Fibrinogen-derived fibrinostatin inhibits tumor growth through anti-angiogenesis

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Angiogenesis involves the degradation of components of the extracellular matrix and then migration, proliferation and differentiation of endothelial cells from pre-existing blood vessels to form sprouts and eventually new capillaries,¹–⁴ whereas hemostasis maintains the blood flow by regulating platelet adherence and fibrin deposition. Both systems normally appear quiescent, but become activated in response to such injury as the leaky blood vessels in solid tumors.⁵

Cancer has long been recognized to be associated with hypercoagulation as a result of imbalanced coagulation (fibrin deposition) and fibrinolysis (fibrin degradation). Fibrinogen, a multimeric molecule containing two of three non-identical chains, the α, β and γ chains, is the soluble circulating precursor of fibrin. It accumulates around leaky blood vessels in solid tumors and at the host–tumor interface, which contributes to the tumor growth and spreading.⁶,⁷ Fibrinogen can be cleaved by plasmin and thrombin and its proteolytic products can either promote or inhibit angiogenesis. Plasmin cleavage generates the carboxyl termini of the paired α, β and γ chains of fibrinogen and the central domain of fibrinogen (fibrinogen E-fragment [FgnE]). FgnE is a potent anti-angiogenic factor capable of inhibiting endothelial cell migration and tubule formation.⁸,⁹ When cleaved by thrombin, fibrinogen is converted into fibrin monomers after the removal of the amino termini of both α and β chains, yielding fibrinopeptides A (FpA) and B (FpB), respectively. The fibrin-E fragment (FnE) of fibrin degraded by plasmin was reported to promote angiogenesis. The main difference between FnE and FgnE lies in its loss of FpA.

We previously found a 15-amino acid (aa) fragment peptide in the sera of gastric cancer patients by surface-enhanced laser desorption/ionization and time-of-flight mass spectrometry (SELDI TOF-MS). This 15-mer peptide (DSGEGD-FLAEGGGVR) was identified as the amino terminal fragment (2-16aa) of the fibrinogen α chain, which was named as fibrinostatin.¹⁰ Compared with FpA, fibrinostatin loses the first alanine from the amino terminus of FpA, which could be a result of the cleavage by exoprotease, because differential exoprotease activities acting on the ex vivo coagulation and complement–degradation pathways could generate cancer type-specific serum peptides.¹¹ We and other groups have found that fibrinostatin is differentially present in the sera of patients in a variety of cancers: higher in gastric, ovarian, hepatocellular and urothelial cancers,¹²–¹⁵ lower in prostate, bladder and thyroid cancers,¹¹,¹⁶ but no difference in breast cancer patients when compared with healthy controls.¹¹ Based on
the endothelial cells were maintained in DMEM (10% FBS) at 37°C in 5% CO2. The amino acid sequences are: DSGEGD\(\text{-}\)LAEGGGVR (fibrinostatin) and GRKKRRQRRRGY (Suzhou, China). The mice were randomly divided into 10 groups and administered with PBS control or 1, 2.5 or 5 mg fibrinostatin for 24 h, then washed twice and fixed in 0.4% methanol at –20°C for 16 h. After staining with the propidium iodide (PI) solution, samples were then analyzed using a FACSAria flow cytometry system (BD Biosciences, San Jose, CA, USA).

DNA ladder assay. Cells were treated with PBS or fibrinostatin (25 μg/mL) for 72 h, then washed twice with ice-cold TBS (pH 7.6) and lysed with lysis buffer (10 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% Triton-100, 10 mM NaCl and KCl, 0.5 mg/mL proteinase K) for 10 min. The 300 μL of saturated hydroxybenzene and chloroform was added for genomic DNA extraction by centrifugation of 12,000 g for 10 min, and the supernatant was collected and two volumes of ethanol with 10% 3 mol/L NaAc was added to precipitate DNA. 20 μL H2O with 1 mg/mL RNase A was used to digest DNA, and the DNA ladder were analyzed in 1.2% agarose gel.

TUNEL assay. Cells were seeded on glass coverslips in 24-well plates. After treatment with fibrinostatin (25 μg/mL) for 72 h, TUNEL assay was performed with a kit from Roche according to the manufacturer’s instructions and observed under a TCS-SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany).

**Fluorescence visualization for entry of peptides.** To assay the entries of FITC-labeled peptides, HUVEC, BGC-823, PG and HT-29 cells were incubated with FITC-conjugated fibrinostatin (25 μg/mL) or TAT (25 μg/mL) for 3 h. The cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% NP-40 in PBS. The dye 4,6-diamidino-2-phenylindole (DAPI, 5 ng/mL) was added to stain nuclei, washed three times with PBS, and observed under a TCS-SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany).

**Cell migration assay.** HUVEC (5 × 104 cells) pretreated with PBS control or 25 μg/mL fibrinostatin for 24 h were plated in the upper chamber of Transwell inserts with 8-μm pores, and the lower chamber contained RPMI-1640, 10% FBS (to control the HUVEC growth) with PBS or fibrinostatin (25 μg/mL). The cells were allowed to migrate for 24 h at 37°C. The mean number of cells per field that migrated across the filter was calculated based on the cell number counted in four fields at ×200 magnification per insert. Each condition was performed in triplicate.

**Cell adhesion assay.** Six-well plates were coated with 200 μL/well of Matrigel (1 mg/mL; BD Biosciences) overnight. The plates were blocked with 500 μL/well of 100 μg/mL BSA for 2 h. Then 1 mL of the HUVEC suspension (2 × 104 cells/mL) pretreated with PBS control or 25 μg/mL fibrinostatin for 24 h, was added to each well and incubated for 2 h at 37°C. After washing twice with PBS, the number of attached cells was counted after methylene blue staining at ×200 magnification. Mean ± SD attached cells was calculated based on four fields per insert. Each condition was performed in triplicate.

**Tubule formation assay.** A total of 200 μL Matrigel (1 mg/mL) was added to each well of a 24-well plate and allowed to polymerized for 20 min at 37°C. A suspension of HUVEC...
Fibrinostatin induces necrosis and decreases microvascular density in tumors. To explore the effects of fibrinostatin on tumor growth and tumor angiogenesis, we dissected and analyzed all the xenografts from the mice by histological H&E staining. We observed necrotic areas in the fibrinostatin-treated tumors, in which more lymphocyte and erythrocyte exudation and some necrotic tumor cells were identified, with significantly less areas of necrosis in the control tumors (Fig. 2a). We then assessed the microvascular density (MVD) by immunostaining endothelial cells with an anti-CD31 antibody and calculated MVD as a percentage of tumor section area using the Chalkley grid counting method.\(^{(24)}\) We found that the MVD in the fibrinostatin-treated group was significantly decreased compared with the control group \((P < 0.05)\) (Fig. 2b). These results suggest that the fibrinostatin’s anti-tumor activity is associated with inhibition of tumor angiogenesis.

Effects of fibrinostatin on angiogenesis in zebrafish. The anti-angiogenic effect of fibrinostatin was further confirmed using a transgenic zebrafish model; zebrafish express enhanced green fluorescent protein (EGFP) in their endothelial cells. Observations demonstrated that both fibrinostatin and Endostar (as a positive control) inhibited the development of subintestinal vein vessels and had no toxicity to zebrafish. Fibrinostatin was administrated at 333.5, 1110 and 3335 ng and the inhibition rates of angiogenesis were 17.6 ± 3.0%, 23.9 ± 3.1% and 33.7 ± 5.0%, respectively (Fig. 2d left and middle panel). When micro-injected with 3335 ng of fibrinostatin, the number of subintestinal vascular vessels was decreased to 3–4, which was less than that of the control group (7–8; Fig. 2d right panel).

Fibrinostatin inhibits endothelial cell migration, adhesion and tubule formation. The above results suggest that fibrinostatin may act on endothelial cells. To investigate this possibility, we isolated HUVEC from the vein of human umbilical cords and tested the effect of fibrinostatin on HUVEC. Migration and adhesion of HUVEC with or without treatment of fibrinostatin was significantly reduced compared with that of the control cells (mean 89 ± 36 cells/ chamber vs mean 265 ± 82 cells/ chamber, \(P < 0.05\); Fig. 3a).

We then coated the 24-well plate with Matrigel and examined the adhesion of HUVEC with or without treatment of fibrinostatin by counting the number of cells attached on the Matrigel membrane. Our data showed an approximate 50% reduction of HUVEC adhesion by fibrinostatin treatment compared with that of the control cells (mean 96 ± 39 vs mean 186 ± 72, \(P < 0.05\); Fig. 3b).

Matrigel tubule formation assay was used as an in vitro model to study the effect of fibrinostatin. HUVEC were pre-treated with fibrinostatin and angiogenesis was assessed after an 18-h incubation period on Matrigel. Untreated cells formed a branching and anastomosing network of capillary-like tubules with multi-centric junctions, whereas the fibrinostatin-treated cells had reduced branching points, tubule number and length. HUVEC treated with 50 \(\mu\)g/mL of fibrinostatin grew into complete single cells and no capillary-like tubule formed (Fig. 3c).

The fibrinostatin-induced inhibition of tubule formation was further confirmed by the rat aortic ring assay. After culturing the aortic rings for 5 days, the tubules grown from the rings were observed in the control group, but the tubule formation was inhibited in the fibrinostatin-treated group in a dose-de-
Fibrinostatin inhibits tumor growth in vivo in mouse xenograft models. Fibrinostatin inhibited tumor growth in BGC-823 gastric cancer cells (a), HT-29 colon cancer cells (b) and PG lung cancer cells (c) bearing BALB/c nude mice. Cyclophosphamide (CTX), a chemotherapeutic agent, was used as a positive control. Bars represent mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

Fibrinostatin inhibits tumor growth in a dosedependent manner. At the dosage of 50 μg/mL fibrinostatin, complete abrogation of capillary growth was observed, whereas the tubule structure was formed even far from the ring in the control (Fig. 3d).

Fibrinostatin has no effect on vascular endothelial growth factor signal pathway. Because VEGF and its receptors play key roles in angiogenesis, we further tested whether fibrinostatin interferes with VEGF signal pathway. The phosphorylations of VEGFR1, VEGFR2, AKT and ERK were increased when treated with VEGF (10 ng/mL) for 15 min. However, pretreatment with fibrinostatin had no inhibitory effect (Fig. 3e). These results reflected that fibrinostatin’s effect is independent of VEGF signal pathway.

Fibrinostatin inhibits the proliferation of endothelial cells but not of cancer cells, possibly by induction of apoptosis. To clarify the possible mechanisms of fibrinostatin on angiogenesis, we tested the effect of fibrinostatin on HUVEC proliferation by MTT assay. We found that fibrinostatin significantly inhibited
HUVEC proliferation in a concentration-dependent manner (10–50 μg/mL). At 20 μg/mL concentration, fibrinostatin almost reached the plateau of inhibition ($P < 0.05$) (Fig. 4a). In contrast, fibrinostatin had no distinguishable effects on proliferations of BGC-823, PG and HT-29 cancer cells. We also tested the inhibitory effect of fibrinostatin on HMEC.
fibroblasts WI-38 cells and HEK-293 cells. We found that fibrinostatin did not inhibit the proliferation of fibroblasts WI-38 cells or HEK-293 cells, but could inhibit the growth of HMEC (Fig. S1). These results demonstrated that fibrinostatin has a specific inhibitory effect on endothelial cells, but not on cancerous or other untransformed cells. By flow cytometry...
Fibrinostatin inhibits proliferation and induces apoptosis of endothelial cells specifically. (a) The effects of fibrinostatin on the proliferation of HUVEC, BGC-823, PG and HT-29 cells were determined with an MTT assay. *P < 0.05, #P > 0.05, ANOVA. (b) Flow cytometry analysis of cells treated with PBS or fibrinostatin and stained with propidium iodide. The percentage of the sub-G1 cells is presented. (c) DNA ladder analysis of cells treated with PBS or 25 μg/mL fibrinostatin.
In analysis, we further found that in comparison with the control cells, fibrinostatin treatment resulted in almost 10-fold increase in the percentage of sub-G1 cell, but had no obvious effect on those of cancer cells (Fig. 4b), indicating that fibrinostatin inhibits HUVEC proliferation by inducing apoptosis. In addition, we found that significantly more DNA fragmentation was generated in fibrinostatin-treated HUVEC than in controls by the DNA Ladder assay (Fig. 4c).

TUNEL assay was further used to confirm these findings. Consistent with flow cytometry analysis and DNA ladder results, fibrinostatin treatment in HUVEC caused 20.51 ± 6.62% positive staining, while almost no positive staining was observed in cancer cell groups (Fig. 5a,b). By Western blot analysis, we found that fibrinostatin upregulated p53 and Bax levels in HUVEC (Fig. 5c).

Fibrinostatin enters endothelial cells but not cancer cells. To investigate why fibrinostatin inhibited the proliferation of endothelial cells specifically, we labeled fibrinostatin with a fluorescence dye FITC and stained the HUVEC and three cancer cell lines. To our surprise, we observed intracellular localization of FITC-fibrinostatin in HUVEC cells, but not in cancer cells (Fig. 6). However, FITC-labeled TAT peptide derived from the transactivator of transcription of human immunodeficiency virus, which is well known as a cell-penetrating peptide, entered both HUVEC and the different cancer cells. These findings further support the endothelial cell-specific functions of fibrinostatin, and the mechanism is under investigated.

Identification of key inhibitory sequence of fibrinostatin. We further synthesized a series of deleted peptides of fibrinostatin and tested their inhibitory effect on tumor growth in a H22 allograft model to identify the key inhibitory motif. We found that three amino acid deletions in the amino or carboxyl terminus did not affect its anti-tumor activity. However, more amino acid deletions in the N-terminal or the C-terminal strongly decreased its anti-tumor activity (Fig. 7a).
results suggested that the six amino acids (DFLAEG) of fibrinostatin comprised the key inhibitory sequence. In addition, we performed tubule formation and migration assays to define the effect of these deleted peptides. Consistent with \textit{in vivo} experiment results, peptides C3d, N3d, N6d and N5C4d also inhibit tubule formation and migration of HUVEC \textit{in vitro} (Fig. 7b,c).

**Discussion**

The hemostatic system has long been shown to regulate angiogenesis involving various hemostatic precursor proteins and cleavage of these proteins by proteolysis, which can generate novel cryptic fragments as either positive or negative factors to control the rate of angiogenesis.\(^{(5,26,27)}\) Some cryptic fragments can antagonize angiogenesis, such as Domain 5 of kininogen, fragment-1 and -2 of prothrombin and cleaved AT-III (serpin antithrombin).\(^{(28–30)}\) Fibrinostatin detected in the serum is the amino terminal fragment (2–16 aa) derived from the precusor fibrinogen \(\alpha\) chain,\(^{(10)}\) differing from FpA in its loss of the first alanine at the amino terminus. Previous studies showed that a linear form of FpA was inactive \textit{in vitro}, but the first 24 amino terminal amino acids (ADSGEGDFLAEGGGVR) of fibrinostatin (DSGEGDFLAEGGGVRGPRVVERH) of the fibrinogen \(\alpha\) chain (named alphastatin) tested as a synthetic peptide showed anti-angiogenesis activity.\(^{(31)}\) Neither the FpA portion (1-16aa) (ADSGEGDFLAEGGGVR) nor the 17–24 amino acids (GPRVVERH) of alphastatin could mimic the inhibitory effect of alphastatin.\(^{(31)}\) In alphastatin, a fragment of 11 amino acids (DFLAEGGGVRG) was identified to be a key sequence containing the activity (termed AHN419).\(^{(32)}\) In our study, we synthesized a series of deleted peptides of fibrinostatin and tested their inhibition of tumor growth in mice. We found that the six amino acids (DFLAEG) of fibrinostatin (DSGEGDFLAEGGGVR) comprised the key inhibitory sequence of fibrinostatin, which was included in AHN419. These results suggest that the addition of extra amino acids at either the amino or the carboxyl terminus of the key sequence (DFLAEG) may contribute to the conformation or stability of the core sequence that subsequently contributes to the biological activity of the key sequence. We speculate that a proper structure may be required for the interaction and stabilization of binding of fibrinostatin with its cellular target protein.

VEGF and its receptors play important roles in tumor angiogenesis.\(^{(33)}\) However, we did not find fibrinostatin to interfere with the VEGF signal pathway. Together with fluorescence visualization results, we propose that fibrinostatin binds to its target protein in cytoplasm and induces apoptosis. Increased p53 and Bax expressions were observed when treated with fibrinostatin, but the mechanism underlying these two molecules’ upregulation and their contributions to fibrinostatin-induced apoptosis remain to be determined. The target of fibrinostatin and the mechanism of fibrinostatin’s specific effect on endothelial cells also deserve further investigation.

Taken together, our study provides evidence for development of a new therapeutic candidate, fibrinostatin, as an anti-angiogenesis inhibitor targeting endothelial cells. Fibrinostatin blocks angiogenesis by inhibiting endothelial cell proliferation, adhesion, migration and tubule formation. We have also demonstrated the anti-tumor effects of fibrinostatin on three cancer types (gastric cancer, lung cancer and colon cancer) in mouse xenograft models, supporting the notion that fibrinostatin may have a broad spectrum of anti-tumor/anti-angiogenesis activities. The toxicity studies demonstrated the good safety profiles in rats and monkeys. These findings support further

**Fig. 6.** Fibrinostatin enters endothelial cell specifically. Cells were incubated with 25 \(\mu\)g/mL FITC-conjugated fibrinostatin (green) or TAT (green, positive control), fixed, stained with DAPI (blue), and observed under a confocal microscope. Scale bar = 25 \(\mu\)m.
| Sequence of deletion peptide | Treatment | Inhibition % ± Standard Deviation |
|------------------------------|-----------|----------------------------------|
| D S G E G D F L A E G G G V R | Control   | 89.9 ± 4.3%                      |
| E G D F L A E G G G V R       | CTX       | 65.4 ± 10.8%                     |
| D S G E D F L A E G G G V R   | N3d       | 63.3 ± 15.1%                     |
| D S G E D F L A E G G G V     | C3d       | 57.6 ± 12.9%                     |
| D F L A E G                     | N5C4d     | 54.9 ± 7.4%                      |
| E G G G V R                   | N6d       | 51.3 ± 18.7%                     |
| D S G E D                      | C9d       | 34.5 ± 22.3%                     |
| E G G G V R                   | N9d       | 34.5 ± 20.8%                     |
| D S G E D F L A               | C6d       | 32.3 ± 29.5%                     |

Fig. 7. Identification of key sequence of fibrinastatin. (a) The inhibitory effects of truncated peptides on tumor growth were assessed in murine H22 allografts. Cyclophosphamide (CTX) was used as a positive control. Inhibition rates were calculated compared with the control group. The inhibitory effects of fibrinastatin deleted peptides on HUVEC tubule formation (b) and migration (c) are presented; mean ± SD *P < 0.05; **P < 0.01; ***P < 0.001 compared with the control group (Student’s t-test).
Fibrinostatin inhibits proliferation of HMEC, but not WI-38 or HEK-293.

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Disclosure Statement

The authors declare no competing financial interests.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Fibrinostatin inhibits proliferation of HMEC, but not WI-38 or HEK-293.