Angiogenesis Inhibitors – Current Strategies and Future Prospects

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Abstract

Angiogenesis has become an attractive target for drug therapy due to its key role in tumor growth. An extensive array of compounds is currently in pre-clinical development, with many now entering the clinic and/or achieving FDA approval. Several regulatory and signaling molecules governing angiogenesis are of interest, including growth factors (e.g. VEGF, PDGF, FGF, EGF), receptor tyrosine kinases, transcription factors such as HIF, as well as molecules involved in MAPK and PI3K signaling. Pharmacologic agents have been identified that target these pathways, yet for some agents (notably thalidomide), an understanding of the specific mechanisms of anti-tumor action has proved elusive. The following review describes key molecular mechanisms and novel therapies that are on the horizon for anti-angiogenic tumor therapy.

Keywords

angiogenesis inhibitors; hypoxia inducible factor (HIF); growth factors; VEGF; kinase inhibitors; anti-angiogenesis

In 1971, Dr Judah Folkman published a landmark paper in the New England Journal of Medicine, with the hypothesis that solid tumors caused new blood vessel growth (angiogenesis) in the tumor microenvironment by secreting pro-angiogenic factors[1]. This publication heralded the beginning of research on angiogenesis and hypoxia and their role in cancer. Over the last four decades, the discovery of a plethora of genes, signaling cascades and transcription factors has revealed the complexity of the angiogenic process and furthered our understanding of this hypothesis.

Inhibition of Angiogenesis for Anti-Cancer Purposes

Because angiogenesis is a key process to tumor growth, and a limited process in healthy adults, developing angiogenesis inhibitors is a desirable anti-cancer target where few side effects might be expected. Resistance to anti-angiogenesis drugs is also unlikely to occur, or at least at a much lower rate than seen with traditional cytotoxic chemotherapeutics, particularly if the genetically stable endothelial cells are targeted[2]. Selecting specifically for tumor endothelial cells and vessels could be achieved by targeting their unique or unusual properties. While physiologic angiogenesis is a tightly orchestrated process that is regulated by a balance of pro- and anti-angiogenic factors, tumor angiogenesis is erratic and irregular, with leaky vessels that are poorly formed (for review see references[3,4,5]). The tumor

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endothelial cells divide more rapidly than non-tumor endothelial cells and also express markers that the normal endothelial cells do not express[2]. Because endothelial cells line the blood vessels, they are also much more accessible to the circulation and therefore pharmacologic treatments than are the tumor cells themselves.

Advances in our understanding of the regulatory mechanisms that govern tumor angiogenesis continues to aid drug development, particularly with the identification of new therapeutic targets. An understanding of how both newly developed and conventional anti-cancer compounds function to inhibit angiogenesis will help further our understanding of how tumor angiogenesis occurs and how it might be successfully limited to halt the growth and spread of a tumor. One interesting finding is that many conventional chemotherapeutics actually possess previously unknown anti-angiogenesis activity. These include cytotoxic chemotherapeutic drugs, hormonal ablation therapies and tyrosine kinase inhibitors (for review see Kerbel *et al.*[6]).

The following review will give a broad overview of the key mechanisms involved in tumor angiogenesis and the various inhibitors that have shown promise for cancer therapy.

Process of Carcinogenesis and Subsequent Tumor Angiogenesis

The process of transformation from a normal cell into a cancer cell involves a series of complex genetic and epigenetic changes. In an influential paper, Hanahan and Weinberg proposed that six essential 'hallmarks' or processes were required for transformation of a normal cell to a cancer cell[7]. These processes include (i) self-sufficiency in growth signals, (ii) insensitivity to anti-growth signals, (iii) evasion of programmed death (apoptosis), (iv) endless replication potential, (v) tissue invasion and metastasis and importantly (vi) sustained angiogenesis[7].

Initially, the growth of a tumor is fed by nearby blood vessels. Once a certain tumor size is reached, these blood vessels are no longer sufficient and new blood vessels are required to continue growth. The ability of a tumor to induce the formation of a tumor vasculature has been termed the 'angiogenic switch' and can occur at different stages of the tumor-progression pathway depending on the type of tumor and the environment[4]. Acquisition of the angiogenic phenotype can result from genetic changes or local environmental changes that lead to the activation of endothelial cells.

One way for a tumor to activate endothelial cells is through the secretion of pro-angiogenic growth factors which then bind to receptors on nearby dormant endothelial cells (ECs) that line the interior of vessels (figure 1). Upon EC stimulation, vasodilation and permeability of the vessels increase and the ECs detach from the extracellular matrix and basement membrane by secreting proteases known as matrix metalloproteinases (MMPs). The ECs then migrate and proliferate to sprout and form new branches from the pre-existing vasculature. The growth factors can also act on more distant cells recruiting bone-marrow derived precursor endothelial cells and circulating endothelial cells to migrate to the tumor vasculature (for an overview see references[4,8]).

The pro-angiogenic growth factors may be overexpressed due to genetic alterations of oncogenes and tumor suppressors, or in response to the reduced availability of oxygen (figure 2). Tumor cell expression of many of the angiogenic factors, including vascular endothelial growth factor (VEGF) is regulated by hypoxia through the transcription factor hypoxia inducible factor (HIF)[9]. As the tumor cells proliferate, oxygen becomes depleted and a hypoxic microenvironment occurs within the tumor. HIF is degraded in the presence of oxygen, so formation of hypoxic conditions leads to HIF activation and transcription of target genes. The strongest activation of HIF results from hypoxia, but several other factors

can contribute to increased expression and activity of HIF, including growth factors and cytokines such as TNF-α[10], IL-1β (interleukin-1β)[11,10], EGF[12,13], and insulin-like growth factor-1 (IGF-1)[14], which lead to increased cell signaling. Along similar lines, oncogenes that trigger increased expression of growth factors and overactive signaling pathways can increase HIF expression and activity. For example, mutant Ras can contribute to tumor angiogenesis by enhancing the expression of VEGF through increased HIF activity[15,16]. The oncogenes *v-Src*[17] and *HER2*[18] and dysregulated PI3K and MAPK signaling pathways[12,13,19,14,20] have also been shown to upregulate HIF expression and HIF transcriptional activity.

Receptor Tyrosine Kinase Signaling

Receptor tyrosine kinases (RTKs) are transmembrane proteins that mediate the transmission of extracellular signals (like growth factors) to the intracellular environment, therefore controlling important cellular functions and initiating processes like angiogenesis. Structurally, the RTKs generally consist of an extracellular ligand binding domain, a single transmembrane domain, a catalytic cytoplasmic tyrosine kinase region and regulatory sequences. RTKs are activated by the binding of a growth factor ligand to the extracellular domain, leading to receptor dimerization and subsequent auto phosphorylation of the receptor complex by the intracellular kinase domain, utilizing ATP[21]. The phosphorylated receptor then interacts with a variety of cytoplasmic signaling molecules, leading to signal transduction and eventually angiogenesis, among other processes involved in cell survival, proliferation, migration and differentiation of endothelial cells (for review see[21,22]).

RTKs that become dysregulated can contribute to the transformation of a cell. The dysregulation can occur through several different mechanisms, including (i) amplification and/or overexpression of RTKs, (ii) gain of function mutations or deletions that result in constitutively active kinase activity, (iii) genomic rearrangements that produce constitutively active kinase fusion proteins, (iv) constant stimulation of RTKs from high levels of pro-angiogenic growth factors and (v) retroviral transduction of a deregulating proto-oncogene that cause RTK structural changes, all of which lead to increased downstream signaling[21].

The complex signaling network uses multiple factors to determine the biological outcome of the receptor activation. While the pathways are often depicted as linear pathways for simplicity, they are actually a network of pathways with various cross-talk and overlapping functions, as well as distinct functions. Some of the known signaling cascades include the PLCγ-PKC-Raf kinase-MEK-MAPK and PI3K-AKT-mTOR pathways and activation of the Src tyrosine kinases[23,24,25,26]. A detailed overview of the individual growth factors and their receptor tyrosine kinases is beyond the scope of this review, but some of the main factors will be briefly covered below.

VEGF—Vascular endothelial growth factor (VEGF) and its receptor tyrosine kinase (VEGFR) play key roles in angiogenesis (figure 3; for review see references[27,28,29,30]). While VEGF is actually a family of at least seven members (table 1), the term VEGF typically refers to the VEGF-A isoform, one of the most studied members and a major mediator of tumor angiogenesis. VEGF-A is a pro-angiogenic factor that plays important roles in cell migration, proliferation, and survival. Four spliced isoforms of VEGF-A are known (VEGF$_{121}$, VEGF$_{165}$, VEGF$_{189}$, and VEGF$_{206}$), with VEGF$_{165}$ being the most predominant form[30]. VEGF-A was initially identified for its ability to increase vascular permeability in guinea pigs, and was termed vascular permeability factor (VPF)[31] and then separately identified for its ability to promote the growth of vascular endothelial cells, naming it VEGF[32]. Cloning the VPF and VEGF genes revealed that they were actually the same[33,34].

When VEGF is secreted from tumor cells, it interacts with cell-surface receptors, including VEGFR-1 and -2, located on vascular endothelial cells and bone-marrow derived cells. VEGFR-2 is believed to mediate the majority of the angiogenic effects of VEGF-A while the role of VEGFR-1 is complex and not fully understood[30]. A soluble form of VEGFR-1 can act as a decoy receptor, preventing VEGF-A from acting on VEGFR-2 and activating signaling pathways. However, there is also evidence that indicates VEGFR-1 plays an important role in developmental angiogenesis[30]. A third receptor, VEGFR-3, is involved in lymphangiogenesis and does not bind VEGF-A[30].

VEGF-A$_{165}$ is commonly overexpressed by a wide variety of human tumors and overexpression has been correlated with progression, invasion and metastasis, microvessel density and poorer survival and prognosis in patients[35,36,37,38]. VEGF-A and VEGFR-2 are currently the main targets for anti-angiogenesis efforts[27].

PDGF—The family of platelet derived growth factors (PDGF) and receptors (PDGFR) are involved in vessel maturation and the recruitment of pericytes[39]. PDGF stimulates angiogenesis *in vivo*[40,41], though the role of PDGF in angiogenesis is not fully understood (for review see references[42,43]). The family of PDGF ligands consists of four structurally related soluble polypeptides, that exist as homo- and hetero-dimers (table 1). There are two forms of the PDGF tyrosine kinase receptors, PDGFR-α and -β[42]. PDGF is expressed by endothelial cells and generally acts in a paracrine manner, recruiting PDGFR expressing cells, particularly pericytes and smooth muscle cells, to the developing vessels[43].

Mutations involving upregulation of PDGF and/or PDGFR have been described in human cancers, though the role of these mutations in cancer has not been fully characterized[43]. Nearly all gliomas tested are positive for PDGF and PDGFR[44,45] and overexpression of PDGFR has been associated with poor prognosis in ovarian cancer[46], indicating a likely role for the PDGF pathway in human cancers.

FGF—The mammalian fibroblast growth factor (FGF) family is composed of 23 different proteins, which are classified into six different groups based on the similarity of their sequences (for review see references[42,47]). The FGF ligands were among the earliest angiogenic factors reported and are involved in promoting the proliferation, migration and differentiation of vascular endothelial cells[48,49]. FGF ligands have a high affinity for heparin sulfate proteoglycans (HSPGs), which act as co-receptors by binding to both FGF and one of the four different fibroblast growth factor receptors (FGFR) simultaneously[47]. The FGF receptor tyrosine kinases are widely expressed and are present on most, if not all, cell types, where they act through a wide range of biological roles[42]. FGFRs are often overexpressed in tumors and mutations of the FGFR genes have been found in human cancers, making it particularly significant that FGFR activation in endothelial cell culture and animal models leads to angiogenesis[42,47]. Overexpression of various FGF ligands in different types of tumors has been documented[47]. FGF-2, in particular, has been shown to possess potent angiogenic activity[50] and is also commonly overexpressed in tumors and has been found to correlate with poor outcome in non-small cell lung cancer (NSCLC) and bladder carcinomas[51,52].

EGF—The epidermal growth factor (EGF) family consists of eleven known members which bind to one of four epidermal growth factor receptors (EGFR)[53]. All of the receptors, except HER3, contain an intracellular tyrosine kinase domain[54]. HER2 does not have any known ligands that bind with high affinity, despite it being a potent oncoprotein (for review see references[53,54]). Activation of EGFR has been linked to angiogenesis in xenograft models[55], in addition to metastasis, cell proliferation, survival, and migration, transformation, adhesion and differentiation[54]. Because activation of the EGFR pathway upregulates the production

of pro-angiogenic factors like VEGF, it can be viewed as more of an indirect regulator of angiogenesis, rather a direct regulator, making the role of the EGF/EGFR system less important to angiogenesis than more direct regulators, such as the VEGF and PDGF systems (reviewed in references[53,54,56,57]).

Other Angiogenic Factors

TGF-β—Transforming growth factor-β (TGF-β) and corresponding receptors are produced by nearly every cell type, though each of the three isoforms of TGF-β (TGF-β1, -β2, -β3) demonstrates a different tissue expression pattern[58]. TGF-β participates in angiogenesis, cell regulation and differentiation, embryonic development, and wound healing and also has potent growth inhibition properties[58]. The TGF-β receptors are classified as type I, II, or III. Type I and II receptors contain serine/threonine kinase domains in their intracellular protein regions, while type III does not possess kinase activity but thought to participate in transferring TGF-β ligands to type II receptors[58]. TGF-β ligands bind to and stimulate type II receptors that recruit, bind and phosphorylate type I receptors, activating downstream signaling proteins known as SMADs, which are believed to be specific to the TGF-β family. Activated SMADs eventually move to the nucleus where they can interact with different transcription factors, regulating gene expression in a cell-specific manner[58]. SMADs have been found to be mutated at a high rate in pancreatic and colorectal cancer, and are found in other cancers as well, indicating that SMAD mutations and aberrant regulation likely contribute to the development of cancers (reviewed in[59]). In addition to the SMADs, signaling mediated by TGF-β can involve activation of downstream targets such as MAPK and PI3K[59].

TGF-β is thought to have both pro- and anti-angiogenic properties, depending on the levels present. Low levels of TGF-β contribute to angiogenesis by upregulating angiogenic factors and proteases, while high doses of TGF-β stimulate basement membrane reformation, recruit smooth muscle cells, increase differentiation and inhibit endothelial cell growth[8]. Tumor cells can also become resistant to TGF-β and will no longer respond to the growth-inhibiting properties, leading to tumor cell proliferation. Tumors that no longer respond to the growth inhibition signals from TGF-β can then exploit the abilities of TGF-β to regulate processes involved in angiogenesis, cell invasion and tumor cell interactions[60].

Overexpression of TGF-β1 has been seen in gastric, breast, colon, hepatocellular, lung and pancreatic cancer and is correlated with tumor angiogenesis in addition to metastasis, progression and poor prognostic outcome[61]. High levels of endoglin, part of the TGF-β receptor complex, have also been detected in cancers and are associated with tumor metastasis[60].

Angiopoietins and Tie Receptors—The angiopoietin ligands and Tie receptor tyrosine kinases play a regulatory role in vascular homeostasis and maintenance of quiescent endothelial cells in adults and are also an essential component of embryonic vessel assembly and maturation[62]. The angiopoietin (Ang) family of ligands (Ang-1, -2, and -3/-4) bind to the receptor tyrosine kinase Tie-2. No ligand has been identified yet for the Tie-1 receptor (for review see references[62,63,64]). Ang-1 behaves as an agonist, activating the Tie-2 receptor, while Ang-2 acts as an antagonist for Tie-2[65], though the role of Ang-2 in tumor angiogenesis is not fully understood and appears to be dependent on the environmental context. In the presence of VEGF-A, Ang-2 will promote angiogenesis, and in the absence of VEGF-A, Ang-2 will cause vessel regression[66,67]. Overexpression of Ang-2 has been found to correlate with increased angiogenesis, malignancy and aggressive tumor growth in some cancers, and in other tumor types, overexpression led to decreased tumor growth and metastasis and vessel regression (for review see[62,63,64]). The involvement of additional

factors in Ang-2 function are likely the cause of conflicting data on Ang-2, indicating the need for further studies of the Ang/Tie system.

Ang-1 overexpression leads to vasculature that is more mature and normal in appearance, explaining the vessel-normalization effect that results from anti-VEGF/VEGFR therapies, as they these effects are mediated through Ang-1[68]. Most studies have shown that Ang-1 possesses mostly anti-tumorgenic effects, though some have indicated that Ang-1 can stimulate tumor growth[62].

While the angiopoietins and Tie receptors appear to play an important role during tumor angiogenesis, the specific mechanisms are controversial. A further understanding of the specific roles of the members of the Angiopoietin/Tie system may enable targeting of this system for anti-angiogenic and anti-cancer purposes.

Attempts at targeting the Tie-2 pathway for angiogenesis inhibition have had more difficultly than some of the other angiogenesis targets, such as VEGF, in part because of the lack of understanding as to the agonistic and antagonistic roles of Ang-1 and Ang-2 on the Tie-2 receptor. There have been some efforts though, and peptide-antibody fusions that bind and neutralize Ang-2 have been shown to decrease tumor growth and angiogenesis, and suppress endothelial cell proliferation in pre-clinical models[69], demonstrating the feasibility of targeting Ang/Tie for anti-angiogenic purposes.

Delta/Jagged-Notch Signaling—The family of Notch receptors (Notch1-4) and their transmembrane ligands Delta-like (Dll1, 3, 4) and Jagged (Jagged1, 2) play important roles in cells undergoing differentiation, acting primarily to determine and regulate cell fate, as well as playing a part in developmental and tumor angiogenesis. In healthy mice, Dll4 is required for normal vascular development and arterial formation, while in tumor angiogenesis, Dll4 and Notch signaling appears to play a role in regulating the cellular actions of VEGF (for review see references[70,71]).

Activation of Notch signaling is dependent upon cell to cell interactions and occurs when the extracellular domain of the cell surface receptor interacts with a ligand found on a nearby cell. Lateral inhibition, one mechanism of Notch signaling, involves binding of a Notch ligand to a Notch receptor on an adjacent cell, which results in activation of the Notch signaling pathway in one cell and suppression in the other cell, resulting in two different fates for each cell[70]. Notch receptors also participate in transcriptional regulation through a unique mechanism involving cleavage of the intracellular domain of the Notch receptor, which then translocates to the nucleus where it can participate in transcriptional regulation[71].

Delta-like 4 (Dll4) and Jagged1 have particularly been implicated in tumor angiogenesis, with strong expression of Dll4 seen in the endothelium of tumor blood vessels, and much weaker expression in nearby normal blood vessels[72,73,74,75]. The expression of Dll4 appears to be regulated directly by VEGF in the setting of tumor angiogenesis; increased levels of VEGF lead to increased expression of Dll4[75,76]. Dll4 then signals to the Notch receptor-expressing endothelial cells to downregulate VEGF-induced sprouting and branching[77]. In this manner, Dll4 acts as a negative modulator of angiogenesis, regulating excessive VEGF-induced vessel branching, allowing vessel formation to occur at a productive and efficient rate[77]. Overexpression of Jagged1, a Notch ligand, is dependent on MAPK signaling[78] and has been associated with angiogenic endothelial cells *in vitro*[79]. Jagged1 is thought to promote angiogenesis, as overexpression in head and neck squamous cell carcinoma (SCC) cells leads to increased vascularization and tumor growth[78].

Attempts to manipulate Notch signaling for anti-cancer purposes have been studied, particularly through inhibition of Dll4. Interestingly, inhibition of Dll4 leads to an increase in tumor vascular density; this increase is likely due to the lack of downregulation of branching and sprouting caused by Dll4[74,80]. However, even though an increase in vascularity is seen, the vascular network is very poorly formed and essentially non-functional (even more so than typical disorganized tumor vasculature) and a significant decrease in tumor size was observed[74,80]. The decrease in tumor size was seen even in tumor models that are resistant to VEGF-blockade, making inhibition of this pathway an attractive alternative for tumors that become resistant to VEGF inhibitors used in the clinic[74,80]. When Dll4 inhibition was combined with VEGF inhibition in tumors with no resistance, additional anti-tumor activity was seen than compared to inhibition of either factor alone[80].

Inhibition of Jagged1 has also been studied. Knockdown of Jagged1 expression in SCC cells inhibits pro-angiogenic effects of the cells *in vitro*, even when the cells were stimulated with growth factors[78]. Another study looked at inhibition of Notch receptor function, using a soluble Notch1 receptor decoy that prevented Dll1, Dll4 and Jagged1 from binding to Notch receptors[81]. The decoy blocked angiogenesis in both *in vitro* and *in vivo* models, as well as causing a decrease in tumor growth using mammary xenografts[81].

Inhibition of specific components of the Notch signaling pathway, such as Dll4 or Jagged1, or more broad inhibition of Notch signaling may prove to be effective for inhibiting functional angiogenesis and neovascularization in tumors and some of the pre-clinical studies appear promising. However, further studies are needed to better understand the role that Notch signaling and its individual components play in tumor angiogenesis before these pathways can be exploited for clinical use.

Hypoxia Inducible Factor—Hypoxia inducible factor (HIF) is a transcription factor involved in cellular adaptation to hypoxia. HIF transcriptional activity is regulated by the presence of oxygen and becomes active in low oxygen conditions (hypoxia). HIF controls a large number of angiogenesis-involved genes (for review see references[9,82]). The active HIF complex consists of an α and β subunit in addition to coactivators including p300 and CBP. The HIF-β subunit (also known as ARNT) is a constitutive nuclear protein with further roles in transcription not associated with HIF-α. In contrast to HIF-β, the levels of the HIF-α subunits and their transcriptional activity are regulated by oxygen availability.

There are three related forms of human HIF-α (-1α, -2α and -3α), each of which is encoded by a distinct genetic locus. HIF-1α and HIF-2α have been the best characterized, possessing similar domain structures that are regulated in a related manner by oxygen, though each isoform does have distinct and separate roles. The role of HIF-3α is not fully understood, though a truncated form of murine HIF-3α known as inhibitory PAS domain protein (IPAS) has been found to act as an inhibitor of HIF via dimerization with HIF-β[83].

Both the HIF-α and HIF-β subunits are produced constitutively, but in normoxia HIF-1α and -2α are degraded by the proteasome in an oxygen-dependent manner. Hydroxylation of two prolines in HIF-α enables HIF-α to bind to the von Hippel-Lindau tumor suppressor protein (pVHL), which links HIF-α to a ubiquitin ligase complex. The ubiquitin ligase catalyzes polyubiquitinylation of HIF-α, targeting it for degradation by the proteasome. In addition, hydroxylation of an asparagine residue in HIF-α disrupts the interaction between HIF-α and the coactivator p300, through a process independent of proteasomal degradation, which leads to reduced HIF transcriptional activity. In this manner, asparaginyl hydroxylation acts as a regulatory switch controlling the activity and specificity of HIF gene expression, as opposed to the prolyl-hydroxylations which control HIF-α stability (for review see[83,84]). In

hypoxia, minimal to no hydroxylation occurs, enabling HIF-α to avoid proteasomal degradation and dimerize with HIF-β and coactivators, forming the active transcription complex on the hypoxia response element (HRE) associated with HIF target genes (figure 4).

Because HIF regulates genes that enable cell survival in a hypoxic environment, including those involved in glycolysis, angiogenesis and expression of growth factors, it holds importance in the biology and regulation of tumor growth. The central role of HIF in the activation of angiogenic-related genes makes it a promising target for the treatment of solid tumors particularly since HIF-1α and/or HIF-2α is reported to be overexpressed in the majority of solid tumors[85,86]. HIF-1α (and sometimes HIF-2α) overexpression in tumors has been found to positively correlate with angiogenesis, aggressiveness, metastasis, and resistance to radiation/chemotherapy and negatively correlate with progression, survival and outcome[87,88,89,90,91,92,93] (for an excellent review see reference[94]).

Anti-Angiogenesis Compounds

Fumagillin and TNP-470

The anti-angiogenic activity of fumagillin was discovered when a dish of cultured endothelial cells was accidentally contaminated with the fungus *Aspergillus fumigatus* Fresenius, a fumagillin producing organism[95]. The contaminated endothelial cells stopped proliferating but showed no outward signs of toxicity[95]. After isolating fumagillin as the source of the activity, a series of synthetic analogues were produced that also inhibited endothelial cell growth and proliferation without cytotoxic effects *in vitro*, and limited tumor-induced angiogenesis in xenograft models[95]. The most potent of the analogues, known as TNP-470, was found to be a significantly more potent anti-angiogenic compound than fumagillin in four different angiogenesis assays[96]. The means by which fumagillin and TNP-470 exert their anti-angiogenic properties are not fully understood. Several mechanism have been proposed, including inhibition of methionine aminopeptidase (MetAP-2)[97] through covalent modification of a histidine[98] and prevention of endothelial activation of Rac1[99]. TNP-470 also affects cell cycle through activation of p53, leading to an increase in the G_1 cyclin-dependent kinase inhibitor p21$^{CIP/WAF}$ and subsequent growth arrest[100,101].

TNP-470 shows broad spectrum anti-cancer activity in animal models[102] and was one of the first anti-angiogenesis drugs to undergo clinical trials (for review see[103]). In early clinical trials, TNP-470 demonstrated anti-tumor activity as a single agent, causing tumor progression to slow or even regress in squamous cell cancer of the cervix[104,105], as well as showing activity in combination with paclitaxel and carboplatin[106]. While TNP-470 appeared promising in terms of anti-cancer activity, it presented several obstacles to further clinical development and use. The primary hurdles included dose-limiting toxicities and a very short plasma half-life[107,108]. Toxicities seen were mainly neurological including problems with motor coordination, short-term memory and concentration, dizziness, confusion, anxiety, depression[107,109]. Later studies showed that neurological symptoms could be eliminated by conjugating TNP-470 to a polymer, preventing the drug from penetrating the blood-brain barrier[110]. However, this formulation still had a short-half life and could not be administered orally[110].

To improve upon the short half-life and oral availability, TNP-470 was conjugated to a di-block copolymer, monomethoxy-polyethyleneglycol-polylactic acid (mPEG-PLA). The polymeric drug is amphiphilic causing self-assembly into micelles with the TNP-470 tails in the center[102]. The micelle formulation improved the properties of TNP-470 in several ways. The micelles protected TNP-470, particularly from the acidic environment of the stomach, making the new formulation, named lodamin, orally available[102]. Additionally, the micelles

increased the half-life, as the micelles hydrolyze over time, allowing slow release of lodamin. This property allowed accumulation of lodamin in tumor tissue (likely due to the permeability of the tumor vasculature), but still prevented penetration of the blood-brain barrier, effectively overcoming several of the major hurdles to clinical use of TNP-470[102]. One interesting observation was that particularly high concentrations of lodamin accumulated in the liver, so lodamin may prove to be especially effective against primary liver cancer or metastases within the liver[102]. Pre-clinical results of lodamin are promising thus far and warrant further investigation. Additional studies on the safety of lodamin in non-human primates may lead to clinical trials in the future. Furthermore, second generation conjugated TNP-470 are in pre-clinical development.

Thalidomide

Thalidomide (Thalomid®) was initially marketed as a safe, non-toxic sedative and anti-emetic in the 1950's in Europe, Australia, Asia and South America (but was not FDA-approved in the USA due to safety concerns)[111]. In countries where it was approved for use, thalidomide became a popular treatment for pregnancy related morning sickness until 1961 when two physicians, William McBride from Australia[112] and Widukind Lenz from Germany[113] noted the link between severe limb defects and other birth defects in babies whose mothers had taken thalidomide during pregnancy. The drug was rapidly removed from the market in Europe in 1961 and from Canada in 1962 due to the previously unknown teratogenic effects of thalidomide[111].

Thalidomide was rediscovered in 1965 as a useful treatment for erythema nodosum leprosum (ENL), due to the immunomodulatory and anti-inflammatory properties of thalidomide; however it did not actually obtain FDA approval for ENL until 1998[111]. The idea of using thalidomide for cancer treatment occurred upon the discovery of its anti-angiogenic properties.

Studies of the effects of thalidomide suggested that the limb defects could be caused by inhibition of blood vessel growth in the limb buds of a developing fetus[114]. Thalidomide displayed anti-angiogenic properties in a rabbit cornea micropocket model, though interestingly thalidomide did not inhibit angiogenesis in a chicken chorioallantoic membrane (CAM) assay[114]. It was proposed that the teratogenic activity of thalidomide may be due to one of the many metabolites of the parent thalidomide model, explaining the lack of activity in the CAM assay[114,115]. Bauer *et al.* examined this proposal using liver microsomes co-incubated with thalidomide in angiogenesis assays[116]. Co-incubation with human or rabbit liver microsomes led to potent anti-angiogenic activity, demonstrating that a metabolite of thalidomide is responsible for the anti-angiogenic activity, and that the metabolite is not produced by rodents[116].

While the mechanism of thalidomide (and metabolites) is not fully understood, some properties and activities of thalidomide are beginning to be deciphered. Thalidomide inhibits the synthesis of TNF-α in monocytes, microglia, and Langerhans cells, which provides thalidomide with its anti-inflammatory properties (for review see references[111,117]). The immunomodulatory and anti-inflammatory effects of thalidomide likely contribute to the anti-angiogenic effects of thalidomide, and add to the anti-cancer activity of thalidomide. Studies of thalidomide in rabbits, an animal species susceptible to thalidomide's teratogenic effects, causes inhibition of mesenchyme proliferation in the developing limb bud of a fetus[118], embryonic DNA oxidation and teratogenicity[119].

Unfortunately the pre-clinical animal studies of thalidomide that led to widespread use in humans throughout the world used rodents, which are resistant to the teratogenic effects of thalidomide. The human tragedies that resulted from thalidomide highlight the importance

of selecting correct animal models, as well as testing in different species when examining new treatments for clinical use[119].

Thalidomide analogues were developed with the goal of improving TNF-α inhibition, leading to lenalidomide (CC-5013, Revimid®) and pomalidomide (CC-4047, Actimid®), the latter of which is 50,000-fold more potent than thalidomide at suppressing endotoxin-induced TNF-α secretion in cell models[117]. Lenalidomide and pomalidomide also lack some of the side-effects seen with thalidomide such as constipation, peripheral neuropathy and the sedative effects[111,117]. In 2006, lenalidomide was approved by the FDA for use in relapsed multiple myeloma in combination with dexamethasone, while pomalidomide is currently in clinical development[120].

Despite the previous damage thalidomide caused (and even, perhaps due to thalidomide's mechanism of action that led to birth defects), thalidomide and analogues are currently in development for anti-angiogenic and anti-cancer use. While the complex metabolism of thalidomide causes many challenges to the development of thalidomide, initial results with analogues appear to indicate that structural alterations can change the side-effect profile, potentially eliminating them, while still maintaining the desired anti-cancer activity and demonstrating promising results in clinical trials.

Inhibitors of Growth Factors, Receptor Tyrosine Kinases and Signaling Pathways

Because growth factors stimulate endothelial cells, leading to angiogenesis, targeting the growth factors, receptors and subsequent signaling cascades make for promising targets in angiogenesis inhibition. The growth factors and receptor tyrosine kinases are particularly attractive targets since it is possible to target them in the extracellular environment, removing drug development hurdles such as permeability of the cellular membrane. Significant progress towards targeting these pathways has been made and a number of drugs have been FDA approved or are in clinical development.

Growth Factor Inhibitors—Bevacizumab (Avastin™) is a humanized monoclonal antibody that binds to VEGF-A, preventing it from binding to receptors and activating signaling cascades that lead to angiogenesis. Initial proof of concept that targeting VEGF-A could inhibit the growth of tumors (despite having no effect on growth rate of the tumor cells *in vitro*) was demonstrated in a mouse model in 1993 using a monoclonal antibody against VEGF-A[121], leading to the clinical development of bevacizumab.

Initial clinical trials in colorectal cancer tested irinotecan, 5-fluorouracil (5-FU) and leucovorin with or without bevacizumab[122]. The addition of bevacizumab significantly increased progression-free survival (PFS), as well as median overall survival (OS)[122], leading to FDA approval of bevacizumab as the first drug developed solely for anti-angiogenesis anti-cancer use in humans.

Anti-cancer activity of bevacizumab across all tumor types has demonstrated some mixed results. Bevacizumab did not provide any benefit to PFS or OS for metastatic breast cancer patients when used in combination with capecitabine[123]. Further studies in a Phase III trial on previously untreated metastatic breast cancer using paclitaxel with or without bevacizumab showed that the addition of bevacizumab increased PFS (11.8 months versus 5.9 months) and increased overall response rates (36.9% versus 21.2% without bevacizumab)[124]. However, there was still no significant increase in overall survival, as had been seen previously with colorectal cancer[122] and NSCLC[125,126]. A beneficial response may masked by the lack of biomarker screening in patients in many of the clinical trials, since bevacizumab is specific for VEGF. By screening for tumors that overexpress VEGF and/or are highly dependent on VEGF signaling, the likelihood of a positive response to

treatment of bevacizumab would probably be increased. Targeted therapies may prove more effective when patients are screened for markers, ensuring that the proper subset of the population is treated with a particular targeted drug.

Bevacizumab is currently being tested in several hundred clinical trials in a variety of different tumor types[127] and as of 2009, bevacizumab is approved for various indications in colorectal cancer, NSCLC, breast cancer, renal cell carcinoma (RCC) and glioblastoma.

Aflibercept (VEGF-trap)—Aflibercept (VEGF-trap, AVE0005) is a soluble fusion protein of the human extracellular domains of the VEGFR-1 and -2 receptors and the Fc portion of human IgG. Aflibercept binds to both VEGF-A and PlGF with a higher affinity than monoclonal antibodies and essentially renders the VEGF-A and PlGF ligands unable to bind and activate cell receptors[128]. Aflibercept was engineered to optimize pharmacokinetic properties while still maintaining the potent VEGF blocking activity that other anti-VEGF antibodies have demonstrated. *In vitro*, aflibercept showed significant anti-proliferative activity and completely blocked VEGF-induced VEGFR-2 phosphorylation when added in a 1.5 molar excess of VEGF[128]. Aflibercept inhibited tumor growth in xenograft models and blocked nearly all tumor-associated angiogenesis, resulting in tumors that appeared nearly avascular[128].

Aflibercept is in clinical trials with some early results reported. In Phase II trials as a single agent in ovarian cancer, 41% of patients had stable disease at 14 weeks[129]. In addition, a reduction of 30% or more in tumor size was seen in 8% of patients[129]. Another Phase II trial of aflibercept in 33 NSCLC patients, showed two partial responses; interim analysis results are not yet available[130]. In contrast, a Phase II trial of aflibercept in metastatic breast cancer showed a response rate of 5% and PFS at six months of 10%, rates that did not meet efficacy goals and were decided too low to continue[131]. Additional clinical trials of aflibercept are ongoing in a variety of cancers including prostate, colorectal, ovarian, thyroid, RCC, and brain cancers.

Receptor Tyrosine Kinase Inhibitors—There are a wide range of receptor tyrosine kinase (RTK) inhibitors in all stages of development (pre-clinical, clinical and FDA-approved). RTK inhibitors are particularly useful in treating cancer because of the dual roles they inhibit; both oncoprotein signal-transduction and the downstream angiogenic processes are blocked. They also often target more than one type of receptor and affect both endothelial cells and cancer cells because the receptors are expressed on both types of cell[3]. Since the target kinase specificity between inhibitors can vary, different compounds have shown varying levels of efficacy and activity between cancers, as well as different side effect profiles. Several approaches to targeting the growth factors and receptors have been undertaken; some of these include compounds that bind to the ATP binding site of the receptor tyrosine kinase, blocking receptor activation, or with antibodies that bind to the growth factors or their receptor, preventing binding and subsequent receptor activation

Sunitinib (Sutent®, SU11248) is an orally available compound that inhibits the VEGFR, PDGFR, Flt-3, c-kit, RET and CSF-1R receptor tyrosine kinases (for review see reference[132]). In a Phase III clinical trial in metastatic RCC patients, sunitinib was compared to interferon-α (IFN-α), with sunitinib providing a statistically significant improvement in both the median PFS (47.3 weeks for sunitinib versus 24.9 weeks for IFN-α) and the objective response rate (24.8% versus 4.9% for IFN-α)[133]. In addition, the interim analysis of a Phase III trial of sunitinib in gastrointestinal stromal tumors (GIST) revealed a significantly longer time to progression (27.3 weeks for sunitinib versus 6.4 weeks for placebo), and at 22 weeks, stable disease was seen in 17.4% of patients versus 1.9% of patients on placebo[134,135]. A partial response was seen in 6.8% of sunitinib patients versus

0% of patients treated with the placebo[134,135]. The results from these trials led to FDA approval of sunitinib in 2006 for GIST and advanced metastatic RCC. Further studies and clinical trials are currently being conducted in additional cancers using sunitinib.

Sorafenib (Nexavar®, BAY 43-9006) is an oral inhibitor of the intracellular Raf kinase (B-Raf, C-Raf), therefore targeting the MAPK and Raf/MEK/ERK signaling pathways. Sorafenib also inhibits VEGFR (-2 and -3), PDGFR-β and c-kit (for review see reference[136]). In most tumor cell lines (colon, pancreatic and breast), but not all (NSCLC), sorafenib potently inhibited the Raf kinase, and blocked phosphorylation of ERK 1/2, an indicator of MAPK pathway blockade[137]. Sorafenib was also shown to possess significant anti-angiogenesis activity *in vitro*[137].

A Phase II trial of sorafenib in advanced RCC showed an increased PFS rate after 12 weeks (50% with sorafenib versus 18% with placebo) and a significantly increased median PFS (23 weeks versus 6 weeks with placebo)[138]. In a Phase III trial of RCC (769 patients) sorafenib increased median PFS from 12 weeks with placebo to 24 weeks and increased PFS after 12 weeks (79% versus 50% with placebo)[139]. Sorafenib was subsequently approved by the FDA for RCC in 2005 and unresectable hepatocellular carcinoma (HCC) in 2007.

Semaxanib (SU5416) was the first tyrosine kinase inhibitor tested in humans and is an inhibitor of VEGFR (for review of semaxanib see reference[140]). Semaxanib was tested in combination with 5-FU/leucovorin compared to 5-FU/leucovorin alone in a Phase III trial against metastatic colorectal cancer (737 patients)[141]. Addition of semaxanib did not improve clinical outcome and additional toxicities were seen in the semaxanib arm, including an increased risk of hematological and thromboembolic events[141]. Semaxanib in combination with cisplatin and gemcitabine also had unacceptable toxicity associated with it, particularly severe thromboembolic events[142], leading to the clinical development of semaxanib to be stopped[5].

Erlotinib (Tarceva®, OSI-774) is an oral inhibitor of the EGFR/HER1 receptor tyrosine kinase. Erlotinib is believed to exert anti-cancer activity at least partially through the inhibition of expression of pro-angiogenic factors[143]. While Phase III clinical trials had some mixed results, with two trials seeing no benefit in treating previously non-treated NSCLC with chemotherapy and erlotinib[144,145], one trial in NSCLC that had failed chemotherapy treatment, showed erlotinib provided an increase in PFS (2.2 months versus 1.8 months with placebo), median duration of response (7.9 months versus 3.7 months with placebo), response rate (8.9% versus less than 1% with placebo), and overall survival (6.7 months versus 4.7 months with placebo)[146]. A Phase III trial of erlotinib in combination with gemcitabine in pancreatic cancer showed significantly improved survival[147]. The results of these trials led to erlotinib being FDA approved for NSCLC that has failed chemotherapy treatment and for pancreatic cancer in combination with gemcitabine.

Cediranib, pazopanib, vandetanib, lapatinib, and motesanib are examples of additional receptor tyrosine kinase inhibitors that are currently in clinical trials for a variety of cancers[148] (table 2).

Imatinib (Gleevec®, STI571) inhibits the cytoplasmic and nuclear protein tyrosine kinase, Abl, as well as the receptor tyrosine kinases PDGFR and c-kit[149]. Imatinib was the first commercially available small molecule tyrosine kinase inhibitor and has been used extensively in the treatment of chronic myelogenous leukemia (CML) because the molecular pathogenesis of CML involves the Bcr-Abl protein and deregulated tyrosine kinase activity[149]. Imatinib demonstrates anti-angiogenesis activity *in vitro*, which is thought to occur through inhibition of PDGFR[150,151].

Monoclonal Antibodies Directed at EGFR—Cetuximab (Erbitux®) and panitumumab (Vectibix, ABX-EGF) are indirect receptor tyrosine kinase inhibitors, using a different approach than the above small molecule inhibitors. Cetuximab is a chimeric monoclonal antibody that binds to the inactive form of EGFR on the extracellular domain[101]. Cetuximab essentially prevents the ligand from being able to bind to the receptor and therefore any downstream signaling activation[152]. It received accelerated FDA approval in 2004 for use in EGFR-expressing metastatic colorectal cancer in combination with irinotecan or as a single agent for irinotecan-intolerant patients, after cetuximab showed activity in clinical trials as a single agent and even more activity when used in combination with irinotecan[153,154,155]. Cetuximab was subsequently tested in combination with radiotherapy for the treatment of unresectable head and neck SCC and showed that the addition of cetuximab to radiotherapy significantly increased median survival compared to radiotherapy alone[156], leading to additional FDA approval for this use. As for the anti-angiogenic activity of cetuximab, studies have shown that EGF/EGFR inhibitors appear cause a reduction in the synthesis of pro-angiogenic cytokines, rather than a direct inhibition of angiogenesis[55]. Panitumumab is also a monoclonal antibody that binds to EGFR, inhibiting phosphorylation and activation of EGFR associated kinases[151].

PI3K/AKT/mTOR Pathway Inhibitors—PI3K signaling contributes to many cell processes, including angiogenesis, cell proliferation, survival and motility, and is initiated by activation of receptor tyrosine kinases (for review see reference[23,24]). Upregulation of the PI3K pathway can increase angiogenesis through multiple pathways, including increasing the levels of HIF-1α under normoxic conditions[12,13,19,14,15,18,20].

Initial evidence that PI3K and AKT were involved in the regulation of angiogenesis *in vivo* was obtained when constitutively active PI3K and AKT were shown to induce angiogenesis and increase levels of VEGF[12]. Cancer genome studies have highlighted the importance of this, demonstrating that components of the PI3K pathway are often mutated in human cancers, increasing the likelihood of inhibitors of this pathway demonstrating efficacy in the clinic[24].

Inhibitors of the PI3K pathway have been found to decrease tumor angiogenesis and demonstrate HIF inhibition, including LY294002 and wortmannin, two compounds that directly inhibit members of the PI3K family[15,13,12,14,11,20]. Both LY294002 and wortmannin showed unacceptable levels of toxicity in animals and therefore were not developed clinically; however a wortmannin analogue, PX-866, and a conjugate version of LY294002, are both currently being tested in Phase I trials[24]. 9-β-*D*-arabinofuranosyl-2-fluoroadenine (FARA-A) is a nucleoside analogue that causes DNA damage in S-phase cells and has been found to inhibit AKT, thereby inhibiting the expression of HIF-1α and VEGF[157]. Perifosine is a lipid-based phosphatidylinositol analogue that inhibits AKT by preventing translocation of AKT to the cell membrane[24]. Perifosine is currently in clinical trials for several different cancers. Rapamycin (sirolimus) acts as an inhibitor of mTOR and was initially used as an immunosuppressive agent[25]. Rapamycin and analogues including temsirolimus (CCI-779), and everolimus (afinitor, RAD001), block tumor angiogenesis *in vivo*, in addition to inhibiting tumor growth[19,158,159,160,161]. The blocked angiogenesis is believed to be due at least partially to the inhibition of HIF-1α caused by the inhibition of mTOR[19,158,159,160]. While rapamycin inhibits HIF-1α *in vitro*, it is unknown to what degree the decrease in HIF-1α actually plays in the anti-tumor activity, since the PI3K/AKT/mTOR pathway plays a role in many cell processes and the anti-tumor activity may stem from acting upon multiple downstream targets.

Clinical trials of temsirolimus and everolimus as single agents improved survival in patients with advanced RCC, leading to FDA approval for this indication. Results of activity in other tumors have initially indicated mixed results and are being further tested[25,24].

MAPK- Farnesyltransferase Rho and Ras Inhibitors—The MAPK signaling pathway is another pathway that can lead to increased angiogenesis and increased levels of HIF-1α, making it a logical anti-angiogenesis target. One approach has been to inhibit Ras and Rho, activators of the MAPK pathway. During Ras activation, a farnesyl group is transferred onto a cysteine residue in the *C*-terminal end of Ras, enabling Ras to interact with intracellular membranes via the farnesyl group[26]. Without farnesylation, Ras can no longer interact with regulatory and effector molecules in the cell membrane and no MAPK pathway activation occurs. Ras is also involved in stabilizing HIF-1α and targeting Ras has been shown to destabilize HIF-1α and decrease HIF transcriptional activity[15,20]. Two farnesyltransferase inhibitors are tipifarnib (zarnestra®, R115777)[162] and lonafarnib (Sarasar®, SCH66336)[163]. Tipifarnib has been the most studied farnesyltransferase inhibitor thus far, with anti-angiogenic, anti-proliferative and pro-apoptotic activity in preclinical studies[164,165]. However, clinical trials of tipifarnib in multiple cancers failed to show significant anti-cancer activity (for review see reference[162]). It remains to be seen whether inhibition of farnesylation may or may not be an effective anti-cancer strategy. Sorafenib, mentioned previously under tyrosine kinase inhibitors, also acts on the MAPK pathway through inhibition of Raf[136].

Interferon-α—Interferon-α (IFN-α) was first discovered to have anti-endothelial activity in 1980, when experiments showed that it inhibited the motility of endothelial cells *in vitro*[166] and inhibited angiogenesis *in vivo*[167,168]. Low doses of IFN-α have been shown to downregulate FGF expression in cancer cells[169], and is probably one of the mechanisms behind the anti-angiogenic effects of IFN-α. In 1989, IFN-α was first used in humans to treat a hemangioendothelioma. After a low-dose daily treatment for seven months, complete regression of lesions and symptoms occurred[170]. These results led to the successful treatment of infant haemangiomas with IFN-α[171], in addition to successful treatment of angioblastomas and giant cell tumors[172,173,174].

2-Methoxyestradiol (2ME2)—2-Methoxyestradiol (2ME2, Panzem®) is a human metabolite of estradiol that inhibits tubulin polymerization, destabilizing the microtubules, and causing cell cycle arrest[175]. 2ME2 has also been found to decrease HIF-1α protein levels by acting at the translational level without affecting rates of HIF-1α gene transcription or HIF-1α proteasomal degradation through a mechanism dependent on the microtubule disrupting properties of 2ME2[175]. 2ME2 possesses potent anti-angiogenic and pro-apoptotic properties and inhibits cell proliferation and migration both *in vitro* and *in vivo*[176]. The anti-angiogenic properties of 2ME2 appear to come from both direct inhibition of endothelial cells, and inhibition of HIF-1α[175]. In clinical trials, little anti-tumor activity was seen in breast and prostate cancers, which may be due to the short half-life and poor bioavailability of 2ME2[177]. Re-formulation has improved upon bioavailability, though the half-life is still sub-optimal[177]. Development of analogues with improved properties may increase the efficacy and potential clinical use for 2ME2. A more thorough understanding of the targets which 2ME2 acts upon and the relationship between microtubules and HIF-1α may provide new therapeutic avenues.

Hypoxia Inducible Factor Pathways and Binding Partners—The central role of HIF in the transcriptional activation of genes under hypoxic conditions, including those involved in angiogenesis and cell proliferation, makes it a promising target for cancer treatment. There is good evidence that treatments targeting HIF, or components of the HIF

regulatory pathway will result in a physiological effect in humans[178]. Work on some of the different approaches focusing directly on HIF and its binding partners is discussed in the following section (figure 5).

Preventing DNA Target Sequence Binding—One method of preventing transcriptional activation of HIF (and other transcription factors) is to block binding to its target DNA site, the HRE, inhibiting gene expression of *VEGFA* and other HIF regulated genes. The natural product echinomycin, has been found to bind a region of the HRE sequence (5′- ACGT -3′), which is also shared with the consensus sequence of c-Myc, another cancer-related transcription factor[179]. Echinomycin inhibited HIF transcriptional activity and VEGF expression in tumor cells *in vitro*, without inhibiting other tested transcription factors[179]. However, clinical trials using echinomycin produced disappointing results[180], which may be due in part to the unclear selectivity and specificity of echinomycin, which binds only a short region of DNA. Despite the lack of clinical success seen with echinomycin, inhibition of the HIF-HRE complex still holds promise as a selective HIF inhibitor.

In an effort to generate a more specific HRE binding inhibitor, a series of polyamides were designed to specifically bind the *VEGFA* promoter sequence 5′- WTWCGW -3′, which did lead to a decrease in VEGF-A mRNA and protein levels *in vitro*[181]. Another model utilized hairpin polyamides designed to bind HRE sequences[182]. While the hairpin polyamides did not have as strong of an effect on HIF-regulated genes as a HIF-1α siRNA model, the results did suggest that polyamides could potentially be designed to affect a select subset of target genes by targeting particular HREs[182] and further validating HRE sites as a molecular target for pharmacological manipulation.

Preventing Cofactor Binding—Another way of blocking HIF activation is preventing HIF from binding to essential transcriptional coactivators. The interaction between the HIF-1α CTAD domain and the CH1 domain of the coactivator p300 has been demonstrated to be a potential drug target using polypeptides corresponding to the CH1 or the CTAD domain, which led to attenuation of HIF transcriptional activity in cell-based models and anti-tumor activity in xenograft models[183].

A small molecule, chetomin, found in a high-throughput screen, blocked binding of p300 to either HIF-1α or HIF-2α in both *in vitro* and *in vivo* assays[184]. In xenograft models, systemic administration of chetomin attenuated HIF-1-mediated gene expression, and caused a significant reduction in tumor size[184]. While high levels of necrosis in tumor tissues were observed, repeated injections led to localized toxicity and coagulative necrosis at sites of tail vein injection. Unfortunately, due to the toxicity, chetomin is unlikely to be pursued as a chemotherapeutic drug, but it did prove that inhibition of HIF:p300 had anti-tumor effects, establishing this as a potential drug target.

Recent structure-activity studies on chetomin led a series of analogues that have shown some activity *in vitro*[185]. Chetomin and analogues were found to disrupt the structure of the CH1 domain of p300, to which the HIF-α subunit binds, by chelating structural zincs bound to p300. The unstructured p300 can then no longer bind HIF-α, preventing transcriptional activation of HIF[185]. Further studies on zinc chelation of p300 and downstream effects on HIF transcriptional activity could utilized for drug development of this target.

Inhibition of HSP90—The chaperone HSP90 (heat shock protein 90) possesses a wide range of functions, assisting in folding and stabilizing many cellular proteins[186]. Client proteins of HSP90 include oncoproteins and/or angiogenic related proteins, for example, HIF-1α, AKT and mutant EGFR[186], making HSP90 a regulatory component of many

oncogenic processes. In addition, HSP90 is often overexpressed in cancer cells, can contribute to malignant transformation of cells and has been associated with decreased survival in breast cancer[187]. It was initially thought that inhibition of HSP90 may not demonstrate selectivity for cancerous cells because there are high levels of HSP90 present in nearly all tissues, and HSP90 interacts with a large number of important cellular proteins in healthy cells; however, experimental evidence revealed that cancer cells were actually more sensitive to HSP90 inhibitors than healthy cells[186].

In vitro evidence has shown that inhibitors of HSP90, such as geldanamycin (GA) and its analogues 17-AAG (17-allylamino-17-demethoxygeldanamycin) and 17-DMAG (17-dimethylaminomethylamino-17-demethoxygeldanamycin), do act on client proteins, for example GA and 17-AAG promote HIF-α degradation[188]. By inhibiting HSP90 from binding to HIF-1α, the protein RACK1 is able to bind HIF-1α, recruiting a ubiquitin ligase complex, inducing ubiquitination, and leading to proteasomal degradation[189]. 17-AAG was the first inhibitor to enter the clinic and showed limited success; subsequent alterations to the formulation and delivery have improved upon the efficacy[186]. Clinical trials of several HSP90 inhibitors are ongoing. While 17-DMAG was halted for clinical development in 2008 due to unfavorable toxicity, 17-AAG is currently in clinical trials for anti-cancer uses and the effect on HIF-1α and angiogenesis is of interest[190]

Thioredoxin Inhibitors—The thioredoxins (Trx) are redox proteins that function to reduce oxidized cysteines in proteins through an NADPH-dependent reaction. One member, thioredoxin-1 (Trx-1) is overexpressed in many human tumors and has been associated with decreased patient survival[191]. Trx-1 participates in the regulation of transcription factors, including HIF-1α[192]. Overexpression of Trx-1 has been shown to increase levels of HIF-1α protein and VEGF expression *in vitro*, and increase angiogenesis *in vivo*, making it an attractive target for HIF and angiogenesis inhibition[193]. Inhibition of Trx-1 by PX-12 and pleurotin prevents accumulation of HIF-1α protein in hypoxic conditions, as well as decreases HIF-regulated gene expression *in vitro* and *in vivo*[194]. PX-12 became the first thioredoxin-1 inhibitor to enter a Phase I trial of 38 patients with various types of solid tumors. PX-12 showed some preliminary anti-tumor activity in the Phase I trial[195], and as of mid-2009, PX-12 is currently being tested in two Phase II trials for advanced/metastatic cancer and advanced pancreatic cancer.

Known and Potential Side Effects from Inhibition of Angiogenesis—While many of the molecular targets proposed for inhibition of angiogenesis appear promising, particularly through targeting HIF and related pathways, caution should always be employed when applying findings from *in vitro* studies to clinical applications. As with most studies using isolated systems and molecular targets, many of the results obtained in preclinical studies have yet to be verified as relevant in a clinical setting. Even the importance of molecular targets, such as HIF, are still unknown in a clinical setting and many of the pre-clinical studies have found conflicting results. For example, studies using embryonic stem cell tumors found that inhibition of HIF increased tumor growth[196,197], and that activation of HIF led to a slower growth rate than the wildtype cells[198], indicating that the biology of HIF is still not completely understood. Caution should be exercised when drawing conclusions as to the role of the HIF system in cancer from results obtained using a limited number of cell types. Similar conclusions apply to other molecular targets in angiogenesis, particularly those that have not yet been targeted in humans, as there is still a significant knowledge gap in our understanding between pre-clinical and clinical studies.

In addition, as the use of angiogenesis inhibitors like bevacizumab becomes widespread, the potential side effects that occur in short- or long-term use of angiogenesis inhibitors are becoming apparent. Some of these side effects include gastrointestinal perforations,

impaired wound healing, bleeding, hypertension, proteinuria and thrombosis (for review see references[199,200,201]). Many of the side effects are actually due to the direct effects of the drugs; cardiovascular complications are thought to be caused by direct effects of angiogenesis inhibitors on the non-tumor-associated endothelial cells[200]. The likelihood for these occurrences has thus far, been unpredictable and further studies are needed to measure the risk for patients, understand the cause for complications and find prophylactic measures to minimize risk. The effects of long-term administration of angiogenesis inhibitors are not fully known, as most long-term studies have not yet been conducted due to the recent development of these drugs[101].

It has also been found that most tumors develop mechanisms of resistance to anti-angiogenic agents[202], and may be a potential consequence of long-term administration of anti-angiogenesis inhibitors. Because multiple signaling pathways are involved in angiogenesis and more than one pathway is often dysregulated in human tumors, there is a certain level of signaling redundancy, and blocking a single pathway may not be highly effective and/or can lead to resistance when the tumor cells develop other angiogenesis mechanisms (for a review of other mechanisms of resistance see Eikesdal *et al.*[202]). By targeting multiple pathways, resistance may be able to be overcome or delayed, making combination drug therapies important in the design of future clinical trials.

Conclusion—The complex molecular pathways that govern tumor angiogenesis are logical targets for pharmacological manipulation given the important role they play in the growth and development of cancers. Initial trials of putative anti-angiogenesis inhibitors have shown some promise in cancer, although this has not always translated to the clinic. A lack of validated biomarkers and patient screening restricts our ability to tailor specific drugs to patient cohorts, and might be seen as one of the largest barriers to success in angiogenesis inhibition. As cancer pharmacology moves away from cytotoxic to so-called molecular targeted drugs that are expected to have minimal side effects and toxicity, predictive biomarkers could be used to screen for patients likely to demonstrate a clinical response. Biomarkers also need to be validated for use as objective response measurements, since anti-angiogenic monotherapies may only act as cytostatic agents, making objective response measurements like tumor shrinkage, less useful for determining the efficacy of a drug. The long time period required for an observable response also demonstrates the need for a rapid biomarker so that response to a treatment can be measured. While some clinical trials have shown that particular surrogate biomarkers of angiogenesis, like circulating VEGF and microvessel density, have value as prognostic markers, more validated biomarkers need to be found so that anti-angiogenic agents can be properly used and evaluated in the clinic (for review see reference[203,204]).

The end goals of anti-angiogenesis inhibitors also need to be determined. It is still not understood if angiogenesis inhibitors will eliminate or shrink tumors or simply inhibit further growth and spread. If angiogenesis inhibitors are used as a cytostatic agent, then further studies are needed to examine the effect of vessel normalization, whereby the tumor vessels become more organized and blood flow is improved and if improved tumor delivery of chemotherapeutics could be achieved. This means more studies using combination therapies with angiogenesis inhibitors, and determining whether angiogenesis inhibitors are more effective in combination with chemotherapy or as single agents. *In vitro* studies have found that combination with traditional chemotherapies and/or radiation increases the anti-tumor efficacy of kinase inhibitors[149] and this seems to be true with many of the anti-angiogenesis inhibitors[205]. It is also thought that combination therapies could be used to provide maximum anti-tumor effect and minimal side effects if a lower dose of each drug were to be used. Treatments could be tailored to target the specific altered pathways in a tumor with a combination of drugs to minimize resistance and provide a more effective

combined treatment. Future clinical studies may provide the information needed to find the best combination of treatments for maximum anti-cancer effect and minimal side effects.

The past decade has led to major advances in the understanding the molecular pathways involved in tumor angiogenesis. This basic research has led to the identification of new targets associated with angiogenesis, leading to the development of an extensive number of pre-clinical anti-angiogenesis agents. Ongoing studies of different approaches are evaluating some of the molecular targets and agents, with some even in clinical trials, and data regarding efficacy and safety is currently emerging.

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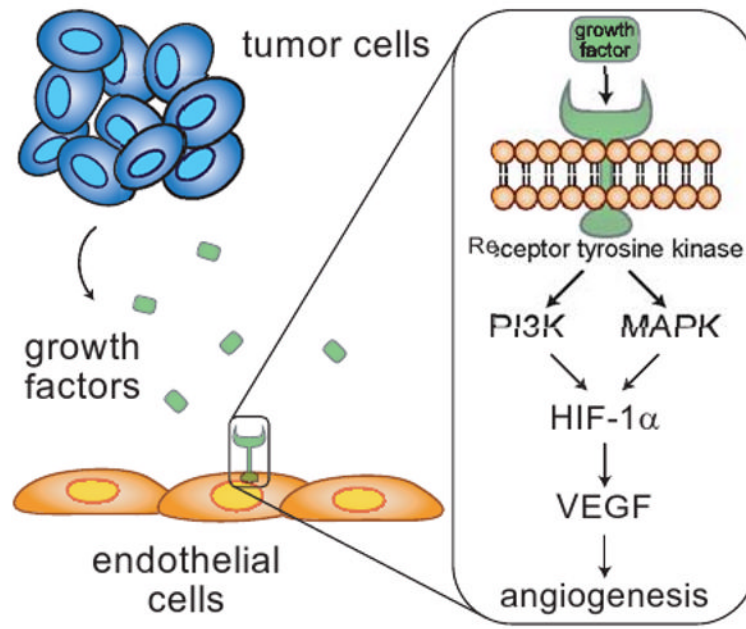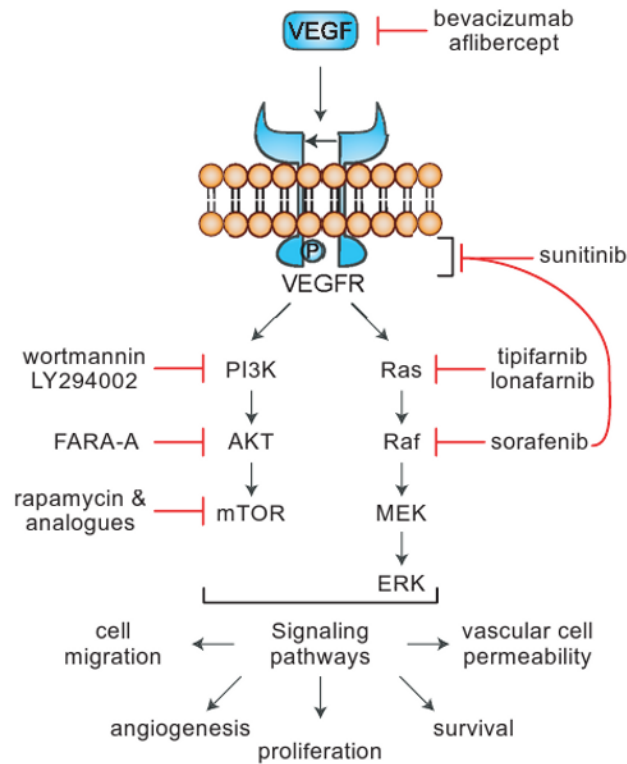
Figure 1.
Tumor cells secrete pro-angiogenic growth factors that bind to receptors on dormant endothelial cells (ECs) leading to vasodilation and an increase in vessel permeability. The ECs migrate and proliferate to form new branches from the pre-existing vasculature by detaching from the extracellular matrix and basement membrane.
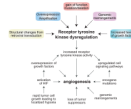
Figure 2.
Pathways and mechanisms that can lead to increased angiogenesis, including overexpression of growth factors and receptor tyrosine kinase dysregulation.
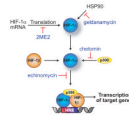
Figure 3.
Vascular endothelial growth factor (VEGF) binds to the VEGF receptor, a receptor tyrosine kinase, leading to receptor dimerization and subsequent auto phosphorylation of the receptor complex. The phosphorylated receptor then interacts with a variety of cytoplasmic signaling molecules, leading to signal transduction and eventually angiogenesis. Examples of both pre-clinical and clinical compounds that inhibit the pathway are shown.

Figure 4.

Both the HIF-α and HIF-β subunits are produced constitutively, but in normoxia the α subunit is degraded by the proteasome in an oxygen-dependent manner. Hydroxylation of two prolines in HIF-α enables HIF-α to bind to the von Hippel-Lindau tumor suppressor protein (pVHL), which links HIF-α to a ubiquitin ligase complex. The ubiquitin ligase catalyzes polyubiquitinylation of HIF-α, targeting it for degradation by the proteasome. In addition, hydroxylation of an asparagine residue in HIF-α disrupts the interaction between HIF-α and the coactivator p300, through a process independent of proteasomal degradation, which leads to reduced HIF transcriptional activity. Hypoxic conditions prevents hydroxylation of the α subunit, enabling the active HIF transcription complex to form at the HRE (hypoxia response element) associated with HIF-regulated genes.

Figure 5.
Examples of inhibitors known to act on HIF and/or HIF regulatory pathways are shown.

Table 1

Select Growth Factors and Receptors

Growth Factor	Function / Role	Members	Receptors
VEGF (vascular endothelial growth factor)	Physiological blood vessel formation and pathological tumor angiogenesis	VEGF-A, -B, -C, -D, -E, and the placental growth factors (PLGF-1 and -2)	VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4)
PDGF (platelet derived growth factor)	Cell growth and division; blood vessel formation; pericyte and smooth muscle recruitment and proliferation	PDGF-A, -B, -C and –D that form the five active homo- and heterodimers: PDGF-AA, -AB, -BB, -CC and -DD	PDGF-α and -β
FGF (fibroblast growth factor)	Vascular endothelial cell proliferation, migration, development and differentiation	23 members (FGF-1 through FGF-23)	FGFR-1, -2, -3 and -4
EGF (epidermal growth factor)	Cell growth, proliferation, differentiation, angiogenesis and survival	EGF (epidermal growth factor), TGF-α (tumor growth factor-α), HB-EGF (heparin-binding EGF-like growth factor), AR (amphiregulin), BTC (betacellulin), epigen, epiregulin, neuregulins 1-4	EGFR (ErbB1), HER2 (ErbB2/Neu), HER3 (ErbB3), and HER4 (ErbB4)
TGF-β (transforming growth factor)	Angiogenesis, cell regulation and differentiation, embryonic development, wound healing, growth inhibition properties	TGF-β1, -β2 and -β3	type I, II, or III
Angiopoietin	Initiation and progression of angiogenesis; vessel maintenance, growth and stabilization	Ang-1, -2 and -3/4	Tie-1 and -2

Table 2

Examples of Kinase Inhibitors (not an exhaustive list)

Inhibitor	Other names	Inhibits
Axitinib	AG013736	VEGFR, PDGFR and c-kit
Canertinib	CI-1033	EGFR, HER-2, HER-3 and HER-4
Cediranib	Recentin™, AZD2171	VEGFR, PDGFR-β and c-kit
Dasatinib	Sprycel, BMS-354825	Abl, Src, Tec
Erlotinib	Tarceva®, OSI-774	EGFR/HER1
Gefitinib	Iressa™	EGFR/HER1
Imatinib	Gleevec®, STI571	Abl, PDGFR and c-kit
Lapatinib	Tykerb, GW-572016	EGFR and HER2
Leflunomide	Arava, SU101	PDGFR (EGFR, FGFR)
Motesanib	AMG 706	VEGFR, PDGFR and c-kit
Neratinib	HKI-272	EGFR and HER2
Nilotinib	Tasigna	Abl, PDGFR and c-kit
Pazopanib	Armala®, GW786034	VEGFR, PDGFR-α and -β and c-kit
Regorafenib	BAY 73-4506	VEGFR-2 and Tie-2
Semaxinib	SU5416	VEGFR
Sorafenib	Nexavar®, BAY 43-9006	Raf, VEGFR (-2 and -3), PDGFR-β and c-kit
Sunitinib	Sutent®, SU11248	VEGFR, PDGFR, Flt-3, c-kit, RET and CSF-1R
Tandutinab	MLN518, CT53518	PDGFR, Flt-3 and c-kit
Toceranib		
Vandetanib	Zactima™, ZD6474	VEGFR-2, PDGFR-β, EGFR and RET
Vatalanib	PTK787	VEGFR, PDGFR-β and c-kit