

The Role of Angiogenesis in Neuroendocrine Tumors

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KEYWORDS

- Angiogenesis • i-MTOR inhibitors
- Vascular endothelial growth factor (VEGF)
- Platelet-derived growth factor (PDGF)
- Human in vitro modeling • Somatostatin receptors

Angiogenesis is the process by which new capillaries develop from previously formed venules. Adult angiogenesis exists in a few normal (physiologic) settings, such as menstruation. Angiogenesis is rare in adults except in pathologic settings, such as rheumatoid arthritis. This size represents proliferative retinopathies, psoriasis, and tumor growth. Both benign and malignant tumors must develop their own blood supply to grow larger than 2 mm in size, the limits of effective simple diffusion of oxygen and nutrients and the removal of metabolic waste products.

The angiogenesis response is triggered by an “angiogenic switch” that occurs when the concentration or actions of proangiogenic agents exceeds those of antiangiogenic agents.¹ The concept of an angiogenic switch and its key role in the modulation of new blood vessel growth has led researchers to search for more than three decades for agents responsible for inducing and for inhibiting angiogenesis.

Somatostatin, or somatomedin release-inhibiting factor (SRIF), was originally described in 1968.² It is an endogenous peptide known for its ubiquitous inhibitory capacity on gastrointestinal function. SRIF inhibits gastrointestinal motility, amine release, peptide release, growth factor synthesis and release, and the secretion of a variety of fluids. Although this inhibitory capacity was attractive to clinicians wishing to inhibit bowel fluid secretion and to decrease bowel motility, SRIF’s short half-life limited the compound’s clinical usefulness. The limited half-life of the native peptide led to the development of longer lasting, more potent analogs or congeners of somatostatin. These novel peptides have been used since the 1980s in a variety of clinical situations, including limiting the peptide hypersecretion from tumors of

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gastroenteropancreatic axis.³ In the late 1980s, reports began to surface that somatostatin analogs could produce not only the relief of clinical symptoms due to amine or peptide excess but also an antitumor effect.⁴ Some researchers hypothesized that this antitumor effect was the result of inhibition of angiogenesis. This seemed like a logical theory considering the nearly universal inhibitory abilities of these somatostatin analogs. Fassler and colleagues⁵ were the first to test the antiangiogenic effects of octreotide. In 1988, they presented preliminary data supporting the antiangiogenic effects of octreotide acetate in a few chicken eggs using the chicken chorioallantoic membrane (CAM) model. These investigators demonstrated that octreotide acetate could inhibit blood vessel growth. This theory was further investigated by Woltering and colleagues⁶ using the CAM. The CAM is an assay popularized by Dr Judah Folkman,⁷ the father of the angiogenesis concept. In the CAM model, fertilized chick eggs are placed in a 37°C incubator. On day 2 or 3 of development, the embryos are removed from their shell and placed in a plastic wrap hammock and reincubated. On day 6 or 7, a methylcellulose disc containing a test substance is placed on the outer third of the CAM. The radius of the zone of inhibition of blood vessels is visually assessed 24 to 48 hours after disc implantation. Woltering and colleagues tested the angiogenic potential of both SMS 201-995 (octreotide) and RC-160 (vapreotide). Both somatostatin analogs inhibited angiogenesis, but RC-160 demonstrated a slightly higher percentage of eggs exhibiting inhibition of angiogenesis and a higher degree of overall growth inhibition. These effects were dose dependent. Another finding from these studies was that the degree of angiogenic inhibition was similar to that of the positive (inhibitory) control, a combination of heparin and a steroid, hydrocortisone 21-phosphate. The Folkman group used this steroid along with a heparin facilitator to inhibit capillary growth and had proposed this mixture as a potential chemotherapeutic.⁸ The observation that Folkman's steroid required the presence of heparin to inhibit capillary growth,⁹ whereas the somatostatin analogs inhibited angiogenesis without such a facilitator, suggested that the effect of somatostatin analogs may occur directly on the cell's membrane, acting through specific somatostatin receptors.

In an effort to better understand the ability of somatostatin to inhibit angiogenesis, Barrie and colleagues¹⁰ compared the angiogenic potential of native somatostatin-14 and eight novel somatostatin analogs in the CAM model. These investigators found that the inhibitory ability of these molecules varied greatly. This variation in potency depended on the structure of the analog and its specific amino acid sequence (**Fig. 1** and **Table 1**). This finding implied that certain analogs bound to specific somatostatin receptor subtypes (sst) with varying degrees of affinity. The most potent drugs in this study were cyclic octapeptides that retained a lysine in position 5 and a cysteine at positions 2 and 7 (forming a cysteine-cysteine bridge). The substitution of the position 5 lysine for ornithine, an amino acid not found in mammalian biosynthesis, rendered the analog biologically inactive. These data also suggested that a specific ligand/receptor interaction was required in order for somatostatin analogs to confer different biologic responses, including their antiangiogenic effects. Furthermore, these studies demonstrated that there was a direct correlation between an analog's effectiveness as antiangiogenic agent and its affinity for sst 2. Those analogs with better growth hormone inhibitory ability also bound to sst 2 with greater affinity and were more potent antiangiogenic agents.¹¹

In an attempt to determine the specific signal transduction mechanisms responsible for somatostatin-induced angiogenic inhibition, Patel and colleagues¹² tested octreotide alone and in combination with blockers of specific postreceptor signal transduction pathways.¹² These investigators found that octreotide's inhibition of angiogenesis was G-protein dependent and adenylate cyclase dependent. Octreotide was also

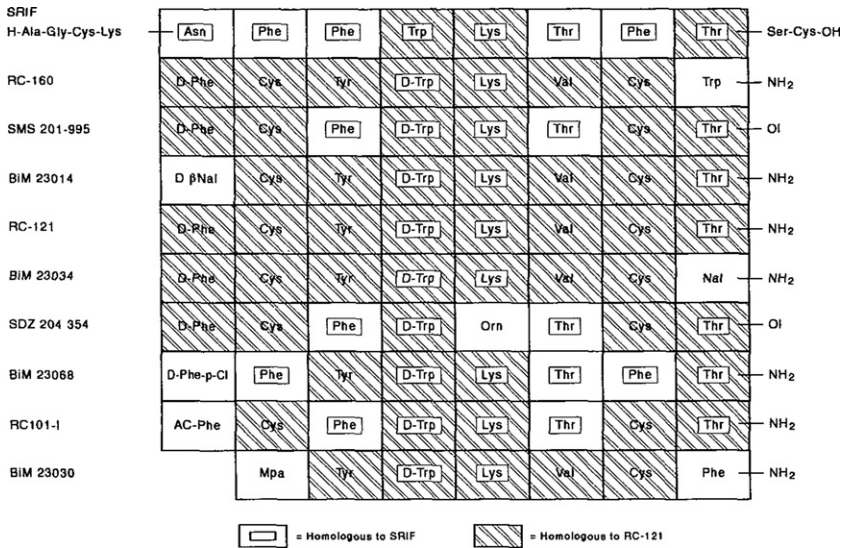


Fig. 1. Amino acid homology of peptide sequences of various somatostatin analogs with native somatostatin-14 (SRIF). (From Barrie R, Woltering EA, Hajarizadeh H, et al. Inhibition of angiogenesis by somatostatin and somatostatin-like compounds is structurally dependent. *J Surg Res* 1993;55(4):446; with permission.)

Test Substance	% Inhibition	RPR
SRIF	26	1
RC-160	68 ^{a,b}	2.6
SMS 201-995	61 ^{a,b}	2.3
BIM 23,014C	46 ^{a,b}	1.8
RC 121	35	1.3
BIM 23,034 ^c	25	1.0
SDZ 204-354	12	0.5
BIM 23,068	10	0.4
BIM 23,030	0	0
Pos. control	71	2.7
Lyoph buffer	14	0.5
Buffer	3	0-1

Relative potency ratios are the percentage of inhibition induced by an analogue divided by the percent inhibition of angiogenesis induced by native somatostatin. SMS 201-995 is octreotide acetate. The positive control is hydrocortisone 21-phosphate.

Abbreviations: Lyoph buffer, lyophilised buffer; NR, not reported; Pos. control, positive control; RPR, relative potency ratio; SRIF, somatostatin release inhibiting factor.

^a Different from SRIF ($P < .05$).

^b Different from buffer ($P < .05$).

^c Opaque disks.

Data from Woltering EA, Watson JC, Alperin-Lea RC, et al. Somatostatin analogs: angiogenesis inhibitors with novel mechanisms of action. *Invest New Drugs* 1997;15:77-86.

found to act along calcium-dependent pathways. Octreotide's angiogenic inhibitory potency was significantly decreased when this analog was combined with either bradykinin, an agent that drives calcium across the cell membrane, or extracellular hypercalcemia. Verapamil, an L-calcium channel blocker, was able to reverse the effects of bradykinin and hypercalcemia and to restore octreotide's ability to inhibit angiogenesis.

Unfortunately, the CAM model has significant limitations. The most obvious limitation of this model is that it uses animal, not human, tissues as its target. Additionally, the blood vessel response that is assessed in this growing chick embryo is more akin to vasculogenesis and embryogenesis than to true angiogenesis. To overcome the issue of embryogenesis, somatostatin analogs were tested against porcine endothelial cells and smooth muscle cells.¹³ Octreotide inhibited cell proliferation of both cell types. Danesi and colleagues¹⁴ demonstrated similar results using human umbilical vein endothelial cells (HUVECs). This group found that octreotide at 10^{-9} M reduced the proliferation of HUVECs by 45% versus untreated controls.

Some of the limitations of the CAM model were overcome with the development of a model that used full-thickness, 3-D, intact mammalian tissues. One of the first such models was a murine model of ex vivo angiogenesis developed by Nicosia and Ottinetti.¹⁵ Briefly, thoracic aortas were harvested from Fischer 344 male rats, sectioned into rings of 1-mm length, and placed into either fibrin or collagen gels. The investigators found that the aortic rings generated branching microvessels using serum-free media in both fibrin and collagen gels. These microvessels were inhibited by the addition of hydrocortisone and upregulated with the addition of medium conditioned with sarcoma 180 cells.¹⁵ Although this model overcame the issue of embryogenesis as was observed in the CAM, it was limited due to the use of animal tissues as its medium.

To counter the limitations imposed by animal-based cell or organ culture systems, Woltering and colleagues¹⁶ developed a unique assay that uses human tissues embedded in a fibrin-thrombin clot as a source of proliferating neovessels. Human placental vein discs are harvested and used as targets in a fibrin-thrombin clot assay to evaluate angiogenesis. These veins normally possess monolayers of quiescent endothelial cells in their intima. When the full-thickness sheets of vein are cut into small discs with a 2-mm skin punch, the mechanical trauma to the vein creates injury and a stressful milieu at the vessel's cut edge. Such an environment is sufficient to turn on the angiogenic switch, stimulating these previously quiescent endothelial cells to begin to proliferate in a manner similar to that which occurs in wounding in vivo. Angiogenic neovessels then proliferate from the cut edge of the vein disc (**Fig. 2**). These neovessel sprouts assays have lumens, and they interconnect and have all of the attributes of human capillaries. Transmission electron microscopy has been used to confirm that placental vein neovessel sprouts are endothelial in nature as they exhibit both Weibel-Palade bodies and tight junctions (**Fig. 3**).¹⁷ The endothelial nature of these sprouts has also been confirmed with immunohistochemical stains for factor VIII.¹⁶ Briefly, 2-mm discs of placental vein are placed into the thrombin-loaded wells and covered with a clot-forming nutrient medium containing fibrinogen. This mixture is incubated at 37°C, allowing a fibrin-thrombin clot to form. Once a tissue-containing clot is formed, control wells are treated with nutrient medium containing fetal bovine serum whereas experimental wells are incubated in an identical nutrient medium plus the test reagent. Tissue-containing wells are examined under an inverted phase microscope after 14 days of incubation. A semiquantitative grading system developed and validated in the Woltering laboratory is used to visually assess the degree of neovascularization.

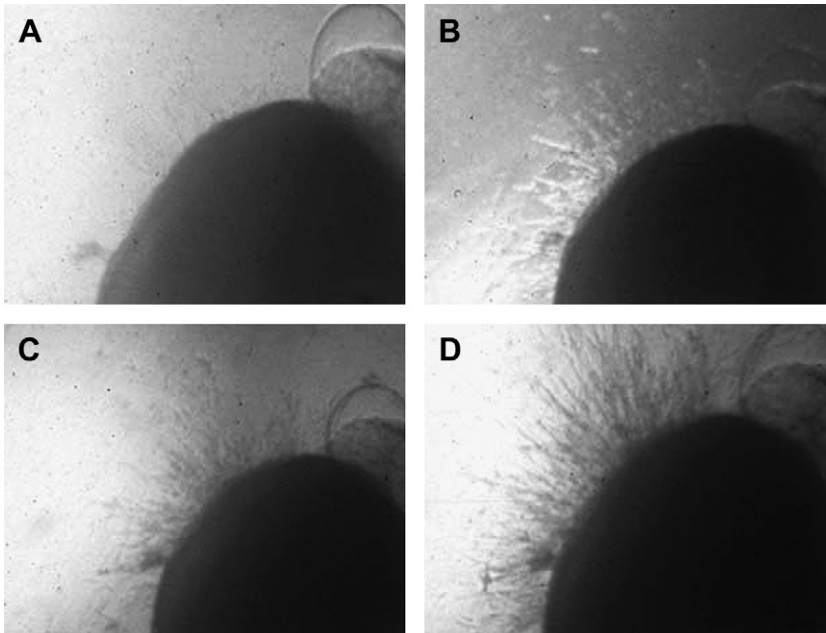


Fig. 2. Evaluation of angiogenesis. Tissue disks were divided into four quadrants with each quadrant given a numeric score from 0 to 4 based on neovessel length, density, and percentage of the quadrant's circumference involved with the angiogenic response. Numeric results from the four quadrants were summed and expressed as a semiquantitative AI (AI, 0–16). Panels A–D depict tissue disks with quadrant AI values of 1–4, respectively. (From Stafford SJ, Schwimer J, Anthony CT, et al. Colchicine and 2-methoxyestradiol inhibit human angiogenesis. *J Surg Res* 2005;125(1):104–8; with permission.)

The knowledge that a somatostatin analog's antiangiogenic effectiveness is directly proportional to its sst 2-binding affinity¹¹ led Watson and colleagues¹⁸ to hypothesize that proliferating human vascular endothelial cells express sst 2 whereas quiescent ones do not. To test this hypothesis, they used human tissues in the previously described fibrin-thrombin clot assay. They embedded placental vein discs from six anonymous donors into fibrin-thrombin clots and assessed their angiogenic response on day 15. Those discs that demonstrated endothelial cell growth from the cut edge of the disc were deemed to be proliferating and those viable discs that lacked endothelial cell growth were deemed to have remained quiescent. These investigators demonstrated for the first time that sst 2 gene expression was universally present in proliferating vascular endothelium but sst 2 expression was uniquely absent in quiescent endothelium derived from tissue-matched placental vein samples.

To confirm these reverse transcriptase-polymerase chain reaction (RT-PCR) results, immunohistochemical staining using anti-sst 2 antiserum was performed in proliferating and nonproliferating discs. These selective stains revealed that proliferating endothelium stained positive for sst 2 receptors whereas quiescent endothelium did not. These observations were extended into an animal model. Nude mice were implanted with neuroblastoma tumor cells that lacked the sst 2 receptor as measured by RT-PCR and *in vitro* assays. When the tumors were approximately 2 cm, mice were injected with ¹²⁵I-WOC4a, a radiolabeled, sst 2-preferring somatostatin analog. Nuclear medicine scans and radiographs were performed in register. Significant

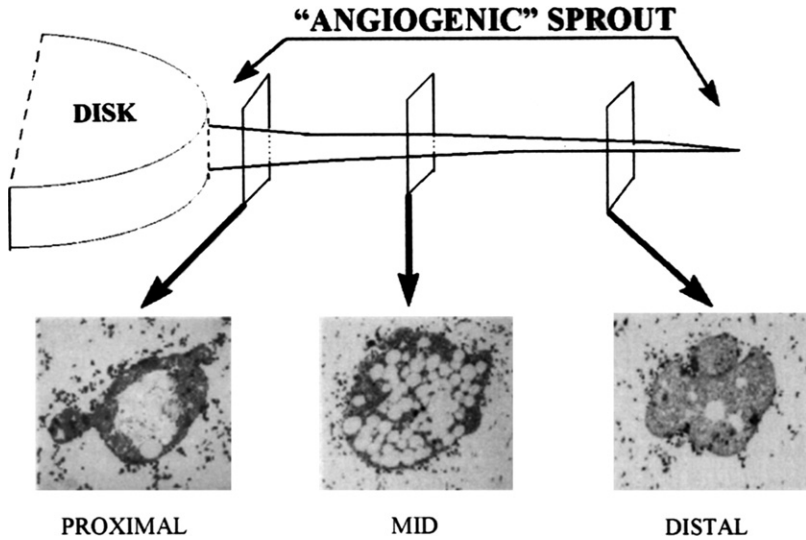


Fig. 3. Electron microscopic examination of microvessel outgrowth from the cut edge of a human placental vein disc. Capillary cross-sections: proximal (original magnification $\times 4200$), mid (original magnification $\times 2000$), and distal (original magnification $\times 2700$). (From Watson JC, Redmann JG, Meyers MO, et al. Breast cancer increases initiation of angiogenesis without accelerating neovessel growth rate. *Surgery* 1997;122:509-14; with permission.)

uptake was observed in the tumor whereas non-tumor-bearing sites did not accumulate radioligand (except for the liver, which was the normal route of excretion for the radiolabeled peptide). The binding seen in the tumors was thought to be the result of sst 2 receptor expression on angiogenesis blood vessels supplying the tumor.

An additional advantage of the fibrin-thrombin clot assay is its ability to test human tumor specimens against a variety of antiangiogenic agents over a wide range of concentrations. Similar to the method used to plate vein discs, fresh human tumors harvested at operation can be made into 1-mm³ fragments and embedded in the fibrin-thrombin clots.¹⁹ As neovessels sprout from the tumor's cut edge, the angiogenic response can then be quantified by the percent of specimens that begin to grow (percent initiation) or the degree of neovessel growth (angiogenic index [AI]) or the overall effect of the drug (overall drug effect). Unlike vein discs, which possess only resting endothelial cells, tumor fragments harbor actively proliferating endothelial cells in their existing angiogenic vessels. This tumor-based fibrin-thrombin clot assay was used to further investigate the presence of sst 2 receptors on angiogenic blood vessels.²⁰ Two different tumor types, one whose tumor cells were sst 2 positive and the other whose tumor cells were sst 2 negative, were implanted in the fibrin-thrombin clot assay. Both were allowed to develop an angiogenic response (presumably sst 2 positive). Then both tumor models were treated with an sst 2-favoring radiolabeled somatostatin analog. Tumorcidal effects were seen only in the sst 2-positive tumor cells, whereas antiangiogenic effects were seen in both tumor types. The investigators concluded that although sst 2s were present on the tumor cells of only one of the tumors, sst 2s were present on the neovessels of both tumors.

To date, many tumor types have been studied in this fibrin-thrombin clot assay. These include breast, colon, and ovarian cancers as well as malignant neuroendocrine tumors (NETs). Carcinoid tumors and islet cell tumors (ICTs) have been extensively

studied in this assay using a wide variety of clinically available agents, such as taxol, vincristine, vinblastine, and octreotide acetate. In addition, a variety of experimental reagents that are still in clinical development have been used against these NET specimens, including 2-methoxyestradiol (2-MeOH), rapamycin, vatalanib (PTK787/ZK222584), everolimus (RAD-001), and pasireotide (SOM-230), an sst 1-, 2-, 3-, and 5-preferring somatostatin analog.

Stafford and colleagues²¹ used this fibrin-thrombin clot assay to study the antiangiogenic profile of tubulin inhibitors against human neovessels. Two tubulin inhibitors that were investigated were colchicine and 2-MeOH. Colchicine, an older drug used commonly in the treatment of gout, has been shown to inhibit tumor growth by triggering cell cycle arrest in the G2/M phase and by inducing apoptosis. A natural metabolite of 17-estradiol, 2-MeOH has been recently discovered to be far more potent than colchicine.²² It has also shown to maintain potent apoptotic activity against rapidly growing tumor cells and endothelial cells.^{23,24} These investigators used placental vein discs from three separate human placentas and encouraged them to develop an angiogenic response in the fibrin-thrombin clot assay. Both colchicine and 2-MeOH were tested against these neovessels over a wide range of concentrations (10^{-6} to 10^{-12} M). The investigators observed that both colchicine and 2-MeOH exhibited antiangiogenic properties. The doses of colchicine required to induce antiangiogenesis, however, was much greater than the maximum tolerated human dose, negating its clinical use as an effective antiangiogenic agent. 2-MeOH inhibited neovessel growth and angiogenic initiation at 10^{-6} M. This inhibitory dose was consistent with the IC₅₀ ($2-5 \times 10^{-6}$ M) observed by other investigators who have tested this drug in animal models.²⁵ Based on these data, Stafford and colleagues proposed that 2-MeOH should be tested as an antiangiogenic in human clinical trials.

Antiangiogenic properties of tubulin inhibitors were further studied in NETs using this assay. Woltering and colleagues¹⁶ hypothesized the epothilone B (Epo), a naturally occurring macrolide that inhibits cell proliferation by stabilizing microtubules, would inhibit angiogenesis. They tested human tissues using two different assay methodologies. In the first set of experiments, eight tumors (including five carcinoids) and four normal tissues were treated with either nutrient medium or drug-containing medium starting on the first day in culture. This was designed to represent the effect of early or neoadjuvant Epo treatment on the development of a tumor- or normal tissue-derived angiogenic response. In a second set of experiments, three tumors (two carcinoids and a parathyroid adenoma) and three normal tissues were allowed to develop an angiogenic response for 14 to 18 days, and then they were treated with either control medium or drug-containing medium for 1 to 2 weeks. This method allowed tumors or normal tissues to develop an angiogenic response before treatment, and it was developed to more accurately determine the therapeutic response of developed, mature neovessel networks, such as those seen in widely metastatic tumors. Epo at drug levels of 10^{-8} M or greater inhibited angiogenesis in the majority of tissues studied. This antiangiogenic effect was seen in both sets of experiments (early and late drug application), and it was observed in carcinoid tumors as well, a group of NETs that are typically unresponsive to chemotherapy *in vivo*.²⁶ The effective drug levels of Epo were consistent with the range of blood levels seen when this drug was tested in phase 1 trials.

Most recently, experiments using this assay have extended into the investigation of the vascular endothelial growth factor (VEGF) pathway in human tissues. Lyons and colleagues²⁷ hypothesized that the addition of VEGF to the placental vein disc or human tumor specimen-containing wells would stimulate angiogenesis and while anti-VEGF regimens would inhibit angiogenesis. To test these hypotheses,

discs of human placental veins (physiologic model) and fragments of human tumors (pathologic model) were embedded in fibrin-thrombin clots and treated with either VEGFA165 or anti-VEGF pathway reagents. The anti-VEGF drugs included bevacizumab, a humanized monoclonal antibody to VEGF; IMC-18F1 and IMC-1121, human monoclonal antibodies directed against VEGF R1 and VEGF R2, respectively; and vatalanib (PTK787/ZK222584; PTK/ZK), a tyrosine kinase inhibitor of all three primary VEGF receptors (VEGF R1, R2, and R3) and several non-VEGF receptors, such as platelet-derived growth factor receptor. VEGF was tested against the vein discs of five separate human placentas and the fragments of eight separate malignant tumors, four of which were carcinoids. To the investigators' surprise, VEGF did not consistently stimulate neovascularization in human, vein-containing wells or in malignant tumor fragments. These experiments were repeated testing placental veins and malignant tumors (including carcinoids) with anti-VEGF reagents. Antibodies targeting VEGF R1 and VEGF R2 did not inhibit neovascularization in human tissues. Bevacizumab, although not affecting placental veins or gynecologic tumors, moderately inhibited angiogenesis in a subset of NETS (carcinoid tumors). In contrast, PTK/ZK significantly inhibited angiogenesis in every tissue type tested at multiple different concentrations. The inhibitory capacity of each molecule was proportional to the number of downstream targets that it affected. Antibodies to VEGF R1 and VEGF R2 affected only one target and had no inhibitory ability. Bevacizumab, an antibody that targets the VEGF-A ligand, affects all the receptors influenced by VEGF-A. This molecule had a modest inhibitory effect, but one that was greater than the antibodies to VEGF R1 and VEGF R2. PTK/ZK directly targets three primary VEGF receptors (R1, R2, and R3) as well as PDGF receptors and other non-VEGF receptors. PTK/ZK's inhibitory capacity was profound across all tissues and tumor types. This led the investigators to conclude that simply stimulating or blocking the VEGF pathway alone does not consistently alter neovascularization in human tissues. Manipulation of human neovessels was more consistently achieved when multiple growth factor pathways were affected simultaneously.

The fibrin-thrombin clot angiogenesis assay has been used extensively to study carcinoid tumors and ICTs. The ability to harvest an individual patient's tumor and to visually assess the angiogenic response of that tumor to a variety of agents potentially offers clinicians several unique opportunities to gauge a tumors' responsiveness to specific agents or pathways. These assays have the potential to make statements about the aggressiveness of a tumor. These assays may also enable clinicians to screen a variety of potentially useful medications and to design a customized drug regimen with the best antiangiogenic potential for that individual.

Methods to screen the effects of chemotherapeutic agents against tumor cells have been widely reported for several decades. These assays were first reported by Hamburger and Salmon,²⁸ who described their *in vitro* soft agar culture system in 1977. The ability of these assays to choose effective chemotherapeutic agents was clinically evaluated when Von Hoff and colleagues²⁹ compared the clinical responses of patients who received a single reagent that was either picked by a clinician or picked by the assay. Patients who received an assay-guided drug enjoyed a 25% response rate whereas those given a clinician-selected reagent experienced a 15% response rate.²⁹ In contrast to chemosensitivity assays, Kern and Weisenthal³⁰ described a chemoresistance assay that exposes tumor specimens to supra-pharmacologic concentrations of reagents. They have shown that drugs failing to suppress tumor growth at these extreme concentrations are likely to clinically fail 90% of the time. None of these chemosensitivity or chemoresistance assays

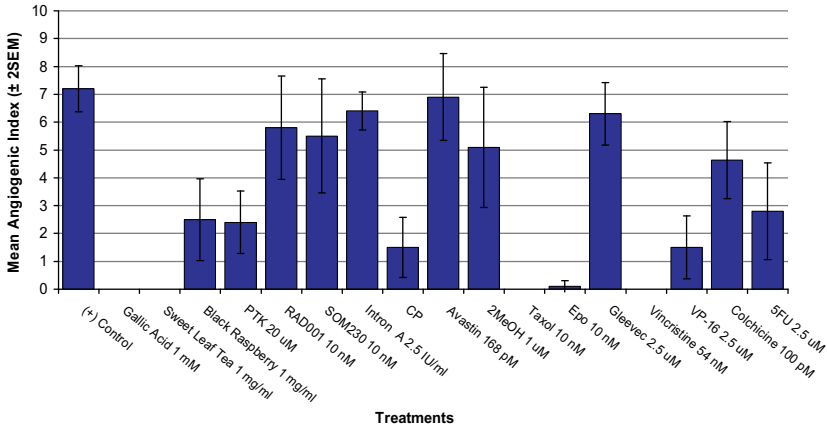


Fig. 4. One human carcinoid liver metastasis was tested against the 17 potential antiangiogenic drugs. Drugs with a lower angiogenic response are the better antiangiogenics in vitro. Avastin, bevacizumab; VP-16, etoposide; Gleevec, imatinib.

considers the blood vessel compartment of the tumor growth, however. The ability of fibrin-thrombin clot assay to assess the effect of therapy on neovessel growth makes this assay unique.

Figs. 4 and **5** outline the types of data that can be obtained from this fibrin-thrombin clot angiogenesis assay. **Fig. 4** illustrates data from one patient with a carcinoid. It outlines the angiogenic response in the fibrin-thrombin clot assay when tested with a panel of 17 different drugs. Drugs with a lower angiogenic response are the better antiangiogenics in vitro. Based on this data, it can be surmised that the most effective antiangiogenics in this carcinoid patient are gallic acid, sweet leaf tea, taxol, Epo, and vincristine, because they all significantly

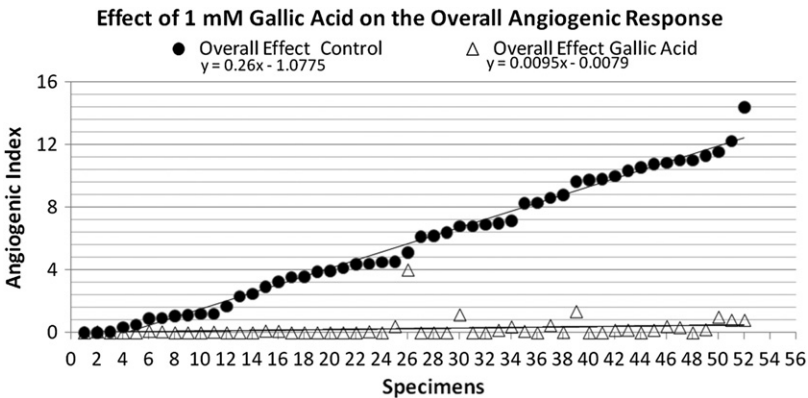


Fig. 5. Effect of 1mM gallic acid on overall angiogenic response. Fifty-two different human NETx were tested against the antiangiogenic drug, gallic acid. These different tumors are represented on the X axis as Specimens. Data were arranged in ascending order according to the control group's overall angiogenic response (0–16). Gallic acid consistently inhibited the overall angiogenic response, even in those specimens that had a robust control angiogenic response.

Table 2
Selected antiangiogenic agents tested in clinical trials

Agent	Carcinoid			Noncarcinoid NET		
	Complete Response	Partial Response	Stable Disease	Complete Response	Partial Response	Stable Disease
Bevacizumab + FOLFOX ³⁴	0/5 (0%)	1/5 (20%)	4/5 (80%)	0/7 (0%)	2/7 (29%)	5/9 (71%)
Bevacizumab + 2methoxyestradiol ³⁵	0/31 (0%)	0/31 (0%)	27/31 (93%)	NR	NR	NR
Bevacizumab + temozolamide ³⁶	0/12 (0%)	0/12 (0%)	11/12 (92%)	0/17 (0%)	4/17 (24%)	12/17 (70%)
Bevacizumab + Interferon ³¹	0/22 (0%)	4/22 (18%)	17/22 (77%)	NR	NR	NR
Imantinib ³⁷	0/27 (0%)	1/27 (4%)	17/27 (63%)	NR	NR	NR
Imantinib ³⁸	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/13 (0%)	0/13 (0%)	2/13 (15%)
Endostatin ³⁹	NR	NR	NR	0/40 (0%) ^a	0/40 (0%) ^a	32/40 (80%) ^a
Vatalanib ⁴⁰	NR	NR	NR	0/10 (0%) ^a	0/10 (0%) ^a	1/10 (10%) ^a
Atiprimod ⁴¹	0/23 (0%)	0/23 (0%)	21/23 (91%)	NR	NR	NR
Gefitinib ⁴²	0/22 (0%)	0/22 (0%)	14/22 (64%)	0/15 (0%)	0/15 (0%)	2/15 (13%)
Sunitinib ³³	0/41 (0%)	1/41 (0%)	34/41 (83%)	0/66 (0%)	11/66 (17%)	45/66 (68%)
Sunitinib ³²	NR	NR	NR	2/86 (2%)	6/86 (7%)	NR
Sorafenib ⁴³	0/41 (0%)	4/41 (10%)	NR	0/41 (0%)	4/41 (10%)	NR
Everolimus + temozolamide ⁴⁴	NR	NR	NR	0/17 (0%)	6/17 (35%)	9/17 (53%)
Everolimus + octreotide ⁴⁵	0/30 (0%)	5/30 (17%)	24/30 (80%)	0/30 (0%)	8/30 (27%)	18/30 (60%)
Pasireotide ⁴⁶	0/11 (0%)	0/11 (0%)	9/11 (82%)	NR	NR	NR
Lanreotide ⁴⁷	NR	NR	NR	0/25 (0%)	1/25 (4%)	7/25 (28%)
Intron A ⁴⁷	NR	NR	NR	0/27 (0%)	1/27 (4%)	7/27 (26%)
Lanreotide + intron A ⁴⁷	NR	NR	NR	0/28 (0%)	2/28 (7%)	5/28 (18%)
Octreotide ⁴⁸	NR	NR	NR	0/42 (0%) ^a	1/42 (2%) ^a	28/42 (66%) ^a
5-fluorouracil + octreotide ⁴⁹	NR	NR	NR	0/29 (0%)	7/29 (24%)	20/29 (68%)
Thalidomide + temozolamide ⁵⁰	0/14 (0%)	1/14 (7%)	NR	1/14 (7%)	6/14 (43%)	19/28 (68%) ^a

Abbreviation: NR, not reported.

^a This includes patients with carcinoid and noncarcinoid NETs because there was no clear distinction.

inhibited the overall angiogenic response. **Fig. 5** depicts the results of several patients (specimens 1–52) whose tumors were tested *in vitro* with the same antiangiogenic drug, gallic acid. In all 52 patients studied, gallic acid consistently inhibited neovascularization, even in those tumors with a robust control angiogenic response. Another notable point is that the specimen depicted in **Fig. 4** was harvested from a patient's liver metastasis. It has been occasionally observed that the same drug yields different antiangiogenic responses within the same patient depending on the harvest location of the specimen. In other words, a patient liver metastasis may significantly respond to gallic acid *in vitro* whereas his lymph node metastasis may not (data not shown). The potential for such intrapatient variability further underscores the importance of pretreatment drug screening.

Several investigators have tested antiangiogenic agents against carcinoids and other NETs in clinical trials. **Table 2** outlines selected clinical data that have been reported to date. Although complete responses to these reagents have been rare, many patients have experienced disease stabilization and some have enjoyed improved progression-free survival (PFS). Yao and colleagues³¹ randomly assigned 44 patients with advanced carcinoid to receive 18 weeks of bevacizumab or pegylated interferon alfa-2b (intron A). Partial responses were observed in four (18%) patients receiving bevacizumab and zero (0%) receiving intron A. Stable and progressive disease was observed in 17 (77%) patients and 1 (5%) patient receiving bevacizumab, respectively, and 15 (68%) and 6 (27%) patients receiving intron A. The PFS rate after 18 weeks was 95% in the bevacizumab arm and 68% in the intron A the arm. Raymond and colleagues³² evaluated another anti-VEGF reagent, sunitinib, versus placebo in patients with pancreatic ICTs. They also observed prolonged PFS in patients receiving antiangiogenic therapy (median PFS was 11 months after sunitinib vs 5 after placebo). Kulke and colleagues³³ evaluated this reagent in patients with both carcinoids and ICTs, and they found more objective tumor responses in patients with ICTs (16% vs 2%) than in patients with carcinoids. The median time to tumor progression was not significantly different between ICT patients and carcinoid patients (7 vs 10 months, respectively) nor was the 1-year survival rate (81% vs 83%, respectively).

The optimal drug regimen for patients with NETs has not yet been identified, but pretreatment *in vitro* drug screening to assess those drugs that are most likely to generate antiangiogenesis may hasten the ability to identify the most clinically efficacious drugs. Further investigations need to be made to clinically validate the angiogenic response observed with the fibrin-thrombin clot assay *in vitro* (ie, better understanding is needed of how a patient's *in vitro* response translates into a clinical outcome). Such information could enable avoiding futile medications in the future and customizing patients' therapy to drugs that are most likely to be affective against a NET.^{31–50}

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