Comparisons of biophysical properties and bioactivities of mono-PEGylated endostatin and an endostatin analog

Shan Wang1,2,3, Yan Fu1,2,3 and Yongzhang Luo1,2,3*

Abstract

Background: Endostatin (ES) is a well-established potent endogenous antiangiogenic factor. An ES variant, called zinc-binding protein-ES (ZBP-ES), is clinically available; however, its use is limited by rapid renal clearance and short residence time. PEGylation has been exploited to overcome these shortcomings, and mono-PEGylated ES (called M2ES) as well as mono-PEGylated ZBP-ES (MZBP-ES) are developed in our study. This study aimed to compare the biophysical properties and biological effects of M2ES and MZBP-ES to evaluate their druggability.

Methods: Circular dichroism and tryptophan emission fluorescence were used to monitor the conformational changes of M2ES and MZBP-ES. Their resistance to trypsin digestion and guanidinium chloride (GdmCl)-induced unfolding was examined by Coomassie staining and tryptophan emission fluorescence, respectively. The biological effects of M2ES and MZBP-ES on endothelial cell migration were evaluated using Transwell migration and wound healing assays, and the uptake of M2ES and MZBP-ES in endothelial cells was also compared by Western blotting and immunofluorescence.

Results: Structural analyses revealed that M2ES has a more compact tertiary structure than MZBP-ES. Moreover, M2ES was more resistant to trypsin digestion and GdmCl-induced unfolding compared with MZBP-ES. In addition, although M2ES and MZBP-ES showed comparable levels of inhibiting transwell migration and wound healing of endothelial cells, M2ES displayed an increased ability to enter cells compared with MZBP-ES, possibly caused by the enhanced interaction with nucleolin.

Conclusions: M2ES has a more compact tertiary structure, is more stable for trypsin digestion and GdmCl-induced unfolding, exhibits increased cellular uptake and shows equivalent inhibitory effects on cell migration relative to MZBP-ES, indicating that M2ES is a more promising candidate for anticancer drug development compared with MZBP-ES.

Keywords: Endostatin, PEGylation, Antiangiogenic therapy, Drug design, Zinc-binding protein-endostatin

Background

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is vital for both physiologic and pathologic conditions. As one of the hallmarks of cancer [1], the process of angiogenesis is exquisitely regulated by a variety of activators and inhibitors [2, 3]. These pro-angiogenic and anti-angiogenic factors constitute a tightly controlled angiogenic balance. Among them, one of the first identified endogenous factors with anti-angiogenic activities was endostatin (ES) [4]. ES, a 22 kD C-terminal fragment of collagen XVIII, can significantly retard tumor growth by targeting endothelial cells in mouse models [5] when assessed by proliferation, migration, tube formation, and numerous signaling pathways. The clinical trials of ES in the United States failed due to insufficient efficacy and problems in the production and formulation [5]. However, mutagenesis of ES by the linkage of MGGSH-HHHH to the N terminus stabilizes ES and has made it...
successfully clinically available [5]. This ES variant, also known as the zinc-binding protein-endostatin (ZBP-ES), was approved by the China Food and Drug Administration (CFDA) to treat non-small cell lung cancer (NSCLC) patients in China in 2005 [6]. As a widely used anticancer drug in China, ZBP-ES has exhibited beneficial therapeutic effects [7–9]. A meta-analysis of clinical data, involving 1953 NSCLC patients, demonstrated that ZBP-ES co-treatment significantly improved the overall response rate and disease control rate of patients who underwent platinum-based doublet chemotherapy by 14.7 and 13.5%, respectively [6]. In another study, the endpoints of progression-free survival (PFS) and overall survival (OS) were evaluated in a total of 110 patients with metastatic malignomas [8]. Significant improvements were observed in favor of the ZBP-ES plus dacarbazine arm compared with the placebo plus dacarbazine arm (PFS, 4.5 vs. 1.5 months; OS, 12.0 vs. 8.0 months). However, hindered by its relatively rapid renal clearance and short circulation half-life in vivo [10], ZBP-ES was recommended to be taken on a daily basis in clinic.

PEGylation, the process of covalent attachment of polyethylene glycol (PEG) to the proteins or peptides, can overcome the short half-life problem. PEGylation masks the surface of the proteins and increases their molecular size, therefore protecting them from protease digestion and renal filtration, while at the same time increasing the protein’s stability and residence time in vivo [11]. The applicability and safety of this strategy have been well documented, and PEGylated proteins have been used in clinics for years. Our group and our partners have developed a second generation of ES by conjugation of a PEG molecule to the N terminus of wild-type ES, which is also known as M2ES, and therefore provide a basis for better understanding the druggability and bioactivities of PEGylated ES variants.

Methods
Proteins, antibodies, and cell culture
The recombinant proteins used in this study were provided by Protgen Co., Ltd. (Beijing, China). ES and ZBP-ES were expressed in *E. coli*, refolded into native forms and purified. The N-terminal site-directed mono-PEGylation was conducted by addition of 20 mmol/L NaBH3CN and incubating the PEG2000 and proteins (PEG2000:protein = 1.5:1) at room temperature for more than 2 h, followed by purification.

We purchased primary antibodies against extracellular regulated protein kinases (Erk), phosphor-Erk, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), PEG (Abcam, Cambridge, UK), and turbo green fluorescent protein (tGFP) (OriGene, Rockville, MD, USA). Anti-ES antibody was from our lab stock.

Human microvascular endothelial cells (HMECs) were from our lab stock and cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA).

Circular dichroism (CD)
The far-ultraviolet (UV) CD spectra were obtained by a Chirascan™-plus CD Spectrometer (Applied photophysics, Surrey, UK). ES or its variants were diluted in 5 mmol/L Tris–HCl, pH 7.4, to a final concentration of 10 μmol/L. For each protein, data were recorded three times and corrected by subtracting the baseline spectrum of the buffer.

Tryptophan emission fluorescence
Tryptophan emission fluorescence is a good probe to monitor the subtle tertiary structural changes of ES [16]. Proteins (1 μmol/L) in Tris buffer were measured as previously described [17].

Proteolysis assay
ES or its variants (0.5 mg/mL) were incubated with trypsin (0.2 mg/mL) in phosphate buffer saline (PBS) buffer (pH 7.4) at 37 °C [18]. At each indicated time point, reaction solutions were quickly removed and mixed with the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer to stop the trypsin digestion reactions. The samples were subsequently subjected to SDS–PAGE and stained with the Coomassie dye.

Guadinium chloride (GdmCl)-induced unfolding
ES or its variants (0.8 μmol/L) were incubated in 5 mmol/L Tris–HCl, pH 7.4, containing GdmCl concentrations ranging from 0 to 6 mol/L. After incubation for 24 h at room temperature, GdmCl-induced denaturation was monitored by the tryptophan emission fluorescence intensity at 318 nm [5, 19]. Data were normalized by subtracting the baseline fluorescence intensity of the buffer. The linear extrapolation method was used to evaluate the value of the free change energy in the absence of the denaturant GdmCl (∆Go°N−U), the apparent slope of the
In vitro protein–protein interactions
The nucleolin-tGFP plasmid was transfected into HMECs. Nucleolin-tGFP proteins were immunoprecipitated following the procedure described in our previous study [21]. Subsequently, ES or its variants were incubated with the nucleolin-tGFP beads for 1 h at 4 °C. After washing with the lysis buffer (150 mmol/L Tris, 50 mmol/L sodium chloride, 1% NP40, protease inhibitor mixture, pH 7.4), the bead-bound proteins were subjected to Western blotting.

Internalization assay
As previously described [22], HMECs were treated with 5 μg/mL ES or its variants for 1 h. Subsequently, cells were washed with acidic buffer (pH 3.5) and ice-cold PBS to remove cell surface-binding proteins. The amount of internalized proteins was examined by Western blotting or immunofluorescence.

Western blotting
Protein samples were separated by SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking for 1 h at room temperature, the membrane was incubated with the indicated primary antibodies overnight and then to the corresponding horseradish peroxidase (HRP)-conjugated goat-anti-mouse or goat-anti-rabbit secondary antibodies for 1 h. The enhanced chemiluminescent substrate was added to the blot, and reactive bands were detected.

Immunofluorescence
Cells were seeded directly on coverslips, fixed, and stained. After blocking with 5% goat serum, cells were incubated with the indicated primary antibodies and fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse or goat-anti-rabbit secondary antibodies. The nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were captured using a Nikon A1 confocal microscope (Nikon Corporation, Tokyo, Japan).

Transwell migration and wound healing
Transwell migration and wound healing assays were conducted as previously described [22]. Cells were treated with the indicated proteins for 6 and 48 h at 37 °C, respectively. All experiments were repeated twice.

Statistical analyses
All data from the experiments were presented as means ± standard deviations (SDs). Differences between two groups were calculated by GraphPad (GraphPad Software, San Diego, CA, USA), and considered significant if the P value was <0.05 using a two-tailed Student’s t test.

Results
Summary of Methods

Structural analyses
The schematic diagram shows the sequence of ES, ZBP-ES, M₂ES, and MZBP-ES (Fig. 1a). The purity of these proteins is shown in Fig. 1b. The proteins were tested for secondary and tertiary structures using CD and tryptophan emission fluorescence. The CD spectra results revealed that no obvious change in the secondary structure was observed between ES and ZBP-ES, whereas slight differences were observed between M₂ES and MZBP-ES (Fig. 1c). The maximal Trp fluorescence emission wavelengths (λ max) of all the ES variants were approximately 318 nm (Fig. 1d), indicating a relatively stable core structure of ES variants. However, the fluorescence intensities of ES and M₂ES were higher than those of ZBP-ES and MZBP-ES, respectively, implying that the tertiary structure of ES is more compact than that of ZBP-ES and that the tertiary structure of M₂ES is more compact than that of MZBP-ES.

Protease digestion
To identify the stability of ES variants, trypsin was incubated with ES variants for the indicated time periods. ES was quickly digested within 20 min, whereas ZBP-ES was not totally digested until 4 h (Fig. 2a). PEGylated proteins were overall more resistant to trypsin digestion compared with intact proteins. Interestingly, after incubation for 6 h, the residual amount of full-length M₂ES was larger than that of MZBP-ES. Quantitative results showed that the initial reaction rate was 0.026 mg/(mL·min) for M₂ES and 0.037 mg/(mL·min) for MZBP-ES. These results showed that M₂ES was more resistant to trypsin digestion than MZBP-ES.

GdmCl-induced unfolding
GdmCl-induced unfolding was used to detect the structural stability and unfolding cooperativity of ES variants. Slight changes were observed between the unfolding curves of ES and ZBP-ES (Fig. 2b, upper panel). These two proteins had a similar C m value, whereas the modified ES exhibited lower values of ΔG⁰ N–U and m (Table 1). Moreover, the unfolding curve of M₂ES had slight shifts towards higher GdmCl concentrations compared with that of MZBP-ES (Fig. 2b, lower panel), with increases in ΔG⁰ N–U, m, and C m (Table 1). These results showed that
both the structural stability and unfolding cooperativity of M2ES were better than those of MZBP-ES (Fig. 2c).

**Biological activities**

We next investigated the effects of ES variants on endothelial cell activities. ES and its variants significantly reduced endothelial cell migration, and PEGylated proteins were more efficient compared with intact proteins (Fig. 3a, b). In the Transwell assay, M2ES and MZBP-ES inhibited endothelial cell migration to an equivalent extent. The wound healing scratch results showed that M2ES was slightly more potent than MZBP-ES in retarding wound healing (Fig. 3c).

ES was shown to bind to the vascular endothelial growth factor receptor 2 (VEGFR2) on the surface of endothelial cells and disrupt vascular endothelial growth factor (VEGF)-induced activation of mitogen-activated protein kinase/extracellular regulated protein kinases (MAPK/Erk) [23]. All ES variants inhibited Erk phosphorylation (Fig. 3d). ZBP-ES was more potent in reducing Erk activation compared with ES, whereas the blockage effect of M2ES was more potent than that of MZBP-ES.

In summary, M2ES and MZBP-ES had comparable effects on the inhibition of cell migration, whereas M2ES was more efficient in disrupting Erk activation.

**Internalization by endothelial cells and interactions with cell surface receptors**

Internalization is important, if not critical, for the biological functions of ES [24]. Increasing the uptake of ES via the addition of cholesterol chelating agents [22] or engineering a peptide to its N terminus [25] dramatically enhanced the therapeutic efficacy of ES. We determined the uptake of ES and its variants by HMECs and found that ZBP-ES was more efficient in entering the cells than ES (Fig. 4a), which validates the data from our previous study [16]. Intriguingly, more M2ES proteins accumulated in the cells than MZBP-ES (Fig. 4a). Consistent results were obtained by immunofluorescence (Fig. 4b).

Nucleolin is a functional receptor of ES and participates in the ES internalization process [26]; therefore, we explored whether the distinction of ES variant internalization was attributed to different binding affinities to nucleolin. Consistent with the internalization results (Fig. 4a, b), ES showed weaker interaction with nucleolin.
than ZBP-ES, whereas M₂ES showed increased interaction with nucleolin compared with MZBP-ES (Fig. 4c). Collectively, M₂ES exhibited an increased cellular uptake than MZBP-ES, possibly caused by the enhanced interaction with its functional receptor nucleolin.

Discussion
In this study, we demonstrate that M₂ES not only has a more compact tertiary structure than MZBP-ES but also exhibits more resistance to trypsin digestion and GdmCl-induced unfolding. Although M₂ES shows comparable inhibitory effects on endothelial cell migration and

Table 1 Parameters of the GdmCl-induced unfolding of ES variants

| Protein      | ΔG_ratio (kJ/mol) | m (kJ·L/mol²) | Cm (mol/L) |
|--------------|------------------|---------------|------------|
| ES           | 29.23            | 12.88         | 2.27       |
| ZBP-ES       | 24.16            | 10.70         | 2.26       |
| M₂ES         | 31.27            | 14.31         | 2.18       |
| MZBP-ES      | 20.57            | 9.65          | 2.13       |

GdmCl guanidinium chloride, ΔG_ratio the free change energy in the absence of GdmCl, m the apparent m value defined by the linear extrapolation model, Cm the concentration of GdmCl at the midpoint of the unfolding transition, ES endostatin, ZBP-ES zinc-binding protein-ES, M₂ES mono-PEGylated ES, MZBP-ES mono-PEGylated ZBP-ES

Fig. 2 Stability of ES variants under certain conditions. a Trypsin digestion profiles of ES variants for the indicated time. b and c Proteins were incubated with different concentrations of guanidinium chloride (GdmCl), and GdmCl-induced denaturation of ES variants was monitored with tryptophan emission fluorescence intensity at 318 nm. b Raw data and fitted data of the unfolding process of ES, ZBP-ES (upper panel), and M₂ES, MZBP-ES (lower panel). c Normalized data of the GdmCl-induced unfolding of ES variants.
wound healing compared with MZBP-ES. M2ES is more efficient in entering the cells than MZBP-ES, possibly due to enhanced interaction with its functional receptor nucleolin.

M2ES is more stable towards trypsin digestion than MZBP-ES (Fig. 2a). Our previous study revealed that the endostatin digestion process contains two stages: cleavage of the first four residues (HSHR) during the first stage and further digestion of the dominant product in the second stage [18]. We therefore supposed that the PEG molecule in M2ES masks the HSHR residues and therefore protects it from degradation by trypsin, whereas the PEG molecule of MZBP-ES covers the MGGSHHHHHH part, instead of the first four residues, making MZBP-ES more susceptible to protease digestion.

Endostatin is well established as an antiangiogenic factor; therefore, our study focused on the direct impacts of ES and PEGylated ES on endothelial cells. However, endothelial cells are not the only target of ES. Our recent study showed that adipocytes were also targeted by ES. ES can inhibit adipogenesis and dietary-induced obesity and its related metabolic disorders, including glucose intolerance, insulin resistance, and hepatic steatosis [27]. Although both intensive and extensive researches were performed on ES, and it appeared that there was not much progress on this protein, this new discovery suggests a potential expanded application of ES for the treatment of obesity. Moreover, this discovery opens new avenues for this protein and indicates that this is only the beginning.

**Fig. 3** Biological activities of ES variants. a and b Representative images and quantified results of the Tranwell migration assay for endothelial cells. Human microvascular endothelial cells (HMECs) were seeded into the upper well of the Boyden chamber and treated with the indicated proteins for 6 h. Cells were stained with crystal violet, photographed, and counted. c In the wound healing assay, the HMEC monolayer was scratched and cultured in serum-free media in the absence or presence of the indicated proteins for 48 h. Cells were photographed at 0 and 48 h, respectively, and the relative wound closure was quantified. d Erk phosphorylation of ES variant treatments examined by Western blotting. Error bars indicate standard deviations (SDs). *P < 0.05, **P < 0.01, ***P < 0.001, vs. control.
Conclusions
Collectively, these findings suggest that M₂ES is a more suitable drug candidate in comparison with MZBP-ES and provides a basis for further endostatin-based drug development.

Authors' contributions
SW designed the research, carried out experiments, interpreted data, and wrote the manuscript. YF participated in data analysis and interpretation, as well as administration and grant support. YL supervised the study, interpreted data, and wrote the manuscript. All authors read and approved the final manuscript.

Author details
1 The National Engineering Laboratory for Anti-tumor Protein Therapeutics, School of Life Sciences, Tsinghua University, Beijing 100084, P. R. China. 2 Beijing Key Laboratory for Protein Therapeutics, School of Life Sciences, Tsinghua University, Beijing 100084, P. R. China. 3 Cancer Biology Laboratory, School of Life Sciences, Tsinghua University, Beijing 100084, P. R. China.

Acknowledgements
We thank Shaoqin Zhang (Tsinghua University) for the circular dichroism experiments and all the Luo laboratory members for their insightful discussions.

YL was supported by the General Programs of the National Natural Science Foundation of China (No. 81171998, 81472667, and 81461148021), the National Science and Technology Major Project for “Major New Drugs Innovation and Development” (No. 2013ZX095090103). YF was supported by the General Programs of the National Natural Science Foundation of China (No. 81272529).

Competing interests
The authors declare that they have no competing interests.

Received: 14 September 2015   Accepted: 25 November 2015
Published online: 20 January 2016

References
1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74. doi:10.1016/j.cell.2011.02.013.
2. Stacker SA, Achen MG. The VEGF signaling pathway in cancer: the road ahead. Chin J Cancer. 2013;32(6):297–302. doi:10.5732/cjc.012.10319.
3. Yan L. Molecular targeted agents—where we are and where we are going. Chin J Cancer. 2013;32(5):225–32. doi:10.5732/cjc.013.10051.
4. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vassios G, Lane WS, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell. 1997;88(2):277–85.

5. Fu Y, Tang H, Huang Y, Song N, Luo Y. Unraveling the mysteries of endostatin. JUBMB Life. 2009;61(6):613–26. doi:10.1038/sj.jub.215.

6. Rong Y, Yang S, Li W, Zhang W, Ming Z. Systematic review and meta-analysis of Endostar (rh-endostatin) combined with chemotherapy versus chemotherapy alone for treating advanced non-small cell lung cancer. World J Surg Oncol. 2012;10:170. doi:10.1186/1477-7819-10-170.

7. Han B, Xiu Q, Wang H, Shen J, Gu A, Luo Y, et al. A multicenter, randomized, double-blind, placebo-controlled study to evaluate the efficacy of paclitaxel-carboplatin alone or with endostar for advanced non-small cell lung cancer. J Thorac Oncol. 2011;6(6):1104–9. doi:10.1097/JTO.0b013e3182166b6b.

8. Cui C, Mao L, Chi Z, Si L, Sheng X, Kong Y, et al. A phase II, randomized, double-blind, placebo-controlled multicenter trial of endostar in patients with metastatic melanoma. Mol Ther. 2013;21(7):1456–63. doi:10.1038/mt.2013.79.

9. Sun Y, Wang JW, Liu YY, Yu QT, Zhang YP, Li K, et al. Long-term results of a randomized, double-blind, and placebo-controlled phase III trial: endostar (rh-endostatin) versus placebo in combination with vinorelbine and cisplatin in advanced non-small cell lung cancer. Thorac Cancer. 2013;4(4):440–8. doi:10.1111/1759-7714.12050.

10. Lee SH, Jeung IC, Park TW, Lee K, Lee DG, Cho YL, et al. Extension of the in vivo half-life of endostatin and its improved anti-tumor activities upon fusion to a humanized antibody against tumor-associated glycoprotein 72 in a mouse model of human colorectal carcinoma. Oncotarget. 2015;6(9):7182–94.

11. Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov. 2003;2(3):214–21. doi:10.1038/nrd1033.

12. Guo L, Geng X, Chen Y, Qi F, Liu L, Miao Y, et al. Pre-clinical toxicokinetics and safety study of M2ES, a monoPEG20000-Endostar. Int J Biol Macromol. 2010;46(3):331–6. doi:10.3892/mco.2014.271.

13. Chen Y, Yu Y, Li P, Wu F, Fu Y, Li Z, et al. Phase I trial of MES, a novel polyethylene glycosylated recombinant human endostatin, plus gemcitabine in advanced pancreatic cancer. Mol Clin Oncol. 2014;2(4):386–90. doi:10.3892/mco.2014.271.

14. Nie Y, Zhang X, Wang X, Chen J. Preparation and stability of N-terminal mono-PEGylated recombinant human endostatin. Bioconjug Chem. 2006;17(4):995–9. doi:10.1021/bc050355d.

15. Tong Y, Zhong K, Tian H, Gao X, Xu X, Yin X, et al. Characterization of a monoPEG20000-Endostar. Int J Biol Macromol. 2010;46(3):331–6. doi:10.1016/j.jmbiomac.2010.01.017.

16. Fu Y, Luo Y. The N-terminal integrity is critical for the stability and biological functions of endostatin. Biochemistry. 2010;49(30):6420–9. doi:10.1021/bi100489x.

17. Wang S, Lu XA, Liu P, Fu Y, Jia L, Zhan S, et al. Endostatin has ATPase activity, which mediates its anti-angiogenic and anti-tumor activities. Mol Cancer Ther. 2015;14(5):1192–201. doi:10.1158/1535-7163-MCT-14-0836.

18. Han Q, Fu Y, Zhou H, He Y, Luo Y. Contributions of Zn(II)-binding to the structural stability of endostatin. FEBS Lett. 2007;581(16):3027–32. doi:10.1016/j.febslet.2007.05.058.

19. Li B, Wu X, Zhou H, Chen Q, Luo Y. Acid-induced unfolding mechanism of recombinant human endostatin. Biochemistry. 2004;43(9):2550–7. doi:10.1021/bi0357863.

20. Santoro MM, Bolen DW. Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl alpha-chymotrypsin using different denaturants. Biochemistry. 1988;27(21):8063–8.

21. Fu Y, Chen Y, Luo X, Liang Y, Shi H, Gao L, et al. The heparin binding motif of endostatin mediates its interaction with receptor nucleolin. Biochemistry. 2009;48(49):11653–63. doi:10.1021/bi901265z.

22. Chen Y, Wang S, Lu X, Zhang H, Fu Y, Luo Y. Cholesterol sequestration by nystatin enhances the uptake and activity of endostatin in endothelium via regulating distinct endocytic pathways. Blood. 2011;117(23):6392–403. doi:10.1182/blood-2010-12-322867.

23. Kim YM, Hwang S, Pyun BJ, Kim YJ, Lee ST, Gho YS, et al. Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. J Biol Chem. 2002;277(31):27872–9. doi:10.1074/jbc.M202771200.

24. Shi H, Huang Y, Zhou H, Song X, Yuan S, Fu Y, et al. Nucleolin is a receptor that mediates antiangiogenic and anti-tumor activity of endostatin. Blood. 2007;110(8):2899–906. doi:10.1182/blood-2007-01-064428.

25. Lim J, Duong T, Lee G, Seong BL, El-Rifai W, Ruley HE, et al. The effect of intracellular protein delivery on the anti-tumor activity of recombinant human endostatin. Biomaterials. 2013;34(26):6261–71. doi:10.1016/j.biomaterials.2013.05.011.

26. Song N, Ding Y, Zhou W, He T, Fu Z, Chen Y, et al. The nuclear translocation of endostatin is mediated by its receptor nucleolin in endothelial cells. Angiogenesis. 2012;15(4):697–711. doi:10.1007/s10456-012-9284-y.

27. Wang H, Chen Y, Lu XA, Liu G, Fu Y, Luo Y. Endostatin prevents dietary-induced obesity by inhibiting adipogenesis and angiogenesis. Diabetes. 2015;64(7):2442–56. doi:10.2337/db14-0528.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at www.biomedcentral.com/submit