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Review Article

In-vitro and in-vivo assays for angiogenesis-modulating drug discovery and development

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Abstract

In the past 35 years, significant findings have been made in relation to angiogenesis, and how this usually normal physiological function is converted into an abnormal state in cancer. To search for agents that can inhibit angiogenesis, and thereby prevent a tumour from proliferation and spread that is ultimately fatal to the patient, various in-vitro assays have been developed. In addition, older assays have been refined usually into high throughput screening formats, mainly by the biopharmaceutical industry in their attempts to develop novel therapeutic molecules and maintain a pipeline of lead candidates. The central aim is to extract more accurate data that would facilitate the birth of innovative mechanisms to defeat aberrant angiogenesis in-vivo. At the same time, better in-vivo models have been established, with the goal to mimic as close as possible the natural progression of various types of neoplasms in response to a good angiogenic response. More clinically relevant models are needed as anti-angiogenesis drug discovery and drug development companies fast track their lead molecules from preclinical investigations to phase I clinical trials.

Brief overview of angiogenesis

Angiogenesis is the process by which blood vessels form from pre-existing vessels (Risau 1998). The primitive vascular network, developed by vasculogenesis in the embryo, is reworked and developed by angiogenesis. This allows maturation, branching and formation of complex vasculature. Angiogenesis involves pruning, intussusception and enlargement of vessels, allowing greater interaction with surrounding endothelial cells in the extracellular matrix, pericytes and smooth muscle cells, thereby creating a stable vascular environment (Zadeh & Guha 2003).

In the normal physiological state vascular endothelial cells in the normal adult organism have a very low turnover, which is usually only crucial in conditions such as pregnancy and wound healing. However, angiogenesis can be enhanced, promoted or inhibited in pathological situations such as solid tumour growth, retinopathy (Risau 1998), and atherosclerosis (Conway 2003). It is in these situations that angiogenesis can be manipulated to prevent further disease progression.

Chronic tissue hypoxia stimulates the angiogenic pathway, by initiating a cascade of factors which stimulate endothelial cell proliferation and/or migration. It is the primary mechanism whereby tumours grow and metastasize (Denko & Giaccia 2001). Since tumour-angiogenesis is a crucial step in cancer, several in-vitro and in-vivo techniques used in cancer disease research and drug development measure response to cell proliferation, apoptosis and tube formation.

Tumour blood flow is abnormal and new tumour blood vessels do not resemble normal blood vessels in healthy tissues. The tumour vasculature has widened lumens, aneurysmal dilatations, fewer associated smooth muscle cells and pericytes, irregular blood flow, regions of stasis, and high permeability. Ironically, while angiogenesis first nourishes a tumour, it later can be the same channel via which various drugs may be delivered to the tumour, even in a selective manner (Dass & Su 2001), and finally, when the vasculature collapses, various central core zones of the tumour undergo necrosis as a result of unrecoverable hypoxia.

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Anti-angiogenic therapy

Anti-angiogenic therapy, first proposed in 1971 (Folkman et al 1971), has become an increasingly popular anticancer strategy. Angiogenesis inhibition approaches have several advantages over the conventional anticancer treatment modalities. Unlike most cancer cells, vascular endothelial cells (VECs) are readily accessible from the circulation and are genetically stable and thus unlikely to develop drug resistance (Kerbel 1997). VECs of the tumour are actively proliferating and less differentiated in comparison with the VECs of normal tissues (Folkman 1995). More than a score of anti-angiogenic drugs are currently undergoing clinical trials. These agents are not expected to be curative in the conventional sense of the word (that is causing tumour regression), but are anticipated to prevent the expansion of the tumour mass. Thus, the tumour may remain in the benign state for years (subject to regular check-ups) or may be eradicated by either surgery or a concomitant form of treatment such as radiotherapy. Indeed, a combination of low dose chemotherapy and different types of anti-angiogenic agents are widely being evaluated in clinical trials (Saaristo et al 2000). The FDA has just recently approved the first anti-angiogenesis drug, Avastin, which is a monoclonal antibody against vascular endothelial growth factor (VEGF). Even with conventional drugs against cancer, no one entity has shown the capacity to work alone in regressing the clinical pathology of cancer.

The molecular mechanisms of angiogenesis seem to vary in differing tumour sites and, as such, anti-angiogenic treatment modalities are likely to display variable effects in different tumours (Saaristo et al 2000). Chronic use of anti-angiogenic agents needs to be weighed against any possible delayed side effects to angiogenic processes in the normal tissues within the patient's body. To date, inhibition of VEGF or its receptor alone has shown some indications of efficacy in a variety of tumour models (Presta et al 1997; Brekken et al 1998; Goldman et al 1998; Kong et al 1998). What remains to be seen is whether tumours are able to activate complementary or alternative angiogenic pathways as a result of such a 'monotherapeutic' approach in long-term studies.

However, clinical trials only occur when the foundation on which they stand is solid. This foundation, at its very base, is the ability to identify potential anti-angiogenic compounds in robust in-vitro cell-based assays. It is nowadays imperative that drug discovery companies implement, maintain and build on their screening assays towards a high-throughput screening (HTS) format. Select companies in fact have taken this a bit further with ultra-high throughput screening (UHTS) formats, but as it is not a simple task to set up cell-based assays in even a 384-well format, UHTS will not be discussed in this review. Once a lead candidate is identified from the anti-angiogenic screens, then disease models with aberrant angiogenesis are used for further testing. The challenge is to establish clinically-relevant models in-house (usually a laborious and costly exercise) or use the services of contract research organizations (CROs) who already have technical proficiency in testing compounds in these models. Several recently used syngeneic murine and human xenograft models which evaluated anti-angiogenesis as a read-out, either directly or indirectly, are listed in Tables 1 and 2. These form the bulk of the tasks involved with pinpointing the potency of anti-angiogenic activity of a lead molecule.

Types of in-vitro assays

Methods that use non-human endothelial cells (ECs) will not be considered in this review, since there are differences between getting anti-angiogenic activity with murine cells for example compared with human endothelial cells. Endothelial cells used in the laboratory are meant to proliferate, whilst those in-vivo generally do not unless pushed, such as in wound healing or during the menstrual cycle. Cells grown in culture are by their very nature different from those in-vivo, as is now common knowledge.

A widely used technique to measure cell proliferation and cell death is via the incorporation of radiolabelled precursor molecules into cellular macromolecules. [^{3}H]Thymidine incorporation can be used as a direct measure of DNA replication. Responses are measured autoradiographically or by liquid scintillation counting. In a study profiling drug candidate ZD4190, cellular proliferation was measured using [^{3}H]thymidine incorporation. The experiment measured rate of proliferation by amount of tritium incorporated into cells using a beta counter (Wedge et al 2000). Autoradiographic detection of thymidine is more suited to malignant cells and is time-consuming. Recently a study demonstrated the capability of a bioactive portion of parathyroid hormone (PTH) molecule to increase the proliferation of human umbilical endothelial cells (HUVECs) using [^{3}H]thymidine incorporation technique (Ding et al 2005).

Table 1 Basic syngeneic murine tumour models used for testing anti-angiogenesis agents in the past five years

Cell line	Mouse strain	Test agent	Treatment details	Reference
Lewis lung	C57BL6	Angiozyme	From day 3, os. pump, for 14 days, intravenously	Pavco et al (2000)
Lewis lung	C57BL6	Cyclophos. + TNP-470	From 3rd day, every 2 days, for 20 days	Browder et al (2000)
Lewis lung	C57BL6	VE-cad antibody	From day 21, for 24 days, 2 ×/week, intraperitoneally	Liao et al (2000)
Lewis lung	C57BL6	Endostatin	From day 14, for 7 days, subcutaneous os. pump	Kisker et al (2001)
A549 carcinoma	C57VL/6J	ST1481	Orally by gavage every	Petrangolini et al (2003)
MeWo melanoma	Swiss nude BALB/c	ST1481	4th day or 5 times/week for 2 weeks	Petrangolini et al (2003)
B16 (melanoma)	C57BL/6	Taxol	Intraperitoneal injection for 3 weeks	Wang et al (2003a)

Table 2 Basic human xenograft tumour models for testing anti-angiogenesis agents in the past five years

Cell line	Mouse strain	Test agent	Treatment details	Reference
PC3 (prostate)	Balb/c nude	Angiostatin + Tumstatin	From day 14, daily, intraperitoneally	Maeshima et al (2000)
A431 (epidermoid)	Balb/c nude	VE-cadherin antibody	From day 21, for 40 days, 2 ×/week, intraperitoneally	Liao et al (2000)
BxPC3 (pancreatic)	SCID	Endostatin	From day 21, for 14 days, 1 ×/day, subcutaneously	Kisker et al (2001)
HT1080 (fibrosarcoma)	SCID	Endostatin	For 14 days, 1 ×/day, Subcutaneously or intraperitoneally	Kisker et al (2001)
MDA-MB435 (breast)	Balb/c nude	Tumstatin	From day 21, 2×/d, for 24 days, intraperitoneally.	Maeshima et al (2001a)
PC3 (prostate)	Balb/c nude	Endostatin + Tumstatin	From day 7, for 2 weeks, subcutaneous os. pump	Maeshima et al (2001b)
PC3 (prostate)	Balb/c nude	VEGF-fused toxin	From day 1, every 2–3 days, for 11 days, intravenously	Veenendaal et al (2002)
Caki (renal)	Ncr nude	VEGF anti-sense	On days 24 and 27, intravenous with cationic liposome, 20 mg kg^{-1}	Shi & Siemann (2002)
MCF7 (breast)	BALB/c nude	Egr-1 deoxy-ribozyme	From day 15, twice/week, four doses each 20 μg, with cationic reagent, intratumorally	Fahmy et al (2003)
A549 (lung)	BALB/c nude	Egr-1 deoxy-ribozyme	From day 15, twice/week, 4 doses each 20 μg, with cationic reagent, intratumorally	Fahmy et al (2003)
AsPC-1 (pancreatic)	nude (Crl:NU/NU-nuBR)	AS-3	From 3 days intraperitoneally per day	Hotz et al (2005)
CHP-134 (neuroblastoma)	Athymic nude	A-357300	From 14 days subcutaneously twice daily	Wang et al (2003b)
HT1080 (fibrosarcoma)	SCID-beige	A-357300	From day 8 subcutaneous injection	Wang et al (2003b)
MDA-435 (breast carcinoma)	SCID-C.B17	A-357300	From day 4 subcutaneous injection	Wang et al (2003b)
PC3 (prostate carcinoma)	BALB/c nu/nu	SU5416	Subcutaneously twice weekly	Abdollahi et al (2003)
U87 (glioblastoma)	BALB/c nu/nu	SU5416	Intraperitoneally twice weekly	Abdollahi et al (2003)
BT474 (breast cancer)	SCID	HER-50, HER-66, HER-70	From 10 weeks one intraperitoneally	Spiridon et al (2004)
Mia Paca-2 (pancreatic carcinoma)	nude	Genistein	Daily intraperitoneal administration	Buchler et al (2004)
Capan-1 (pancreatic carcinoma)	nude	Genistein	Daily intraperitoneal administration	Buchler et al (2004)
A2780 (ovarian carcinoma)	athymic CD-1	IDN 5390	From day 7, intravenously, subcutaneously or orally daily	Petrangolini et al (2004)
SNB19 (glioblastoma)	athymic nude	uPAR + MMP siRNA plasmids	From day 10, for 2 weeks, subcutaneous os. pump	Lakka et al (2003)

The resazurin assay (Promega) is an indirect high throughput method of assessing cell viability. This commercially available product is based on the ability of healthy cells to reduce resazurin to resorufin. Proliferation can be measured either spectrophotometrically or through measurement of fluorescence, which is much more sensitive (O'Brien et al 2000). Apart from the avoidance of dealing with radiolabels, advantages of this assay over conventional radioactive assays include: homogeneity; simplicity; less cost; non-labour intensive; rapidity of assessment of proliferation of large number of samples; non-toxicity; usefulness in determining the kinetics of cell growth of hybridomas; and non-interference of secretion of antibodies by a hybridoma cell line (Ahmed et al 1994). However, there are a few concerns with this assay, including the possibility of accumulation of fluorescent product in the medium, which may cause an overestimation of the cell population (O'Brien et al 2000).

Apoptosis is the process of programmed cell death which occurs in normal physiological function, and caspases play an integral part in the cascade of events which results in apoptosis. Caspase 3 is an effector caspase and has a major role in apoptotic events including processing caspase 7. Commercially available, various forms of the caspase 3/7 whole cell assay exploit the apoptosis cascade. This assay detects caspase 3 and 7 activity within whole cells by introducing the substrate Z-DEVD-R110, conversion of which is then fluorometrically measured. Earlier studies used a similar peptide as substrate to measure time-resolved fluorescence (Preaudat et al 2002). The apoptosis assay has given insight into how the fraction of interest causes a decrease in cell viability. Various companies are looking at ways to combine both proliferation and apoptosis assays on the same well in a HTS format. On the other hand, there is the choice of using the lactate dehyrogenase (LDH) release assay, which is now available in homogeneous formats, for measurement of cell necrosis. However, the kits are not as straightforward to use as the ones for apoptosis.

One of the most relevant in-vitro assays for angiogenesis is the tube formation assay. Endothelial cell migration can be assessed using extracellular matrices like laminin, collagen, fibrin and Matrigel (Lawley & Kubota 1989). Currently in our research group, Matrigel-based tube formation assays using human microvascular endothelial cells 1 (HMEC-1) cells are being developed to investigate materials of interest and their effect on cellular function. Endothelial cells can form lumen containing tubule structures. The affects of potential anti-angiogenic compounds are assessed visually in their ability to interfere with this process. The method can be adapted to a 96-well HTS format as has been done in our laboratory with read-out possible within 2 h of cell seeding (Figure 1). Normal times for tube formation range from 6 h (Polykratis et al 2005) to overnight. This technique has worked for a variety of different murine endothelial cell lines plated in a 48-well format (Walter-Yohrling 2004). The advantages of this method are reproducibility, time-efficiency, fluorescent staining capability and the option of software analysis which, however, increases the cost of analysis.

The limitation of assays using monolayers (or suspensions) is that the setup is artificial and does not yield an accurate predictive model which gives insight into the clinical efficacy of the compound (Kunz-Schughart et al 2004). A realistic model of avascular solid tumour growth should include all factors of mitosis, apoptosis and necrosis, processes dependent on the local pressure as well as growth factor and nutrient concentrations (Dormann & Deutsch 2002). Recently, a 3-dimensional (spheroid) method for growing cells to emulate the in-vivo environment has been proposed (Ahammer et al 2001), but this still is very much an artificial set-up. Thus, the in-vivo study is the only way to increase the chances of definitely identifying an anti-angiogenic compound.

One of the better models available is the in-vitro culture of fragments of human placental blood vessel (Brown et al 1996). In this technique, a fragment of human placental blood vessel is embedded in a fibrin gel in microculture plates and it gives rise to a complex network of microvessels during a period of 7 to 21 days in culture. The response does not require the addition of exogenous growth factors, providing a convenient system for testing substances for their ability to stimulate or inhibit a human angiogenic response. RT-PCR strongly implicated aFGF, bFGF, and VEGF as having an important role in the neovascularization response. This was further confirmed by the ability of neutralizing antibodies to aFGF, bFGF, and VEGF to inhibit the angiogenic response to varying extents. This assay, which can be performed in microcultures, was also shown to be an excellent method for screening for potential inhibitors and enhancers of human angiogenesis. However, probably due to technical difficulties and variation between vessels from different donors and

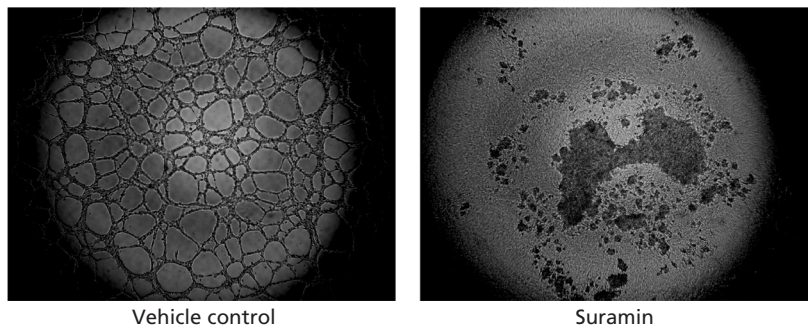

Figure 1 Tube formation and its inhibition with suramin. HMEC-1 cells were seeded at a concentration of 50 000 cells/well in a 96-well format. Treatment was administered immediately thereafter and microphotographs taken 2 h later. Microscope magnification 40×. Data is representative of triplicate samples analysed on two different days by each of two operators.

intra-donor differences along different sections of the placenta vessels, this method has not been adopted readily for routine testing.

A similar approach using tumour tissue was taken by Gulec & Woltering (2004). Fragments of tumour tissue were embedded in fibrin gels containing basal medium, endothelial growth factors, fetal bovine serum, and epsilon-aminocaproic acid. Tumour implants sprouted angiogenic vessels that progressively grew into the fibrin matrix. The differential growth pattern of tumour cells and angiogenic vessels in the fibrin gel matrix separated the angiogenic vessels and the tumour stroma into independently observable regions (vessel and tumour compartments). However, exploiting such a difference in culture is already introducing an artificial phenomenon into the assay dynamics. In any case, all tumour fragments studied showed angiogenic sprouting into the fibrin matrix. Availability of human tumour specimens would be a major hurdle to the routine use of this method.

Types of in-vivo assays

Directed in-vivo angiogenesis assay (DIVAA) is a reproducible and quantitative in-vivo method of assaying angiogenesis. It involves subcutaneous implantation of semi-closed silicon cylinders (angioreactors) – filled with 18 μL of extracellular matrix premixed with or without angiogenic factors – into nude mice (Guedez et al 2003). Vascularization response is measured by intravenous injection of fluorescein isothiocyanate (FITC)-dextran before their recovery, followed by spectrofluorimetry. The technique allows accurate dose–response analysis and identification of effective doses of angiogenesis-modulating factors in-vivo. High levels of FITC-dextran are not significantly influenced by vascular permeability, inhibition correlates with decreased endothelial cell invasion. The DIVAA assay can compare potencies of angiogenic factors or inhibitors and profiling molecular markers of angiogenesis in-vivo (Guedez et al 2003).

A traditional method used is that of the chick chorioallantoic membrane (CAM). Day 7–9 chick embryos are exposed by making an opening in the egg shell, and the tissue and organ grafts placed directly on CAM. Window-sealed eggs are re-incubated, and the grafts are recovered after a given incubation period. Imaging techniques give measurements of the bifurcation points in the designated area around the test site. One limitation of this assay is that the response is in accordance to the individual chicken cells' response. The CAM may rapidly change morphologically and gradual change in the rate of endothelial cell proliferation during the course of embryonic development may lead to confounding results (Auerbach et al 2003).

The corneal angiogenesis assay is the preferred 'gold standard' method in many laboratories. The cornea is avascular, therefore vessels in the cornea after stimulation by angiogenesis-inducing factors are new vessels. A stromal pocket is made in the cornea, test tumours/tissues are introduced into the pocket, and this brings about the formation of ingrown new vessels from peripheral limb vasculature. Growth factors (VEGF or bFGF) are immobilized in a slow release form in an inert hydron pellet of approximately 1–2 μL volume, or alternatively in sponge, ELVAX (ethylene vinyl copolymer), or nitrocellulose membranes (Fahmy et al 2003; Zhang et al 2004). Over a five- to seven-day period angiogenic factors stimulate the ingrowth of vessels from the adjacent vascularized corneal limbus. Anti-angiogenic compounds may be administered systemically or as an eye droplet. A photographic record is created by slit lamp photography. The appearance, density and extent of these vessels are evaluated and scored. Vessels are evaluated for length, density and the radial surface of the limbus from which they emanate (expressed as clock-faced hours).

Sustained administration by osmotic pump implant has also been performed in the corneal neovascularization assay (Ambati et al 2002; Yu et al 2004). One popular method for quantification is by FITC-labelled high molecular weight dextran. The advantages of this assay are that progress of angiogenesis can be monitored, as there is the absence of an existing background vasculature in cornea, and the ability to cost-effectively test in mice (if surgical proficiency is achieved). However, the disadvantages include demanding surgery, as only six animals can be comfortably implanted in a day, the space available for introducing test material is limited, and sometimes inflammatory reactions are difficult to avoid. One other weakness of the method is that it is an atypical site because the cornea is avascular (Auerbach et al 2003).

The other commonly used method is that of the Matrigel plug. Matrigel containing test cells or substances is injected subcutaneously where it solidifies to form a plug. The plug is recovered after 7–21 days and examined histologically to investigate the extent of the blood vessels within. Fluorescence measurement of plasma volume using fluorescein isothiocyanate (FITC)-labelled dextran or measurement of the amount of haemoglobin in the plug allows a certain degree of quantitation. This assay can be misleading, as blood content is affected by size of the vessels. The method is also difficult to control since plugs in different mice may vascularize differently. An alternative is to use the sponge/Matrigel in mice, where a sponge or tissue fragment is inserted into the plug. New vessels are measured by injection of FITC-dextran described for the corneal assay. A major disadvantage of the sponge/matrigel method is that it is more time-consuming than the standard Matrigel plug assay.

A tumour growth assay and subsequent angiogenesis assay on the dissected tumour can also be performed and is quite clinically relevant. Tumours are generated by injection of appropriate tumour cells into C57 mice (mouse tumours, example B16 melanoma) or into immune-compromised SCID or nude mice (human tumours, well-vascularized types). Treatments are given systemically and typical treatments continue for 2–4 weeks. Data collected includes: tumour volume (length $\times$ width $\times$ height in mm^3) during study, tumour weight at the end of study, and histology sections. Blood vessels may be highlighted in sections using EC-specific immunostains such as antibodies against CD31. Digital calipers are essential for accurate and time-efficient measurements (Zhang et al 2004; Mitchell et al 2004).

Another clinically relevant assay is the metastasis establishment assay. Murine (e.g. B16) or human cancer cells (e.g. PC3M) are injected into the tail veins of mice. Mice are treated systemically, by subcutaneous or intraperitoneal injection. After a certain period, the animals are killed and the lungs are weighed to determine tumour growth. Tumour nodules on the surface of lungs are enumerated using a dissecting microscope. Lung micrometastases are enumerated using haematoxylin and eosin (H&E) staining and microscopy. A further, though challenging, assay would take into account intravasation of metastatic cells from the primary site of the tumour in the test animal into the circulation, and then spread to distal sites of secondary growths. Such orthotopic or transgenic models are usually difficult to control due to interanimal differences not only in tumour and metastatic growth kinetics, but also in the way different test treatments are handled by each animal.

Quantitation of tumour angiogenesis, based on microvessel density, is increasingly being used to estimate the extensiveness and aggressiveness of a given tumour as well as predicting its response to various forms of treatment (Sokoloff & Chung 1999). Endothelial cell markers such as CD31 are used to correctly identify VECs in sections of the tumour. The higher the microvessel count, the lower the rate of survival of cancer patients (Weidner et al 1991). However, the same authors (Weidner et al 1992) cautioned that mean vascular density (MVD) was rather heterogeneous in tumours and that histosection analysis might lead to false negative results if regions of low vascular density were examined. In any case, more sections may need to be analysed to allow a better representation of the tumour tissue microvessel architecture. Ideally, a 3-dimensional mapping of the tumour in terms of vessel density will need to be done to acquire a true picture of the extent of vascularization.

The use of tissue-isolated tumours fed by a single artery and connected with a single vein has allowed research into perfusion rates, drug pharmacokinetics and metabolism in tumours in mice (Kristjansen et al 1994, 1996) and man (Less et al 1997). A modified Sandison rabbit ear chamber permits great optical data acquisition since the tumours and suppliant vessels are both readily visible and accessible (Dudar & Jain 1983). The modified Algire mouse dorsal chamber enables researchers to evaluate both angiogenesis and tumorigenesis in immunodeficient and genetically engineered animals, thus increasing the power of such studies significantly (Leunig et al 1994). The use of the cranial window in mice and rats (Yuan et al 1994) has enabled the monitoring of angiogenesis and tumorigenesis induced by the administration of different growth factors in a real-time mode. Single vessels of a tumour may be perfused with the use of these windows (Lichtenbeld et al 1996).

Thus, in-vivo assays are of utmost importance in evaluating the true potential of an anti-angiogenic compound. However, the assay has to be chosen carefully with due diligence since choosing the wrong model can lead to wasted efforts and loss of productive time. There are no hard and fast rules, but if it is cancer that is being targeted, then an assay looking at inhibition of angiogenesis is required. The clinical relevance of each assay must be considered and balanced against cost-effectiveness and the value that needs to be added to the lead candidate. The beauty about angiogenesis assays such as the Matrigel plug is that if a pro-angiogenic effect is discovered (some examples of such agents are listed in Table 3) in contrast to angiogeneic inhibitors (common naturally occurring agents are listed in Table 4), then the indication could be cardiovascular disease or wound repair.

Table 3 Common angiogenic growth factors

Angiogenin
Fibroblast growth factors: acidic (aFGF) and basic (bFGF)
Granulocyte colony-stimulating factor (G-CSF)
Interleukin-8 (IL-8)
Leptin
Placental growth factor (PlGF)
Platelet-derived endothelial cell growth factor (PD-ECGF)
Platelet-derived growth factor-BB (PDGF-BB)
Transforming growth factor-alpha (TGF-α)
Transforming growth factor-beta (TGF-β)
Tumour necrosis factor-alpha (TNF-α)
Vascular endothelial growth factor (VEGF)/ vascular permeability factor (VPF)

Table 4 Common naturally occurring angiogenic inhibitors

Angiostatin (plasminogen fragment)
Antiangiogenic antithrombin III (aaATIII)
Canstatin
Cartilage-derived inhibitor (CDI)
Endostatin (collagen XVIII fragment)
Fibronectin fragment
Human chorionic gonadotropin (hCG)
Interferon alpha/beta/gamma
Interleukin-12 (IL-12)
Kringle 5 (plasminogen fragment)
Metalloproteinase inhibitors (TIMPs)
2-Methoxyestradiol (2-ME)
Pigment epithelial-derived factor (PEDF)
Prolactin 16 kDa fragment
Thrombospondin-1v
Transforming growth factor-beta
Tumistatin
Vasculostatin
Vasostatin (calreticulin fragment)

Summary

Several in-vitro and in-vivo angiogenesis assays have been established over the past three decades. Old assays have been continuously refined, and new ones are quickly adopted by anti-angiogenic drug R & D companies attempting to find better drug candidates to inhibit angiogenesis, especially in in-vivo assays. It is imperative that clinically-relevant models are used to facilitate a smoother transition from preclinical to clinical testing. With better technologies, including high-throughput screening, it is hoped that more efficacious anti-angiogenic compounds will be discovered and developed in the future.

References

Abdollahi, A., Lipson, K. E., Han, X., Krempien, R., Trinh, T., Weber, K. J., Hahnfeldt, P., Hlatky, L., Debus, J., Howlett, A. R., Huber, P. E. (2003) SU5416 and SU6668 attentuate the angiogenic effects of radiation-induced tumour cell growth factor production and amplify the direct anti-endothelial action of radiation in vitro. *Cancer Res*. **63**: 3755–3763

Ahammer, H., DeVaney, T. T., Tritthart, H. A. (2001) Fractal dimension of K1735 mouse melanoma clones and spheroid invasion in vitro. *Eur. Biophys. J*. **30**: 494–499

Ahmed, S. A., Gogal, R. M., Walsh, J. E. (1994) A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J. Immunol. Methods* **170**: 211–224

Ambati, B. K., Joussen, A. M., Ambati, J., Moromizato, Y., Guha, C., Javaherian, K., Gillies, S., O'Reilly, M. S., Adamis, A. P. (2002) Angiostatin inhibits and regresses corneal neovascularization. *Arch. Ophthalmol*. **120**: 1063–1068

Auerbach, R., Lewis, R., Shinners, B., Kubai, L., Akhtar, N. (2003) Angiogenesis assays: a critical overview. *Clin. Chem*. **49**: 32–40

Brekken, R. A., Huang, X., King, S. W., Thorpe, P. E. (1998) Vascular endothelial growth factor as a marker of tumour endothelium. *Cancer Res*. **58**: 1952–1959

Browder, T., Butterfield, C. E., Kraling, B. M., Shi, B., Marshall, B., O'Reilly, M. S., Folkman, J. (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res*. **60**: 1878–1886

Brown, K. J., Maynes, S. F., Bezos, A., Maguire, D. J., Ford, M. D., Parish, C. R. (1996) A novel in vitro assay for human angiogenesis. *Lab. Invest*. **75**: 539–555

Buchler, P., Reber, H. A., Buchler, M. W., Friess, H., Lavey, R. S., Hines, O. J. (2004) Antiangiogenic activity of genistein in pancreatic carcinoma cells is mediated by the inhibition of hypoxia-inducible factor-1 and the down-regulation of VEGF gene expression. *Cancer* **100**: 201–210

Conway, E. M. (2003) Angiogenesis: a link to thrombosis in atherothrombotic disease. *Pathophysiol. Haemost. Thromb*. **33**: 241–248

Dass, C. R., Su, T. (2001) Particle-mediated intravascular delivery of oligonucleotides to tumours: associated biology and lessons from genotherapy. *Drug Deliv*. **8**: 191–213

Denko, N. C., Giaccia, A. J. (2001) Tumour-hypoxia, the physiological link between Trousseau's syndrome (carcinoma-induced coagulopathy) and metastasis. *Cancer Res*. **61**: 795–798

Ding, K. H., Zhong, Q., Xie, D., Xu, J., Bollag, W. B., Isales, C. M. (2005) Carboxy-terminal PTH fragments stimulate [3H] thymidine incorporation in vascular endothelial cells. *Peptides* **26**: 853–862

Dormann, S., Deutsch, A. (2002) Modeling of self-organized avascular tumor growth with a hybrid cellular automaton. *In Silico Biol*. **2**: 393–406

Dudar, T. E., Jain, R. K. (1983) Microcirculatory flow changes during tissue growth. *Microvasc. Res*. **25**: 1–21

Fahmy, R. G., Dass, C. R., Sun, L. Q., Chesterman, C. N., Khachigian, L. M. (2003) Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumour growth. *Nat. Med*. **9**: 1026–1032

Folkman, J. (1971) Tumour angiogenesis: therapeutic implications. *N. Engl. J. Med*. **285**: 1182–1186

Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med*. **1**: 27–31

Goldman, C. K., Kendall, R. L., Cabrera, G., Soroceanu, L., Heike, Y., Gillespie, G. Y., Siegal, G. P., Mao, X., Bett, A. J., Huckle, W. R., Thomas, K. A., Curiel, D. T. (1998) Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumour growth, metastasis, and mortality rate. *Proc. Natl. Acad. Sci. USA* **95**: 8795–8800

Guedez, L., Rivera, A. M., Salloum, R., Miller, M. L., Diegmueller, J. J., Bungay, P. M., Stetler-Stevenson, W. G. (2003) Quantitative assessment of angiogenic responses by the directed in vivo angiogenesis assay. *Am. J. Pathol*. **162**: 1431–1439

Gulec, S. A., Woltering, E. A. (2004) A new in vitro assay for human tumour angiogenesis: three-dimensional human tumour angiogenesis assay. *Ann. Surg. Oncol*. **11**: 99–104

Hotz, H. G., Hines, O. J., Masood, R., Hotz, B., Foitzik, T., Buhr, H. J., Gill, P. S., Reber, H. A. (2005) VEGF antisense therapy inhibits tumour growth and improves survival in experimental pancreatic cancer. *Surgery* **137**: 192–199

Kerbel, R. S. (1997) A cancer therapy resistant to resistance. *Nature* **390**: 335–336

Kisker, O., Becker, C. M., Prox, D., Fannon, M., D'Amato, R., Flynn, E., Fogler, W. E., Sim, B. K., Allred, E. N., Pirie-Shepherd, S. R., Folkman, J. (2001) Continuous administration of endostatin by intraperitoneally implanted osmotic pump improves the efficacy and potency of therapy in a mouse xenograft tumour model. *Cancer Res*. **61**: 7669–7674

Kong, H. L., Hecht, D., Song, W., Kovesdi, I., Hackett, N. R., Yayon, A., Crystal, R. G. (1998) Regional suppression of tumour growth by in vivo transfer of a cDNA encoding a secreted form of the extracellular domain of the flt-1 vascular endothelial growth factor receptor. *Hum. Gene Ther*. **9**: 823–833

Kristjansen, P. E., Roberge, S., Lee, I., Jain, R. K. (1994) Tissue-isolated human tumour xenografts in athymic nude mice. *Microvasc. Res*. **48**: 389–402

Kristjansen, P. E., Brown, T. J., Shipley, L. A., Jain, R. K. (1996) Intratumor pharmacokinetics, flow resistance, and metabolism during gemcitabine infusion in ex vivo perfused human small cell lung cancer. *Clin. Cancer Res*. **2**: 359–367

Kunz-Schughart, L. A., Freyer, J. P., Hofstaedter, F., Ebner, R. (2004) The use of 3-D cultures for high-throughput screening: the multicellular spheroid model. *J. Biomol. Screen*. **9**: 273–285

Lakka, S S., Gondi, C. S., Yanamandra, N., Dinh, D. H., Olivero, W. C., Gujrati, M., Rao, J. S. (2003) Synergistic down-regulation of urokinase plasminogen activator receptor and matrix metalloproteinase-9 in SNB19 glioblastoma cells efficiently inhibits glioma cell invasion, angiogenesis, and tumour growth. *Cancer Res*. **63**: 2454–2461

Lawley, T. J., Kubota, Y. (1989) Induction of morphologic differentiation of endothelial cells in culture. *J. Invest. Dermatol*. **93**: 59S–61S

Less, J. R., Posner, M. C., Skalak, T. C., Wolmark, N., Jain, R. K. (1997) Geometric resistance and microvascular network architecture of human colorectal carcinoma. *Microcirculation* **4**: 25–33

Leunig, M., Yuan, F., Berk, D. A, Gerweck, L. E., Jain, R. K. (1994) Angiogenesis and growth of isografted bone: quantitative in vivo assay in nude mice. *Lab. Invest*. **71**: 300–307

Liao, F., Li, Y., O'Connor, W., Zanetta, L., Bassi, R., Santiago, A., Overholser, J., Hooper, A., Mignatti, P., Dejana, E., Hicklin, D. J., Bohlen, P. (2000) Monoclonal antibody to vascular endothelial-cadherin is a potent inhibitor of angiogenesis, tumour growth, and metastasis. *Cancer Res*. **60**: 6805–6810

Lichtenbeld, H. C., Yuan, F., Michel, C. C., Jain, R. K. (1996) Perfusion of single tumour microvessels: application to vascular permeability measurement. *Microcirculation* **3**: 349–357

Maeshima, Y., Colorado, P. C., Torre, A., Holthaus, K. A., Grunkemeyer, J. A., Ericksen, M. B., Hopfer, H., Xiao, Y., Stillman, I. E., Kalluri, R. (2000) Distinct antitumor properties of a type IV collagen domain derived from basement membrane. *J. Biol. Chem*. **275**: 21340–21348

Maeshima, Y., Yerramalla, U. L., Dhanabal, M., Holthaus, K. A., Barbashov, S., Kharbanda, S., Reimer, C., Manfredi, M., Dickerson, W. M., Kalluri, R. (2001a) Extracellular matrix-derived

peptide binds to alpha(v)beta(3) integrin and inhibits angiogenesis. *J. Biol. Chem*. **276**: 31959–31968

Maeshima, Y., Manfredi, M., Reimer, C., Holthaus, K. A., Hopfer, H., Chandamuri, B. R., Kharbanda, S., Kalluri, R. (2001b) Identification of the anti-angiogenic site within vascular basement membrane-derived tumstatin. *J. Biol. Chem*. **276**: 15240–15248

Mitchell, A., Dass, C. R., Sun, L. Q., Khachigian, L. M. (2004) Inhibition of human breast carcinoma proliferation, migration, chemoinvasion and solid tumour growth by DNAzymes targeting the zinc finger transcription factor EGR-1. *Nucl. Acids Res*. **32**: 3065–3069

O'Brien, J., Wilson, I., Orton, T., Pognan, F. (2000) Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem*. **267**: 5421–5426

Pavco, P. A., Bouhana, K. S., Gallegos, A. M., Agrawal, A., Blanchard, K. S., Grimm, S. L., Jensen, K. L., Andrews, L. E., Wincott, F. E., Pitot, P. A., Tressler, R. J., Cushman, C., Reynolds, M. A., Parry, T. J. (2000) Antitumor and antimetastatic activity of ribozymes targeting the messenger RNA of vascular endothelial growth factor receptors. *Clin. Cancer Res*. **6**: 2094–2103

Petrangolini, G., Pratesi, G., De Cesare, M., Supino, R., Pisano, C., Marcellini, M., Giordano, V., Laccabue, D., Lanzi, C., Zunino, F. (2004) Antiangiogenic effects of the novel camptothecin ST1481 (gimatecan) in human tumour xenografts. *Mol. Cancer Res*. **1**: 863–870

Polykratis, A., Panagiotis, K., Courty, J., Papadimitriou, E. (2005) Characterization of heparin affin regulatory peptide signaling in human endothelial cells. *J. Biol. Chem*. **280**: 22454–22461

Preaudat, M., Ouled-Diaf, J., Alpha-Bazin, B., Mathis, G., Mitsugi, T., Aono, Y., Takahashi, K., Takemoto, H. (2002) A homogeneous caspase-3 activity assay using HTRF technology. *J. Biomol. Screen*. **7**: 267–274

Presta, L. G., Chen, H., O'Connor, S. J., Chisholm, V., Meng, Y. G., Krummen, L., Winkler, M., Ferrara, N. (1997) Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumours and other disorders. *Cancer Res*. **57**: 4593–4599

Risau, W. (1998) Angiogenesis is coming of age. *Circ. Res*. **82**: 926–928

Saaristo, A., Karpanen, T., Alitalo, K. (2000) Mechanisms of angiogenesis and their use in the inhibition of tumour growth and metastasis. *Oncogene* **19**: 6122–6129

Shi, W., Siemann, D. W. (2002) Inhibition of renal cell carcinoma angiogenesis and growth by antisense oligonucleotides targeting vascular endothelial growth factor. *Br. J. Cancer* **87**: 119–126

Sokoloff, M. H., Chung, L. W. (1999) Targeting angiogenic pathways involving tumour-stromal interaction to treat advanced human prostate cancer. *Cancer Metastasis Rev*. **17**: 307–315

Spiridon, C. I., Guinn, S., Vitetta, E. S. (2004) A comparison of the in vitro and in vivo activities of IgG and F(ab′)2 fragments of a mixture of three monoclonal anti-Her-2 antibodies. *Clin. Cancer Res*. **10**: 3542–3551

Veenendaal, L. M., Jin, H., Ran, S., Cheung, L., Navone, N., Marks, J. W., Waltenberger, J., Thorpe, P., Rosenblum, M. G. (2002) In vitro and in vivo studies of a VEGF121/rGelonin chimeric fusion toxin targeting the neovasculature of solid tumours. *Proc. Natl. Acad. Sci. USA* **99**: 7866–7871

Walter-Yohrling, J., Morgenbesser, S., Rouleau, C., Bagley, R., Callahan, M., Weber, W., Teicher, B. A. (2004) Murine endothelial cell lines as models of tumour endothelial cells. *Clin. Cancer Res.* **10**: 2179–2189

Wang, F, Cao, Y., Liu, H. Y., Xu, S. F., Han, R. (2003a) Anti-invasion and anti-angiogenesis effect of taxol and camptothecin on melanoma cells. *J. Asian Nat. Prod. Res*. **5**: 121–129

Wang, J, Sheppard, G. S., Lou, P., Kawai, M., BaMaung, N., Erickson, S. A., Tucker-Garcia, L., Park, C., Bouska, J., Wang, Y. C., Frost, D., Tapang, P., Albert, D. H., Morgan, S. J., Morowitz, M., Shusterman, S., Maris, J. M., Lesniewski, R., Henkin, J. (2003b) Tumour suppression by a rationally designed reversible inhibitor of methionine aminopeptidase-2. *Cancer Res*. **63**: 7861–7869

Wedge, S. R., Ogilvie, D. J., Dukes, M., Kendrew, J., Curwen, J. O., Hennequin, L. F., Thomas, A. P., Stokes, E. S. E., Curry, B., Richmond, G. H. P., Wadsworth, P. F. (2000) ZD4190: an orally active inhibitor of vascular endothelial growth factor signaling with broad-spectrum antitumor efficacy. *Cancer Res*. **60**: 970–975

Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E. N., Moore, D. H., Meli, S., Gasparini, G. (1992) Tumour angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J. Natl. Cancer Inst*. **84**:1875–1887

Yu, Y., Moulton, K. S., Khan, M. K., Vineberg, S., Boye, E., Davis, V. M., O'Donnell, P. E., Bischoff, J., Milstone, D. S. (2004) E-selectin is required for the antiangiogenic activity of endostatin. *Proc. Natl. Acad. Sci. USA* **101**: 8005–8010

Yuan, F., Salehi, H. A., Boucher, Y., Vasthare, U. S., Tuma, R. F., Jain, R. K. (1994) Vascular permeability and microcirculation of gliomas and mammary carcinomas transplanted in rat and mouse cranial windows. *Cancer Res*. **54**: 4564–4568

Zadeh, G., Guha, A. (2003) Molecular regulators of angiogenesis in the developing nervous system and adult brain tumours. *Int. J. Oncol*. **23**: 557–565

Zhang, G., Dass, C. R., Sumithran, E., Di Girolamo, N., Sun, L. Q., Khachigian, L. M. (2004) Effect of deoxyribozymes targeting c-Jun on solid tumour growth and angiogenesis in rodents. *J. Natl. Cancer Inst*. **96**: 683–696