Therapeutic Efficacy of Endostatin Exhibits a Biphasic Dose-Response Curve

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Abstract

We show here that recombinant endostatin protein has a biphasic effect on the inhibition of endothelial cell migration in vitro. In tumor-bearing animals, there is a similar biphasic effect on the inhibition of tumor growth and on circulating endothelial cells after once-daily s.c. injections. This biphasic effect is revealed as a U-shaped curve in which efficacy is optimal between very low and very high doses depending on the tumor type. This result may be applicable to other inhibitors of endothelial growth and to angiogenesis. Furthermore, these results have important implications for clinicians who administer angiogenesis inhibitors for cancer or other angiogenesis-dependent diseases. When these results are taken together with two previous reports of angiogenesis inhibitors with a U-shaped dose-response, they suggest that other regulators of endothelial growth may display a similar pattern. (Cancer Res 2005; 65(23): 11044-50)

Introduction

Tumor growth is angiogenesis-dependent (1). In order to stimulate angiogenesis, tumors up-regulate the production of a variety of angiogenic factors, including the fibroblast growth factors [acidic and basic fibroblast growth factor (bFGF)] and vascular endothelial cell growth factor (VEGF)/vascular permeability factor (1). However, some tumors also generate angiogenesis inhibitors. It is becoming more apparent that the angiogenic phenotype is the result of a net balance between positive and negative regulators of angiogenesis (2-4). A new class of drugs called angiogenesis inhibitors can suppress angiogenesis and therefore inhibit tumor growth, in part, by changing the balance of angiogenic activity in the tumor (5).

One of these inhibitors is endostatin, a 20 kDa COOH-terminal proteolytic fragment (183 amino acid) of collagen XVIII (6). Endostatin is a specific endogenous inhibitor of endothelial cell proliferation, migration (7), and vascular permeability (8). Although it has been shown that endostatin interacts with several receptors and pathways such as α5β1 integrin (9), cell surface glypicans (10), c-myc (11), cyclin-D1 (12), and VEGF signaling pathways (13, 14), additional mechanisms of action are being described (15, 16).

We show here that the in vitro and in vivo activities of endostatin follow a biphasic dose-response curve. The endostatin-induced inhibition of endothelial cell proliferation and migration in vitro increases proportionally with endostatin concentrations; however, further increases in dose result in reduced activity. Endostatin therapy of tumor-bearing mice reveals a similar pattern, i.e., increasing doses correlate with the increasing efficacy of tumor inhibition until an optimal dose is reached beyond which further dose increases result in less activity, a biphasic effect that has a U-shaped configuration.

Materials and Methods

Cell culture and reagents. Human pancreatic cancer cells, BxPC-3 and AsPC-1 (American Type Culture Collection, Rockville, MD), were grown in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Life Technologies) and 1% glutamine-penicillin-streptomycin (Irvine Scientific, Santa Ana, CA). Cells were maintained in T-75 tissue culture flasks (BD Falcon, Becton Dickinson, Franklin Lakes, NJ) or in 60 cm2 roller bottles and grown in 5% CO2/95% air at 37°C.

Cell proliferation assay. Human pancreatic cancer cell lines (BxPC-3 and AsPC-1) were maintained as described above. For the proliferation assay, cells were washed with PBS and dispersed in a 0.05% trypsin/EDTA solution. A cell suspension (10,000 cells/mL) was added to the bottom wells with or without VEGF (3 ng/mL; R&D Systems, Bad Nauheim, Germany) diluted in ECG Medium with 0.05% gelatin and cells were incubated for 6 hours at 37°C. Cells were washed once with PBS and the cells that had not migrated were bound to the bottom of the tissue culture inserts were incubated for 1.5 hours in 400 μL of a 1% Triton X-100 solution at 37°C. Medium (300 μL) was added to the bottom wells with or without VEGF (3 ng/mL; R&D Systems, Bad Nauheim, Germany) diluted in ECG Medium with 0.05% gelatin and cells were incubated for 6 hours at 37°C. Cells were washed once with PBS and the cells that had not migrated were removed from the top membrane by scraping with a cotton swab. Cells that had migrated were quantified using a colorimetric assay as follows: cells bound to the bottom of the tissue culture inserts were incubated for 1.5 hours in 400 μL of acid phosphatase substrate [10 mmol/L p-nitrophenol phosphate, 10 mmol/L sodium acetate, and 0.1% Triton X-100].
X-100 (pH 5.8) at 37 °C. The reaction was then quenched with 100 μL of 1 N NaOH, and the absorbance of the solution was read at 405 nm. Data (n = 3) were calculated as the percentage of inhibition compared with the difference of negative control (without VEGF) subtracted from positive control (stimulated with 3 ng/mL VEGF).

Animal studies. All animal work was done in the animal facility at the Children’s Hospital, Boston, MA in accordance with federal, local, and institutional guidelines. Male severe combined immunodeficiency (SCID) mice (Massachusetts General Hospital, Boston, MA) 6 to 8 weeks old (22-23 g) were used. They were acclimated, caged in groups of five in a barrier care facility, and fed with animal chow and water ad libitum. Animals were anesthetized via inhalation of isoflurane (Baxter, Deerfield, IL) before all surgical procedures, and were observed until they had fully recovered. At the end of each experiment, animals were euthanized by a lethal dose of carbon dioxide asphyxiation.

Tumor cell implantation and measurement. Before tumor cell injection, mice were shaved and the dorsal skin was cleaned with ethanol. Tumor cells were grown in cell culture as described above. A tumor cell suspension (BxPC-3 or AsPC-1) of 4.0 × 10^6 cells in 0.2 mL RPMI 1640 was injected into the s.c. dorsa of mice in the proximal midline. The mice were weighed and tumors were measured every third to fifth day in two diameters with a dial-caliper and the tumor volume was determined using the formula a^2 × b × 0.52 (a = shortest, b = longest diameter). The observers were masked to the identity of the mice. At the end of each experiment, the mice were sacrificed in accordance with institutional guidelines and the resected tumors were weighed and fixed in buffered Formaldehyde-Fresh (Fisher Scientific, Fair Lawn, NJ). During the whole experiment, the room temperature was recorded.

Treatment of tumor-bearing mice with human endostatin. When the tumor volume was 90 to 110 mm^3, mice were randomized into six groups for BxPC-3-bearing mice (n = 7/group) and five groups for AsPC-1-bearing mice (n = 7/group). Endostatin treatment was done by single bolus s.c. injections for BxPC-3 tumor–bearing mice (50, 100, 250, 500, and 1,000 mg/kg/d) and single bolus s.c. injections for AsPC-1 tumor–bearing mice (100, 250, 500, and 1,000 mg/kg/d). The control groups for both experiments received comparable bolus injections of vehicle (s.c.). The s.c. injections for BxPC-3 tumor–bearing mice (50, 100, 250, 500, and 1,000 mg/kg/d) and single bolus s.c. injections for AsPC-1 tumor–bearing mice (100, 250, 500, and 1,000 mg/kg/d). The control groups for both experiments were filled with endostatin using a 20-gauge 1.5-inch needle (BD Microlance L). Injection (s.c.) of endostatin was immediately (0-4 °C) in an ice-bath until application. Tuberculin syringes were filled with endostatin using a 20-gauge 1.5-inch needle (BD Microlance L, Becton Dickinson, Franklin Lakes, NJ). Injection (s.c.) of endostatin was done using a 30-gauge 1.5-inch needle (BD Microlance L). During the entire experiment, the room temperature in the animal facility was maintained at −24 °C; the optimum room temperature for mice. At lower temperatures, mice become cold. They then conserve heat by vasconstriction of skin vessels. This may reduce blood flow to a s.c. tumor and also reduce absorption of drugs injected s.c.7

Flow cytometry. Circulating endothelial cells (CEC) in peripheral blood were evaluated using three-color flow cytometry as previously described (17). Briefly, venous blood was obtained from the retro-orbital plexus and red cell lysis was done using ammonium chloride lysis buffer. Cells were then incubated with antibodies against murine CD45 conjugated to FITC (fluorescein) and Flk-1 conjugated to phytoerythrin (both from Becton Dickinson). 7-Aminoactinomycin D was used to assess viability and was purchased from Sigma (St. Louis, MO). Flow cytometry was done using a FACScalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) with analysis gates designed to remove platelets and cellular debris. For each mouse, 50,000 to 100,000 events were typically counted. Mouse blood added to hemangioendothelioma (EOMA) cells expressing Flk-1 was used as a positive control.

Statistics. All data (tumor volumes) were expressed as the mean ± SD or SE. Assuming that the data was not normally distributed (non-Gaussian application of endostatin and was centrifuged at 3,000 rpm for 10 minutes. Serum was carefully separated and stored at −70 °C. Serum endostatin levels were measured by competitive enzyme immunoassay (Accucyte Human Endostatin, Cytimmune Sciences, Rockville, MD) according to the manufacturer’s recommendations. The minimum detection limit for this endostatin kit is 1.95 ng/mL.

Endostatin. Clinical grade soluble human recombinant endostatin was a generous gift from EntreMed Corporation (Rockville, MD). The recombinant protein was formulated in potassium sucrose octasulfate to a concentration of 130 mg/mL, lyophilized, and stored at 4 °C. The lyophilized protein was diluted in double-distilled water in a first step, vortexed for 30 minutes until completely resolved, followed by further dilutions using PBS to prepare a dilution for the needed dosages. The prepared solutions were cooled immediately (0-4 °C) in an ice-bath until application. Tuberculin syringes were filled with endostatin using a 20-gauge 1.5-inch needle (BD Microlance L, Becton Dickinson, Franklin Lakes, NJ). Injection (s.c.) of endostatin was done using a 30-gauge 1.5-inch needle (BD Microlance L). During the entire experiment, the room temperature in the animal facility was maintained at −24 °C; the optimum room temperature for mice. At lower temperatures, mice become cold. They then conserve heat by vasconstriction of skin vessels. This may reduce blood flow to a s.c. tumor and also reduce absorption of drugs injected s.c.7

Figure 2. Tumor growth under endostatin therapy in BxPC-3 tumor–bearing mice. Mice were treated over a treatment period of 20 days (group size, n = 7). Tumors were measured every 3 to 5 days. PBS-control (●), 1,000 mg/kg/d (▲), 50 mg/kg/d (○), 500 mg/kg/d (△), 250 mg/kg/d (□), and 100 mg/kg/d (△). T/C, tumor volumes of treated versus control groups; *, P < 0.001, significantly different compared with the control group.
Results

Efficacy of different dosages of recombinant endostatin in vivo. In all experiments, the initial range of the tumor volume was 90 to 110 mm³, 12 to 14 days after implantation. At the end of treatment (20 days), the tumor volume in the control group for BxPC-3, a slow-growing tumor, was 978 ± 214 mm³ (Fig. 1). Tumor volumes of 519 ± 134, 152 ± 23, 297 ± 81, 480 ± 98, and 606 ± 110 mm³ were observed in the endostatin-treated group with 50, 100, 250, 500, and 1,000 mg/kg/d, respectively. All of the tumor volumes in the treated groups were significantly different (P < 0.001) compared with the control group (Fig. 1). The treatment/control ratio decreased progressively during the treatment and, at day 20 was 0.53, 0.16, 0.30, 0.49, and 0.62 for endostatin at dosages of 50, 100, 250, 500, and 1,000 mg/kg/d, respectively. These results represent a 4-fold difference between the 100 and 1,000 mg/kg/d groups. Tumors in mice that were treated with endostatin with 100 mg/kg/d exhibited little or no growth over the course of the treatment compared with the other groups (Fig. 2).

In the second experiment, mice were inoculated with AsPC-1, a fast-growing pancreatic tumor. These mice were also treated with endostatin at different dosages (100, 250, 500, and 1,000 mg/kg/d; Fig. 3). At the end of the treatment, the tumor volume in the control group was 1,425 ± 180 mm³. This experiment had to be terminated after 16 days due to the large tumor sizes in the control group. Tumor volumes of 547 ± 83, 201 ± 40, 110 ± 26, and 520 ± 26 mm³ were observed in the endostatin-treated groups with 100, 250, 500, and 1,000 mg/kg/d, respectively. All of the tumor volumes in the treated groups were significantly different (P < 0.001) compared with the control group (Fig. 3). The treatment/control ratio decreased progressively during the treatment and at day 16 was 0.38, 0.14, 0.08, and 0.36 for endostatin at dosages of 100, 250, 500, and 1,000 mg/kg/d, respectively.

Serum endostatin levels in tumor-bearing mice. To obtain information about circulating (systemic) serum endostatin levels under daily s.c. injections of human endostatin in SCID mice in our experiment, blood was drawn at the end of each experiment (24 hours after the last injection of endostatin). Maximum serum endostatin levels in the 1,000 mg/kg/d dose group were 190 ± 20.4 ng/mL for the AsPC-1 group and 144 ± 19.9 ng/mL for the BxPC-3. The endostatin levels in untreated controls were 4.8 ± 2.4 ng/mL for AsPC-1 and 8.6 ± 3.4 ng/mL for BxPC-3 (Fig. 5). For BxPC-3, at the maximally effective dose of 100 mg/kg/d, serum endostatin was 9.14 ± 4.6 ng/mL. For AsPC-1, at the maximally effective dose of 500 mg/kg/d, the serum concentration was 62.2 ± 22.9 ng/mL (Fig. 5).

In vivo proliferation of BxPC-3 and AsPC-1 tumors in severe combined immunodeficiency mice. In order to investigate the in vivo proliferation of both tumor cell lines used, the tumor volumes in BxPC-3- and AsPC-1-bearing mice in the control groups were determined at day 16 after the start of the experiment. This time point was chosen because the tumor volumes in AsPC-1-bearing mice reached 1,425 ± 180 mm³ and the experiment was terminated for this group. Comparison of the tumor volumes at that time point revealed a significantly faster (2-fold) in vivo tumor growth for AsPC-1 (1,425 ± 180 mm³) compared with BxPC-3 (701 ± 53 mm³) bearing mice (P < 0.001; see Fig. 6).

In vitro proliferation rate of pancreatic tumor cells (BxPC-3 and AsPC-1). To explain the observed differences in the in vivo tumor growth (see above) and to determine in vitro differences of tumor cell proliferation, proliferation assays using BxPC-3 and AsPC-1 tumor cells were done (see above). The in vitro proliferation rate over 4 days for the two pancreatic tumor cell lines was not significantly different (Fig. 7).
In vitro inhibition of endothelial cell migration by endostatin. It has been previously shown that endostatin inhibits endothelial cell migration (18–20). Because clinical grade endostatin was used in our experiment, we tested this endostatin formulation for antiendothelial cell migration activity. Antiendothelial cell migration activity was determined in a VEGF-stimulated (3 ng/mL) migration assay with HUVEC using several endostatin concentrations (0.03-20 μg/mL). Endostatin inhibits the migration of VEGF-stimulated (3 ng/mL) HUVEC in a dose-dependent manner (0.03-20 μg/mL endostatin) following a bell-shaped curve of inhibitory activity of endostatin (Fig. 8). The maximum inhibitory effect was obtained using 0.2 μg/mL of endostatin. Lower and higher concentrations led to a decrease in inhibition activity. These results were consistent with our previous publications.

Inhibition of circulating endothelial cells by endostatin. Previous studies have suggested that CECs may be a useful marker for the biological activity of endostatin in murine models of cancer (21) and in patients with pancreatic neuroendocrine tumors (22). We have also recently established that endostatin inhibits the VEGF-induced mobilization of CECs (17). To investigate the correlation between changes in CECs and the antitumor efficacy of endostatin, blood was drawn from the retro-orbital plexus at the time of sacrifice and CECs were measured as previously described (17). At a dose of 100 to 500 mg/kg/d, a 50% to 60% reduction in CECs was observed as compared with vehicle controls. At the highest dose of 1,000 mg/kg/d, however, an increase in CECs was observed (Fig. 9).

Discussion
Cancer is an angiogenesis-dependent disease. Angiogenesis inhibitors have been shown to inhibit tumor growth in numerous animal models and in patients. In fact, recently, Avastin was the first angiogenesis inhibitor to receive Food and Drug Administration approval to treat advanced colon cancer (23). Throughout the history of chemotherapy, the maximum tolerated dose was always considered the most effective dose. However, because of the relative lack of toxicity of antiangiogenic therapy, the maximum tolerated dose is no longer useful. For antiangiogenic therapy, effective dosing requires a knowledge of whether or not the dose-response curve is linear and also requires a surrogate marker.

In this report, we show that human endostatin inhibits tumor growth in mice not in a linear relationship, but in a biphasic dose-response curve, which is U-shaped. For BxPC-3, optimal inhibition of tumor growth was obtained at a dosage of 100 mg/kg/d by s.c. bolus injection. In contrast, for AsPC-1 (human pancreatic cancer), the optimal inhibition of tumor growth was obtained at 500 mg/kg/d s.c. bolus injection. For both tumors, higher and lower dosages of endostatin showed a less inhibitory effect. For both tumors, the therapeutic efficacy of endostatin was represented by a U-shaped curve, but for the faster-growing tumor (AsPC-1), a proportionally higher dose of endostatin was required.

AsPC-1 tumors exhibited an increase in volume ~2-fold faster compared with BxPC-3 tumors (1,425 ± 180 versus 701 ± 53 mm³ after 16 days; Fig. 6). It is unlikely that this difference in growth rate of tumor mass is due to the proliferation rate of tumor cells because both AsPC-1 and BxPC-3 have previously been shown to have an average proliferating cell nuclear antigen rate of 60% (3). In addition, we showed that the in vitro proliferation rates of BxPC-3 and AsPC-1 were not statistically different (see Fig. 7).

However, the contrast in growth rate may be due to the angiogenic phenotype of each tumor. The microvessel density of AsPC-1 is ~160 vessels/hpf compared with BxPC-3 of 120 vessels/hpf (3). In both tumors, the production of the proangiogenic factors, VEGF and bFGF, did not differ significantly (BxPC-3: VEGF, 6,300 pg/mL; bFGF, 6.2 pg/mL; AsPC-1: VEGF, 5,219 pg/mL; bFGF, 5.6 pg/mL; ref. 24). On the other hand, it has been shown that BxPC-3 tumors generate at least four endogenous angiogenesis inhibitors (angiostatin, ref. 24), cleaved AT III (24), latent AT III (24), and DBP-maf (25) thereby suppressing other tumors in the double-sided tumor model (25). AsPC-1 tumors are not capable of inhibiting the growth of other tumors. At present, only the generation of angiostatin by AsPC-1 tumor cells has been reported, and it is unclear whether this tumor generates any other endogenous angiogenesis inhibitors (26). We therefore hypothesize that BxPC-3 has a greater capacity to produce negative regulators of angiogenesis.

Although tumor inhibition was dose-dependent, exhibiting maximum efficacy at intermediate endostatin doses, we found a linear correlation between increasing doses of endostatin and...
endostatin serum levels. It should be noted, however, that the samples were drawn 24 hours after the application of the last dose, which might have resulted in a slight underestimation of the true peak levels of endostatin (Fig. 5).

After these experiments were conducted, it was reported by others that platelets sequester endostatin (27). Therefore, in future clinical studies, it may be prudent to measure the serum and plasma levels of endostatin, especially because endothelial cells are exposed only to plasma levels.

It should be noted that human endostatin was used in these experiments, which may account for the relatively higher doses of endostatin required to inhibit tumor growth compared with our previous reports which employed murine endostatin (6, 19). This finding has potential clinical implications. It is conceivable that serum or plasma endostatin levels in a patient receiving high doses of endostatin (e.g., $\sim 250$ mg/m$^2$/d) for a prolonged period of time, could slowly increase over time, eventually reaching less effective endostatin levels. This possible scenario could be misinterpreted as “drug resistance.” In fact, our preliminary observations$^8$ suggest that either lowering the dose or temporarily discontinuing the administration of endostatin would lower endostatin serum levels to the levels that are optimally therapeutic based on murine studies (5, 19).

It is of interest that a genome-wide microarray analysis of endostatin treatment of human microvascular endothelial cells reveals that the expression of several genes which normally up-regulate endothelial proliferation, such as VEGF and hypoxia inducible factor-1a, are not simply down-regulated. Instead the down-regulation follows a $U$-shaped pattern whereby a low dose or short-time incubation with endostatin is more effective than a very high dose or prolonged incubation (16).

This finding also has implications for gene therapy. Some investigators reported that endostatin gene therapy was poorly effective against tumor growth in mice (28), despite the fact that serum levels reached >20,000 ng/mL. Based on the results of our study, the poor activity of endostatin which Eisterer et al. reported is most likely the result of the exceedingly high levels of circulating endostatin. Eisterer et al. also reported the failure of endostatin gene therapy in mice (29, 30), in which endostatin levels of 300 ng/mL were reported. They were operating at the high end of the $U$-shaped curve.

In contrast, Shi et al. reported highly effective tumor inhibition by endostatin gene therapy in mice in which the serum concentrations reached only 35 to 40 ng/mL (31). When these results are viewed in light of the effects of endostatin on $in vitro$ migration of endothelial cells and on tumor growth $in vivo$, endostatin seems to exert its biphasic effect directly on endothelial cells.

Effect of endostatin on circulating endothelial cells. Several recent studies have established that in both murine models and patients, changes in CECs are observed after treatment with endostatin (17, 21, 22). Consistent with these previous studies, we observed an $\sim 50\%$ reduction in CECs after treatment with endostatin doses which led to maximal antitumor effect (100-500 mg/kg/d) as compared with vehicle controls. Paradoxically, at a higher dose of 1,000 mg/kg/d, there was an increase in CECs compared with controls. There are several possible explanations for this observation. Recent evidence suggests that there are at least two distinct populations of endothelial cells detectable in the circulation: bone marrow–derived endothelial precursors, which are mobilized by VEGF and can contribute to neo-vascularization (32, 33), and endothelial cells shed from the preexisting vasculature (34, 35). We have recently established that endostatin inhibits the VEGF-induced mobilization of endothelial precursors in non–tumor-bearing mice. Preliminary evidence suggests, however, that antiangiogenic agents may cause an increase in the shedding of endothelial cells from the tumor vasculature (36, 37). Consistent with this hypothesis, a transient increase in phenotypically mature CECs is observed in endostatin-treated patients during the first month of therapy (22). It is possible that at higher doses of endostatin, an increase in endothelial shedding would be observed from established vasculature.

Endostatin is not the first drug to exhibit a $U$-shaped or “biphasic” concentration-response curve. IFN-$\alpha$, as reported by Slaton et al. (38), exhibits a $U$-shaped curve in tumor-bearing animals in which low doses of IFN-$\alpha$ are more effective at inhibiting tumor growth, and reducing bFGF serum levels and microvessel

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$^8$ Unpublished observations.
density (38). Furthermore, other inhibitors of endothelial cell migration and/or proliferation have recently been reported to display a biphasic or U-shaped dose response curve. These include rosiglitazone, a PPAR-γ ligand that inhibits angiogenesis and tumor growth (39), thrombospondin (40), angiostatin (41), endostatin (18, 42), a 27-amino acid peptide starting at the NH2 terminus endostatin (43), and rapamycin (44, 45). Furthermore, in a recently published report of a phase I dose-finding clinical study using recombinant human endostatin (15-600 mg/m²) in patients with solid tumors, biphasic (U-shaped) response curves were also determined by analyzing biomarkers (e.g., changes in microvessel density and tumor blood flow) to define an optimal biological dose for endostatin (46). These results are consistent with our findings of a biphasic (U-shaped) dose-response curve in vitro and in vivo for endostatin.

The mechanism(s) for endostatin’s activity, and its U-shaped dose-response curve, remain uncertain. Endostatin has been reported to bind to a number of different cell surface proteins, including integrin α(v)β3 (9, 15, 47), heparan sulfate proteoglycans, glypicans, and vascular endothelial growth factor receptor-2 (9, 10, 13, 48, 49). For ligands that induce receptor dimerization, a biphasic dose-response curve has also been observed with optimal receptor activation at intermediate doses, and self-antagonism occurring at higher doses at which monomeric ligand-receptor complexes predominate. A similar relationship may exist between endostatin and a key receptor. It is also possible that endostatin may signal through multiple pathways simultaneously, and that optimal output from this antiangiogenic signaling network (16) requires doses which only activate certain pathways.

Recent evidence shows that endostatin is sequestered in platelets (27). Therefore, it may be prudent to measure plasma and serum levels in patients because a fixed dose of endostatin may accumulate in platelets and then spill over into plasma. The clinical implications are that oncologists who employ any drug, which does not have a linear dose-response curve, need to pay careful attention to serum and or plasma levels.

Understanding the mechanism(s) by which endostatin exerts its activity should reveal additional insights into the observed dose-response relationship and provide important guidance for the clinical application of this agent as well as other angiogenesis inhibitors.

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