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The Rationale and Future Potential of Angiogenesis Inhibitors in Neoplasia

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Abstract

Malignant tumours are angiogenesis-dependent diseases. Several experimental studies suggest that primary tumour growth, invasiveness and metastasis require neovascularisation. Tumour-associated angiogenesis is a complex multistep process under the control of positive and negative soluble factors. A mutual stimulation occurs between tumour and endothelial cells by paracrine mechanisms. Angiogenesis is necessary, but not sufficient, as the single event for tumour growth. There is, however, compelling evidence that acquisition of the angiogenic phenotype is a common pathway for tumour progression, and that active angiogenesis is associated with other molecular mechanisms leading to tumour progression. Experimental research suggests that it is possible to block angiogenesis by specific inhibitory agents, and that modulation of angiogenic activity is associated with tumour regression in animals with different types of neoplasia.

The more promising angiosuppressive agents for clinical testing are: naturally

occurring inhibitors of angiogenesis (angiostatin, endostatin, platelet factor–4 and others), specific inhibitors of endothelial cell growth (TNP-470, thalidomide, interleukin-12 and others), agents neutralising angiogenic peptides (antibodies to fibroblast growth factor or vascular endothelial growth factor, suramin and analogues, tecogalan and others) or their receptors, agents that interfere with vascular basement membrane and extracellular matrix [metalloprotease (MMP) inhibitors, angiostatic steroids and others], antiadhesion molecules antibodies such as anti–integrin $\alpha_v\beta_3$, and miscellaneous drugs that modulate angiogenesis by diverse mechanisms of action. Antiangiogenic therapy is to be distinguished from vascular targeting. Gene therapy aimed to block neovascularisation is also a feasible anticancer strategy in animals bearing experimental tumours.

Antiangiogenic therapy represents one of the more promising new approaches to anticancer therapy and it is already in early clinical trials. Because angiosuppressive therapy is aimed at blocking tumour growth indirectly, through modulation of neovascularisation, antiangiogenic agents need to be developed and evaluated as biological response modifiers. Therefore, adequate and well designed clinical trials should be performed for a proper evaluation of antiangiogenic agents, by determination and monitoring of surrogate markers of angiogenic activity.

Therapy aimed at direct targeting of tumour cells by cytotoxic agents has for several years represented the main therapeutic pharmacological strategy against human advanced malignant tumours. Many cytotoxic agents, with different mechanisms of action, have been developed and for most patients with solid tumours only transient remissions have been obtained even when using combined multidrug regimens and by optimising the schedules of administration.

Only for a few tumour types is chemotherapy curative: acute leukaemias, Hodgkin's disease, some types of lymphomas, gestational choriocarcinoma, testicular tumours and some paediatric neoplasias such as Wilm's tumour and Burkitt lymphoma, all reviewed in Chabner.[1]

A better understanding of both the molecular events involved in tumour progression and the inherent characteristics of tumour cells have permitted the identification of the causes of therapeutic failure of chemotherapy. Acquired drug resistance which depends on the genetic instability, heterogeneity and high mutational rate of tumour cells has been identified as the major cause of inefficacy of cytotoxic agents in neoplasia.[2,3]

In the last decade new therapeutic antitumoural strategies have been identified and developed, including new forms of immunotherapy, neutralisation of specific tumour growth factors, gene therapy (reviewed in Karp and Broder[4,5]) and last but not least, inhibition of angiogenesis.[6]

The paradigm of cancer eradication by cytotoxic drugs is being substituted by the paradigm of cancer control by using biologically driven treatments. We are at the beginning of a new era of anticancer drug development. The main concept is that major clinical advances will come not from further refinements of traditional cytotoxic and hormonal agents but rather from new treatments targeting precise molecular abnormalities involved in the malignant phenotype.

1. Inhibition of Angiogenesis as Anticancer Therapy: Biological Rationale and Targets

Angiogenesis, as summarised in table I, is a complex multistep process. The term angiogenesis was first proposed by Judah Folkman[6] in 1971 to define the phenomenon leading to neovascularisation, that is, the formation of new blood vessels from the pre-existing vascular network.

Tumour-associated endothelium is activated by

Table I. The complex sequential process of angiogenesis

Step	Mechanism
1. Endothelial cell activation	Angiogenic 'switch' mediated by soluble endothelial cell growth factors
2. Basement membrane and extracellular matrix degradation	Secretion of proteolytic enzymes by endothelial cells
3. New capillary-tubes formation	Endothelial cell proliferation and migration
4. Vascular lumen and deposition of basement membrane	Endothelial cell differentiation and synthesis of the new basement membrane
5. Linkage to pre-existing vessels and formation of new capillary loops and of the intratumoural vascular network	Active neovascularisation with involvement of adhesion molecules

the production of soluble growth factors by tumour and stromal cells.[7-11] Tumour-associated endothelial cells have no genetic alterations, are homogeneous and have a low rate of spontaneous mutations.[12,13] Emerging data suggest that there are phenotypic differences between normal blood vessels and those associated with tumours. Intratumoural vessels are characterised by increased fenestration and leakiness of the vessels[14] and present an abnormal architecture with arteriovenous shunts, multiple loops, fan and spiral motifs.[15]

Tumour endothelial cells may divide up to 50 times more frequently than endothelial cells of normal tissues.[8-15] Furthermore, tumour-induced, activated, vessels preferentially express specific molecules such as integrin $\alpha_v\beta_3$,[16,17] E-selectin,[18] endoglin,[19] endosialin,[20,21] and vascular endothelial growth factor (VEGF)-receptors.[22-24] Finally, tumour-associated endothelial cells may acquire the capability of secreting certain growth factors and cytokines that sustain their own growth (by autocrine mechanisms) and stimulate the growth of the parenchymal component of the tumour as well (by paracrine mechanisms). Therefore, between the 2 components of a tumour (parenchyma and stroma) a mutual growth stimulation may operate mediated by soluble factors, which cause a positive feedback loop[3] that sustains tumour growth, progression and metastasis.

The molecular mechanisms for acquisition of the angiogenic phenotype are only partially known;[25] however, it is presumed that the angiogenic 'switch' is the result of the alteration of the balance of positive and negative molecular regulators of angiogenesis. The angiogenic phenotype may be the result of up-regulation of endothelial cell growth factors or of down-regulation of naturally occurring inhibitors of angiogenesis,[26,27] the latter predominating in the tissues of the adult under physiological conditions.[28] The evolution from non-angiogenic to angiogenic phenotype may occur in malignant progression by 1-step 'switch' or by a gradual increase of angiogenic potential as normal tissue converts to a benign neoplasm and, later, a malignant tumour.

Several lines of experimental evidence suggest that tumours are angiogenesis-dependent diseases. A solid neoplasm is unable to grow beyond a critical volume without neovascularisation.[29] In tissues, the oxygen diffusion limit corresponds to a distance of 100 to 200μm between the capillary and cells to be perfused. For solid tumours this is in the range of 3 to 5 cellular lines around a single vessel.

In an integrated biological model the tumour cell-capillary system should be considered as a functional unit for tumour growth. Angiogenesis is necessary not only for growth and local invasiveness of the primary tumour, but also for development of metastasis.[30,31] Activated endothelial cells are the primary target for inhibition of angiogenesis, and their therapeutic targeting present several advantages over therapy directed against tumour cells. First, under physiological conditions endothelium is quiescent in normal adult tissues (with the exception of the female reproductive organs), whilst tumour-induced vessels are activated, proliferating and migrating.[16-24] This knowledge suggests it may be possible to inhibit tumour vascularisation selectively without affecting normal vasculature and not causing systemic adverse effects on the normal vascular network. In fact, a good safety profile has been observed in animals treated with most inhibitors of angiogenesis.[32,33]

Second, endothelial cells are normal, diploid and genetically stable cells, and represent a uniform target as compared with tumour cells.[12,13] Third, endothelium is a cellular target that can be easily reached by antiangiogenic agents administered systemically. In contrast, activity of chemotherapy may be impaired, in part by the presence of specific barriers for its delivery including: the chaotic blood supply of tumours, permeability of the vessel wall and the interstitial hypertension that characterises solid tumours.[34] Fourth, the molecular sequence of several endothelial cell growth factors and of naturally occurring inhibitors of angiogenesis is known. Therapeutic approaches based on pharmacological neutralisation of angiogenic peptides[35,36] as well as on external administration of therapeutic doses of naturally occurring inhibitors of angiogenesis[37,38] are, therefore, a feasible therapeutic strategy, at least in experimental models. Finally, some inhibitors of angiogenesis have synergistic effects when combined with current anticancer drugs.[39]

2. Angiogenesis: A Pivotal Mechanism of Tumour Growth Connected with Other Biological Pathways

Angiogenesis is necessary but not sufficient as a single biological mechanism for tumour growth and progression.[29,40] Compelling evidence suggests that angiogenesis is connected to other molecular mechanisms involved in the development and growth of tumours.

2.1 Angiogenesis and Tumour Suppressor Genes

A major pathway links p53/p21, thrombospondin-1 (TSP-1) and VEGF.[41] Wild type p53 is an oncosuppressor gene that encodes a protein inhibiting tumour progression by complex molecular mechanisms involving: genetic stability, apoptosis, cell cycle arrest, differentiation as well as modulation of angiogenesis (reviewed in Gasparini and Harris[42]). Tumour neovascularisation may be suppressed by wild type p53 in 2 ways: through enhanced production of TSP-1[43,44] and reduced secretion of VEGF.[45,46] The relevance of the above mechanisms is tumour specific and depends on the cell type in which wild type p53 is expressed.[42,45] Opposite findings are induced by altered, mutated p53 which down-regulates TSP-1 and enhances secretion of VEGF.[47-49] Therefore, in specific experimental models, treatments aimed at restoring wild type p53, indirectly, also affect angiogenesis.

Another important example is Von Hippel-Lindau tumour suppressor gene whose expression maintains low levels of secreted VEGF in renal cells.[50]

2.2 Angiogenesis and Oncogenes

In experimental models, certain oncogenes including V-HA-RAS, V-RAF, K-RAS, FOS, SRC and HER-2-neu stimulate angiogenesis by up-regulation of angiogenic peptides, mainly VEGF.[48,49] Some oncogenes are themselves angiogenic factors. One example is the proto-oncogene c-met also known as scatter factor,[51] and, in some cases oncogenes can down-regulate production of the secreted naturally occurring inhibitors of angiogenesis via the c-jun pathway.[52] For example, down-regulation of TSP-1 plays an important role in the maintenance of the malignant phenotype in polyoma middle T transformed NIH3T3 cells[52] or in cultured rat embryo fibroblasts.[53]

2.3 Angiogenesis and Tumour Invasiveness

Common pathways link the process leading to the formation and penetration of newly formed vessels during pathologic angiogenesis and tumour invasiveness. The adhesion molecule integrin $\alpha_v\beta_3$ seems to play a pivotal role in both angiogenesis[17] and tumour invasiveness.[54] Both the pathways of degradation of plasminogen and modulation of activity of matrix metalloproteases (MMPs) are regulated by integrin $\alpha_v\beta_3$.[55-57] An excess of VEGF activates integrin $\alpha_v\beta_3$ and a cascade of enzymatic pathways leading to the alterations of the extracellular matrix that, ultimately, permit neovascularisation and tumour invasiveness. Similarly, basic fibroblast growth factor (bFGF), another endothelial cell growth factor, activates the plasminogen

system.[58,59] Treatment with either integrin $\alpha_v\beta_3$ antagonists[60] or MMP inhibitors[61] blocks tumour growth indirectly by inhibition of angiogenesis and, directly, by not allowing tumour cells to invade the extracellular matrix and to colonise distant tissues.

2.4 Angiogenesis, Apoptosis and Dormant Metastasis

Experimental studies suggest that the bcl-2 gene prevents apoptosis in bFGF-deprived endothelial cells,[62] and that tumour necrosis factor-α (TNF-α) induces endothelial cell apoptosis *in vitro*.[63] Braf gene, localised on chromosome 10, plays a critical and necessary role in the integration of mechanisms regulating the differentiation of angioblasts (precursor of endothelial cells), the survival of differentiated endothelial cells and formation of the vascular system by the Ras signalling pathway.[63] Wojnowski et al.[64] demonstrated that Braf-deficient mice die of vascular defects during mid-gestation because of apoptotic death of differentiated endothelial cells. These data document that Braf is a critical signalling factor for vasculogenesis and, perhaps, angiogenesis as well.

Overall, the findings of these and other studies[65-67] suggest it is possible to interfere on angiogenesis by genes regulating endothelial cell apoptosis.

Angiostatin, one of the more potent naturally occurring inhibitors of angiogenesis up to now discovered and sequenced, sustains metastasis dormancy by suppression of angiogenesis and causing an enhanced apoptotic rate of tumour cells.[68] It has been found in experimental models that both angiostatin[69] and TSP-1[70] induce apoptosis of endothelial cells. Thus, these angiogenesis inhibitors exert their antiangiogenic effect, in part, by driving activated endothelial cells into apoptosis. However, there is no direct evidence on the literature that this is indeed the case and so far this mechanism of action is to be considered speculative.

2.5 Angiogenesis and the Immune System

During angiogenesis, VEGF and bFGF play opposite effects on the regulation of natural killer cell adhesion to tumour endothelium. In fact, VEGF promotes whilst bFGF inhibits this type of adhesion.[71] Thus, VEGF may facilitate lymphocyte recognition of angiogenic vessels by upregulating the expression of certain extracellular molecules including intercellular adhesion molecule-1, VCAM-1 and E-selectin.[71] The balance between VEGF and bFGF in the local microenvironment of a tumour may be critical for adhesion of cytotoxic lymphocytes to tumour-associated endothelial cells.[72]

A different effect of VEGF has been recently found on the functional maturation of dendritic cells. Human cancer cells release soluble factors that dramatically affect maturation of dendritic cells from precursors. It has been found that VEGF causes inadequate presentation of tumour antigens by host dendritic cells with consequent impaired immune response.[73] Therapeutic blockage of VEGF functions may block tumour growth by inhibiting neovascularisation and, indirectly, improving the immunologic response of the host.[72] Efficacy of immunotherapy using cultured tumour-infiltrating lymphocytes (TILs) depends upon infused TILs migrating into tumour-bearing tissue. For TILs to reach tumour mass, the first step is the binding to endothelium. This process is mediated by adhesion molecule such as integrins, CD31 and E-selectins which are expressed at high levels during active angiogenesis.[74]

The above are a few examples of the complex interrelationships of angiogenesis with other known molecular pathways that cooperate in tumour progression.[75] The central role of angiogenesis in the regulation of the mechanisms involved in tumour growth suggests that therapies aimed at blocking abnormal angiogenesis may also interfere with the cascade of complex molecular events that concur to tumour growth. Antiangiogenic therapy combined with other specific biologically driven treatments might improve curability of tumours. Antiangiogenic agents may be useful in clinical

practice but not before they are adequately tested on animals.

3. The Most Relevant Studies on Experimental Angiosuppressive Treatments

3.1 Antiangiogenic Therapy

The first inhibitor of angiogenesis was discovered in cartilage, a physiological poorly vascularised tissue, by Brem and Folkman[76] in 1975, but it was not possible at that time to completely purify the agent nor to obtain sufficient amounts for testing its antitumour activity.

In 1982, Taylor and Folkman[77] found that protamine exerts an angiosuppressive effect. Protamine is a 4.3 kD arginine-rich cationic heparin-binding protein that inhibits endothelial cell migration and proliferation. Subsequently, it was documented that certain steroids (tetrahydrocortisol, hydrocortisone and others) inhibit angiogenesis[78] as well as did some heparin-binding compounds such as β-cyclodextrin tetradecasulfate.[79] All these agents suppress neovascularisation by altering the basement membrane turnover in growing blood vessels.[80]

In 1990, during routine culturing of capillary endothelial cells, Ingber et al.[81] observed a fungal contamination which caused a local gradient of endothelial cell-rounding. They isolated the fungus, *Aspergillus fumigatus fresienus*, and then purified the active angiosuppressive antibiotic, named fumagillin. This antibiotic was clinically in use for therapy of amoebiasis and it was active in suppressing endothelial cell growth *in vitro* and tumour-induced angiogenesis *in vivo*.[81] Because fumagillin presented systemic toxicity, several analogues were tested, one of the more potent being AGM-1470 (angioinhibin, fumagillol, TNP-470) which is 50 times more active than the parent compound, but with fewer adverse effects.[82]

Several authors studied the mechanisms of action of AGM-1470, which prevents entry of endothelial cells into the G_1 phase of the cell cycle,[83-85] and it was found that it induces regression of both primary tumours and metastasis in different experimental models.[86-88] Recent studies demonstrate that combined administration of AGM-1470 with other antiangiogenic agents as well as with cytotoxic drugs or radiation therapy has enhanced antitumour activity compared with the administration of each single agent.[89-92]

Thalidomide is a sedative drug that has teratogenic effects, mainly dysmelia, observed in babies born during the 1960s from mothers who took the drug during pregnancy. The mechanisms by which thalidomide caused the above effects remained unknown. D'Amato et al.[93] demonstrated that orally administered thalidomide is a moderate inhibitor of angiogenesis as observed in a model of bFGF-induced neovascularisation in the rabbit cornea micropocket assay. The antiangiogenic effect of thalidomide requires metabolic activation, which is species-dependent.[94] The effect of thalidomide on angiogenesis is mediated, in part, by suppression of macrophage-produced TNFα. Subsequently, it was found that thalidomide has antitumour activity, particularly evident when administered together with 5,6-dimethylxanthenone-4-acetic acid, an investigational antitumour agent.[95]

TSP-1 is the first isolated and sequenced 'pure' naturally occurring inhibitor of angiogenesis.[96] An elegant experiment by Sheibani and Frazier[97] showed that TSP-1 is an important regulator of endothelial cell phenotype, necessary for maintaining the quiescent, differentiated state, and that transfection of TSP-1 in bEDN.3 cells (a transformed endothelial cell line) restores the normal phenotype and the ability of cells to form cords *in vitro*. TSP-1 is capable of suppressing endothelial cell tumourigenesis.[97] Further studies provided evidence that transfection of TSP-1 induces tumour regression *in vivo* in experimental models.[98] An elegant experimental study by Volpert et al.[99] demonstrated that, when returned into the blood stream of mice bearing TSP-negative tumours, purified human TSP-1 halts the growth of experimental lung metastasis, providing clear evidence of its efficacy as an antitumour agent.

Another strategy to suppress angiogenesis is neutralisation of specific angiogenic peptides. Hori et al.[35] first documented that an antihuman bFGF monoclonal antibody suppressed, in part, neovascularisation and induced partial regression of solid tumours *in vivo*.

Similar results were obtained by other authors[36,100-102] using anti-VEGF monoclonal antibodies. A general finding of these studies is that it was not possible to obtain complete angiosuppression and, of consequence, a complete disappearance of experimental tumours *in vivo* with such a therapeutic strategy. These results may be related to the fact that a tumour may concurrently produce more endothelial growth factors and that neutralisation of only a single one is not sufficient for a complete blockade of the angiogenic pathways. In fact, it has been proved that cosecretion of more angiogenic peptides may have synergistic effects in promoting angiogenesis.[103-105] Furthermore, being tumour cells and therefore genetically unstable, they may switch to the production of a different angiogenic stimulus or stimuli and thus escape the treatment with neutralising antibodies and regrow.

MMPs are a family of proteolytic enzymes involved both in invasiveness of tumour cells and in penetration of new blood vessels within extracellular matrix.[106-109] Inhibitors of MMP induce tumour regression by interfering with both angiogenesis and tumour cell invasiveness.[110] Several agents that modulate this enzymatic activity have been discovered, some of which are active antitumoural drugs in experimental models.[111]

A recent discovery is of a noncatalytic MMP fragment with integrin $\alpha_v\beta_3$-binding activity, termed PEX, which is a natural inhibitor of MMP-2 activity, thereby regulating the invasive behaviour of new blood vessels.[112]

Retinoic acid and derivatives are differentiating agents that are promising for chemoprevention of certain solid tumours.[113] One of the possible mechanisms of action of *trans*-retinoic acid is inhibition of angiogenesis[114] by rendering endothelial cells refractory to angiogenic stimuli[115] or by blocking secretion of angiogenic factors by tumour cells in *in vitro* studies on cultured oral squamous cell carcinoma cells.[116]

Interleukin-12 (IL-12) demonstrated potent antitumour and antimetastatic activities in murine tumour models that were attributed to its ability to boost host immunity, especially by activation of cytotoxic T lymphocytes.[117] Voest et al.[118] demonstrated that intraperitoneal administration of IL-12 strongly inhibited bFGF-induced corneal neovascularisation, via mechanisms mediated by other cytokines such as interferon (IFN)- α, -β and -γ, and by stimulating the production of inducible protein-10 which has direct angioinhibitory activity.

In the last 4 years, Folkman's group has discovered and sequenced 2 highly biologically active naturally occurring inhibitors of angiogenesis: angiostatin[119] and endostatin.[38] Both these agents are internal fragments of larger proteins, but the parent molecules are not active in angiogenesis.[38,119] The recognition that there are cryptic naturally occurring antiangiogenic molecules suggests that they are involved in physiological processes, and that they probably act via specific receptors (presently unknown).

The earliest report on the existence of such a novel class of inhibitors which are fragments of larger proteins was published in 1991, concerning the study of the 16 kD fragment of prolactin.[120] A list of the naturally occurring inhibitors of angiogenesis up to now discovered is reported in table II.[121-135] These molecules specifically inhibit proliferating and migrating blood vessels, but do not affect resting endothelial cells, nor other cell types including tumour cells.[38,119]

Discovery of the more recent naturally occurring inhibitor molecules is the fruit of novel research strategies based on: (i) identification of the angiosuppressive molecule down-regulated at the switch to the angiogenic phenotype during tumourigenesis; (ii) transfection of an angiogenic tumour with a tumour suppressor gene interfering with angiogenic activity; and (iii) purification from serum or urine of mice bearing a tumour of the circulating

Table II. Naturally occurring inhibitors of angiogenesis

Inhibitor	Molecular characteristic	Source	Mechanism of action	Reference
Angiostatin	38 kDa protein	Plasminogen	↓EC proliferation, ↑EC apoptosis	119
Endostatin	20 kDa protein	Collagen XVIII	↓EC proliferation, ↑Apoptosis	38
Thrombospondin-1	450 kDa glycoprotein	Platelets, fibroblasts	↓collagen synthesis, ↓EC migration and proliferation, ↑EC apoptosis	96
Kringle-5	?	Plasminogen	↓EC proliferation	121
IFNα	8 to 20 kDa glycoproteins	Lymphocytes, phagocytes	↓EC proliferation and migration, ↓bFGF-induced angiogenesis	122
IFNβ	23 kDa glycoprotein	Fibroblasts, epithelial cells	↓EC proliferation	122
IFNγ	20 to 25 kDa glycoprotein	T cells, natural killer cells	Cytotoxic to EC proliferation	123
Interleukin-12	75 kDa glycoprotein	Phagocytes B cells, mast cells	↑IFNγ, IP-10	118
Platelet factor 4	28 kDa heparin-binding protein	Platelets	↓FGFR, ↓collagenase	124, 125
Prolactin-fragment	16 kDa N-terminal protein	Prolactin	↓EC DNA synthesis	120
Protamine	43 kDa arginine-rich protein	Sperm	Remodelling vessels	77
Retinoic acid	300D protein	?	Transcriptional regulator	114, 116
Tissue inhibitor of metalloproteases-1 and -2	8.5 kDa and 21 kDa proteins	Cartilage	↓Collagenase	126, 127
Transforming growth factor-β	25 kDa protein	Platelets, bone, tumour cells	↓EC motility	128, 129
TNF-α	17 kDa protein	Macrophages	↓bFGF	130, 131
Thrombospondin-2	?	?	↓EC proliferation and migration	132
Vascular endothelial cell growth inhibitor	Protein (30% homology with TNF proteins)	?	↓VEGF, ↓capillary tube formation	133
Giloma-derived angiogenesis inhibitor	Protein	?	?	134
Leukaemia inhibitor factor	Protein	?	↓EC proliferation migration, ↓extracellular proteolysis	135

EC = endothelial cell; **bFGFR** = basic fibroblast growth factor; **IFN** = interferon; **TNF** = tumour necrosis factor; **VEGF** = vascular endothelial growth factor; **?** = unknown; ↓ = decrease; ↑ = increase.

antiangiogenic molecule which suppresses metastasis.[136]

Angiostatin was purified as a 38 kD protein and is an internal fragment of human plasminogen.[119] Studies indicated that angiostatin inhibits endothelial growth *in vitro*, neovascularisation in the chick embryo, growth of metastases as well as growth of different types of primary tumours in mice.[119] Gately et al.[137,138] documented that the human prostatic cancer cells PC-3 release enzymatic activity that converts plasminogen to angiostatin. One pathway involves a plasminogen-angiostatin converting enzyme, and other enzymatic activities include urokinase and free sulphydryl donors. Dong et al.[139] found that Lewis lung carcinoma as well produces angiostatin by a macrophage-derived metalloelastase. These enzymatic activities generate bioactive angiostatin from human plasminogen or plasmin.

Holmgren et al.[68] provided evidence that dormancy of micrometastases is in part modulated by angiosuppression. They found that metastases remain dormant when tumour cell proliferation is balanced by an equivalent rate of apoptosis. Indeed, both the systemic administration to animals bearing tumour metastases of recombinant angiostatin or of exogenous inhibitors of angiogenesis, such as AGM-1470, maintains dormancy of metas-

tases by a mechanism enhancing the rate of apoptosis in tumour cells.[68] In a subsequent study, the same group[140] showed that systemic administration of human angiostatin potently suppresses the growth of human and murine primary carcinomas in mice. Tumours regressed to microscopic dormant foci in which, in absence of angiogenesis, tumour cell proliferation is balanced by apoptosis. Angiostatin did not cause systemic toxicity. The above mentioned studies suggest that angiostatin, by mechanisms not yet completely identified, induces tumour dormancy in both primary tumours and metastasis.

Endostatin is a 20 kD C-terminal fragment of collagen XVIII isolated from cultures of mouse haemangioendothelioma that specifically inhibits endothelial proliferation, angiogenesis and tumour growth *in vivo*.[38] The enzymes involved in proteolytic cleavage of collagen XVIII have not yet been identified. Systemic therapy with endostatin causes primary tumour regression to dormant microscopic lesions without relevant toxicity.[38]

Endostatin modulates apoptosis as angiostatin does, but it is 30 times more potent than angiostatin in suppressing angiogenesis.[38] The results of a paper published by Boehm et al.,[141] documented that antiangiogenic therapy with endostatin blocks tumour growth with no acquired resistance after multiple cycles of therapy. In the experimental study design, endostatin was administered to mice bearing 3 different types of tumours. Cyclic treatment was discontinued when tumours had regressed. As expected, tumours regrew and therapy with endostatin was resumed. After multiple cycles of therapy tumour dormancy was obtained, no tumour recurred after endostatin was stopped again and the animals had a long survival. In contrast, when the animals bearing the same tumours were treated with the cytotoxic agent cyclophosphamide, acquired resistance precluded the success of therapy, and the animals rapidly died of tumour progression. This study underscores a relevant theoretical advantage of antiangiogenic therapy over chemotherapy because the major cause of treatment failure using cytotoxic agents is development of acquired resistance.[142]

Another interesting result of this experimental study is that when mice bearing Lewis lung carcinoma were treated with the combination of angiostatin and endostatin, tumours regressed completely, whereas minimal residual disease was observed in the animals treated using endostatin as single agent.[141] The capability of the combined therapy to obtain the cure of experimental tumours was followed by extensive international mass media coverage focused on the potential clinical applications of antiangiogenic therapy.

The US Food and Drug Administration has applied a rapid procedure for evaluation of the antiangiogenic agents angiostatin and endostatin, with patient enrolment foreseen by Autumn 1999 for the latter.

Bergers et al.,[143] using the RIP1-Tag2 transgenic mouse model of pancreatic islet cells, a well defined model of multistage tumorigenesis and with known angiogenic characteristics, studied the effects of different antiangiogenic agents in 3 distinct stages of carcinogenesis. The first stage consisted of evaluating early treatment, at the hyperplastic stage, to block the angiogenic switch before formation of the tumour (i.e. chemoprevention).

At this stage both AGM-1470 and angiostatin had modest impact, whilst the MMP inhibitor BB-94 and endostatin alone or combined with angiostatin significantly reduced the percentage of angiogenic switching cells. In the second stage, mice bearing small and asymptomatic solid tumours were treated (intervention trial). At this stage all 4 antiangiogenic agents tested were efficacious, endostatin being the single most active drug. Finally, the third stage consisted of treating mice with substantial tumour burdens and short life expectancy (<2 weeks)[regression trial]. Both AGM-1470 and the combination of angiostatin plus endostatin caused regression of tumour volumes. The other antiangiogenic agents only showed tumour growth.

This study suggests, for the first time, that different antiangiogenic agents have diverse efficacies depending on the stage of carcinogenesis. In-

deed, none of the agents tested completely prevented the angiogenic switch, blocked the growth of small tumours or induced complete remission in advanced disease.

This information may help to better design clinical protocols, to identify the angiogenesis inhibitors of choice for the treatment of different clinical situations, and emphasises that the anticancer therapeutic activity of these compounds is not absolute and that, perhaps, to obtain the maximum benefit they should be combined with cytotoxic agents and/or radiation therapy.

Finally, brief mention must be made on the fact that few cytotoxic agents, in particular taxanes[144-145] and camptothecines,[146] have moderate angiosuppressive activity.

Studies pioneered by Teicher suggest that the strategy of the 2 compartment targeting (tumour cells and stromal cells) gives synergic antitumour effects in animal models.[39]

The list of targets, the agents and their mechanisms of action for therapeutic inhibition of angiogenesis are summarised in table III. Recently, Dr Folkman proposed, on the basis of observations made in experimental models, a simplified classification for antiangiogenic agents in 3 categories: drugs of first generation that slow tumour growth in animal models without frequent major responses such as IFNs, AGM-1470, thalidomide and MMP inhibitors; drugs of second generation that induce stable disease and frequent partial tumour regressions such as anti-VEGF antibody and anti-integrin $\alpha_v\beta_3$ antibody, and drugs of third generation that are 'curative' such as angiostatin and endostatin.[157]

3.2 Vascular Targeting

This is a therapeutic strategy distinct from inhibition of angiogenesis. This therapy is directed against tumour vasculature and is aimed at inducing the coagulation cascade within intratumoural neovessels resulting in infarction of the tumour mass, without relevant immunogenicity or systemic adverse effects.[147]

An example is the use of an antibody against the truncated form of tissue factor, a human coagulation-initiating protein, that in an experimental model produced major tumour regression with large necrotic areas histologically recognised.[148] Another strategy of vascular targeting consists in the use of antibodies with direct biological activity or conjugated with toxins, binding to molecules selectively expressed on tumour-activated vascular endothelium and absent on normal endothelium. An example is systemic administration to animals bearing tumours of an immunotoxin obtained by conjugating the M5/114 antibody to the ricin-A-chain (a protein synthesis inhibitor) that was active in suppressing tumour growth in an experimental model.[149] Potential target molecules for development of new vascular targeting treatments or immunotoxins are: $\alpha_v\beta_3$ integrin, fibronectin ED-B domain, endoglin, VEGF-receptors complexes, E-selectin and VCAM-1.

4. Strategy for Preclinical Development of a New Inhibitor of Angiogenesis and General Principles of Antiangiogenic Therapy of Tumours

4.1 Preclinical Development

Inhibitors of angiogenesis are biological response modifiers (BRMs) that affect tumour growth indirectly, by targeting the steps involved in the process leading to formation of neovessels within a tumour. 'Pure' antiangiogenic agents do not inhibit tumour growth in *in vitro* assays.[38,119] Therefore, specific strategies and assays are needed to identify, characterise and to test the efficacy of antiangiogenic agents, as a novel class of anticancer drugs.[158]

In the development of a new angiosuppressive agent, the chemical purification and definition of the spatial structure is the first step. Preliminary evaluation of the angiosuppressive effect could be carried out using specific *in vitro* assays, where in cultures of endothelial cells, such as human umbilical vascular endothelial cells, it is possible to test the effects of the new agent on: cell growth and proliferation, migration, invasiveness and the ability of endothelial cells to form cords and tube-like

Table III. Antiangiogenic therapy. Agents and targets (adapted from Gasparini[33] with permission)

Target	Agent	Mechanism of action
Activated endothelial cells	*Endogenous angiogenesis inhibitors (see table II for references)* Angiostatin, endostatin, thrombospondin-1, platelet factor 4 and others	↓EC proliferation, ↓angiogenic 'switch'
	Inhibition of endothelial cell growth and migration[50-53,81-95,117,118,143-145,156,162,174,176] AGM-1470 (TNP-470), linomide, thalidomide, interferons, CAI, interleukin-12, retinoids, nitric oxide synthase inhibitors, carboxyaminotriazole, CM 101, taxol/paclitaxel, camptothecin analogues (CPT-11, 9-AC, topotecan)	↓Cycling EC, ↓migration EC
Neutralisation or sequestration of angiogenesis mediators and their receptors	*Antibodies to angiogenic peptides*[35,36,100-102] Anti-VEGF monoclonal antibody, anti-bFGF monocolonal antibody, anti-angiogenin monoclonal antibody	↓Angiogenesis promoting factors
	Antigrowth factor agents[33,178,179] Chimeric soluble Flt-IgG heavy chain protein, SU5416, inhibitor of the Flk-1 tyrosine kinase blocking and Flk-1/KDR-VEGF receptor SU101, inhibitor of the signalling for the PDGF receptor, pentosan polysulphate, tecogalan (sulphated polysaccaride-peptidoglycan complex), suramin and analogues	Neutralisation of angiogenic peptides and interference with receptors for angiogenic mediators
Inhibition of functions of basement membrane and extracellular matrix	*Metalloprotease inhibitors*[61,110-112] Batimastat, marimastat (BB-2516), BAY 12-9566, MMPI, CGS 27023A, PEX	↓EC invasiveness and migration, ↓degradation MMP dependent of extracellular matrix
	Antibiotics and angiostatic steroids[33] Minocycline, tetracyclines, sulphated carboxymethil chitin, heparin-steroid complexes, Halofuginone	↓Synthesis and turnover of basement membrane, ↓collagen type I synthesis
Other mechanisms:	*Various*[33,60,147-149,175,178,181]	
Inhibition of endothelial cell adhesion molecules	Anti-integrin $\alpha_v\beta_3$ monoclonal antibody	↓Vessel formation and growth
Vaccines	Antiangiogenic vaccine by T cell immunity against endothelial T cell epitopes such as TEK	Activated EC death
Vascular targeting	VEGF-diphtheria toxin conjugates, antitissue factor (truncated form) monoclonal antibody, M5/114 antibody-ricin-A-chain-conjugate	Direct EC injury
Cytokine-mediated or unknown mechanisms	CM101	↑Cytokine-driven inflammatory response with activation of complement C_3
	Methoxyestradiol	Angiosuppressive *in vitro*
	Squalamine	Interferes with the metabolism of embryonic vessels

bFGF = basic fibroblast growth factor; **EC** = endothelial cell; **MMPI** = metalloprotease inhibitor; **PDGF** = platelet-derived growth factor; **VEGF** = vascular endothelial growth factor; ↓ = decreased; ↑ = increased.

capillary structures on gelatinised disks.[84,159] However, the use of human capillary endothelial cells is recommended for the *in vitro* assays because these cells are more representative of tumour endothelium than large vessel cells that differ for their receptor representation and response to angiogenic stimuli.

The subsequent step is to perform *in vivo* angiogenesis assays and to define the pharmacokinetic parameters, the different modalities of administration, therapeutic dosages etc., to provide indications on the more appropriate schedule. It should be established whether the compound needs metabolisation to be biologically active. The direct

antiangiogenic effect of the new agent can be displayed using various assays. The chicken chorioallantoic membrane (CAM) is a semi-quantitative method that allows to verify the ability to inhibit the growth of capillaries by implanting tumours onto the CAM and by comparing tumour growth and vascularisation with or without the administration of the antiangiogenic agent.[160]

Another test is matrigel, an extract of basement membrane proteins, containing collagen. Subcutaneous implants are created containing test substances combined with heparin. The addition of angiogenic peptides induces an angiogenic response that can be quantitated histologically or by determination of the haemoglobin content. It is therefore feasible to test inhibitors of angiogenesis by a matrigel assay and to assess their effect in relation to the specific angiogenic stimuli.[161]

The corneal neovascularisation test permits the direct visualisation of microvessels in the cornea of animals. In the corneal assay a slow release pellet containing test substances is placed into a micropocket surgically created 1 to 2mm from the avascular limbus. In some instances the pellet can be replaced by inoculation of tumour cells. By placing angiogenic inhibitors directly into the pellet it is possible to quantify and grade the inhibition of neovascularisation by monitoring the density, length and calibre of the vessels.[162,163] The angiosuppressive compound can also be administered to the animal systemically.[150]

Interestingly, using the corneal assay it is possible to quantify the inhibition on neovessels as well as on pre-existing vasculature by changing the time of administration of the angiosuppressive agent after having placed the angiogenic growth factor locally.[150] The use of this assay for quantification purposes is limited as minor variations in the distance that separates the micropocket from the limbus considerably affects the magnitude of angiogenic response. The effects of blocking neovascularisation on tumour growth can be observed by using a variety of *in vivo* experimental models in animals bearing tumours,[38,119] transgenic mouse models of tumour growth inclusive.[164,165]

Table IV. Development of a new inhibitor of angiogenesis: preclinical steps

Chemical purification of the agent
Evaluation of the angiosuppressive effect:
in vitro assays
endothelial cell growth and proliferation
endothelial cell migration
endothelial cell invasiveness
endothelial cell capillary-like tubes formation
in vivo assays
on different experimental tumours
xenografts
nude mice
primary tumour growth
metastasis models
corneal neovascularisation ('pure' antiangiogenic effect)
chicken chorioallantoic membrane
matrigel
Pharmacokinetics and metabolism
Inhibition and pre-existing tumour vasculature
Inhibition of tumour neovascularisation
Mechanism of action and identification of the specific step of angiogenesis inhibited
Definition of the related-factors for the activity of the agent
Angiosuppressive activity of the serum and urine of the treated animals
Development of tissue and sera surrogate marks predictive of the activity of the agent (i.e. levels of the suppressed target such as integrin $\alpha_v\beta_3$; matrix metalloproteases, angiogenesis nodulating receptors, etc.)
In vivo dynamic monitoring of the effects induced by the agent on tumour vascularisation

The potency of the inhibitor of angiogenesis on the primary tumour as well as on metastasis can be observed by comparing the animals treated with those receiving a placebo. The angiosuppressive activity of the serum and urine of the treated animals may be detected, and then tested for the biological activity *in vitro*. The *in vivo* assays also allow the development of surrogate markers predictive of the efficacy for specific antiangiogenic agents.

The main preclinical steps for development of a new inhibitor of angiogenesis are summarised in table IV.

4.2 General Principles of Antiangiogenic Therapy

Some principles derived from the preclinical studies up to now performed of antiangiogenic

therapy may be useful in planning future clinical trials in cancer patients.

4.2.1 Modality of Administration

Angiogenesis needs to be inhibited for a long time to induce primary tumour and metastasis dormancy[68,140] or to prevent development of new metastatic foci. Antiangiogenic therapy has its optimum efficacy if given over a long period of time and in the development of new inhibitors of angiogenesis a compound active by oral or subcutaneous administration is preferred.

For those antiangiogenic agents which are proteins sensitive to gastrointestinal degradation another active modality of administration is gene therapy (table V).[166]

4.2.2 Combined Therapy

Some experimental studies suggest that associations of two or more inhibitors of angiogenesis, with different mechanisms of action, lead to synergistic antitumoural effects and better results than the administration of each single agent.[167,168] Furthermore, combinations of antiangiogenic therapy and cytotoxic agents[39] or radiation therapy[151] have enhanced efficacy in tumour-bearing animals, for which each single drug induces only partial tumour remission.

4.2.3 Pharmacological Advantages Over Cytotoxic Therapy

Antiangiogenic therapy presents several theoretical advantages over chemotherapy. Inhibitors of angiogenesis have, in general, moderate systemic toxicity and, in particular, do not cause bone marrow suppression, gastrointestinal mucose alterations or hair loss, effects often observed with cytotoxic agents.[28] Because angiosuppressive therapy is mainly directed at activated endothelial cells, its cellular target can be easily reached by systemic administration.[34] Last but not least, therapy with some antiangiogenic agents is not impaired by development of acquired drug resistance.[141]

4.2.4 Antimetastatic Activity

It has been observed that certain inhibitors of angiogenesis are more active against metastasis than on the primary tumour.[68,87] This finding is potentially of great relevance because metastasis is the major cause of mortality in cancer patients. For example, in patients with breast cancer, chemotherapy is more active on the primary tumour (neoadjuvant therapy) than on metastasis[169] and it is only partially useful in preventing tumour recurrence when given as adjuvant therapy.[170]

4.2.5 Monitoring Efficacy

To assess antiangiogenic effects, noninvasive methods may be used to monitor the pharmacological effect.[171] These include quantitation of angiogenic growth factors in serum, urine[172-175] or in the cytosol[176] of neoplastic lesions.

Other possibilities are the use of techniques measuring blood flow *in vivo* using color doppler, magnetic nuclear resonance, and by positron emission tomography.[177]

Table V. Inhibition of angiogenesis by gene therapy (adapted from Kong & Crystal,[159] with permission)

Gene	Delivery system	Experimental model
Thrombospondin-1	Calcium phosphate transfection	*Ex vivo* transfection of breast cancer cells, followed by implantation into nude mice
Vascular endothelial growth factor antisense	Phosphorothioate oligonucleotides	Primary glioblastoma model in nude mice
Dominant-negative Flk-1 mutant receptor	Retrovirus vector	Various primary tumour models in mice
Soluble platelet factor 4	Adenovirus and retrovirus vectors	Primary glioma models in nude mice
Soluble Flt-1 receptor	Adenovirus vector	Primary and metastatic tumour models in mice
Angiostatin	Adenovirus and retrovirus vectors	Primary glioma models in nude mice
Cytostatic drug-inducible vector for tumour necrosis factor-α	PM3mdr-phTNF vector	MCF-7 tumour-bearing animals also treated with doxorubicin
Angiostatin-Endostatin fusion protein	Retrovirus vector	Human neuroblastoma cells (SKNAS) in nude mice

5. Clinical Development of Angiogenesis Inhibitors

Proper and well designed studies are crucial for a valid clinical development of antiangiogenic therapy. The understanding that antiangiogenic agents are BRMs has certain implications for clinical study design.

The development of antiangiogenic agents into novel anticancer therapeutics is an important opportunity to expand our possibilities in the battle against cancer. However, it presents major challenges since traditional criteria are inadequate for a valid evaluation of their efficacy. When patients are selected for experimental clinical trials, surrogate markers for angiogenesis should be tested to determine the patients that are more likely to benefit of specific antiangiogenic compounds that are to be chosen on the basis of the angiogenic characterisation of the tumour.

For phase I clinical trials the identification of the 'optimal biological dose' should be the specific end-point rather than the maximal tolerable dose as determined for conventional cytotoxic agents. Therefore, it is suggested that the development of surrogate marker/s for monitoring the biological effect be an integral part of the study design.[33,171] For specific treatments, directed at the inhibition of molecular defined targets, the definition of the 'target inhibiting dose range' is the appropriate end-point for phase I studies, besides definition of the pharmacokinetic parameters. The feasibility and tolerability of schedules of combined therapy of diverse antiangiogenic drugs and of these with chemotherapy is another matter for investigational clinical research.

Conventional phase II trials performed in patients with advanced disease probably are not useful for testing angiosuppressive compounds.[33,171] It is important to realise that long lasting stable disease is an alternative end-point that is to be considered as a positive response to antiangiogenic therapy along with a major objective response of tumour lesions. The changes induced by antiangiogenic therapy in the degree of vascularity, certain enzymatic activities (MMP, collagenases etc.) or levels of angiogenic peptides in the biological fluids may serve as intermediate determinants of efficacy of inhibitors of angiogenesis.

The more appropriate clinical testing for evaluation of antiangiogenic therapy is performing randomised phase IIb or phase III studies for chemoprevention, neoadjuvant, adjuvant therapy and maintenance therapy by comparing the new therapeutic strategy with conventional therapy.[33]

Experimental studies suggest that antiangiogenic therapy may prevail over angiogenic stimuli produced by the tumour[178] as well as prevent the development of metastasis.[119,179] Therefore, inhibitors of angiogenesis alone or in combination with conventional anticancer treatments are particularly promising to prevent cancers and to reduce the risk of recurrence of disease in patients who have undergone radical surgery and with highly metastatic tumours such as node-positive breast cancer or Dukes stage C colorectal cancer.

The more promising agents for clinical testing should be able to interfere with tumour-associated angiogenesis without affecting normal endothelium. A particularly promising category of compounds is that of naturally occurring inhibitors, including angiostatin and endostatin. The more potent antiangiogenic drugs up to now identified belong to this category. They are highly specific for activated endothelial cells, have low toxicity and do not cause immunological response.[38,119]

Because naturally occurring inhibitors of angiogenesis are proteins, most of which are biologically active after enzymatic cleavage, a potential problem may be in their long term administration to patients. In fact, it is possible that some of these compounds are not active by oral administration because of gastric enzymatic activities and that they need to be given systemically via subcutaneous or intravenous infusion. Another approach for drug delivery of these compounds is gene therapy (table V).

Regarding angiostatin and endostatin, EntreMed, Inc., in collaboration with Dr Folkman's laboratory is preparing a more appropriate pharmaceutical preparation for administration of the drugs to can-

cer patients which is planned to begin phase I clinical trials in 1999.

Certain questions regarding the use of antiangiogenic agents for the treatment of human tumours remain open: such as what inhibitors are most effective for specific tumour types, and whether multiple inhibitors have additive effects in inhibiting angiogenesis? It is also possible that there are tumour-derived factors that ablate the activity of antiangiogenic agents even though such activities have not yet been documented.

The knowledge of the crystal structure of the naturally occurring inhibitors of angiogenesis as well as the identification of their specific targets or receptors may help in the development of smaller peptides with enhanced activity.

The primary target of naturally occurring inhibitors of angiogenesis as well as of other drugs including: AGM-1470, IL-12 and thalidomide is the activated endothelial cell of neovessels within tumours that have acquired the angiogenic phenotype.

AGM-1470 possesses a wide spectrum of activity as found in animals bearing murine and human tumours. In phase I to II trials starting at a dose of 70 mg/m^2 for intravenous infusion, a reversible CNS toxicity with encephalopathy, dizziness, ataxia and tremors was observed. The drug demonstrated some activity against prostate, cervix and breast cancers. Active therapeutic doses ranged between 50 and 70 mg/m^2 for intravenous infusion, with a maximum tolerated dose of 177 mg/m^2.[152] An active metabolite, AGM-1883, with a short plasma half-life has been identified.[153]

In a phase I study, 18 patients with refractory, advanced squamous cell carcinoma of the uterine cervix were treated with AGM-1470. A patient with multiple lung metastases achieved a complete remission, 3 patients had long lasting stable disease, whereas the other 14 patients had progressive disease. Toxicity consisted of transient rise in aminotransferase levels and mild anorexia.[154] Currently, phase II trials are ongoing in patients with glioblastoma, pancreatic, renal and cervical cancers. Phase I studies of AGM-1470 in combination with taxol have been started in patients with advanced breast cancer (DE Hayes, personal communication) and in patients with advanced lung cancer (Herbst R, personal communication).

A different class of agents, those directed to neutralise endothelial growth factors such as bFGF and VEGF, is also of interest for clinical testing. Humanised anti-VEGF monoclonal antibody by Genentech, Inc., has been tested in phase I trials without important systemic toxicity and phase II to III studies are planned for refractory tumours.[180]

Other agents, such as the small peptide SU5416 by Sugen, Inc., interfere with activity of the Flk-1, a receptor for VEGF. SU5416 is potent, selective and inhibits a wide variety of experimental tumours including those growing slowly.[155] It is already entered in clinical phase I to III studies.[181]

Clinical use of agents inhibiting specific angiogenic peptides requires the knowledge of which is the more active pathway of angiogenesis of each single tumour. Therefore, determination of expression of VEGF in a certain tumour is to be done prior of planning therapy with specific anti-VEGF treatments. However, targeting VEGF may result in development of tumour cell variants that may be able to stimulate neovascularisation by secreting alternative growth factors and so developing acquired resistance to VEGF-targeting agents. More angiogenic peptides may be involved in tumour progression,[103-105] so that blocking only one of these may lead to only a partial control of tumour growth.[36,100-102] Finally, angiogenesis is presumed to be the result of the equilibrium between antiangiogenic and proangiogenic factors.

It seems reasonable to hypothesise that combined administration of naturally occurring inhibitors of angiogenesis together with angiogenic peptides-neutralising agents may lead to synergistic effects. This type of therapy requires preclinical testing in animals to design an optimal therapeutic approach for cancer patients.

Up to now only a limited number of targets expressed by endothelial cells forming new blood vessels, but not on quiescent endothelium, have been identified. One of such molecules is the in-

tegrin $\alpha_v\beta_3$ adhesion receptor, necessary for neovascularisation and favouring the anchorage of migrating endothelial cells to other extracellular matrix components including vitronectin, fibronectin and fibrinogen.[16] Antagonists of integrin $\alpha_v\beta_3$ interferes with adhesion-dependent signals causing apoptosis of activated endothelial cells.[182] Integrin $\alpha_v\beta_3$ activity can be inhibited in experimental models by a neutralising anti-integrin $\alpha_v\beta_3$ monoclonal antibody developed by Cheresh's group,[60] which is presently under early clinical testing,[156] as well as by TNFα and IFNγ, that disrupt tumour vasculature in part by suppressing integrin $\alpha_v\beta_3$ activity.[183] Also in this case, a prerequisite for assignment of therapy neutralising integrin $\alpha_v\beta_3$ is the documentation of its expression on tumour-associated vasculature.

In a large series of immunohistochemical stainings we have determined the expression of integrin $\alpha_v\beta_3$ in invasive breast cancer. Tumour vasculature stained positively for this adhesion molecule, but displayed a varying degree of positivity with the monoclonal antibody LM 609. The expression of integrin $\alpha_v\beta_3$ was predictive of clinical outcome, with the patients having highly vascularised LM 609–positive tumours having the worst prognosis.[184] These findings suggest that, perhaps, not all the patients with invasive breast cancer will gain the same benefit of therapeutic neutralisation of such an adhesion molecule.

MMPs are a multigene family of enzymes involved in degradation of a wide range of matrix protein substrates including collagens, laminins, fibronectin and elastin.[106-109] Several MMP inhibitors are under early clinical investigation and their potential therapeutic applications in the clinical setting is promising.[153] Among these agents, marimastat is orally active.[185] Phase I studies have defined the levels of tolerated doses, and the toxicity profile. A musculoskeletal syndrome with local pain, stiffness and discomfort in about one-third of the patients who received marimastat was the major adverse effect. The oral dose of marimastat 5 to 10mg twice daily gives biologically active plasma concentrations of 40 to 80 mg/ml and significantly reduced serum tumour markers over baseline values in patients with a variety of solid tumours. Phase III randomised studies are in progress in patients with breast cancer, small cell lung cancer, pancreatic cancer and glioblastoma in ECOG or EORTC trials.[153]

Another orally active agent under extensive clinical evaluation is BAY-12-9566. It has good bioavailability, is characterised by high protein binding, long plasma half-life and minimal toxicity on soft-tissues.[186] A general finding from early clinical studies with this MMP inhibitor is that long term therapy is quite well tolerated and that it induces long stable disease with promising activity, particularly in patients with bone metastasis.

Recent studies presented at the 1998 Meeting of the American Society of Hematology have highlighted the promising antitumour activity of thalidomide in patients with refractory high risk multiple myeloma. The largest study evaluated 89 patients who received a dose escalating schedule of the drug from 200mg to 800mg daily. Thalidomide induced a total response rate of 34% (30 out of 89 cases) associated with resolution of bone marrow plasmocytosis in 11 of the 24 evaluable patients. Only 42 patients experienced tumour progression, while the other 15 patients had stable disease lasting more than 3 months. The major toxicities were neurological, gastrointestinal or constitutional, all of these being generally of moderate degree, with only 8 patients requiring suspension of therapy because of toxicity.[187]

6. Conclusions and Future Prospects for Research

During 1998, the extensive press coverage of two new antiangiogenic agents, angiostatin and endostatin, gave new hopes to the cancer patients around the globe and sparked a heated debate among researchers. In spite of being cautioned by the most prominent researcher in the field, Dr Judah Folkman, the public was inclined to believe antiangiogenesis to be a long anticipated miraculous cure for cancer. A more realistic way to view it is that antiangiogenic drugs whilst not a 'magic

bullet', are a promising new approach to cancer therapy, and a powerful aid for the existing anticancer strategies, that require accurate clinical testing before routine use.[188-192]

Because cancer treatment history has often been characterised by new drugs that cure experimental tumours, but that have no or moderate efficacy in humans, it should be emphasised that the journey from the laboratory to the clinical setting is a long, delicate and painstaking process.

Inhibitors of angiogenesis are cytostatic agents that, to be rationally used, need proper clinical evaluation as well as the development of biomarkers and surrogate end-points of their biological activity. A recent study suggested, for the first time, that some subtypes of human non–small cell lung cancer grow through nonangiogenic mechanisms, being perfused by pre-existing alveolar vessels.[193] The patients with the above tumours should be identified because, probably, they may not gain benefit of angiosuppressive therapy. Furthermore, for an appropriate approach to inhibition of angiogenesis by molecular medicine, delivery of therapeutics is a substantial step to reach optimum quantities of drugs in the target cells (activated endothelium) and in the *in vivo* microenvironment, to properly affect the stromal compartment of the tumour.[6,194]

The only way to determine the relevant antiangiogenic agent for each individual tumour is to characterise a particular tumour systematically at the time of surgery or biopsy. Another possibility is to determine the changes in angiogenic phenotype that parallel malignant transformation of an individual tumour type. However, in the absence of such information priority should be given to treatment with inhibitors of angiogenesis that would target specifically the activated endothelial cells and thus potentially effective against multiple tumour types. This strategy would also significally decrease the cost of treatment.

Once phase I studies identify active antiangiogenic agents with a good toxicological profile, future prospectives of translational research include studies of combined therapy. The first possibility is to test combinations of two or more antiangiogenic agents with different mechanisms of action. The second strategy foresees combinations of antiangiogenic drugs with conventional anticancer cytotoxic, treatments that may be able to block, in a synergistic manner, the mutual stimulation operating between the parenchymal and stromal components of a tumour.[195] The third option is the testing of combined treatments of antiangiogenic agents with other biologically driven therapies, such as inhibitors of tumour growth factors, differentiating agents etc. All these approaches require phase II to III comparative trials. However, combination therapy strategies offer many opportunities of drug development of new antiangiogenic drugs, that are difficult to explore in brief time and that need of large clinical trials for comparison.

An improvement of clinical trial designs for the development of BRMs along with the recommendation by Gasparini[33] is important because new therapeutic approaches for therapy of cancer may fail if non-appropriate clinical studies, that could properly exploit the attributes of the new agent, are performed. Von Hoff, in his 'Richard and Hinda Rosenthal Foundation Award Lecture',[196] emphasised that 'there are no bad anticancer agents, only bad trial designs'. This provocative statement should stimulate a more rationale and rigorous approach for the clinical development of anticancer treatments based on novel mechanisms of action.

In conclusion, just how much antiangiogenic therapy will be a valid therapeutic tool for the cure of cancer patients in the years to come depends on further advances in the understanding of the molecular mechanisms involved in angiogenic activity of human tumours, the development of standardised methods to assess surrogate markers predictive of response, proof of the clinical effectiveness and tolerability of antiangiogenic agents, and on the capability of performing well designed clinical studies.

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