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14. ABSTRACT Our overall goal is to determine if human breast cancer can be prevented from becoming angiogenic when it is still at a microscopic size (< ~ 1 mm ³). To date we have made the following progress: (1) We have cloned three different human breast cancers that undergo the angiogenic switch at predictable times. (2) We have found that the angiogenic switch time is modified by host stroma: two-fold earlier for tumors in the mammary fat pad, compared to tumors implanted in subcutaneous tissue. (3) We have found that the angiogenic switch is preceded by repression of stromal expression of thrombospondin-1. Angiogenic tumor cells continue to secrete a novel thrombospondin-1 repressing factor. This protein has been purified and partially sequenced. (4) For one of the breast cancers, the angiogenic switch can be detected at a microscopic size by a significant increase in bFGF in platelet alpha granules. (5) We have determined that the BRCA1 gene (breast cancer susceptibility gene), appears to regulate a ratio of thrombospondin-1 to VEGF in breast cancer cells. The lower the thrombospondin-1/VEGF ratio, the sooner the tumor cells will spontaneously switch to the angiogenic phenotype and grow large tumors in SCID mice.					
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I: Introduction:

After a primary breast cancer is surgically removed, the recurrence rate is approximately 1% per year for 27 years. This means that microscopic dormant metastases remain viable for years. During the support from this grant, we have shown that blocked angiogenesis is a common mechanism for microscopic tumor dormancy. We have also shown that a small percentage of such dormant tumors can eventually switch to the angiogenic phenotype. Development of non-toxic drugs that could prevent the switch to the angiogenic phenotype when micrometastases are still in the harmless, non-angiogenic, dormant state, could potentially prevent tumor recurrence in breast cancer, or at least markedly reduce the risk of recurrence.

II: Body:

Our data suggest that human breast cancer can remain in a harmless, dormant state for prolonged periods of time by upregulating expression of a potent angiogenesis inhibitor, thrombospondin-1. Furthermore, we have found that a normal function of the breast cancer susceptibility gene (BRCA1), may be to sustain the expression of thrombospondin-1. When the breast cancer susceptibility gene is mutated or abnormally over-expressed, the anti-angiogenic activity of thrombospondin-1 is decreased and breast cancer cells switch to the angiogenic phenotype. This permits rapid growth of a primary tumor or of its metastases. These results provide a lead for the development of novel angiogenesis inhibitors which would prevent the suppression of thrombospondin-1 in tumor cells, or in the stromal fibroblasts of the tumor bed, or both.

III. Key Research Accomplishments:

(i) Characterization of human breast cancer cell lines that form dormant, non-angiogenic tumors upon orthotopic implantation into SCID mice.

- During the past year we have demonstrated that two human breast cancer cell lines *induce* the expression of the endogenous angiogenesis inhibitor thrombospondin-1 in the surrounding stroma. This contributes to a non-angiogenic phenotype for these tumor cells. They remain dormant at a microscopic size of $\sim 1 \text{ mm}^3$.
- When derivatives of these breast cancer cell lines spontaneously switch to the angiogenic phenotype, they then repress the expression of thrombospondin-1 in stromal fibroblasts, while simultaneously stimulating expression of the c-Myc oncogene.

- We have also shown that activation of c-Myc in fibroblasts from the mammary fat pad can override the stimulation of thrombospondin-1 by the non-angiogenic, dormant line of breast cancer cells. This results in suppression of thrombospondin-1 in the surrounding stroma.
- Activation of the inducible dominant negative MYC-ER fusion protein in mammary fibroblasts interfered with the repression of thrombospondin-1 that was mediated by angiogenic tumor cells.

(ii) Investigation of the breast cancer susceptibility gene (BRCA1), and its regulation of angiogenesis.

- During the past year we have continued to elucidate the mechanism of how the breast cancer susceptibility gene (BRCA1) could mediate growth of breast cancer. We have increasing data to indicate that BRCA1 can regulate tumor angiogenesis. In BRCA1 mutant tumor cell lines, preliminary data reveals that over-expression of BRCA1 can lead to inactivation of tumor-induced thrombospondin-1, and thus increased tumor angiogenesis.

We have previously described four different tumor cell lines that we are using to elucidate the role of BRCA1 in the switch to the angiogenic phenotype:

HCC1937 (breast cancer cell line with BRCA1 mutation).
HCC1937+BRCA1 (with wild-type BRCA1 over-expression).
UWB289 (ovarian cancer cell line with BRCA1 mutation).
UWB289+BRCA1 (with wild-type BRCA1 over-expression).

- The switch to the angiogenic phenotype and onset of tumor growth after orthotopic injection into SCID mouse directly correlated with the ratio of thrombospondin-1 to VEGF produced by each tumor cell line. The lower the ratio, the earlier the tumor cell line spontaneously switched to the angiogenic phenotype (i.e., months instead of weeks). Angiogenic switch times were as follows:

HCC1937+ BRCA1 = 50 days.
HCC1937 (wild type cells with BRCA1 over-expression) = 220 days.
UWB289 (ovarian cancer cell line with BRCA1 mutation) = 290 days.
UWB289+BRCA1 (wild-type cells with BRCA1 over-expression) = still has not switched after 300 days observation.

- We have demonstrated decreased expression of the angiogenesis inhibitor maspin, a serpin protease family member, that is upregulated in normal breast tissue. Maspin is significantly downregulated in all BRCA1 tumor cell lines as compared to MCF10A cells or breast epithelial cells with two wild-type copies of BRCA1.
- The matrix metalloproteinase, ADAMTS1 is significantly downregulated in BRCA1 tumor cell lines as compared to MCF10A cells. ADAMTS1 has been demonstrated to cleave thrombospondin-1 into its anti-angiogenic fragments.

Therefore, under physiological conditions, we hypothesize that normal breast epithelial cells secreting the enzyme ADAMTS1 could cleave anti-angiogenic fragments from thrombospondin-1 and thus maintain suppression of angiogenesis, i.e., a non-angiogenic dormant phenotype.

- cDNA array data demonstrates a 9.5-fold decrease in ADAMTS1 cDNA in UWB289+BRCA1 cells that switch to the angiogenic phenotype significantly earlier than UWB289 cells that become angiogenic later or not at all.

IV. Reportable Outcomes.

- Normal human mammary fibroblasts expressing an inducible dominant-negative MYC transgene have been generated.
- BRCA1 mutant tumor cell lines with ectopic over-expression of wild-type BRCA1 have been generated.
- Manuscripts for both of these projects are currently in preparation. Due to the length of time necessary for the BRCA1 tumors to undergo an angiogenic switch, these experiments require up to a year for their completion.

V. Conclusions.

a. Summary:

Our work suggests that upregulation of stromal thrombospondin-1 by human breast cancer cells is one mechanism by which these cells can maintain a prolonged non-angiogenic, dormant state which restricts their growth to the size of *in situ* carcinoma (i.e., ~ 1 mm³). One mechanism by which breast cancer cells can switch to the angiogenic phenotype, is to repress thrombospondin-1 expression.

Conversely, we have demonstrated that tumor-mediated repression of thrombospondin-1 can be blunted or reversed by activation of dominant negative MYC in angiogenic breast cancer cells.

Taken together, these results imply that disruption of MYC activity in the stroma of breast cancer patients may be a potential therapy to inhibit tumor angiogenesis and progression. We will explore these concepts in this upcoming year.

Our data also suggests that the breast cancer susceptibility gene, BRCA1 may directly regulate tumor angiogenesis. Our work during the past year indicates that BRCA1 may function by regulating expression of the endogenous angiogenesis inhibitor, thrombospondin-1, either in stromal fibroblasts in the tumor bed, or in tumor cells themselves, or both.

While we have previously demonstrated that BRCA1-mutant tumor cell lines express significantly lower levels of thrombospondin-1, our data does not implicate direct transcriptional regulation of thrombospondin-1, by BRCA1. Our future studies for this year include treatment of BRCA1 tumors in SCID mice with the anti-angiogenic fragment of thrombospondin-1, to determine whether upregulation of the angiogenesis inhibitor thrombospondin-1 is sufficient to prevent or regress BRCA1-mediated tumor growth. We will also determine whether over-expression of either Maspin or ADAMTS1 in BRCA1 tumor cells will alter production of either thrombospondin-1 or VEGF. Similarly, we will investigate whether ectopic over-expression of Maspin or ADAMTS1 by BRCA1 tumor cells will delay or prevent the switch to the angiogenic phenotype.

b. “So what section.”

By understanding how microscopic (*in situ*) human breast carcinoma can maintain its non-angiogenic, dormant state, it may be possible to develop a drug that would prevent the switch to the angiogenic phenotype. From our recent experimental results, such a drug would up-regulate the tumor's induction of thrombospondin-1 in stromal fibroblasts in the tumor bed, or would maintain expression of thrombospondin-1 in the tumor cells *per se*. Alternatively a drug could be developed that would stabilize the enzyme ADAMTS1 so that it would maintain high levels of the anti-angiogenic fragment of thrombospondin-1.

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VII. Appendix.

- Folkman, J. Angiogenesis: an organizing principle for drug discovery? Nature Reviews Drug Discovery (2007), 6:273-286.

OPINION

Angiogenesis: an organizing principle for drug discovery?

Judah Folkman

Abstract | Angiogenesis — the process of new blood-vessel growth — has an essential role in development, reproduction and repair. However, pathological angiogenesis occurs not only in tumour formation, but also in a range of non-neoplastic diseases that could be classed together as ‘angiogenesis-dependent diseases’. By viewing the process of angiogenesis as an ‘organizing principle’ in biology, intriguing insights into the molecular mechanisms of seemingly unrelated phenomena might be gained. This has important consequences for the clinical use of angiogenesis inhibitors and for drug discovery, not only for optimizing the treatment of cancer, but possibly also for developing therapeutic approaches for various diseases that are otherwise unrelated to each other.

The term angiogenesis is generally applied to the growth of microvessel sprouts the size of capillary blood vessels, a process that is orchestrated by a range of angiogenic factors and inhibitors (FIG. 1). Although proliferating endothelial cells undergoing DNA synthesis are a common hallmark of angiogenic microvascular sprouts, extensive sprouts can grow for periods of time, mainly by the migration of endothelial cells¹. Physiological angiogenesis is distinct from arteriogenesis and lymphangiogenesis and occurs in reproduction, development and wound repair. It is usually focal, such as in blood coagulation in a wound, and self-limited in time, taking days (ovulation), weeks (wound healing) or months (placentation). By contrast, pathological angiogenesis can persist for years. Pathological angiogenesis is necessary for tumours and their metastases to grow beyond a microscopic size and it can give rise to bleeding, vascular leakage and tissue destruction. These consequences of pathological angiogenesis can be responsible, directly or indirectly, for the symptoms, incapacitation or death associated with a broad range of ‘angiogenesis-dependent diseases’². Examples of such diseases include cancer, autoimmune diseases, **age-related macular degeneration** and atherosclerosis (TABLE 1).

The concept of angiogenesis-dependent diseases originated in 1972 with the recognition that certain non-neoplastic diseases, such as the chronic inflammatory disease psoriasis, depend on chronic neovascularization to provide a conduit for the continual delivery of inflammatory cells to the inflammatory site^{3–5}. Subsequently, other non-neoplastic diseases were recognized to be in part angiogenesis dependent, for example, **infantile haemangiomas**⁶, peptic ulcers⁷, ocular neovascularization⁸, **rheumatoid arthritis**⁹ and atherosclerosis^{3,10,11}. This led to a more general understanding that the process of angiogenesis itself could be considered as an ‘organizing principle’. Organizing principles are common in the physical sciences, and are now starting to be recognized in biology — other examples might be inflammation or apoptosis, which are also aspects of many otherwise unrelated diseases. The heuristic value of such a principle is that it permits connections between seemingly unrelated phenomena. For example, the discovery of a molecular mechanism for one phenomenon might be more rapidly demonstrated for a second phenomenon if one understands *a priori* that the two are connected. Furthermore, when the mechanisms underlying different diseases can be related in this way, the development

of therapeutics for one disease could aid the development of therapeutics for others. Although it remains to be determined to what extent treating pathological angiogenesis in different angiogenesis-dependent diseases will be successful, the recent approval of ranibizumab (Lucentis; Genentech) — an antibody fragment based on the anti-angiogenic cancer drug bevacizumab (Avastin; Genentech) — for age-related macular degeneration suggests that such strategies merit investigation.

Here, I provide an overview of the current state of drug development of angiogenesis inhibitors, as well as certain drugs that have varying degrees of anti-angiogenic activity in addition to their other functions, and highlight examples of anti-angiogenic strategies in unrelated diseases. Furthermore, I discuss burgeoning new directions in angiogenic research, the optimization of anti-angiogenic strategies and how viewing angiogenesis as an organizing principle might uncover fruitful connections for future drug discovery.

A brief history of angiogenesis inhibitors

The attempt to discover angiogenesis inhibitors became possible after my group and others had developed bioassays for angiogenesis during the 1970s. These included the long-term culture of vascular endothelial cells¹², the development of the chick-embryo chorioallantoic-membrane bioassay¹³, the development of sustained-release polymers¹⁴ and the implantation of these polymers as pellets in the rabbit¹⁵ and murine¹⁶ cornea to quantify the angiogenic activity of tumour-derived proteins.

The first angiogenesis inhibitors were reported in the 1980s from the Folkman laboratory, during a study that continued over 25 years^{17,18} (TIMELINE). No angiogenesis inhibitors existed before 1980, and few scientists thought at that time that such molecules would ever be found. However, the effort to isolate and purify them was driven by preliminary data that led to the 1971 hypothesis that tumour growth is dependent on angiogenesis¹⁹. This effort was also informed by preliminary data that the removal of an angiogenic sustained-release pellet from the rabbit cornea led to a rapid regression (weeks) of neovascularization that was induced by the pellet²⁰.

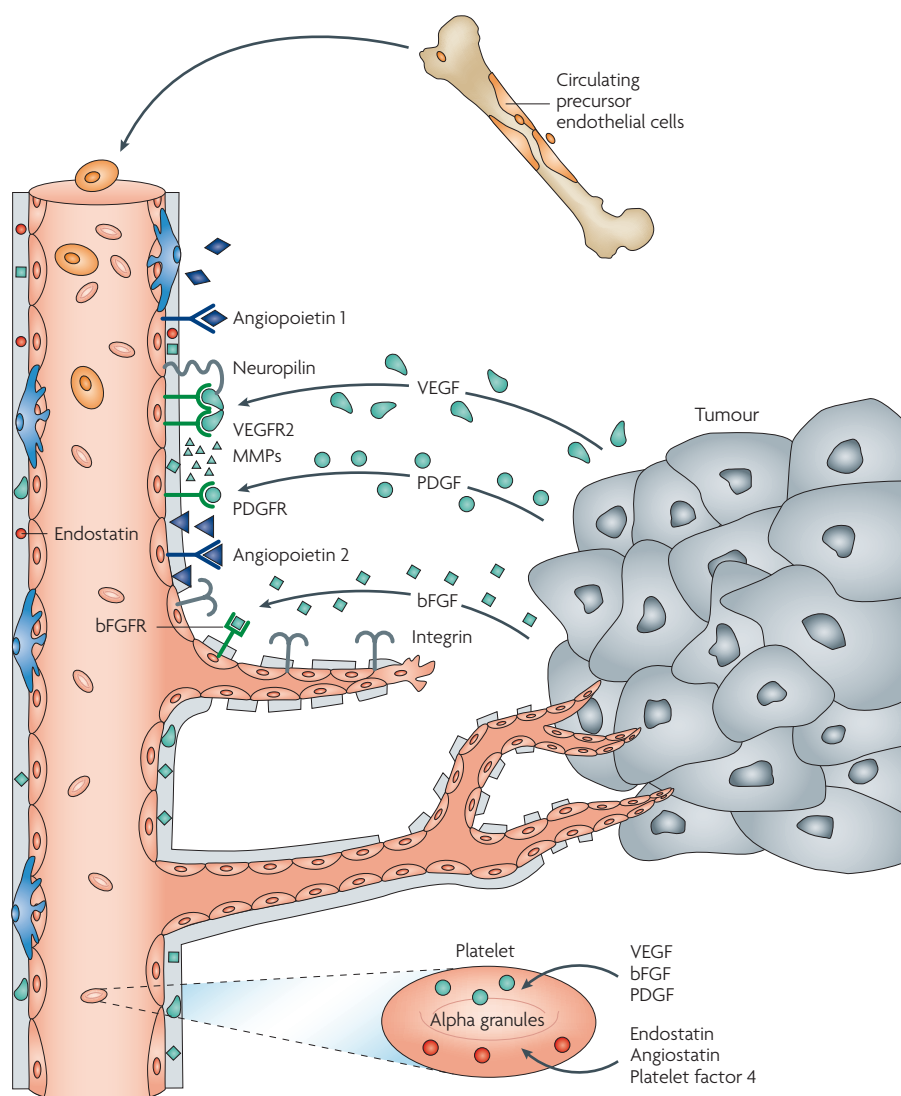


Figure 1 | Key steps in tumour angiogenesis. Angiopoietin 1 (ANGPT1), expressed by many cells, binds to the endothelial TIE2 (also known as TEK) receptor and helps maintain a normalized state in blood vessels. Vascular endothelial growth factor (VEGF) is secreted by tumour cells and binds to its receptor (VEGFR2) and to neuropilin on endothelial cells. It is the most common of at least six other pro-angiogenic proteins from tumours. Matrix metalloproteinases (MMPs) are released from tumour cells, but also by VEGF-stimulated endothelial cells. MMPs mobilize pro-angiogenic proteins from stroma, but can also cleave endostatin from collagen 18 in the vessel wall and participate in the cleavage of angiostatin from circulating plasminogen. Tumour cells secrete angiopoietin 2 (ANGPT2), which competes with ANGPT1 for binding to the endothelial TIE2 receptor. ANGPT2 increases the degradation of vascular basement membrane and migration of endothelial cells, therefore facilitating sprout formation. Platelet-derived growth factor (PDGF), an angiogenic protein secreted by some tumours, can upregulate its own receptor (PDGFR) on endothelial cells. Basic fibroblast growth factor (bFGF; also known as FGF2) is secreted by other tumours. Integrins on endothelial cells carry signals in both directions. Integrins facilitate endothelial cell binding to extracellular membranes, a requirement for the cells to maintain viability and responsiveness to growth regulatory proteins. Endothelial cells are among the most anchorage-dependent cells. Certain pro-angiogenic proteins upregulate endothelial integrins and are thought to sustain endothelial cell viability during the intermittent detachments that are required to migrate towards a tumour and to simultaneously increase their sensitivity to growth regulators — both mitogenic (VEGF or bFGF) and anti-mitogenic (endostatin). New endothelial cells do not all originate from neighbouring vessels. A few arrive as precursor bone-marrow-derived endothelial cells. Endothelial growth factors are not all delivered to the local endothelium directly from tumour cells. Some angiogenic regulatory proteins (both pro- and anti-angiogenic) are scavenged by platelets, stored in alpha granules and seem to be released within the tumour vasculature. It was recently discovered that pro- and anti-angiogenic proteins are stored in different sets of alpha granules (depicted in green and red respectively)⁶³.

After the mid-1980s, we and others began to discover additional angiogenesis inhibitors^{21–29} (TIMELINE). By the mid-1990s, new drugs with anti-angiogenic activity entered clinical trials. These drugs began to receive Food and Drug Administration (FDA) approval in the United States by 2003. Bevacizumab, which received FDA approval for colorectal cancer in 2004, was the first drug developed solely as an angiogenesis inhibitor³⁰. However, certain non-endothelial cells (haematopoietic-derived cells that colonize tumour stroma and some cancer cells, such as those in pancreatic cancer) can also express receptors for vascular endothelial growth factor (VEGF; also known as VEGFA), raising the possibility that this drug might also have direct antitumour effects^{31,32}. At the time of writing this article, 10 new drugs — in which anti-angiogenic activity is considered to be central to their therapeutic effects — have been approved by the FDA in the United States, and by equivalent agencies in 30 other countries, for the treatment of cancer and age-related macular degeneration (TABLE 2). At least 43 other drugs that have varying degrees of anti-angiogenic activity are currently in clinical trials in the United States for different types of cancer, ten of which are in Phase III (TABLE 3). Other FDA-approved drugs revealed anti-angiogenic activity in addition to anticancer activity directed against tumour cells. For example, bortezomib (Velcade; Millennium Pharmaceuticals), approved as a proteasome inhibitor for the treatment of multiple myeloma, was subsequently demonstrated to also have potent anti-angiogenic activity³³.

As the treatment range of angiogenesis inhibitors covers not only many types of cancer, but also unrelated diseases such as age-related macular degeneration and possibly others, angiogenesis inhibitors, or drugs that have varying degrees of anti-angiogenic activity, might be defined as a class of drugs that specifically target an organizing principle in biomedicine.

Angiogenesis as an organizing principle

Clinical advantages to understanding angiogenesis as an organizing principle.

There are important clinical advantages to viewing angiogenesis as an organizing principle. For example, if a clinician recognizes that a patient's disease might be partly angiogenesis-dependent, it is conceivable that an angiogenesis inhibitor approved for one type of tumour could be used for a different type of tumour, or even used off-label for a different disease.

Table 1 | **Angiogenesis-dependent diseases**

Disease	Symptoms
Diabetic retinopathy	Loss of vision
Rheumatoid arthritis ²	Pain and immobility from destroyed cartilage
Atherosclerotic plaques ³	Chest pain, dyspnoea
Endometriosis ^{4,5}	Abdominal pain from intraperitoneal bleeding
Crohn's disease ⁶	Intestinal bleeding
Psoriasis ⁷	Persistent severe itching
Uterine fibroids	Vaginal bleeding, abdominal pain
Benign prostatic hypertrophy	Urinary retention
Cancer	Bleeding, thrombosis, anaemia, abdominal ascites, bone pain, seizures from cerebral oedema around a tumour and others

macular degeneration have been treated with pegaptanib since its approval, and in the past year more than 50,000 patients have been treated with either intravitreal ranibizumab or off-label bevacizumab.

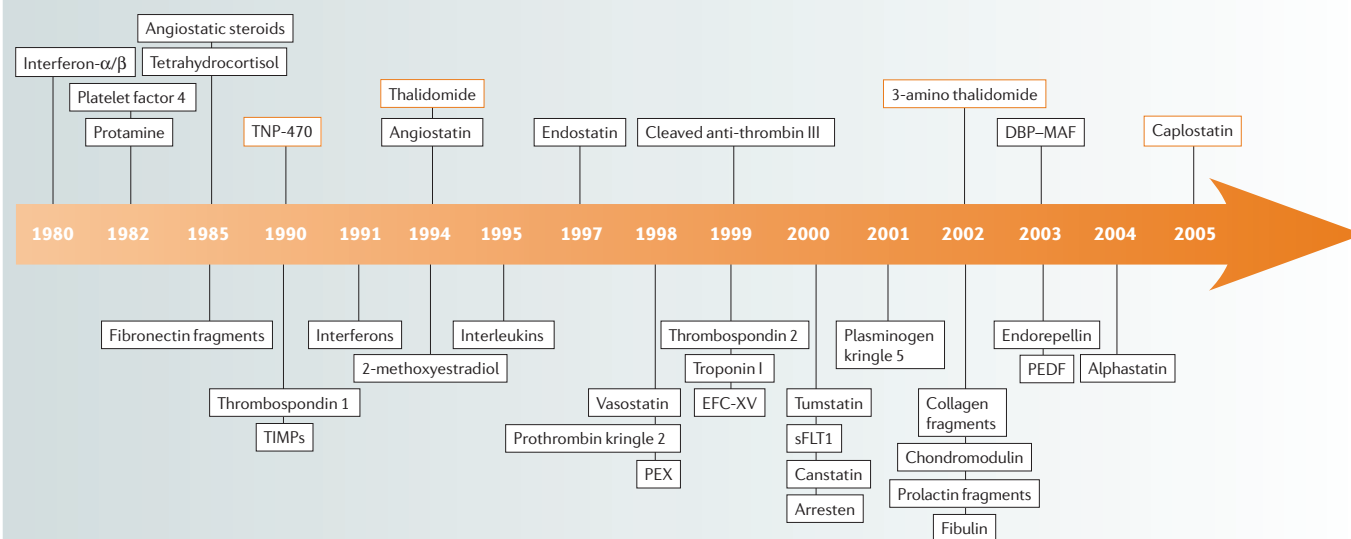
This might be the first time that a relatively non-toxic anticancer drug has been injected into the eye to treat ocular neovascularization. It is rare to treat diseases as diverse as colorectal cancer and age-related macular degeneration with the same agent — with the exception that the target for each was known to be VEGF^{41–44}.

Discovery of dual roles for cancer drugs. The cancer drugs erlotinib (Tarceva; Genentech, OSI Pharmaceuticals, Roche), cetuximab (Erbix; Bristol-Myers Squibb, Merck) and vandetanib were originally developed as inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase. For this reason, they are also known as anti-oncogene protein signal-transduction inhibitors⁴⁵. However, they were subsequently found to also inhibit tumour angiogenesis by blocking the VEGF receptor. Cetuximab, an anti-EGFR agent, produces an antitumour effect *in vivo* that is due to the direct blockade of the EGFR-dependent mitogenic pathway and in part to the inhibition of secretion of various pro-angiogenic proteins such as VEGF, basic fibroblast growth factor (bFGF; also known as FGF2) and transforming growth factor- α (TGF α)⁴⁶.

An example of the former is the use of bevacizumab in colorectal cancer and also in **non-small-cell lung cancer**, and an example of off-label use is its use for age-related macular degeneration. Oncologists might also benefit from knowing that certain anticancer drugs (for example, cyclophosphamide) that were originally developed to target cancer cells also have anti-angiogenic activity.

A connection between colorectal cancer and macular degeneration. Bevacizumab is an antibody that neutralizes VEGF and was approved by the FDA for colorectal cancer in 2004 (REFS 30,34). Ranibizumab is a fragment of bevacizumab. In randomized clinical trials, ranibizumab injected into the eye at monthly

intervals showed dramatic success in patients with age-related macular degeneration. In patients who were legally blind, with an average visual acuity of ~20/300, approximately 40% recovered their sight and improved to a visual acuity of 20/40 (sufficient for some to drive a car). In ~90–95% of patients, the disease was arrested, and there was no further loss of sight. By contrast, patients who were treated with a placebo continually lost visual acuity over a 12-month period, as was expected^{35–40} (FIG. 3a). Pegaptanib (Macugen; OSI Pharmaceuticals), an anti-VEGF aptamer, was the first anti-VEGF drug to be approved by the FDA (2004) for the treatment of age-related macular degeneration. More than 75,000 patients with age-related

Timeline | **Discovery of angiogenesis inhibitors**

Synthetic angiogenesis inhibitors (orange keyline) and endogenous angiogenesis inhibitors that were identified in the Folkman laboratory are depicted above the timeline. Examples of additional endogenous angiogenesis inhibitors discovered in other laboratories are depicted below the timeline. The first drugs with anti-angiogenic activity were approved in 2003 (TABLE 2). DBP-MAF, vitamin-D-binding protein-macrophage-activating factor; EFC-XV, endostatin-like fragment from type XV collagen; PEDF, pigment epithelium-derived factor (also known as SERPINF1); PEX, haemopexin C domain autolytic fragment of matrix metalloproteinase 2; sFLT1, soluble fms-related tyrosine kinase 1; TIMP, tissue inhibitors of matrix metalloproteinase.

With this knowledge of their dual role⁴⁵, these drugs might be used more effectively by oncologists who could follow guidelines for dose-efficacy of angiogenesis inhibitors, which differ from conventional cytotoxic chemotherapies (see below).

Emerging research directions

The usefulness of recognizing an underlying organizing principle during angiogenesis research is illustrated by several fascinating insights into diverse biological processes. Some examples of these are new insights

into platelet biology⁴⁷, metastases²², endothelial control of tissue mass^{48,49,72}, the concept of oncogene dependence⁵⁰ and the surprising discovery that some of the ligand–receptor pairs that mediate axon-pathway finding also mediate

Table 2 | **Anti-angiogenic drugs approved for clinical use and phase of clinical trials for other indications**

Drug (Trade name; company)	Approved*	Phase III	Phase II	Phase I
Bortezomib (Velcade; Millennium Pharmaceuticals)	Multiple myeloma (2003)	NSCLC, multiple myeloma, NHL	Multiple myeloma, NHL, NSCLC, lymphoma, gliomas, melanoma, Waldenstrom's macroglobinaemia, prostate, head and neck, breast, liver, nasopharyngeal, gastric, pancreatic, colorectal, cervical/vaginal cancer, and others	Lymphoma, myelodysplasia, multiple myeloma, NHL, solid tumours, head and neck, cervical, colorectal, ovarian, prostate cancer, and others
Thalidomide (Thalomid; Celgene Corporation)	Multiple myeloma (2003 [†])	Multiple myeloma, brain metastases, SCLC, NSCLC, prostate, kidney, ovarian, hepatocellular cancer	Soft tissue sarcoma, multiple myeloma, ALS, melanoma, neuroendocrine tumours, leukaemia, glioma, glioblastomas, paediatric neuroblastoma, NSCLC, NHL, paediatric solid tumours, myelo- fibrosis, myelodysplastic syndrome, AML, CLL, SCLC, Hodgkin's disease, paediatric brain stem, liver, colorectal, kidney, neuroendocrine, endometrial, thyroid, uterine, ovarian cancer, and others	Solid tumours, glioma
Bevacizumab (Avastin; Genentech)	Colorectal cancer (2004), lung cancer (2006)	NSCLC, GIST, diabetic retinopathy, vascular occlusions, retinopathy of prematurity, colorectal, breast, ovarian, peritoneal, pancreatic, prostate, kidney cancer	Glioblastoma, glioma, mesothelioma, NSCLC, AML, CLL, CML, lymphoma, angiosarcoma, melanoma, biliary tumours, SCLC, Kaposi's sarcoma, sarcomas, NHL, carcinoid, oesophagogastric, gastric, renal cell, head and neck, rectal, hepatocellular, bladder, pancreatic, gall bladder, breast, neuroendocrine, cervical, ovarian, endometrial cancer, and others	NSCLC, pancreatic, solid tumours, head and neck tumours, VHL, retinal tumours
Erlotinib (Tarceva; Genentech, OSI Pharmaceuticals, Roche)	Lung cancer (2004)	NSCLC, colorectal, pancreatic, ovarian, head and neck, oral cancer	NSCLC, mesothelioma, glioblastoma, glioma, gall bladder, GIST, biliary tumours, bladder cancer prevention, malignant peripheral nerve sheath tumours, endometrial, colorectal, pancreatic, breast, renal cell, prostate, ovarian, head and neck, gastric/oesophageal, liver cancer, and others	NSCLC, glioblastoma, solid tumours, colorectal, pancreatic, head and neck cancer
Pegaptanib (Macugen; OSI Pharmaceuticals)	Age-related macular degeneration (2004)			
Endostatin (Endostar)	Lung cancer (2005 [‡])			
Sorafenib (Nexavar; Onyx Pharmaceuticals)	Kidney cancer (2005)	Kidney, melanoma, hepatocellular cancer	Melanoma, glioblastoma, GIST, SCLC, thyroid, neuroendocrine, mesothelioma, soft tissue sarcoma, NSCLC, CLL, multiple myeloma, cholangiocarcinoma, NHL, kidney, colorectal, prostate, ovarian, peritoneal, pancreatic, breast, gastric, head and neck, uterine, gall bladder, bladder cancer, and others	Solid tumours, melanoma, glioblastoma, NHL, glioma, multiple myeloma, Kaposi's sarcoma, ALL, CML, MDS
Lenalidomide (Revlimid; Celgene Corporation)	Myelodysplastic syndrome (2005)	Multiple myeloma, myelodysplastic syndrome	NSCLC, NHL, multiple myeloma, CLL, myelofibrosis, myelodysplastic syndrome, glioblastoma, ocular melanoma, AML, mantle-cell lymphoma, Waldenstrom's macroglobinaemia, ovarian/ peritoneal, thyroid, prostate cancer	Multiple myeloma, prostate cancer, melanoma, myelodysplastic syndrome, solid tumours, paediatric CNS tumours
Sunitinib (Sutent; Pfizer)	GIST, kidney cancer (2006)	Renal cell cancer, GIST	Melanoma, VHL/solid tumour, NSCLC, GIST, hepatocellular, colorectal, prostate, breast, renal cell, gastric, neuroendocrine cancer, and others	Melanoma, solid tumours, colorectal, breast cancer
Ranibizumab (Lucentis; Genentech)	Age-related macular degeneration (2006)			

*Year of first approval by the US Food and Drug Administration, unless stated otherwise. [†]Australia, approved by US Food and Drug Administration in 2006. [‡]China State Food and Drug Administration. ALS, amyotrophic lateral sclerosis (or Lou Gehrig's disease); ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; CNS, central nervous system; GIST, gastrointestinal stromal tumour; MDS, myelodysplastic syndromes; NSCLC, non-small-cell lung cancer; NHL, non-Hodgkin's lymphoma; SCLC, small-cell lung cancer; VHL, von Hippel Lindau.

angiogenesis⁵¹. Furthermore, genetic variations in the expression of angiogenic proteins between different groups of individuals⁵² provide further clues about the role of these angiogenesis-regulatory proteins in different diseases.

Endothelium and neurons share regulatory proteins. In 1998, Klagsbrun and colleagues reported that neuropilin, a cell-surface protein originally identified as a receptor for a signal that guides growing nerves, is also a receptor for VEGF^{53,54}. This marked the beginning of a merger between the fields of neural guidance and angiogenesis. It was discovered that various ligand–receptor pairs that mediate axon-pathway finding also mediate angiogenesis⁵¹.

Also, during development, sensory nerves determine the pattern of arterial differentiation in blood-vessel branching in the skin⁵⁵. It was found that in the highly vascular dorsal root ganglia, neuronal VEGF interacts with endothelial cell VEGF receptor 2 (VEGFR2; also known as **KDR**)⁵⁶, which is necessary for endothelial survival. As the interactions of growth and motility proteins for neurons and endothelial cells are gradually uncovered, they might have important roles in drug discovery, for example, for drugs that can repair spinal-cord injuries, reverse **Alzheimer's disease** or broaden the efficacy of currently approved angiogenesis inhibitors.

New platelet biology. In a review in 2001, my colleagues and I assembled the reports that showed that most of the endogenous angiogenesis-regulatory proteins known at that time were contained in platelets or were on the platelet surface⁵⁷. Several studies subsequently reported that circulating platelets in mice take up and sequester angiogenesis regulatory proteins, such as VEGF, bFGF and connective-tissue-activating peptide, when a microscopic human tumour is present in a mouse^{58–60}.

The angiogenesis-regulatory proteins are sequestered in alpha granules of platelets at a significantly higher concentration than in plasma. In fact, when radiolabelled VEGF is implanted subcutaneously in a Matrigel pellet in mice, platelet lysates take up virtually all of the radiolabelled VEGF and none is found in plasma⁵⁸. Mouse platelets live for ~3–4 days. Nevertheless, platelets seem to recycle the angiogenesis-regulatory proteins they have scavenged, because the concentration of these proteins increases in the platelets over time (weeks to months), as long as the source of an angiogenesis-regulatory protein is present. Also, a single

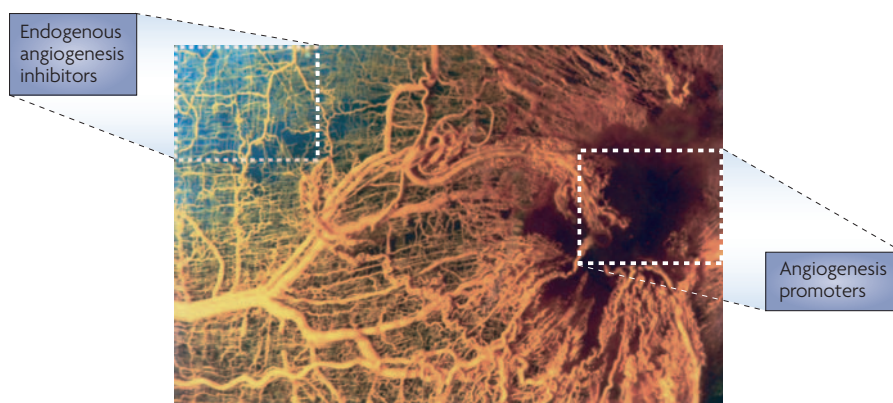


Figure 2 | Angiogenesis in rat sarcoma. In this micrograph, blood vessels grow towards a sarcoma (dark area at right) in rat muscle. This contrasts with the normal grid-like pattern of blood vessels that appears at the upper left. (Courtesy of L. Heuser and R. Ackland, University of Louisville, USA)¹⁴¹.

intravenous injection of thrombospondin 1 (**THBS1**) (2 µg) into *THBS1*-null mice continues to appear in platelet lysates for weeks (S. Ryeom, personal communication). Furthermore, it was recently reported that in patients with cancer who were receiving bevacizumab, the antibody was taken up by platelets where it was bound to VEGF⁶¹.

This new platelet property, quantifiable by mass spectroscopy of platelet lysates, might permit the development of a biomarker for early detection of tumour recurrence. In tumour-bearing mice, the platelet-angiogenesis proteome detects microscopic tumours at a millimetre size, before they have become angiogenic, but when they are generating angiogenic proteins (VEGF, bFGF and platelet-derived growth factor; PDGF) and anti-angiogenic proteins (endostatin or *THBS1*)^{58,62}.

Italiano *et al.* have recently discovered that angiogenesis-regulatory proteins are in fact segregated among two sets of alpha granules in platelets: positive regulators of angiogenesis in one set of alpha granules and negative regulators in the other set⁶³. This previously unknown function of platelets links them with the process of angiogenesis. A new opportunity lies ahead to determine whether and how platelets release pro-angiogenic proteins at a wound site and then later release anti-angiogenic proteins. Furthermore, the putative role of platelet release of angiogenesis-regulatory molecules in tumours remains to be elucidated. It might also be possible to develop drugs that selectively release anti-angiogenic proteins from platelets trapped in haemangiomas or in cancer. It is likely that in the future novel angiogenesis-regulatory molecules that could be developed into drugs will be discovered in platelets.

A new mechanism for site specificity of metastasis. It is known that *THBS1* is a potent angiogenesis inhibitor⁶⁴ that is expressed by fibroblasts and other stromal cells in many tissues. It is also clear that reduction of *THBS1* expression in a tumour bed is a necessary prerequisite for induction of neovascularization and for a microscopic tumour to become neovascularized and to grow^{65,66}. Watnick and colleagues recently found that certain human tumours produce a novel protein that specifically represses *THBS1* in the stromal tissue to which the tumour is subsequently able to metastasize⁶⁷. Suppression of anti-angiogenic activity at the future metastatic site facilitates the initiation of angiogenesis by metastatic tumour cells. If this discovery can be generalized to other tumours, it could be the basis for the development of drugs such as antibodies that could neutralize the *THBS1* suppressor protein produced by the primary tumour.

When a new angiogenesis-based metastatic mechanism is uncovered, it is prudent to ask whether the new cancer mechanism could have a physiological counterpart. As in normal tissues, *THBS1* is highly expressed under normal conditions in the endometrium⁶⁸. It is not known whether *THBS1* is suppressed before the implantation of a fertilized ovum or of a blastocyst, and if so, by what mechanism. This is a topic of current investigation, and there are potential clinical implications. More than 10% of all pregnancies miscarry early in the first trimester. Some women have repeated early miscarriages and are unable to carry a baby to term. *In vitro* fertilization often requires multiple cycles of ovum implantation. Could these problems be the result of insufficient suppression of endometrial *THBS1*, or of some other endogenous angiogenesis

Table 3a | **Clinical trials of drugs that have shown anti-angiogenic activity in preclinical models**

Inhibitor (Company)	Target/mechanism	Clinical development
2-methoxyestradiol-EntreMed (EntreMed)	Inhibits HIF1 α and tubulin polymerization	- Phase I: Breast cancer and solid tumours - Phase II: Glioblastoma, multiple myeloma, neuroendocrine, renal cell, prostate, ovarian cancer
A6 (Angstrom Pharmaceuticals)	Binds to uPA cell-surface receptor	Phase II: History of ovarian cancer with rising CA125
Abergrin (MedImmune)	Anti α v β 3 antibody	- Phase I: Melanoma, solid tumours, colorectal cancer - Phase II: Melanoma, prostate cancer, psoriasis, arthritis
ABT-510 (Abbott Laboratories)	Thrombospondin 1 receptor CD36	- Phase I: Head and neck cancer, solid tumours - Phase II: Lymphoma, renal cell, head and neck, NSCLC, soft tissue sarcoma
Actimid (Celgene Corporation)	Downregulates TNF	Phase II: Prostate cancer (completed)
AG-013736 (Pfizer)	VEGFR, PDGFR	- Phase I: Breast cancer - Phase II: NSCLC, melanoma, thyroid, breast, pancreatic, renal cell cancer
AMG706 (Amgen)	VEGFR, PDGFR, KTR, RETR	- Phase I: Lymphoma, solid tumours, NSCLC, breast, colorectal cancer - Phase II: Solid tumours, NSCLC, gastrointestinal stromal tumours (GIST), breast, thyroid cancer
AP23573 (Ariad Pharmaceuticals)	mTOR, VEGF	- Phase I: Glioma, sarcoma, solid tumours, multiple myeloma - Phase II: Endometrial cancer, prostate cancer, haem malignancies
AS1404 (Antisoma)	Vascular disrupting agent, releases TNF and vWF	Phase II: Prostate cancer
ATN-161 (Attenuon)	α 5 β 1 antagonist	Phase II: Renal cell cancer, malignant glioma
AZD2171 (AstraZeneca)	VEGFR1, VEGFR2, VEGFR3, PDGFR	- Phase I: NSCLC, AML, colorectal, head and neck cancer, CNS tumours (child) - Phase II: Solid tumours, NSCLC, glioblastoma, melanoma, mesothelioma, neurofibromatosis, ovarian, CLL, colorectal, breast, kidney, liver, SCLC - Phase III: NSCLC
BMS-275291 (Bristol-Myers Squibb)	MMP inhibitor	- Phase I: Kaposi's sarcoma - Phase II: Kaposi's sarcoma, prostate cancer, NSCLC - Phase III: NSCLC
CCI-779 (Wyeth)	mTOR, VEGFR	- Phase I: Solid tumours, prostate cancer, CML, and others - Phase II: CLL, melanoma, glioblastoma, multiple myeloma, GIST, SCLC, NHL, NSCLC, neuroendocrine tumours, breast, pancreatic, endometrial cancer, and others
CDP-791 (Imclone Systems)	VEGFR2, KDR	Phase II: NSCLC
Celecoxib (Pfizer)	Increases endostatin	- Phase I: NSCLC, pancreatic, prostate cancer, solid tumours - Phase II: Head and neck cancer prevention, breast cancer prevention, lung cancer prevention, NSCLC, paediatric solid tumours, Ewing's sarcoma, glioma, skin cancer prevention, basal cell nevus syndrome, Barrett's oesophagus, hepatocellular, oesophogael, prostate, cervical, colorectal, head and neck, breast, thyroid, nasopharyngeal cancer, and others - Phase III: Colon, prostate, bladder cancer, NSCLC, and others
Cilengitide (EMD Pharmaceuticals)	α v β 3 and 5 antagonist	- Phase I: Solid tumours, lymphomas, paediatric brain tumours - Phase II: Glioblastoma, gliomas
Combretastatin (Oxigene)	VE-cadherin	- Phase I: Solid tumours - Phase II: Solid tumours, anaplastic thyroid cancer
E7820 (Eisia Medical Research Inc.)	Inhibits integrin α 2 subunit on endothelium	- Phase I: Lymphoma - Phase II: Colorectal cancer
Everolimus (Novartis)	VEGFR, mTOR	- Phase I: Breast cancer, solid tumours, lymphoma - Phase II: NSCLC, melanoma, AML, ALL, CML, lymphoma, glioblastoma, prostate, colorectal, neuroendocrine, breast, endometrial, kidney cancer, paediatric tumours, solid tumours - Phase III: Islet cell pancreas II/III, and others
Genistein (National Cancer Institute (NCI), USA)	Suppresses VEGF and neuropilin and MMP9 in tumour cells, upregulates CTAP	Phase I: Melanoma, kidney, prostate, bladder, breast cancer
Homoharringtonine (ChenGenex Therapeutics)	Downregulates VEGF in leukaemic cells	- Phase II: CML, APML - Phase III: CML
IMC-1121b (Imclone Systems)	VEGFR2, KDR	Phase I: Solid tumours
INGN 241 (Introgen Therapeutics)	MDA7, VEGF	Phase II: Melanoma

Table 3b | **Clinical trials of drugs that have shown anti-angiogenic activity in preclinical models**

Inhibitor (Company)	Target/mechanism	Clinical development
Interleukin-12 (NCI)	Upregulates IP10	• Phase I: Solid tumours, melanoma, paediatric neuroblastoma, kidney, breast cancer • Phase II: Melanoma, NHL, multiple myeloma, breast, ovarian, peritoneal, prostate cancer
Enzastaurin (Eli Lilly and Company)	VEGF	• Phase I: Solid tumours, gliomas • Phase II: Gliomas, lymphoma, brain tumours, NSCLC, pancreatic, colorectal cancer • Phase III: Lymphoma prevention, glioblastoma
Neovastat (Aeterna Zentaris)	MMP inhibitor	• Phase II: Multiple myeloma • Phase III: Kidney, NSCLC
NM-3 (Genzyme Corporation)	Inhibits VEGF expression by tumour cells, inhibits endothelial proliferation	• Phase I: Solid tumours
NPI-2358 (Nereus Pharmaceuticals)	β -tubulin	• Phase I: Solid tumours
Phosphomannopentaose sulphate (Progen Industries, Medigen Biotechnology)	bFGF, stimulates release of TFP1	• Phase II: Melanoma, NSCLC, prostate, hepatocellular cancer
PKC412 (Novartis)	VEGFR2	• Phase I: AML • Phase II: Mast-cell leukaemia
PPI-2458 (Praecis)	METAP2	• Phase I: Solid tumours, NHL
Prinomastat (Agouron Pharmaceuticals)	MMP inhibitor	• Phase II: Glioblastoma
PXD101 (CuraGen Corporation)	HDAC inhibitor	• Phase I: Solid tumours, haem malignancies • Phase II: Multiple myeloma, myelodysplastic syndrome, lymphoma, AML, NHL, ovarian/peritoneal, liver cancer
Suramin (NCI)	IGF1, EGFR, PDGFR, TGF β , inhibits VEGF and bFGF	• Phase I: Bladder, breast, kidney cancer • Phase II: Glioblastoma, breast, kidney, adrenocortical cancer • Phase III: Prostate cancer
Tempostatin (Collard Biopharmaceuticals)	Extracellular matrix proteins	• Phase I: Solid tumours • Phase II: Kaposi's sarcoma
Tetrathiomolybdate (Sigma-Aldrich)	Copper chelator	• Phase II: Prostate, oesophageal, breast, colorectal cancer • Phase III: Psoriasis
TKI-258 (Novartis, Chiron Corporation)	FGFR3, VEGFR	• Phase I: Multiple myeloma, AML, melanoma
Vatalanib (Novartis)	VEGFR1,2, PDGFR	• Phase I: Solid tumours, NSCLC, gynaecologic tumours • Phase II: GIST, AML, CML, solid tumours, NSCLC, VHL, haemangioblastoma, mesothelioma, breast, prostate, pancreatic, neuroendocrine cancer, glioblastoma, meningioma, myelodysplastic syndrome, multiple myeloma, age-related macular degeneration • Phase III: Colorectal cancer
VEGF Trap (Regeneron Pharmaceuticals)	VEGF	• Phase I: NHL, age-related macular degeneration, diabetic macular oedema • Phase II: Kidney, ovarian cancer, NSCLC, age-related macular degeneration • Phase III: Ovarian cancer
XL184 (Exelixis)	MMET, VEGFR, RTK, FLT3, TIE2	• Phase I: Solid tumours
XL880 (Exelixis)	C-met, RTK	• Phase I: Solid tumours • Phase II: Papillary renal cell carcinoma
XL999 (Exelixis)	VEGFR, PDGFR, EGFR, FLT3, Src	• Phase I: Solid tumours • Phase II: Multiple myeloma, colorectal, ovarian, renal cell cancer, AML, NSCLC
ZD6474 (AstraZeneca)	VEGFR2, EGFR	• Phase I: Glioma • Phase II: Breast cancer, NSCLC, SCLC, thyroid, gliomas, multiple myeloma • Phase III: NSCLC

AML, acute myeloid leukaemia; APML, acute promyelocytic leukaemia; bFGF, basic fibroblast growth factor; CML, chronic myeloid leukaemia; CLL, chronic lymphocytic leukaemia; CNS, central nervous system; CTAP, connective tissue activation peptide; EGFR, epidermal growth factor receptor; FGFR3, fibroblast growth factor receptor 3; FLT3, fms-related tyrosine kinase 3; HDAC, histone deacetylase; HIF1, hypoxia-inducible factor 1; IGF1, insulin-like growth factor 1; IP10, inducible protein 10; KDR, kinase insert domain receptor; MDA7, interleukin-24; METAP2, methionyl aminopeptidase 2; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PDGFR, platelet-derived growth factor receptor; RETR, ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease) receptor; SCLC, small-cell lung cancer; TFP1, transferrin pseudogene 1; TGF β , transforming growth factor- β ; TNF, tumour-necrosis factor; uPA, urokinase-type plasminogen activator; VE-cadherin, vascular/endothelial-cadherin; VEGF(R), vascular endothelial growth factor (receptor); vWF, von Willebrand factor.

inhibitor in the endometrium? If so, could this condition be diagnosed by the measurement of THBS1 in the vaginal fluid? Could endometrial THBS1 then be suppressed, for example, by a vaginal suppository containing a putative short-acting THBS1-suppressor protein? Another intriguing finding is that haemangiomas, benign tumours of infancy, have the gene signature of cells of the fetal placental endothelium, implying that they might originate from the fetal placenta^{69,70} (BOX 1). As haemangiomas usually regress spontaneously, they might reveal important clues about the molecular mechanisms of spontaneous regression of new blood vessels.

Endothelial cell control of tissue mass. When approximately 70% of the liver is removed in a rat (hepatectomy), the original mass regenerates completely in approximately 10 days⁷¹. Hepatocyte proliferation and endothelial cell proliferation are initiated the day after surgery. At approximately day 8, there is a wave of endothelial cell apoptosis, following which hepatocyte proliferation ceases⁴⁸. The liver stops growing at ~10 days. However, if an angiogenic protein, such as VEGF or bFGF, is administered systemically, endothelial cells continue to proliferate and the liver continues to grow beyond its normal size. By contrast, if a specific inhibitor of endothelial proliferation is administered, liver regeneration is prevented and the liver remains at 30% of its normal size. Discontinuation of the endothelial inhibitor is followed immediately by liver regeneration that is complete by 10 days⁴⁸. These experiments indicate that normal tissue and organ regeneration are controlled in part by the microvascular endothelium.

Growth and regression of fat is controlled by endothelial proliferation or apoptosis, respectively⁴⁹. Leptin-deficient mice gain up to approximately 1 gram per day, mainly in fat. Adipocyte enlargement and proliferation is accompanied by endothelial proliferation that is restricted to fat. Systemic administration of an angiogenesis inhibitor (TNP-470 or endostatin) specifically induces endothelial apoptosis and a decrease in fat accompanied by rapid weight loss. When the normal weight for age is reached, weight loss stops. A similar result is obtained when endothelial cells in fat are specifically targeted by a genetically regulated inhibitor of proliferation⁷². Growth of normal prostate is also under endothelial control⁷³, and so is bone growth⁷⁴. Therefore, it seems that microvascular endothelial cells can control tissue mass, regardless of whether the cells in this mass have a normal genome or a cancer

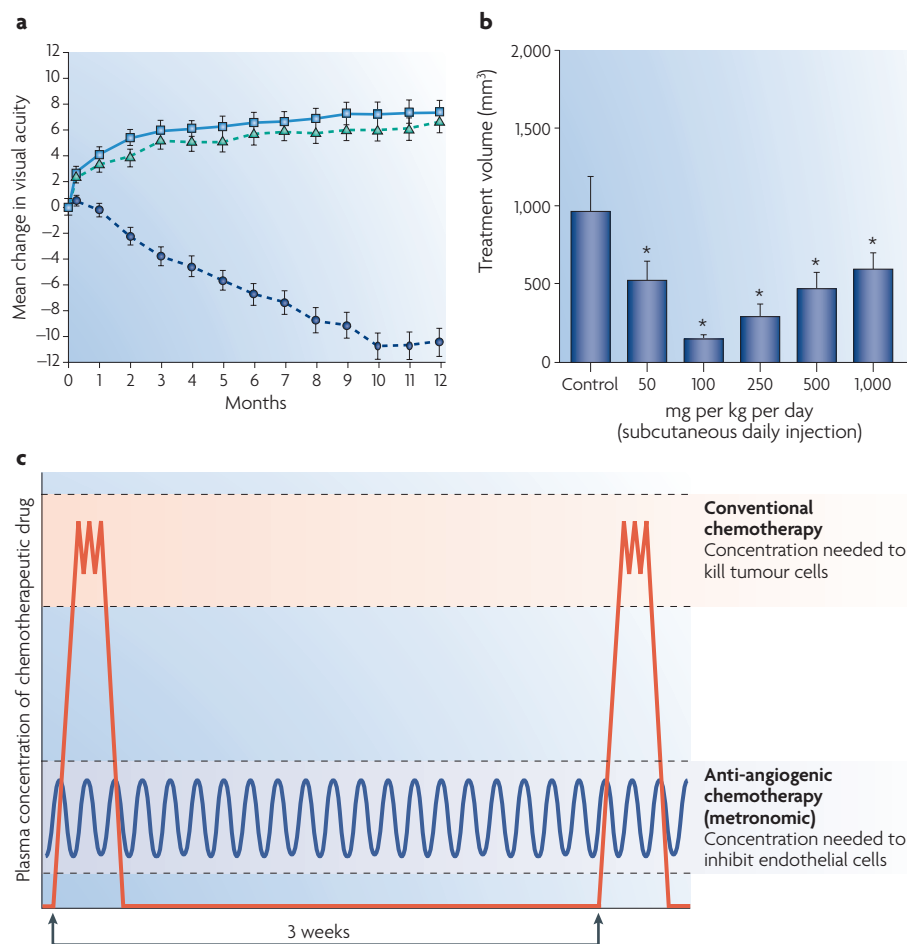


Figure 3 | Examples of anti-angiogenic therapy. **a** | Phase III clinical trial of Lucentis (ranibizumab; Genentech), a fragment of Avastin, an antibody to vascular endothelial growth factor (VEGF). Lucentis is used for intra-ocular injection in patients with age-related macular degeneration³⁷. **b** | A biphasic (U-shaped) dose-efficacy curve for human pancreatic cancer in immunodeficient mice treated with endostatin. The tumour cells are also deficient in p53 (adapted from REF. 119). **c** | Dosing schedule differences between conventional chemotherapy (red) and anti-angiogenic (metronomic) chemotherapy (blue) (adapted from REF. 131 and from discussions with R. Kerbel).

genome⁷⁵. This raises a provocative question: Is there some type of set-point or feedback mechanism in endothelial cells that tells them when a normal organ, such as liver, has reached its normal mass? If so, do tumour cells override this mechanism, and how?

What are the implications of this general principle for drug discovery? There is the possibility that specific endothelial inhibitors might be used to control obesity⁷², as well as overgrowth of other tissues, such as uterine fibroids, overgrowth of bone caused by lymphangiogenesis and ectopic bone growth (fibrodysplasia ossificans progressiva)⁷⁶. Specific endothelial inhibitors might also be used to control vascular malformations that grow rapidly after puberty or after attempts at surgical excision, and for which there are currently no drugs. The endocrine-specific angiogenesis-regulatory proteins, such as

Bombina variegata peptide 8 kDa protein⁷⁷ (Bv8; also known as **PROK2**; **testicular-cancer-specific**), are of particular interest.

Is oncogene dependence angiogenesis dependent? The recognition that endothelial cells control tumour mass is crucial for a more complete understanding of how oncogenes initiate tumour growth. The conventional wisdom is that oncogene activation in a cell leads directly to the formation of large lethal tumours in mice. This concept is reinforced by experiments in which *Ras* or *Myc* oncogenes, under the control of the doxycycline promoter, induce rapid tumour growth when the oncogene is activated, leading to rapid tumour regression when the oncogene is inactivated^{78–83}. This phenomenon is called oncogene dependence or oncogene addiction⁸⁴.

However, my group has found that during oncogene-induced tumour growth there is intense tumour angiogenesis associated with suppression of THBS1 in the tumour bed. When an oncogene is inactivated, the expression of THBS1, a potent angiogenesis inhibitor, is increased in the tumour bed, leading us to propose that oncogene addiction is angiogenesis dependent⁸³. This hypothesis has now been supported by the deletion of THBS1 in the tumour and the host. In these mouse models, an activated oncogene induces more rapid tumour growth than in wild-type mice, but tumours do not regress after the inactivation of the oncogene⁸². Restoration of THBS1 expression in the tumour results in tumour regression upon oncogene inactivation⁵⁰.

How could this change in thinking about oncogene addiction provide new opportunities in drug discovery? Conventional wisdom (FIG. 4) suggests that the development of drugs targeted against oncogenes should be sufficient to control cancer. Imatinib (Gleevec; Novartis), which targets the product of the *BCR-ABL* oncogene, has demonstrated proof-of-concept by its success in the treatment of **chronic myeloid leukaemia**. Furthermore, imatinib targets the product of the oncogene *cKIT*, and has also proved successful in treating gastrointestinal stromal tumours in which this protein has a key role^{85,86}. However, many patients eventually develop drug resistance⁸⁷, and there are numerous other oncogenes that could be responsible for inducing expression of redundant growth factors in these tumours. The imatinib experience also suggests that drugs will need to be developed against combinations of many oncogenes. A single angiogenesis inhibitor, especially a broad-spectrum angiogenesis inhibitor such as endostatin²⁸ or caplostatin, or a combination of angiogenesis inhibitors might block the effect of a large family of oncogenes, as the blockade of angiogenesis can prevent tumour growth downstream of oncogene activation. Analysis of 15 of the most studied oncogenes revealed that the majority of them increase the expression of VEGF (and/or bFGF) and decrease the expression of THBS1 in tumour cells^{88,89}.

Genetic regulation of angiogenesis. Although we all carry endogenous angiogenesis inhibitors in our blood and tissues (at least 29 at the time of writing)^{24,25,90}, different individuals reveal distinct genetic differences in their angiogenic response to a given stimulus. For example, individuals with **Down syndrome** are protected against diabetic retinopathy,

Box 1 | Are infantile haemangiomas metastases from the placenta?

Haemangiomas are benign tumours made of capillary blood vessels that appear in 1 out of 100 newborns and usually begin to undergo spontaneous regression at approximately the end of the first year⁶. Some haemangiomas can be life threatening if they occur in the brain, airway or liver. The mechanism of haemangioma regression is unclear. Haemangiomas provide the possibility that they might reveal a clue about molecular mechanisms of spontaneous regression of new blood vessels. It was recently reported that all infantile haemangiomas express the glucose receptor GLUT1 (also known as SLC2A1), and that this receptor is also found on the endothelium of the placenta⁶⁹. This observation led to a gene array analysis of endothelial cells from haemangioma and other tissues, which revealed that gene expression of haemangioma endothelium is identical to gene expression of fetal placental endothelium, but not to any other tissue analyzed⁷⁰. The implication is that haemangiomas might be metastases from the fetal placenta. A further implication is that putative endogenous angiogenesis inhibitors that control the regression of placental vasculature at term might also be involved in the regression of haemangiomas. This speculation remains to be tested, but it illustrates how viewing a given process as part of an organizing principle can be useful.

although they have a similar incidence of diabetes as individuals without Down syndrome^{52,91}. They also have higher levels of circulating endostatin (~1.6-fold) than normal individuals because of an extra copy of the gene for the endostatin precursor (collagen XVIII) on chromosome 21 (REFS 91,92). Interestingly, they seem to be among the most protected of all humans against cancer. Although testicular cancer and a megakaryocytic leukaemia have been reported for individuals with Down syndrome, they have the lowest incidence of the other ~200 human cancers compared with age-matched controls^{52,91}. Conversely, individuals with a polymorphism in endostatin (specifically arginine substituted for alanine at N104) have a significantly higher risk of breast cancer⁹³. The correlation between endostatin levels and cancer susceptibility was demonstrated in mice. Mice that were engineered to genetically overexpress endostatin to mimic individuals with Down syndrome have tumours that grow 300% slower⁹⁴, and in mice that had THBS1 deleted, tumours grow approximately 300% more rapidly, and more quickly still if two angiogenesis inhibitors are knocked out (tumstatin and THBS1)⁹⁴.

Another interesting finding is that African Americans rarely develop the 'wet' form of age-related macular degeneration. They usually do not have intravitreal haemorrhages and do not go blind from the 'dry' form of this disease⁹⁵. By contrast, African Americans have a similar incidence of diabetic retinopathy. In age-related macular degeneration, neovascularization is in the choroidal layer that is surrounded by melanocytes containing melanin. In diabetic retinopathy, neovascularization arises from the retina. The retinal pigmented epithelial cells contain a lighter form of oxidized melanin, which differs from melanin in

the choroid or in the skin. Also, African American infants rarely develop cutaneous haemangiomas compared with white infants.

These correlations suggest that factors linked to pigmentation and melanin are producing an inhibitory influence on the angiogenic balance in the melanin-rich tissues. However, there is no melanin in prostate or breast tissue, and African Americans are not protected from cancer of these organs.

This hypothesis was examined in animal experiments. When the gene for tyrosinase (in the melanin pathway) was deleted from mice, the albino relatives C57Bl/6J-Tyr^{-/-} showed intense iris neovascularization and haemorrhage (hyphaema) compared with weak neovascularization and no haemorrhage in the pigmented iris of wild-type mice. The amount of corneal neovascularization was not significantly different between these two strains because the cornea is not a pigmented tissue^{96,97}.

Genetic variations of angiogenic factors have important consequences for the clinical treatment of angiogenesis-dependent

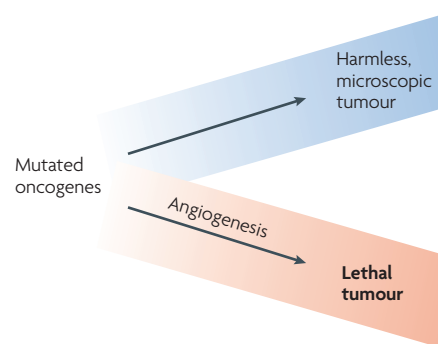


Figure 4 | Oncogene addiction is angiogenesis dependent. An oncogene-induced tumour that cannot recruit new blood vessels will remain as a harmless microscopic tumour in experimental animals^{50,83}.

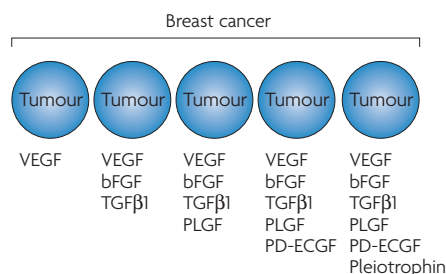


Figure 5 | Angiogenic proteins in breast cancer. Human breast cancer can cause the expression of at least six different angiogenic proteins (adapted from REF. 142). bFGF, basic fibroblast growth factor (also known as FGF2); PD-ECGF, platelet-derived endothelial cell growth factor (also known as ECGF1); PLGF, placental growth factor (also known as PGF); TGFβ1, transforming growth factor-β1; VEGF, vascular endothelial growth factor.

diseases. For example, some tumours that seem poorly vascularized and have a low microvessel density will be inhibited by a significantly lower dose of an angiogenesis inhibitor than is required for a highly vascularized tumour with a significantly higher microvessel density⁹⁸. This may be counter-intuitive to clinicians who might inform a patient that their tumour is not very vascular and therefore will not respond to anti-angiogenic therapy. In fact, these tumours might be expressing their own angiogenesis inhibitors^{99,100} and might respond to a lower therapeutic dose of angiogenesis inhibitor than would be required for a highly vascularized tumour.

The genetic heterogeneity of the angiogenic response is another reason for the pressing need to develop blood or urine biomarkers¹⁰¹ to optimize the dosing of anti-angiogenic therapy. Furthermore, when mice are used for preclinical studies of angiogenesis inhibitors, it is crucial to know the genetic background of the mice in regards to their angiogenic responsiveness.

Optimizing anti-angiogenic therapy

Insights into the molecular mechanisms and significance of angiogenesis in different biological contexts are creating exciting new opportunities for drug discovery. However, as in some cases including cancer, anti-angiogenic therapies can also be used in combination with existing drugs. It is important to understand the difference between anti-angiogenic and cytotoxic drugs to optimize efficacy.

Anti-angiogenic therapy and cytotoxic chemotherapy. In February 2004, when the FDA approved bevacizumab for colorectal cancer, M. McClellan, then FDA Commissioner, said: “Anti-angiogenic therapy can now be considered the fourth modality for cancer treatment.”¹⁰². It is a different modality because there are certain notable differences about chemotherapy that do not always readily transfer to anti-angiogenic therapy.

Importantly, anti-angiogenic therapy primarily targets the activated microvascular endothelial cells in a tumour bed rather than the tumour itself. It can accomplish

this directly by preventing endothelial cells from responding to angiogenic proteins, as endostatin²⁸ and caplostatin^{103,104} do. Anti-angiogenic therapy can also inhibit endothelial cell proliferation and motility indirectly by suppressing a tumour’s production of angiogenic proteins, as erlotinib does¹⁰⁵, or by neutralizing one of these proteins, as bevacizumab does.

Also, although chemotherapy is usually more effective on rapidly growing tumours than on slowly growing tumours, the opposite is often true of anti-angiogenic therapy. More rapidly growing tumours can require higher doses of anti-angiogenic therapy⁹⁸. Furthermore, chemotherapy is optimally given at a maximum tolerated dose, with off-therapy intervals of 1–3 weeks to rescue bone marrow and intestine. Anti-angiogenic therapy might optimally require that endothelial cells be exposed to steady blood levels of the inhibitor¹⁰⁰. Therefore, daily dosing is optimal for those angiogenesis inhibitors with a short half-life. However, certain antibodies such as bevacizumab can be administered every 2 weeks because of long-lasting antibody levels in plasma, and perhaps because of neutralization of VEGF in platelets by bevacizumab that enters the platelets and binds with VEGF⁶¹. Zoledronate (Zometa; Novartis) is an amino-bisphosphonate that has been shown to inhibit angiogenesis¹⁰⁶ by targeting matrix metalloproteinase 9 (MMP9)¹⁰⁷, by reducing circulating levels of pro-angiogenic proteins in the circulation¹⁰⁸ or by suppressing multiple circulating pro-angiogenic factors in patients with cancer¹⁰⁹. It accumulates in bone and can therefore be administered every month. However, after prolonged use, zoledronate may need to be administered less frequently to avoid osteonecrosis of the jaw.

Another important difference concerns the side effects of anti-angiogenic therapy compared with chemotherapy. Bone-marrow suppression, hair loss, severe vomiting and diarrhoea, and weakness are less common with anti-angiogenic therapy, and endostatin has shown minimal or no side effects in animals¹¹⁰ and in humans¹¹¹. It has to be noted though, that certain angiogenesis inhibitors increase the incidence of thrombotic complications, such as thalidomide (Thalomid; Celgene)¹¹² and bevacizumab. The risk of thrombosis is increased when these angiogenesis inhibitors are administered together with conventional chemotherapy¹¹³. Other side effects of inhibitors of VEGF include hypertension, intratumoural bleeding and bowel

Table 4 | Three types of angiogenesis inhibitors

Mechanism	Drug	Action
Type I		
Blocks one main angiogenic protein	Avastin (Avastin; Genentech)	Blocks VEGF
	VEGF Trap (Regeneron Pharmaceuticals)	Blocks VEGF
Type II		
Blocks two or three main angiogenic proteins	Sutent (Sutent; Pfizer)	Downregulates VEGF receptor 2, PDGF receptor, cKIT receptor
	Tarceva (Tarceva; Genentech, OSI Pharmaceuticals, Roche)	Downregulates VEGF production, bFGF production, TGFα by tumour cell
Type III		
Blocks a broad range of angiogenic regulators	Endostatin	Downregulates VEGF, bFGF, bFGF receptor, HIF1α, EGF receptor, ID1, neuropilin Upregulates thrombospondin 1, maspin, HIF1α, TIMP2
	Caplostatin	Broad anti-angiogenic and anticancer spectrum

bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; HIF1α, hypoxia-inducible factor 1α; ID1, inhibitor of DNA binding 1, dominant negative helix-loop-helix protein; PDGF, platelet-derived growth factor; TIMP2, tissue inhibitor of metalloproteinase 2; TGFα, transforming growth factor-α; VEGF, vascular endothelial growth factor.

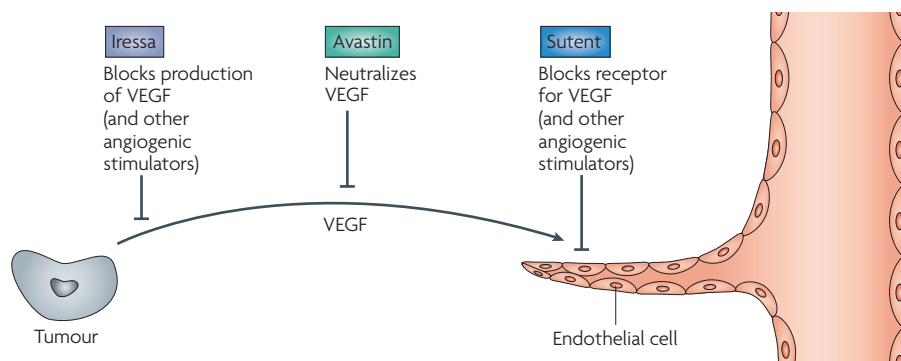


Figure 6 | Three general mechanisms of angiogenesis inhibitors currently approved by the FDA. Iressa blocks tumour expression of an angiogenic factor. Avastin blocks an angiogenic factor after its secretion from a tumour. Sutent blocks an endothelial cell receptor. VEGF, vascular endothelial growth factor.

perforation, especially in cases in which the intestine contains a tumour. Thalidomide has a slightly higher incidence of thromboembolic complications, as well as constipation and peripheral neuropathy — these are usually reversible upon discontinuation of thalidomide. Lenalidomide (Revlimid; Celgene), an FDA-approved derivative of thalidomide, has significantly reduced side effects. Side effects need to be carefully considered, especially when anti-angiogenic and cytotoxic medications are combined. So far, there are almost no data that allow a direct comparison of clotting risk for anti-angiogenic therapy alone, compared with cytotoxic therapy or combination therapy.

However, there can also be unexpected benefits from combining angiogenesis inhibitors, or drugs that have varying degrees of anti-angiogenic activity, with conventional chemotherapy. For example, Jain has shown that bevacizumab, by decreasing vascular leakage in a tumour, can lower intratumoural-tissue pressure and increase delivery of chemotherapy to a tumour¹¹⁴. In other words, anti-angiogenic therapy might ‘normalize’ tumour vessels¹¹⁵. Teicher *et al.* showed that anti-angiogenic therapy could decrease intratumoural pressure, which resulted temporarily in increased oxygenation to a tumour with subsequent increased sensitivity to ionizing radiation¹¹⁶.

Biphasic dose efficacy of anti-angiogenic therapy. Dose efficacy is generally a linear function for chemotherapy. By contrast, several angiogenesis inhibitors have been reported to follow a biphasic, U-shaped dose-efficacy curve (known as hormesis¹¹⁷). For example, interferon- α (IFN α) is anti-angiogenic at low doses, but not at higher doses¹¹⁸. Similarly, rosiglitazone (Avandia;

GlaxoSmithKline), a peroxisome proliferator-activated receptor- γ (PPAR γ) ligand, as well as endostatin protein therapy¹¹⁹ (FIG. 3b) and endostatin gene therapy¹²⁰ inhibit angiogenesis with a U-shaped dose-efficacy curve¹²¹. Before the U-shaped dose-efficacy response was recognized for anti-angiogenic gene therapy, my group had observed that gene therapy of endostatin could produce such high blood levels that all anti-angiogenic activity was lost¹²². It is now clear that blood levels of certain angiogenesis inhibitors (such as endostatin) that are too high or too low will be ineffective, and that the biphasic dose-efficacy curve offers the best explanation for why endostatin gene therapy of murine leukaemia failed^{123,124}.

Even the effect of endostatin on the gene expression (for example, hypoxia-inducible factor 1 α ; HIF1 α) of fresh human endothelial cells *in vitro* reveals a U-shaped dose-efficacy pattern²⁸. This is important information for drug discovery. For example, in the ranibizumab trial for age-related macular degeneration, a higher dose did not increase efficacy over a lower dose.

Anti-angiogenic therapy and drug resistance. Tumours might become refractory to anti-angiogenic therapy, especially if a mono-anti-angiogenic therapy targets only one angiogenic protein (for example, VEGF)¹²⁴. Endothelial cells seem to have a lower probability for developing resistance to anti-angiogenic therapy, even though mouse endothelial cells in a tumour bed can become genetically unstable^{80,125}. Although VEGF is expressed by up to 60% of human tumours, most tumours can also express five to eight other angiogenic proteins — for example, human breast cancers can express up to six angiogenic proteins (FIG. 5). High-grade brain tumours might express more

angiogenic proteins than other tumours. When the expression of one angiogenic protein is suppressed for a long period, the expression of other angiogenic proteins might emerge¹²⁶. The mechanism of this ‘compensatory’ response is unclear. Some angiogenesis inhibitors target up to three angiogenic proteins, whereas others target a broad range of angiogenic proteins (TABLE 4). Certain tumours, such as high-grade giant-cell tumours and angiosarcomas, produce bFGF as their predominant angiogenic protein and do not seem to deviate from this. For this reason, low-dose daily IFN α therapy for 1–3 years is sufficient to return abnormally high levels of bFGF in the urine of these patients to normal. IFN α has been reported to suppress the production of bFGF by human cancer cells¹¹⁸. This treatment regimen has produced long term complete remissions (up to 10 years) without drug resistance (at the time of writing; see REFS 127–129 and L. Kaban, personal communication).

Currently, the majority of FDA-approved angiogenesis inhibitors, as well as those in Phase III clinical trials, neutralize VEGF, target its receptor or suppress its expression by tumour cells (FIG. 6). When drug resistance develops to some of these inhibitors, they are often perceived to represent the whole class of angiogenesis inhibitors. It remains to be seen if broad-spectrum angiogenesis inhibitors will develop less drug resistance than angiogenesis inhibitors that target against a single angiogenic protein. In experimental tumours, TNP-470, a synthetic analogue of fumagillin and caplostatin, its derivative^{103,130}, did not induce drug resistance when administered to mice for prolonged periods of time¹⁰⁴.

Anti-angiogenic chemotherapy (metronomic therapy). Browder *et al.* first reported that when murine tumours were made drug resistant to cyclophosphamide and then cyclophosphamide was administered on a conventional chemotherapy maximum-tolerated dose schedule, all mice died of large tumours¹³¹. However, if cyclophosphamide was administered more frequently at a lower dose, the tumours were potentially inhibited because of endothelial apoptosis. If an angiogenesis inhibitor (TNP-470)¹⁰⁴ was added, which by itself could only inhibit the tumours by 50%, the drug-resistant tumours were eradicated¹³¹. This experiment demonstrated a new principle: a cytotoxic chemotherapeutic agent could be redirected to an endothelial target by changing its dose and frequency of

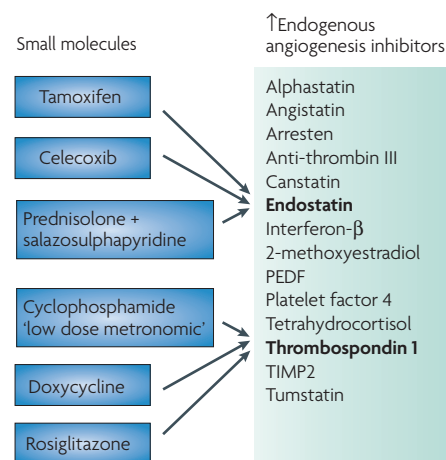


Figure 7 | Small molecules to increase endogenous angiogenesis inhibitors. Examples of small molecules that are orally available and might induce increased levels of endogenous angiogenesis inhibitors in the blood or joint fluid. PEDF, pigment epithelium-derived factor; TIMP2, tissue inhibitor of matrix metalloproteinase 2 (REF. 124).

administration. Browder *et al.* called this regimen anti-angiogenic chemotherapy. Klement *et al.* confirmed this approach with a different chemotherapeutic agent¹³². Bocci *et al.*¹³³ further showed that anti-angiogenic chemotherapy increased circulating THBS1, and that deletion of the *THBS1* gene in mice completely abrogated the antitumour effect of this anti-angiogenic therapy. These results suggested that THBS1 acts as a mediator of anti-angiogenic chemotherapy¹³³. The optimization of chemotherapy to treat vascular endothelium in the tumour bed is also called 'metronomic' therapy¹³⁴ (FIG. 3c) and has entered clinical trials for brain tumours and other tumours that were refractory to conventional chemotherapy. Kieran *et al.* recently studied 20 children with different types of brain tumours refractory to surgery, radiotherapy and chemotherapy, who were treated for 6 months with daily oral thalidomide and celecoxib (Celebrex; Pfizer), plus daily low-dose oral cyclophosphamide alternated every 21 days with daily low-dose oral etoposide¹³⁵. Twenty-five percent of the patients were progression free more than 2.5 years from starting therapy. Forty percent of patients completed the 6 months of therapy, resulting in prolonged or persistent disease-free status. Sixteen percent of patients showed a radiographic partial response. Only elevated THBS1 levels in the blood correlated with prolonged response. This is consistent with the elevated circulating THBS1 levels observed in tumour-bearing

mice treated with anti-angiogenic (metronomic) cyclophosphamide¹³³. It is possible that angiogenesis inhibitors, such as bevacizumab, might be augmented by low dose anti-angiogenic (metronomic) chemotherapy with fewer side effects than conventional dosing of chemotherapy.

New pharmacology: oral drugs that increase endogenous angiogenesis inhibitors. The clinical finding that individuals with Down syndrome have an elevated circulating level of endostatin approximately 1.6-fold higher than normal individuals⁹¹ is provocative. It suggests that small elevations of one or more endogenous angiogenesis inhibitors in the blood might protect against recurrent cancer, or might prevent the switch to the angiogenic phenotype in women at high risk for breast cancer. It is also possible that other genes on chromosome 21 have anti-angiogenic activity.

It has been found that certain orally available small molecules can upregulate expression of specific endogenous anti-angiogenic proteins, opening the way for a new field of pharmacology (FIG. 7). Endostatin is increased by tamoxifen¹³⁶, celecoxib¹³⁷ and (in joint fluid) by prednisolone plus salazosulphapyridine¹³⁸. THBS1 is upregulated by low dose cyclophosphamide¹³³, doxycycline¹³⁹ and rosiglitazone¹²¹. This unifying concept points to future drug discovery in which the known endogenous angiogenesis inhibitors could be screened for small-molecule inducers that would increase the circulating level of one or more of them.

Outlook

Angiogenesis inhibitors are now being approved and introduced into medical practice throughout the world. At the same time, a need for molecular biomarkers is being met by an expanding worldwide research effort to develop gene-based and protein-based molecular signatures in blood, platelets and urine for very early diagnosis of recurrent cancer. One can speculate that if these two fields intersect, it might someday be possible to diagnose microscopic tumours at a millimetre size, at about the time of the angiogenic switch but perhaps years before they are symptomatic, or before they can be visualized by any conventional methods.

For example, today most individuals with the diagnosis of colon cancer are operated on. At least 50–60% of these patients are cured by the surgery. In the other patients, cancer will recur in approximately 4–6 years. Physicians are helpless to do

anything until symptoms (such as pain and jaundice) occur, or until the recurrent cancer can be located by ultrasound, magnetic resonance imaging or CAT (computed axial tomography) scan. However, sensitive and specific molecular biomarkers that are being developed today could be used in the future to diagnose the presence of a microscopic recurrent tumour even before it could be anatomically located. Once these biomarkers are validated in clinical trials, then physicians could 'treat the rising biomarker' with non-toxic angiogenesis inhibitors until the biomarker returns to normal. A paradigm shift would be that recurrent cancer would be treated without waiting to see it, when it is still relatively harmless with low or no metastatic potential (that is, before the switch to the angiogenic phenotype). It might also be possible to use angiogenesis-based biomarkers to monitor the progression or regression of certain angiogenesis-dependent diseases that are non-neoplastic. These could include atherosclerosis, endometriosis, Crohn's disease and rheumatoid arthritis, among others.

There might be an analogy in the history of the treatment of infection. Before 1930, there were virtually no drugs for any infection, and most infections progressed to abscesses. Surgeons had to wait until the abscess was large enough to be located by X-rays so that the abscess could be surgically drained. The surgical textbooks of that era instructed surgeons how to locate an abscess: above the liver, behind the liver, in the mastoid, and so on. The term 'laudable' pus was commonly used to mean that if a surgeon could successfully drain an abscess the patient might live. After 1941, when antibiotics were introduced, it was no longer necessary to precisely locate an infection. Today the treatment of most infections is simply guided by blood tests (white-blood-cell count or blood cultures). As we continue to gain insight into angiogenesis and the role of angiogenic factors in seemingly unrelated diseases, the consequent potential of angiogenic modulators could see P. Carmeliet's prediction in the December 2005 issue of *Nature*¹⁴⁰ becoming prophetic: "Angiogenesis research will probably change the face of medicine in the next decades, with more than 500 million people worldwide predicted to benefit from pro- or anti-angiogenesis treatments"¹⁴⁰.

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Competing interests statement

The author declares no competing financial interests.

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Judah Folkman's homepage: http://www.childrenshospital.org/cfapps/research/data_admin/5105/mainpage5105P0.html
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TOC Blurbs

Pathological angiogenesis plays a role in a wide range of diseases. Folkman argues that viewing angiogenesis as an 'organizing principle' in biology can lead to novel insights into the molecular mechanisms of seemingly unrelated phenomena, and facilitate the development of new therapeutic approaches.