Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 951–954

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Design and Synthesis of Antiangiogenic/Heparin-Binding Arginine Dendrimer Mimicking the Surface of Endostatin

Soko Kasai,[a] Hideko Nagasawa,[a] Mariko Shimamura,[b] Yoshihiro Uto[a] and Hitoshi Hori[a,*]

[a]*Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Minamijosanjimacho-2, Tokushima 770-8506, Japan*

[b]*Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan*

Received 27 July 2001; accepted 19 January 2002

Abstract—We designed and synthesized the antiangiogenic arginine-rich dendrimers, TX-1943 and TX-1944, which mimic the surface structure of endostatin. TX-1944 containing 16 arginine residues is more similar in surface structure to endostatin than TX-1943 with eight arginine residues, and has stronger in vivo antiangiogenic activity at 10 μg/CAM in the chicken embryo chorioallantoic membrane (CAM) assay. TX-1944 also has stronger activity to bind heparin and to inhibit the growth of rat lung endothelial (RLE) cells than TX-1943.

Angiogenesis, which is the formation of new blood vessels from established vessels, is essential for the growth and metastasis of solid tumors.[1] Therefore, tumor angiogenesis is a potential molecular target of chemotherapy.[2,3] Tumor angiogenesis is induced by hypoxia.[4] Recently, we designed and synthesized bifunctional hypoxic cell radiosensitizers with antiangiogenic and antimetastatic activities.[5–7]

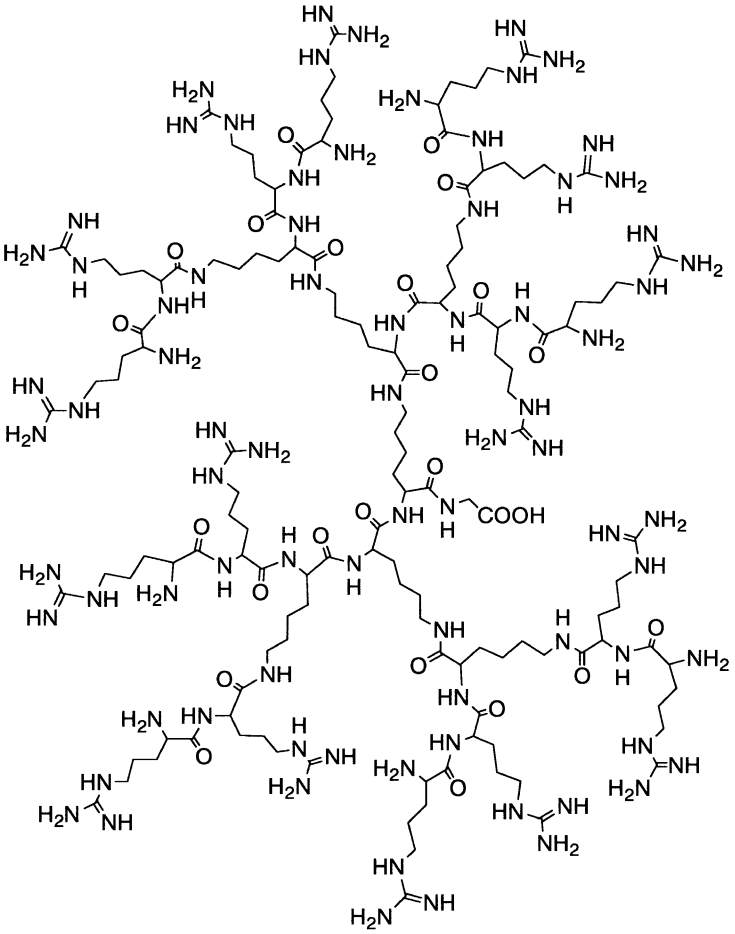
Angiogenesis is stimulated by various cytokines, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2). The interaction of these cytokines with their receptors on the endothelial cell surface depends on the presence of heparin or heparan sulfate proteoglycan (HSPG) which is an extracellular macromolecule.[8–10] On the other hand, angiogenesis is suppressed by endogenous inhibitors such as angiostatin[11] and endostatin.[12] X-ray analysis of the structure of mouse endostatin demonstrated a compact globular fold with one exposed positive-charge-rich face composed of 15 arginine residues.[13] Some sites of the clustered arginines in endostatin participate in binding to heparin or HSPG. The interaction may play a role in its antiangiogenic activity.[14] A possible mechanism for its inhibition of angiogenesis is competition for the heparin or HSPG binding sites, which act as co-receptors for several cytokines. Thus, for most antiangiogenic molecules it is important to interact with heparin and HSPG.

These suggest that heparin and HSPG are potential molecular targets for antiangiogenic drugs. Previously, Fromm et al. reported the importance of arginine peptide for interaction with heparin and heparan sulfate.[15] Furthermore, arginine-rich hexapeptides that interfered the interaction of VEGF with its receptor inhibited tumor growth and metastasis by blocking angiogenesis.[16] The basic hexapeptide regions of hepatocyte growth factor, which is one of the heparin-binding angiogenic cytokines, exhibited antiangiogenic activity.[17]

Our attempts to design antiangiogenic drugs have focused on the characteristic positive-charge-rich surface of endostatin and the interaction between its arginine residues and either heparin or HSPG. To model on the surface structure of a globular protein, we tried to use dendrimers that are synthetic three-dimensional dendritic macromolecules. It is known that dendrimers are prepared in a step-wise manner from simple branched monomer units, the nature and functionality of which can be easily controlled.[18] The later the generation of

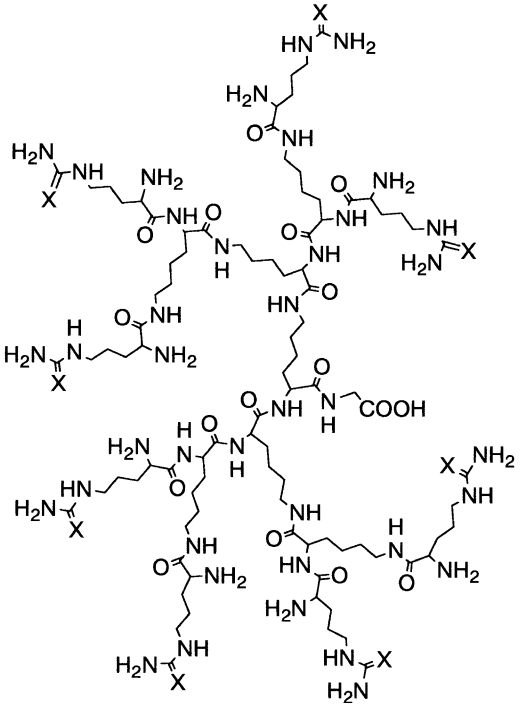
*Corresponding author. Tel.: +81-88-656-7514; fax: +81-88-656-9164; e-mail: hori@bio.tokushima-u.ac.jp

0960-894X/02/$ - see front matter
PII: S0960-894X(02)00066-5



TX-1944

$R_8R_8K_4K_2KG\text{-}OH$



TX-1943

X = NH

$R_8K_4K_2KG\text{-}OH$

TX-1993

X = O

$Cit_8K_4K_2KG\text{-}OH$

Figure 1. Structure of arginine and citrulline dendrimers.

dendrimer, the more the sterically-crowded and branched surface takes on a globular conformation. This unique architecture significantly improves the physical and chemical properties of traditional linear polymers. Then, we designed an arginine dendrimer having a similar globular structure to endostatin. We report here for the first time the design and synthesis of new anti-angiogenic/heparin-binding inhibitors having a dendritic peptidyl core structure mimicking the surface structure of endostatin. We evaluated their in vitro and in vivo antiangiogenic activities, heparin-binding activities, and the correlation between them.

In the synthesis of protein-like dendritic macromolecules, L-lysine is a good building block, acting as a branching unit in the solid-phase peptide synthesis. Dendrimers composed of L-lysine as a branching unit were originally used as the core of the multiple antigen peptide system (MAP).[19] We have designed two dendrimers, TX-1943 ($R_8K_4K_2$KG-OH) with a coarse surface consisting of eight arginine residues and TX-1944 ($R_8R_8K_4K_2$KG-OH), which has a tightly packed surface consisting of 16 arginine residues. The radius of a branching unit in these dendrimers as estimated by molecular modeling is about 20Å. Both dendrimers were synthesized from L-glycine connected to Wang resin by Fmoc-solid-phase peptide synthesis using L-lysine (branching unit) and L-arginine (surface group) in the presence of condensation agents such as diisopropylcarbodiimide (DIC) and *N*-hydroxybenzotriazole (HOBt).[20] Deprotection and cleavage from the resin were achieved by treatment with trifluoroacetic acid (TFA) in the presence of thioanisole and *m*-cresol at room temperature for 10 h. The crude dendrimer was first purified by gel-filtration and reversed-phase HPLC to obtain the powdered product, which was further purified by reversed-phase HPLC to afford the pure product. The chemical structures, synthetic yields and the physical data of arginine dendrimers and citrulline dendrimer (TX-1993: $Cit_8K_4K_2$KG-OH), a negative control of arginine dendrimer, are shown in Figure 1 and Table 1.

Table 2 shows the in vivo antiangiogenic activity of these dendrimers in the CAM assay.[21] Both TX-1944 ($R_8R_8K_4K_2$KG-OH) and TX-1943 ($R_8K_4K_2$KG-OH) inhibited angiogenesis dose-dependently. TX-1944, with its arginine-rich and closely-packed surface, had the more potent activity. In antiangiogenic activity, the two were comparable to angiostatin (100% inhibition at approximately 100 μg/disc)[11] and endostatin (potent inhibition at 10–20 μg/disc, $n=5$/group).[12] A citrulline dendrimer ($Cit_8K_4K_2$KG-OH) (TX-1993) showed weak antiangiogenic activity at 10 μg/CAM. These results suggested that the basicity of arginine ($pK_a=12.48$) and the closely packed surface of the dendrimer play an important role in determining the potency of the antiangiogenic activity. Nevertheless, TX-1993 having neutral citrulline residues in its side chain was also an antiangiogenic inhibitor (its inhibitory activity was 53.4 +18.9% at 100 μg/CAM). It seems that its alpha-amino group (pKa = 9.41) also contributes to its antiangiogenic activity at a higher concentration. On the other hand, TX-1944 moderately inhibited the RLE cell growth, and TX-1943 did not inhibit serum-stimulated RLE cell growth as shown in Figure 2.[22]

To elucidate the relation between heparin-binding activity and antiangiogenic activity, we next determined the activity of these dendrimers to bind heparin using heparin–agarose affinity column.[15] Table 3 shows that

Table 1. Yields and physical data of arginine and citrulline dendrimers

Dendrimer	Total yield (%)	MS (MALDI-TOF)	HPLC[a] retention time (min)
TX-1944 ($R_8R_8K_4K_2$KG-OH)	15.4	3470.9 (MH^+) 3469.3 (calcd)	13.4
TX-1943 ($R_8K_4K_2$KG-OH)	28.5	2221.9 (MH^+) 2220.5 (calcd)	8.2
TX-1993 ($Cit_8K_4K_2$KG-OH)	23.4	2228.7 (M^+) 2228.4 (calcd)	15.8[b]

[a]HPLC condition: ODS-80Ts column (4.6 mm×15 cm), elution condition was a linear gradient of 0 to 70% acetonitrile in 0.1% TFA for 15 min at a flow rate of 1.0 mL/min with UV detection at 210 nm.
[b]ODS-80Ts column (4.6 mm×25 cm), elution condition was a linear gradient of 0 to 90% acetonitrile in 0.1% TFA for 20 min at a flow rate of 1.0 mL/min with UV detection at 210 nm.

Table 2. Antiangiogenic activity of the arginine dendrimer, TX-1943 and TX-1944, and citrulline dendrimer TX-1993 in CAM assay

Dendrimer	100 μg/CAM	10 μg/CAM
TX-1944 ($R_8R_8K_4K_2$KG-OH)	70.0±15.1[a]	45.4±17.5
TX-1943 ($R_8K_4K_2$KG-OH)	69.2±16.8	25.3±10.7
TX-1993 ($Cit_8K_4K_2$KG-OH)	53.4±18.9	16.7±0.0

[a]% inhibition of angiogenesis (mean±SD).

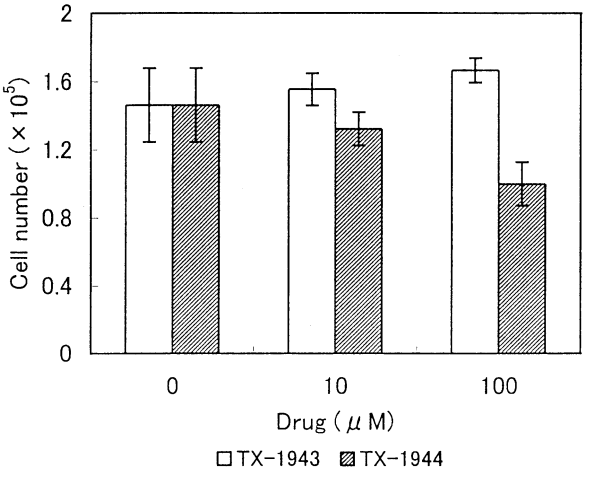


Figure 2. Effects of the arginine dendrimer TX-1943 and TX-1944 on the growth of RLE cells.

Table 3. Heparin-binding activity of the arginine dendrimers TX-1943 and TX-1944, and citrulline dendrimer TX-1993

Dendrimer	NaCl (M)[a]
TX-1944 ($R_8R_8K_4K_2$KG-OH)	1.2
TX-1943 ($R_8K_4K_2$KG-OH)	0.9
TX-1993 ($Cit_8K_4K_2$KG-OH)	0.3

[a]Salt concentration required for release from the heparin–agarose column.

arginine dendrimers have stronger heparin-binding activity than citrulline dendrimer. TX-1944, containing 16 arginine residues, has the strongest heparin-binding activity of the three dendrimers tested. This result suggests that dendritic arginine residues are important for heparin-binding activity the same as the linear peptidic structure.[15] The citrulline dendrimer TX-1993 showed weaker heparin-binding activity than the arginine dendrimers. We calculated the molecular orbitals of the protonated delta-guanidino group and the ureido group to reduce the difference between their LUMOs or HOMOs (protonated delta-guanidino group, LUMO: 1.0274 eV, HOMO: −9.9381 eV; ureido group, LUMO: 1.0299 eV, HOMO: −9.8114 eV). Therefore, the weak heparin-binding activity of TX-1993 is probably caused by the weaker hydrogen bonding of its ureido group than tonic bond of the protonated delta-guanidino group in the arginine dendrimer. The positive relationship between the heparin-binding activity and antiangiogenic activity of arginine dendrimers suggests that the former is in part responsible for the antiangiogenic activity of TX-1943 and TX-1944.

Although the antiangiogenic mechanism is not clear, these arginine dendrimers as endostatin-mimics would interact with heparin and HSPG to exhibit their antiangiogenic activity. Now, we are studying further the antiangiogenic mechanism of arginine dendrimers and have plans to design more hopeful antiangiogenic dendrimers.

Acknowledgements

We thank M. Nakamura, E. Okayama, and K. Yamashita of our faculty, as well as Dr. H. Satake, Center for Cooperative Research, for TOF-MS. We also thank E. Niimoto, for technical assistance.

References and Notes

1. Folkman, J. *Nat. Med.* **1995**, *1*, 27.
2. Gourley, M.; Williamson, J. S. *Curr. Pharm. Des.* **2000**, *6*, 417.
3. Gastl, G.; Hermann, T.; Steurer, M.; Zmija, J.; Gunsilius, E.; Unger, C.; Kraft, A. *Oncology* **1997**, *54*, 177.
4. Maxwell, P. H.; Dachs, G. U.; Gleadle, J. M.; Nichollas, L. G.; Harris, A. L.; Stratford, I. J.; Hankinson, O.; Pugh, C. W.; Ratcliffe, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8104.
5. Shimamura, M.; Ashino, H.; Oikawa, T.; Iwaguchi, T.; Nagasawa, H.; Hori, H.; Inayama, S. *Recent Adv. Chemother.* **1994**, 912.
6. Hori, H.; Jin, C.-Z.; Kiyono, M.; Kasai, S.; Shimamura, M.; Inayama, S. *Bioorg. Med. Chem.* **1997**, *5*, 591.
7. Kasai, S.; Nagasawa, H.; Yamashita, M.; Masui, M.; Kuwasaka, H.; Oshodani, T.; Uto, Y.; Inomata, T.; Oka, S.; Inayama, S.; Hori, H. *Bioorg. Med. Chem.* **2001**, *9*, 453.
8. Tessler, S.; Rockwell, P.; Hicklin, D.; Cohen, T.; Levi, B. Z.; Witte, L.; Lemischka, I. R.; Neufeld, G. *J. Biol. Chem.* **1994**, *269*, 12456.
9. Yayon, A.; Kiagsbrun, M.; Esko, J. D.; Leder, P.; Ornitz, D. M. *Cell* **1991**, *64*, 841.
10. Klagsbrun, M. *Semin. Cancer Biol.* **1992**, *3*, 81.
11. O'Reilly, M. S.; Holmgren, L.; Shing, Y.; Chen, C.; Rosenthal, R. A.; Moses, M.; Lane, W. S.; Cao, Y.; Sage, E. H.; Folkman, J. *Cell* **1994**, *79*, 315.
12. O'Reilly, M. S.; Boehm, T.; Shing, Y.; Fukai, N.; Vasios, G.; Lane, W. S.; Flynn, E.; Birkhead, J. R.; Olsen, B. R.; Folkman, J. *Cell* **1997**, *88*, 277.
13. Hohenester, E.; Sasaki, T.; Olsen, B. R.; Timple, R. *EMBO J.* **1998**, *17*, 1656.
14. Sasaki, T.; Larsson, H.; Kreuger, J.; Salmivira, M.; Claesson-Welsh, L.; Lindahi, U.; Hohenester, E.; Timple, R. *EMBO J.* **1999**, *18*, 6240.
15. Fromm, J. R.; Hileman, R. E.; Caldwell, E. E. O.; Weiler, J. M.; Linhardt, R. J. *Arch. Biochem. Biophys.* **1997**, *343*, 92.
16. Bae, D.-G.; Gho, Y.-S.; Yoon, W.-H.; Chae, C.-B. *J. Biol. Chem.* **2000**, *275*, 13588.
17. Fazekas, K.; Janovics, A.; Dome, B.; Koska, P.; Albini, A.; Timar, J. *Microvasc. Res.* **2001**, *62*, 440.
18. Tomalia, D. A.; Naylor, A. M.; Goddard, W. A., III *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 138.
19. Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5409.
20. The dendrimer was prepared by a stepwise solid-phase peptide synthesis on Wang resin using the Fmoc-strategy. In brief, Fmoc-Gly-OH (4.0 equiv) was coupled to the Wang resin (loading; 1.1 mmol/g) with DMAP (2.0 equiv) and DIC (4.0 equiv) in DMF followed by deprotection with 20% piperidine/DMF. The synthesis of the first and every subsequent level of the carrier core was performed using Fmoc-Lys(Fmoc)-OH (2.0, 4.0, 8.0 equiv consecutively) with HOBt and DIC in DMF at rt for 2 h to give octa-branched core matrix. After removing Fmoc group, the H-Lys_4-Lys_2-Lys-Gly-resin (0.034 mmol) was coupled with Fmoc-Arg(Mtr)-OH (16.0 equiv) or Fmoc-Cit-OH (16.0 equiv) under the same conditions. For TX-1944, the coupling reaction with Fmoc-Arg(Mtr)-OH (16.0 equiv) was repeated twice. After deprotection, the resin was washed with diethyl ether (20 mL×2) and dried in vacuo. The dendrimer was cleaved from resin by TFA/thioanisole/*m*-cresol (13:1.8:0.3) treatment at rt for 10 h and then, purified by gel filtration on Sephadex G-25 using 0.1% TFA solution as an eluent and lyophilized. The powder dendrimer was further separated by reverse phase HPLC (column: TOSOH ODS-80Ts, 4.6 mm×15 cm; elution condition: a linear gradient of 0 to 70% acetonitrile in 0.1% TFA for 15 min at a flow rate of 1.0 mL/min with UV detection at 210 nm) to afford the desired product.
21. Antiangiogenic activities of dendrimers were assayed with chicken embryo chorioallantoic membranes (CAM). On day 4 of the fertilized chicken embryo (Ohmiya Kakin Laboratories, Japan) in a shell, 10 μL aliquots of samples mixed in 1% methyl cellulose/0.9% NaCl were applied into the ring placed on the surface of the CAM. After 48 h exposure at 37 °C, fat emulsion was injected into the CAM to visualize blood vessels clearly. The angiogenic inhibition was indicated by an avascular zone around the ring of 3 mm or more in diameter. The results were expressed as the percentages of embryos showing inhibition.
22. Rat lung endothelial cells were seeded to 24-well plates at 3×10^4 cells per well in Eagle's minimal essential medium containing 10% fetal calf serum (FCS). After 24 h of culture the medium in each well was replaced with fresh medium containing 2% FCS and the samples in triplicate were added to each well. The cells were cultured for 48 h trypsinized and counted with a Coulter counter.