicator of reduced interaction with solvent, due to the intramolecular hydrogen bonding.[28,29] Chemical shift of amino protons in the side chain of Lys1 in Ang1 and Ang2 retained in the almost same positions with the change of temperature and this resulted in temperature coefficients smaller than 2.0 ppb/deg.[12] 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. However, this amino proton in Ang3 has a big temperature coefficient (7.0 ppb/deg) and these results imply that this amino proton is not involved in intramolecular hydrogen bondings. Furthermore, Ang4 does not have Lys1 because of substitution with X, Ang4 cannot form intramolecular hydrogen bonding.

Structure-Activity Relationships. 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

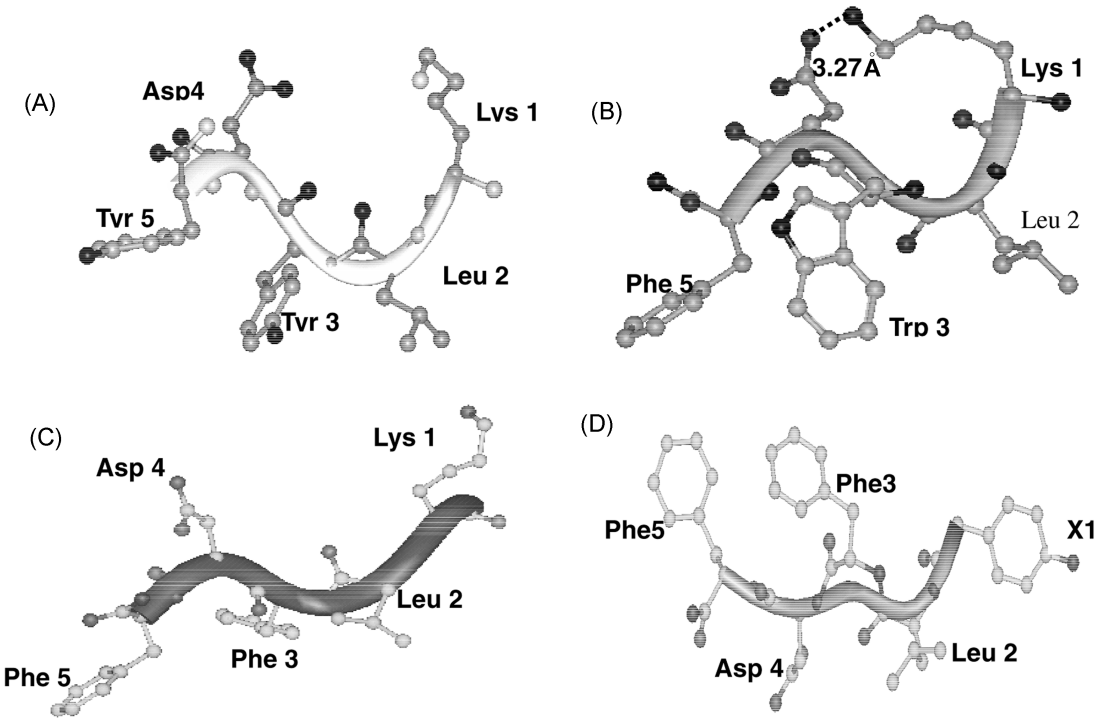


Figure 2. The lowest energy structures of (A) Ang1, (B) Ang2, (C) Ang3, and (D) Ang4 in DMSO.

binding capability is not responsible for its bioactivity.[11,30] Even though Abbott has patented KL(R)YD peptides as anti-angiogenesis agents,[31] there has been no report regarding conformational study of this peptide. 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. KLYDY is involved in its bioactivity of kringle5. Distance between the carboxyl oxygen in Asp73 and the side chain amino nitrogen in Lys70 is 3.34 Å in the crystal structure.

Peptide with five residues cannot adopt single predominant conformation in solution and they do not converge well. Figure 2 show the ribbon diagrams of the lowest energy structures of Ang3 and Ang4 with all heavy atoms, respectively. For the comparison, structures of Ang1 and Ang2, which are reported previously, are shown in this figure, too.[12] Even though Ang1 has the same amino acid sequence to that of active fragment of kringle 5, Ang1 has more extended solution structure than the crystal structure. Ang2 has two sets of structures while Ang1, Ang2, and Ang3 show more diversity of structures in solution than Ang2.[12] Lowest energy structure of Ang2 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 2B. Ang3 (KLFDF) has more extended structure as shown in Figure 2 and does not have angiogenic activities. This implies that Phe does not have effective hydrophobic interaction with other Phe. In Ang4, terminal Lys was substituted by nonnatural amino acid X to increase hydrophobicity as shown in Table 1. However, Ang4 has three aromatic rings in Ang4 have stable arrangement in one side of the peptide and this arrangement results in an extended structure.

Ang2 has more rigid solution structure than other inhibitors because of an intramolecular hydrogen bond. Indole ring in Trp3 is much bigger than the phenyl ring of Phe or Tyr and may have better face-to-edge interaction with Phenyl ring in Tyr5 at the C-terminus, enforcing more rigid and constrained conformational features of Ang2. At the other side of indole rings of Trp in Ang2, there is an intramolecular hydrogen bond between the side chain amino protons of Lys1 and the carboxyl oxygen of Asp4 and it results in a stable structure. This agrees well with the result of small temperature coefficient data.[12]

Conclusion

Since angiogenesis is essential for the growth and persistence of solid tumors and their metastases, anti-angiogenesis could lead to the suppression of tumor growth; angiogenesis inhibitors have received attention for their therapeutic potential. In this study, two angiogenic inhibitors, Ang3 (KLFDF) and Ang4 (XLFDF) were designed based on the bioactive sequence from the kringle 5, and were compared to Ang1 (KLYDY) and Ang2 (KLWDF), designed previously. Ang1 and Ang2 show similar angiogenic inhibition activities, while Ang3 and Ang4 show no angiogenic inhibition activities. Therefore, Phe rings do not have effective hydrophobic interactions with other aromatic residues in Ang3 and Ang4. The representative structure of Ang2 has a stable intramolecular hydrogen bond. According to the structure-activity relationship of these angiogenic inhibitors, it can be concluded that structural rigidity obtained from the intramolecular hydrogen bonding might be more important than the hydrophobic interaction in activities of these peptides.

Since all the peptides have diversity of structures in solution, small temperature coefficients of amino protons of Lys side chains are the strong evidence of the intramolecular hydrogen bondings in Ang2. Conformationally constrained structure with intramolecular hydrogen bondings can offer a chance to overcome the problems of flexibilities of peptides and improved the angiogenic activities. More rigid structure, which can be expected to have higher activities and better match with the receptor bound conformations, can be obtained with a constrained cyclic structure. Further peptido-mimetic approaches should be tried to develop angiogenesis inhibitors.

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