

Antiangiogenic Drugs and Tyrosine Kinases

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Abstract: Various cancer types have different molecular and biological strategies for vascularization: neoangiogenesis, postnatal vasculogenesis, glomeruloid angiogenesis, intussusceptive microvascular growth, vessel cooption and vascular mimicry. The majority is still relatively obscure, which limits the development of more successful antivascular agents. It is not a surprise that, as our knowledge is deepest in case of tumor-induced neoangiogenesis, the first successful antiangiogenic drugs have been developed in this area. As neoangiogenesis involves growth factor receptors, most of them tyrosine kinases (KIT, Flt-3, VEGFRs, PDGFR, TIE2, FGFR1, EGFR and MET), several of these novel agents are tyrosine kinase inhibitors. This review summarizes our recent knowledge on various forms of cancer vascularization, the molecular mechanisms behind, depicting the “drugable” targets. In a short overview, we demonstrate the array of antiangiogenic approaches focusing on the tyrosine kinase inhibitors and summarize their preclinical activities. Finally we review the clinically available antiangiogenic tyrosine kinase inhibitors and demonstrate their current application and future perspectives. Further development in this field may depend on the identification of novel inhibitors targeting kinases that cannot be modulated yet by the available agents and on the development of vascularization strategy-specific design of these antivascular therapies.

Key Words: Tumor vascularization, antivascular therapy, tyrosine kinases, kinase inhibitors.

INTRODUCTION

Clinical oncology used for decades classical cell proliferation inhibitor cytotoxic drugs with variable success in various malignancies. Combination chemotherapy and radio-chemotherapy reached the limit of their efficacy therefore further improvement in clinical management of cancer patients required novel approaches. Molecular biology is fundamentally affecting developments in cancer biology and oncology by identification of an array of potentially drugable targets. Moreover, discovery of new biological pathways in cancers could also help to find novel approaches. The idea that cancer cannot grow beyond a few mm without developing its vasculature, similar to normal tissues is now 40 years old [1]. Though this observation became an unquestioned dogma, the application of this knowledge to clinical practice took several decade of basic and clinical research. Today we have to realize that various cancer types have different molecular and biological strategies for vascularization, the majority is still relatively obscure [2], which limits the development of more successful antivascular agents. It is not a surprise that, as our knowledge is deepest in case of tumor-induced neoangiogenesis, the first successful antiangiogenic drugs have been developed in this area. As neoangiogenesis involves growth factor receptors, most of them tyrosine kinases, several of these novel agents are tyrosine kinase inhibitors. This review summarizes our recent knowledge on various forms of cancer vascularization, the molecular mechanisms behind, depicting the “drugable” targets. A short overview demonstrates the array of antiangiogenic approaches focusing on the tyrosine kinase inhibitors and will summarize their preclinical activities. Finally, we summarize the clinically available antiangiogenic tyrosine kinase inhibitors and review their current application and future perspectives.

MECHANISMS AND MOLECULAR REGULATION OF TUMOR VASCULARIZATION

Until recently, vascularization of solid malignancies was considered exclusively as a result of endothelial sprouting (i.e. directed capillary ingrowth). However, latest advances in angiogenesis research have shown that the simplistic model of an invading microvessel sprout is not sufficient to cover the whole spectrum of morphogenic and molecular events that are required to form a

tumor capillary network [2]. Accordingly, it is now well established that tumor vascularization is not synonymous with endothelial sprouting, instead, solid tumors can acquire their blood supply by co-option of pre-existing vessels, intussusceptive microvascular growth, postnatal vasculogenesis (bone marrow-derived endothelial progenitor cells), glomeruloid angiogenesis or vasculogenic mimicry. It is important to note that these mechanisms are not excluding the appearance of each other, but in most cases they are able to run hand in hand, and to participate concurrently in physiological as well as in pathological angiogenesis. While the alternative mechanisms of tumor vascularization have some common molecular features and may be regulated in part by analogous factors, a variety of differences also exists.

Endothelial Sprouting

The best-known mechanism by which cancers induce their own vascularization is the sprouting of pre-existing host tissue and tumor capillaries. According to the widely accepted model described by Paku and Paweletz [3], the first step of this process is the dilatation of the “mother vessel” and the gel-sol transition of the basal membrane over the entire circumference of the dilated capillary, in response to nitric oxide [4] and vascular endothelial growth factor (VEGF) [5]. This is accompanied by the onset of fenestrations, the development of vesiculo-vacuolar organelles and the redistribution of platelet endothelial cell adhesion molecule (PECAM)-1 [6] and vascular endothelial (VE)-cadherin [7], and involves ANG1 and SRC kinases [8]. The so-called gel-sol transition of the basement membrane, mediated by matrix metalloproteases (MMPs) [9], gelatinases [10] and the urokinase plasminogen activator system [11], can be partly responsible for the initiation of endothelial cell (EC) proliferation and migration. However, a vast number of molecules stimulate endothelial proliferation and migration, including TGFβ1 [12], TNFα [13], members of the chemokine system [14] and the VEGF [15], fibroblast growth factor (FGF) [16] and platelet-derived growth factor (PDGF) [17] families. Following further migration of endothelial cells (which are maintaining their basal-luminal polarity and forming a slit-like lumen) proliferating pericytes of the mother vessel migrate along the basement membrane of the capillary bud, resulting in complete pericyte coverage of the new vessel. ANG2 (a mediator of TIE2 signaling) is involved in this process and regulates the migration of pericytes [18]. Interactions between the members of these soluble molecule families can activate the members of different endothelial cell adhesion molecule families, including integrins, cadherins, cell membrane proteoglycans, selectins and the members of the immunoglobulin super-

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family. It could be argued that the integrins represent the most important adhesion receptors in the vascular system. Accordingly, a wide variety of integrins have been shown to be expressed during sprouting, including $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 5$ and $\alpha v\beta 3$ [19]. Perhaps $\alpha v\beta 3$ has the main role, which can mediate the migration of endothelial cells in the fibrin-containing tumor matrix [20], and is able to keep the sol state of the basement membrane as a result of its capability to bind MMP-2 [21]. In summary, sprouting is tightly regulated by proangiogenic factors and inhibitors. While a proangiogenic molecule promotes endothelial budding, migration or lumen formation, an inhibitor interferes and modulates these mechanisms.

Postnatal Vasculogenesis: Endothelial Progenitor Cells

Until recently, it was generally accepted that in the adult the formation of new blood vessels results from the migration and proliferation of preexisting, fully differentiated endothelial cells (a process referred to as angiogenesis). Vasculogenesis defined as the in situ differentiation of vascular ECs from primitive precursor cells, has been thought to occur only in the early phases of vascular development. Recent studies, however, demonstrated that circulating bone marrow (BM)-derived endothelial progenitor cells (EPCs) home to sites of neovascularization and differentiate into ECs [22]. EPCs therefore resemble embryonic angioblasts, which are motile, anchorage independent cells that have the capacity to migrate, proliferate and differentiate into mature ECs. The life cycle of EPCs from BM to the vessel walls can be divided into three major periods. First, elevated serum levels of angiogenic growth factors in cancer patient may activate and mobilize BM-derived precursors into the circulation for the purpose of supporting local microvessel growth. These cells may be mobilized by VEGF and other tumor tissue-derived cytokines from the BM by a mechanism recently described by Asahara *et al.* [23] or by signaling through the Stroma Cell-Derived Factor (SDF) cytokine, which binds to the chemokine receptor CXCR-4, highly expressed on EPCs [24]. Secondly, additional local stimuli may promote the activation of local endothelium to express adhesion molecules to recruit endothelial progenitors. Thirdly, after homing, i.e. after adhesion and insertion into the monolayer of surrounding mature vascular ECs, this process may be completed by mechanisms still not elucidated. In summary, the role of EPCs in the vascularization of tumors has been demonstrated by several groups. It is not clear yet, however, whether they are essential for the blood supply of tumors or what the relative contribution of EPCs is compared with that of in situ proliferating ECs. Moreover, it has yet to be elucidated whether EPCs can be targeted to halt angiogenesis, or, alternatively, as they have the ability to home to the tumor vasculature - they can be used to deliver toxins or vascular targeting agents.

Vessel Co-Option

Vessel co-option refers to tumor vascularization by incorporation of pre-existing host capillaries into the tumor tissue and subsequent survival of the co-opted blood vessels. This mechanism has been described in cutaneous melanoma [25] where tumor cells proliferate by co-opting the peritumoral vascular plexus present at the base of the tumors, in non-small cell lung cancer [26] where “non-angiogenic” tumors grow by filling the alveoli, entrapping, but not destroying the co-opted alveolar capillaries, and in liver metastases of human colorectal carcinomas with “replacement” growth pattern [27] which can preserve the original architecture of the liver. Maintenance of incorporated mature microvessels depends on the survival of ECs which is thought to be tied to the presence of various survival-promoting cytokines, extracellular matrix proteins and to the intact function of pericytes. Thus, the molecular mechanisms that ECs may use to survive during vessel co-option are diverse and may vary for a given tumor type or host environment. However, the key molecules that regulate vessel co-option are angiopoietins and VEGF. According to the model of vessel co-option described by

Holash *et al.* [28], ANG1 activates TIE2 and induces subsequent signaling pathways favoring EC survival, endothelial quiescence and tumor vessel maintenance. On the contrary, ANG2 is a natural antagonist for ANG1, opposing the effect of ANG1-mediated endothelial survival by acting as a competitive antagonist at the TIE2 receptor. In co-opted host capillaries, the upregulation of ANG2 disrupts the interaction between TIE2 and ANG1, which in turn causes the detachment of pericytes from the endothelial tube. Once ECs are separated from pericytes, they become particularly vulnerable. In the presence of VEGF, EC stabilization and development of new capillaries are promoted; however, lack of stimulatory factors results in the regression of destabilized vessels. This antiapoptotic and survival function of VEGF seems to depend on an interaction between VEGFR2, β -catenin and VE-cadherin [29]. In summary, although molecules responsible for EC stabilization could be the key players, their precise role in initiation and maintenance of vessel co-option is yet to be elucidated.

Vasculogenic Mimicry

Vasculogenic mimicry was initially described in 1999 by Maniotis and colleagues [30]. Investigating aggressive uveal and cutaneous melanomas, the authors discovered a new apparatus by which tumors may acquire a blood supply: the tumor cells themselves were able to metamorphose into blood-containing channels. In their experiments, they found, that connective tissue septa of human melanomas, highlighted by PAS staining, contain channels circumscribed with basement membrane without endothelial lining, but filled with erythrocytes and externally covered by tumor cells. Subsequent studies demonstrated that the PAS-positive patterns may provide clinically useful information [31], and that tumor cells can line vessel-like spaces in non-melanoma tissues, such as aggressive prostate [32], breast [33], and ovarian [34] tumors. Moreover, there is growing body of evidence that many of the genes that are upregulated by aggressive tumor cells include those that are involved in angio- and vasculogenesis, such as the genes encoding vascular-endothelial (VE)-cadherin, Ephrin-A2, laminin $\gamma 2$ chain, thrombin receptor, Flt-1 (VEGF Receptor-1), Flt-1 (VEGFR2), Flt-4 (VEGFR3), TIE1/TIE2 (angiopoietin receptors), CD31, endoglin, angiopoietin-1, CD34 and prostaglandin I₂ synthase [35]. The expression of the above genes by the tumor cells implies a possible reversion to an embryonic-like phenotype with potential stem cell plasticity. An impressive example of this plasticity was demonstrated by Sefror *et al.* in an ischemic limb model: aggressive melanoma cells, when placed in an ischemic milieu, can be influenced to form normal vasculature, possibly recapitulating an embryonic program [36].

All in all, tumor cell-lined vascular structures offer an easy admission to the circulation without the struggle of the complex process of intravasation. On the other hand, such channels might also promote survival of the poorly vascularized areas in a given tumor, and alternatively, their presence can explain the disappointing results of some angiogenesis inhibitors in clinical trials.

Glomeruloid Angiogenesis

Glomeruloid bodies (GBs) are composed of several closely associated capillaries surrounded by variably thickened basal membrane within which pericytes are embedded. They are associated with aggressive tumor behavior and significantly reduced survival in cancer patients [37]. In an animal model, the authors injected an adenoviral vector engineered to express VEGF-A¹⁶⁴ into adult murine brain, and found that VEGF-A¹⁶⁴ inoculation evoked changes in the nascent vasculature, resulting in the sequential formation of mother vessels, glomeruloid bodies, and daughter vessels [38]. They hypothesized that, in the absence of neoplastic cells, high levels of VEGF-A may be necessary to affect mother vessel to GB transformation as characteristic of malignant tumors, and that transient expression of VEGF-A may invoke but not sustain glomeru-

loid bodies. In contrast to this theory, based on our previous results in the first experimental tumor model of glomeruloid angiogenesis [39], we believe that the proliferating and migrating tumor cells are able to pull the capillaries and the adjacent capillary branching points into the tumor cell nests. This process leads to the appearance of simple coiled vascular structures which later develop into chaotic and tortuous vascular structures with multiple narrowed afferent and efferent capillaries and with a superficial resemblance to renal glomeruli. However, whether GBs represent an accelerated form of angiogenesis or a dysfunctional, possibly abortive one, remains an open question. Nevertheless, one can assume that “active” and “passive” type of glomeruloid angiogenesis can function simultaneously in different cancers.

Intussusceptive Microvascular Growth (IMG)

For the period of birth through young adult age, the pulmonary capillary volume increases more than 30 fold [40]. As endothelial sprouting is a relatively slow process relying primarily on cell proliferation, such a rapid expansion cannot be derived merely from sprouting angiogenesis. Although in 1950 Short [41] has already proposed that capillaries in the lung might grow “by insertion of slender columns of interstitial tissue, thus dividing the vessel lumen”, the above mentioned assumption has led to the first description of IMG in the rapidly growing capillary bed of neonatal lungs only in 1986 [42]. Since that, intussusception has been observed in a wide variety of normal and malignant tissues [43, 44, 45]. IMG has four main phases: 1./ The vessel walls are protruding into the lumen until an area of contact is created between the opposing points of endothelial bilayer. 2./ Following reorganization of the inter-EC junctions, a central perforation of the lumen is formed. 3./ The fused ECs form a transluminal cylinder invaded by myofibroblasts and pericytes, which cells subsequently deposit extracellular matrix (ECM) material. 4./ The increase of the perimeter of the transluminal pillars results in the partitioning of the capillaries without undergoing any further change in their basic structure.

Although the molecular regulation of IMG is poorly understood, the role of some molecules is gradually becoming clearer. Major candidates are those capable of transmitting information between endothelial cells or from endothelial to mural cells, such as angiopoietins and their Tie receptors, PDGF-BB, monocyte chemoattractant protein-1, TGF β , and ephrins and EphB receptors. In addition, it has been demonstrated recently that local stimuli, such as intravascular shear stress, might also trigger new capillary development by tissue pillar formation [46].

MOLECULAR TARGETS OF ANTIANGIOGENIC AGENTS

Endothelial Geno/Phenotype

Endothelial mitogens initiate signaling cascade mechanisms leading to reprogramming quiescent endothelial cells to recapitulate embryonic genetic programs. Several of these molecular mechanisms are potentially drugable targets for anti-angiogenic therapies. Selectivity of antiangiogenic drugs rely on the identification of endothel-specific markers and processes. Global genomic approaches helped us to discover the genetic and phenotypic background of this cell lineage. Classically vascular endothelial cells are characterized by factor-VIII production, expression of CD34/CD31 and some other endothelial adhesion molecules such as VE-cadherin and V-CAM [47]. On the other hand, lymphatic endothelium is characterized by CD31 expression. More recently, it was discovered that the specific markers of the lymphatic endothelium are podoplanin and the hyaluronan receptor LYVE-1 [48]. However, microvasculature contains pericytes as well, characterized by desmin- and smooth muscle actin-containing cytoskeleton and PDGFR [49] and angiotensin receptor-1 [50] expressions.

Genomic research identified several novel pan-endothelial marker genes such as CTGF, angiomodulin, CD146 (MUC-18), and

SPARC [51]. Interestingly, tumor-associated endothelial cells were characterized by a few more genes such as MMP-2/MMP-11, nidogen, endo180 mannose receptor and BMP-1 [51]. *In silico* cloning supported previous data in respect of selective expression of several known endothelial marker genes in endothelial cells such as F-VIII, endothelin-1, CD31 and VE-cadherin [52]. Neoangiogenesis is most commonly induced by VEGF activation; therefore expression profile of mitogen-stimulated endothelial cells may have a critical importance from pharmacological point. Data indicate that VEGF-stimulated HUVEC cells overexpress around 100 new genes, among them 5 known to be positive regulators (COX-2, HB-EGF, EGFR1, CYR61 and ANG2) and another 5-gene set containing genes involved in angiogenesis (ACE, TM, Flt-1, PKC δ and C3G) [53]. SAGE libraries of proliferating endothelial cells identified factor-VIII, VEGFR1, VEGFR2, TIE1 and TIE2 expression. Furthermore, a few genes in proliferating endothelial cells were found to be signaling components of various pathways such as MAPK8, RAR β and BAX [52].

Angioblastic Mitogens and Receptors

Bone marrow stem cells are characterized by constitutive expression of WT1 and c-kit and the adhesion molecule CD34. Although the ligand is unknown, this cell population expresses Flt-3 kinase as well. This cell population is therefore responsive to bone marrow SCF ligand. Hemangioblasts maintain c-kit/WT1/CD34 expressions but harboring VEGFR2/TIE2 as well and become responsive to VEGF-A/VEGF-C and ANG1/2. It is a novel finding that hemangioblastic lineage cells express EPOR as well and are responsive to EPO. During the differentiation of hemangioblasts to endothelial precursors in the circulation these cells loose c-kit expression, but maintain all the other mitogen receptors [2,22-24].

Endothelial Mitogens and Receptors [54]

The most common endothelial mitogen is VEGF which is a family growth factors, VEGF-A, -B, -C and -D. Receptors for VEGF-A is Flt-1/VEGFR1, kdr/VEGFR2, both are tyrosine kinases, and neuropilin-1, a non-kinase receptor. VEGF-B binds Flt-1 exclusively. VEGF-C and -D are dual growth factors, since they have two receptors, on vascular endothelial cells (kdr) and another one on lymphatic endothelial cells (flt-4/VEGFR3) which is also a tyrosine kinase receptor. Vascular fate is determined by ephrins, since EphB4 ligand is expressed by endothelial cells destined to arteries while its tyrosine kinase receptor, EphB2 is expressed on endothelial cells to become veins [55].

A ubiquitous angiogen is bFGF the receptor of which is FGFR-1, -2, -3 and -4, all belonging to the tyrosine kinase family. Another non-specific but ubiquitous angiogen is HGF, the receptor of which is the tyrosine kinase, c-met. Recently it was discovered that the common epithelial mitogen, EGF, has also receptor on endothelial cells, another tyrosine kinase, EGFR [56]. Several inflammatory cytokines are endothelial mitogens such as IL-1, IL-6, IL-8 or TNF α , mostly with G-protein coupled receptors.

Some extracellular matrix proteins are endothelial mitogens as well, such as thrombin and fibrin, acting through their receptors which are non-kinases (α v β 3 integrin and thrombin receptor). Certain physiological angiogenesis inhibitors have also kinase receptors, such as NK1-4 variant of HGF (c-met), TGF β (TGF β R1-2), or ANG-2 (TIE2). Accordingly, these kinases may serve useful targets to develop angiogenesis-modulating drugs.

Pericyte/Smooth Muscle Cell Mitogens

The formation of neo-microvasculature requires proliferation and incorporation of pericytes into the new endothelial tubes. Pericytes may derive from local mesenchyme or bone marrow expressing the tyrosine kinase receptor PDGFR β and a G-protein coupled receptor, AT1R. Accordingly, the main mitogens for these cells are PDGF β produced by endothelial cells and ATII. Pericytes respond

to the mitogenic stimuli of other endothelium-derived cytokines, such as TGF β , HGF or ANG1; some of their receptors are also tyrosine kinases (c-met and TIE2). [57].

Signaling Characteristics of Vascular Mitogens

The best characterized signaling pathway is the one associated to VEGFR2/kdr. This tyrosine kinase receptor activated upon VEGF-A or -C initiate signaling of 3 pathways, the PLC γ -PKC-RAF-MEK-MAPK mitogenic-, the PI3K-AKT survival- and the TSAD-SRC mitogenic one [58]. Much less is known about the activation of Flt-1/VEGF-A or -B system, where there is available data only on the PLC γ /PKC activation arm. VEGF-C ligation of VEGFR3 induces heterodimerization with VEGFR2 and/or signaling through SHC/GRB2 adaptors toward the MAPK cascade. EGF activation of EGFR in endothelial cells can initiate a diverse array of signaling cascades such as the MAPK cascade, the cytokine-like JAK-STAT one, the PI3K-AKT survival-, and the mitogenic PLC γ -PKC or RAS-RAC-RHO pathways [59]. Activation of the c-met receptor by paracrine HGF ligand may activate both mitogenic RAS-RAF-MAPK or the mito- and mitogenic p120-SRC/FAK cascade [59]. Stimulation of angioblasts by SCF activates c-kit signaling leading to mitogenic activation of the MAPK cascade [59], while ligation of the EPOR by EPO may activate a cytokine-like signaling through JAK2 and STAT5 [59]. PDGF β ligation of the PDGFR β may initiate homo- or heterodimerization and trigger several types of signaling pathways such as the PLC γ -PKC-MAPK, SRC, the PI3K-AKT survival-, and the STAT-involving cytokine signaling ones [59]. It is now evident that cellular responses to these mitogens are dependent on the concentration and pattern of mitogens, on the activation state of the target cell and on its differentiation level.

TYROSINE KINASE-TARGETS IN VASCULARIZATION MECHANISMS OF CANCER (TABLE 1)

Since various cancer types may adopt various vascularization strategies, selection of tyrosine kinase targets must be based on their molecular characteristics. The most common vascularization strategy in cancer is neoangiogenesis where in proliferating endothelial cells VEGFR2, while in pericytes PDGFR β are the predominant mitogenic receptors [60], although as it was shown above, these cells express several other growth factor receptors as well. When cancer activates postnatal vasculogenesis by producing cytokines able to mobilize bone marrow angioblastic progenitors, obvious tyrosine kinase targets are c-kit, Flt-3 and VEGFR2 [61]. It is now also evident that this type of vessel development requires mesenchymal precursor mobilization to produce pericytes where PDGFR β seems to be the essential target [49].

Table 1. Kinase Targets in Various Cancer Vascularization Forms

Vascularization Form	Kinase Target	Cellular Target
neoangiogenesis	VEGFR1, VEGFR2 FGFR1 PDGFR	endothelial cell endothelial cell pericyte
postnatal vascularization	c-kit Flt-3 VEGFR2	hemangioblast, CEP hemangioblast (hemangioblast) CEP
vessel co-option	TIE2	endothelial cell
vasculogenic mimicry	EphA2, FAK	cancer cell
glomeruloid angiogenesis	?	endothelial cell
Intussusceptive angiogenesis	?	endothelial cell

CEP= circulating endothelial precursor.

Certain cancer types can use the vessels of the host tissue especially in cases where their density is extremely high such as in lung

or skin [2]. Since in these cases microvessel survival is the critical factor, survival signal provided by ANG1/2 and mediated by the TIE2 receptor is the obvious target [28].

Cancers can make advantage of their extreme genetic plasticity to recapitulate embryonic programs such as the angioblastic one, by which they can adopt a microvascular phenotype called vasculogenic mimicry [30]. This feature is not only interesting but also supports the nutrient supply for the tumor tissue. Since EphA2 and FAK kinases have been shown to be key regulators of this mimicry, they are feasible targets for intervention [2].

ANTIVASCULAR CANCER THERAPIES (TABLE 2)

Recognition of the significance of vascularization of cancer tissue by Folkman and its followers in the early seventies of the last century provided the basis of developing treatment strategies of cancer [1]. Today there are two major approaches to this goal, antiangiogenic modalities [62] and vascular targeting therapies [47]. The first approach aims the inhibition of the development of new vasculature induced by cancer, while the other is to target the tumor vasculature itself. Since cancers develop variable vascularization strategies, the antivascular therapies must be able to compete with all the possible mechanisms. The antiangiogenic approaches are primarily targeting the tumor-induced neoangiogenic pathways, while the antivascular ones are broader in their antivascular mechanisms, since they combat the mature tumoral vessels developed by either mechanisms (neoangiogenesis, postnatal vasculogenesis, vessel co-option, glomeruloid vascularization or vasculogenic mimicry).

Today the angiosuppressive strategy is the most developed, containing diverse forms of interferences [63, 64]. The first class of the angiosuppressive agents targets the primary angiogenic cytokine in cancer, VEGF itself by monoclonal antibodies (such as bevacizumab, a humanized anti-VEGF-A antibody), VEGF-trap or VEGF-antisense. The second approach is to target VEGF receptors. Interestingly, anti-receptor antibody therapy is in the early phase of development and most of the available agents are small molecular VEGFR signal transduction inhibitors. Since VEGF is not the only pro-angiogenic cytokine produced by cancers, it would be necessary to develop other strategies, but these are almost completely missing. An exception is thalidomide, which targets the production of various angiogenic cytokines (VEGF, bFGF, TNF α), and it may be the reason why it is so active clinically in various cancer types [65]. Another feasible target is α v β 3 integrin on proliferating endothelial cells where both anti-integrin antibodies (such as Vitaxin) or ligand-mimetics (such as Cilengitide) are available [62-64]. Furthermore, angiostatic endogenous inhibitors are also available such as endostatin which targets the angiostatic integrin α 5 β 1. Since neoangiogenesis requires limited proteolysis of the basement membranes and surrounding matrix, MMP-2/9 inhibitors have been developed and tested with limited success [62-64].

Antivascular strategies require the identification of tumoral endothelium-specific surface markers, signaling mechanisms or other cytoplasmic properties. As it was shown above, our knowledge is relatively limited on the pheno- and genotype of tumor-associated endothelium. Their fibronectin receptor(s), α v β 3 and α 5 β 1, can be targeted by a ligand-mimetic drug L19 or using α v β 3-ligand to deliver signal transduction inhibitor ATP μ -RAF. Other surface receptors selected for such a strategy are endoglin/CD105 targeted by a monoclonal antibody-toxin conjugate, V-CAM1 targeted by a TF-conjugated antibody or CD44 targeted by an antibody linked to a proliferation inhibitor. Intracellular tumor-associated endothelial inhibitors are small molecules mostly targeting cytoskeleton of the endothelial cells, such as combretastatin analogues (actin polymerization inhibitors), or ZD6126 and ABT-751 microtubule inhibitors [2, 47].

It is now recognized that the development of the angiogenic phenotype of cancers is frequently mediated by the hypoxia-

Table 2. Antivascular Strategies

Angiosuppression		Vascular Targeting Therapy	
Target	Strategy	Target	Strategy
VEGF	antibody, antisense "trap"	$\alpha v\beta 3$ and $\alpha 5\beta 1$	ligand-mimetic inhibitor conjugate
bFGF, $TNF\alpha$	SMI	CD105	antibody-toxin conjugate
VEGFR	antibody SMI	VCAM-1	antibody-TF conjugate
$\alpha v\beta 3$ integrin	ligand-mimetic antibody	CD44	antibody-inhibitor conjugate
$\alpha 5\beta 1$ integrin	Ligand-mimetic protein inhibitor	actin	SMI
MMP-2/MMP-9	SMI	tubulin	SMI

SMI= small molecular inhibitor, TF= tissue factor.

induced adaptation mechanisms [66], but these are only theoretical targets for antivascular strategies since specific inhibitors are not available yet.

ANTIANGIOGENIC TYROSINE KINASE INHIBITORS (TABLES 3 AND 4)

As it has been shown above, tumor-induced neoangiogenesis and postnatal vasculogenesis involve several tyrosine kinase receptors functional in hemangioblasts, endothelial precursors and proliferating endothelial cells. Furthermore, endothelial cell survival, important during vessel co-option of tumor tissue, may also involve certain tyrosine kinases, therefore these molecular pathways are attractive targets for drug development. In the past decade pharmacology provided an array of small molecular inhibitors, more or less specific for various classes of tyrosine kinases. Although several of them are relatively specific for a given kinase, the majority have several targets. Furthermore, these agents generally have multiple cellular targets in tumor tissue.

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Table 3. Small Molecule Antivascular Drugs

Agent	Structure
Thalidomide	2-[2,6-dioxo-3-piperidyl]isoindole-1,3-dione
ZD6126	N-Acetylcolchicinol-O-Phosphate
ABT-751	N-[2-[(4-Hydroxyphenyl)amino]-3-pyridinyl]-4-methoxybenzenesulfonamide
imatinib mesylate (Gleevec®)	4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]phenyl]benzamide
sunitinib malate (Sutent®)	5-[5-fluoro-2-oxo-1,2-dihydroindol-(3Z)-ylidenemethyl]-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylaminoethyl)amide
sorafenib/Bay-43-9006 (Nexavar®)	4-(4-{3-[4-chloro-3-(trifluoromethyl)phenyl]ureido}phenoxy)-N2-methylpyridine-2-carboxamide-4-methylbenzenesulfonate
AG013736	6-[2-(methylcarbamoyl)phenylsulfanyl]-3-E-[2-(pyridin-2-yl)ethenyl]indazole
Pazopanib (GW786034)	5-({4-[(2,3-dimethyl-2H-indazol-6-yl)methylamino]pyrimidin-2-yl}amino)-2-methylbenzenesulfonamide
SU5416	3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-indolin-2-one
SU6668	Z)-3-[2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrol-3-yl]-propionic acid
Vatalanib (PTK787/ZK222584)	N-(4-chlorophenyl)-4-(pyridin-4-ylmethyl)phthalazin-1-amine
Gefitinib (Iressa®)	N-(3-Chloro-4-fluorophenyl)-7-methoxy-6-[3-(4-morpholinyl)propoxy]-4-quinazolinamine
Erlotinib (Tarceva®)	N-(3-Ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine Monohydrochloride
Lapatinib (Tykerb®)	N-{3-chloro-4-[(3-fluoro-benzyl)oxy]phenyl}-6-[5-({2-(methylsulfonyl)ethyl}amino)methyl]-2-furyl]-4-quinazolinamine
ZD6474 (Zactima®)	(N-(4-bromo-2-fluorophenyl)-6-methoxy-7-((1-methylpiperidin-4-yl)methoxy)quinazolin-4-amine

Table 4. Kinase Inhibitory Profiles of Various Agents in Clinical Development

	KIT	FLT-3	VEGFR1	VEGFR2	VEGFR3	PDGFR	FGFR1	EGFR	MET
Gleevec	+	-	-	-	-	+	-	-	-
Sunitib	+++	+++	-	+++	-	++	+	-	-
Sorafenib	++	++	-	++	++	++	+	-	-
PTK787	+	-	++	++	+	+	-	-	-
AG-137736	+++	-	+++	+++	+++	+++	-	-	-
GW-786034	+	-	++	++	++	++	+	-	-
ZD6474	-	-	++	+	-	-	-	+	-

Potency of the inhibitory activity is expressed in the range of - to +++ (most potent) and based on IC_{50} values determined in cell-based assays (see in ref. [68]).

The first tyrosine kinase inhibitor clinically successful in cancer is imatinib mesylate (Gleevec) with a narrow spectrum of inhibitory activity for c-kit and PDGFR [67]. Gleevec has a potential to inhibit hematopoietic precursors and circulating endothelial precursors as well as pericytes, but data on such activity are missing. Later several more potent c-kit inhibitors have been identified and tested preclinically and in human cancers, such as sunitinib maleate (Sutent), sorafenib tosylate/Bay-43-9006 (sorafenib), AG013736 or GW786034 (Pazopanib) [68].

Although several Flt-3 kinase inhibitors have been identified, Sutent and sorafenib are those to be considered clinically to inhibit hemangioblasts and endothelial precursors [68].

The development of VEGFR inhibitors started with a class of 3-substituted indolin-2 ones such as SU5416 with preference for VEGFR1 followed by VEGFR2, c-kit and PDGFR [69]. One of its derivatives, SU6668 was a c-kit/PDGFR inhibitor like Gleevec, but lately a novel version, SU11248 (Sutent) was developed with highly potent inhibitory activity for c-kit, Flt-3, VEGFR2 and PDGFR [70]. Some novel agents, AG013736 and GW786034 are at least as potent VEGFR inhibitors than Sutent but have equipotency for all the three VEGFR types, unlike Sutent [68]. PTK787/ZK22-2584 (Vatalanib) is also a potent VEGFR1/VEGFR2/VEGFR3 inhibitor [68].

The most potent pericyte PDGFR inhibitor is AG013736, closely followed by Sutent, sorafenib and the novel agent, GW786034 [68].

There are only a few FGFR1 inhibitors available, although it is evident that after VEGF, bFGF is the second most common cancer angiogenic cytokine. It is interesting from this point that both Sutent and sorafenib are relatively competent FGFR1 inhibitors [68-70].

It was mentioned above that tumor-associated endothelial cells express EGFR [56] and c-met [54], especially in activated state, therefore EGFR or MET-kinase inhibitors would be important in this respect as well. There are several EGFR kinase inhibitors developed and some of them already in clinical practice, such as gefitinib, erlotinib or lapatinib [71], but they do not possess other kinase inhibitory potential important in angiogenesis. Therefore it is interesting that ZD6474 (Zactima) is a dual VEGFR2 and EGFR inhibitor with activity against VEGFR3 as well [72]. Unfortunately, there are no potent c-met kinase inhibitors developed yet, although Sutent is the most potent in this respect among clinically tested inhibitors.

If we consider the various vascularization strategies of cancers and the tyrosine kinases involved in them, the following targets can be considered (Table 1). In case of neoangiogenesis, VEGFR2, VEGFR1 and PDGFR are the primary targets if VEGF is the mitogen, while in case of bFGF-mediated angiogenesis, FGFR1 is the right target. In case of postnatal vasculogenesis, beside VEGFR2 and PDGFR, c-kit and Flt-3 play additional role. In case of vessel co-option, TIE2 receptor for ANG1/2 is the target, while in case of vasculogenic mimicry FAK and EphA2. Compared to this short list, the available kinase inhibitors described above are only effective against tumor-induced neoangiogenesis or postnatal vasculogenesis, and there are no kinase inhibitors developed against vasculogenic mimicry, and only some new agents are reported as inhibitors for TIE2 with no robust preclinical data which could be applied for vessel-co-option [73].

CLINICAL ACTIVITY OF ANGIOSUPPRESSIVE TYROSINE KINASE INHIBITORS

The first clinically successful antiangiogenic agent was anti-VEGF antibody, bevacizumab, active in colorectal- NSCLC lung-, renal- and breast cancers [74]. Its efficacy against such diverse cancer forms encouraged further development of antiangiogenic agents. Bevacizumab has a single molecular target, but it was evident that the complex cellular and molecular mechanisms behind

neoangiogenesis may require simultaneous targeting of various pathways.

Sunitinib with its multiple kinase targets (c-kit, Flt-3, VEGFR2 and PDGFR) was an ideal agent to test in various cancer models [68]. Preclinical studies indicated a wide range of antitumoral effects in lung-, colorectal-, epidermoid- and renal cancers, gliomas, malignant melanoma, neuroendocrine cancer and leukemia. VEGFR and PDGFR inhibitory *in vivo* concentrations required daily dosing with 20-40 mg/kg. Sunitinib inhibited endothelial cell proliferation and induced apoptosis *in vitro*, while in colorectal carcinoma models inhibited tumor vascular density and vascular permeability. Sunitinib-treated colon cancer tumor tissue responded with characteristic gene expression changes including upregulation of cadherin-11, PI3K and EphB4 and downregulation of CDK2 and MUC-1. Phase I studies identified that sunitinib is metabolized by cytochrome-P450-3A4, accordingly, P450-inducers or inhibitors should be avoided in patients under treatment. The tolerable toxicity profile of sunitinib helped to develop Phase II trials. Since antiangiogenic agent bevacizumab has shown some activity against renal cell cancer, and this tumor is one of the most vascularized human cancers due to the VHL mutation/inactivation and consequent HIF1 α upregulation and VEGF, PDGF and bFGF production, sunitinib was tested against the cytokine refractory form of the disease. It demonstrated significant and previously unprecedented clinical activity against renal cell cancer, with 40% response rates and almost 30% stabilized disease, resulting in a median time to progression of 8.7 months and a median survival of 16.4 months [68, 75].

Sunitinib was tested as first-line therapy in Phase III trial as well, comparing it to IFN α in renal cell cancer. Again, it doubled median progression-free survival of renal cancer patients as compared to IFN α because of a highly significant increase in response rates. Molecular markers of sunitinib therapy were decrease of soluble VEGFR2 and increase of VEGF-A and PlGF in the sera of cancer patients. In renal cancer patients sunitinib is also on trial in combination with gefitinib, a selective EGFR inhibitor, with encouraging early results [68].

Sunitinib is approved by FDA against Gleevec-resistant GIST, and is on trial in metastatic breast cancer where, beside the previously mentioned markers, decrease in soluble KIT levels was also observed. Sunitinib seems to be also active in previously treated islet cell carcinomas as well, but has shown much less impressive activity against NSCLC lung- and colorectal cancers [68].

Sorafenib was developed as a RAF inhibitor, but it also has a multiple tyrosine kinase inhibitory profile similar to sunitinib, with an additional target, VEGFR3 [76]. Initially sorafenib was tested on B-RAF mutated preclinical cancer models (colorectal, NSCLC, ovarian, pancreatic, breast, melanoma) where it demonstrated significant activity inducing inhibition of the RAF/MEK/ERK cascade. In endothelial cells sorafenib inhibits proliferation due to VEGFR2/3 inhibition while in smooth muscle cells it prevents ligand-induced PDGFR phosphorylation, resulting in proliferation inhibition [69, 70]. *In vivo*, sorafenib inhibited renal cancer growth due to decrease in tumor vascularization (CD31+ endothelial and SMA+ pericyte-labeled vessels). The good toxicity profile of sorafenib allowed the initiation of Phase II studies in refractory renal cell cancer, where it demonstrated highly significant clinical activity, inducing fourfold increase in progression-free survival at a twice-daily dose of 400 mg. In another trial, sorafenib was tested on untreated renal cell cancer patients and demonstrated 75% clinical benefit. In a Phase III trial on unresectable or metastatic renal cell cancer patients, sorafenib doubled median progression-free survival and induced 39% improvement in overall survival [70, 76]. In NSCLC, sorafenib was tested in several Phase II trials in advanced progressive disease and induced stable disease with high frequency but rarely partial responses [68].

Other Multi-Kinase Inhibitors

Vatalanib (PTK787/ZK222584) has a similar inhibitory profile to Sunitinib (VEGFR1, VEGFR2/VEGFR3, PDGFR and KIT) and was extensively tested in various Phase II and III trials on metastatic colorectal cancer in combination with FOLFOX4 regime (5-FU/leucovorin/oxaliplatin). Despite the expectations, vatalanib did not improve FOLFOX4 efficacy significantly [68]. AG013736 is another broad range kinase inhibitor with high efficacy for VEGFR1/2/3 and PDGFR, it has also been tested clinically on advanced renal cell cancer, and found to induce high frequency of response rate (46%) and similarly frequent stable disease [68].

All the agents discussed above are typical angiostatic drugs targeting various kinases involved in endothelial and pericyte proliferation. More recently, a novel idea was developed based on the assumption that in a significant proportion of cancers EGFR is overexpressed and even mutated [71]. Furthermore, studies also indicated that EGFR-driven signaling pathways are responsible for the development of the angiogenic phenotype [15]. Based on these premises, dual targeting of EGFR in cancer cells and VEGF/VEGFR in tumor-associated microvessels was established [72]. Trials are initiated to combine anti-growth factor antibodies (EGFR: cetuximab, VEGF: bevacizumab), with kinase inhibitors (bevacizumab and erlotinib, or bevacizumab with gefitinib) in various cancer types including colorectal-, renal cell- and lung cancers [72].

Till now there is only one kinase inhibitor available, which is able to inhibit EGFR as well as VEGFR2 and VEGFR3, that is Zactima® (vandetanib, ZD6474) [78]. This agent inhibits EGFR-driven cancer cell proliferation and VEGF-driven endothelial cell proliferation *in vitro*, and was shown to be active in NSCLC lung- and head and neck cancer preclinical models demonstrating antitumoral and antiangiogenic activity [78-80]. Furthermore, vandetanib was also active in combination with irradiation and chemotherapy in a preclinical pancreatic carcinoma model; meanwhile data on clinical activity in cancer patients are not available yet.

CONCLUSION

Antivascular therapy of cancer became a reality of the everyday practice in clinical oncology, based on the development of molecularly targeted agents. Since several tyrosine kinases are involved in various forms of tumor vascularization, they serve as validated molecular targets for small molecular inhibitors. Although the battery of such inhibitors is still limited, a few of them already demonstrated unprecedented clinical activity in various cancer types, demonstrating the proof of principle of the idea of using angiostatic tyrosine kinase inhibitors. Further development may depend on the identification of novel inhibitors targeting kinases that cannot be modulated yet by the available agents and on the development of vascularization strategy-specific design of these antivascular therapies.

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ABBREVIATIONS

ANG1	=	Angiotensin-1
BM	=	Bone marrow
EC	=	Endothelial cells
EGF	=	Epidermal growth factor

EGFR	=	Epidermal growth factor receptor
Eph	=	Ephrin
EPO	=	Erythropoietin
EPOR	=	Erythropoietin receptor
FAK	=	Focal adhesion kinase
FOLFOX	=	Oxaliplatin+Leucovorin+5-Fluorouracil
FDA	=	Food and Drug Administration
GIST	=	Gastrointestinal stromal tumor
HIF	=	hypoxia-induced transcription factor
IL	=	Interleukin
NSCLC	=	Non-small cell lung cancer
PDGF	=	Platelet-derived growth factor
SAGE	=	Serial analysis of
TF	=	Tissue factor
TGF	=	Transforming growth factor
TNF	=	Tumor necrosis factor
VHL	=	Von Hippel-Lindau

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