REVIEW PAPER

Anti-angiogenic tyrosine kinase inhibitors: what is their mechanism of action?

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Abstract Tyrosine kinases are important cellular signaling proteins that have a variety of biological activities including cell proliferation and migration. Multiple kinases are involved in angiogenesis, including receptor tyrosine kinases such as the vascular endothelial growth factor receptor. Inhibition of angiogenic tyrosine kinases has been developed as a systemic treatment strategy for cancer. Three anti-angiogenic tyrosine kinase inhibitors (TKIs), sunitinib, sorafenib and pazopanib, with differential binding capacities to angiogenic kinases were recently approved for treatment of patients with advanced cancer (renal cell cancer, gastro-intestinal stromal tumors, and hepatocellular cancer). Many other anti-angiogenic TKIs are being studied in phase I-III clinical trials. In addition to their beneficial anti-tumor activity, clinical resistance and toxicities have also been observed with these agents. In this manuscript, we will give an overview of the design and development of anti-angiogenic TKIs. We describe their molecular structure and classification, their mechanism of action, and their inhibitory activity against specific kinase signaling pathways. In addition, we provide insight into what extent selective targeting of angiogenic kinases by TKIs may contribute to the clinically observed anti-tumor activity, resistance, and toxicity. We feel that it is of crucial importance to increase our understanding of the clinical mechanism of action of anti-angiogenic TKIs in order to further optimize their clinical efficacy.

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Introduction

Cancer development is characterized by uncontrolled cell growth and proceeds via genetic changes resulting in numerous biological alterations. Essential hallmarks that drive tumorigenesis as described by Hanahan and Weinberg include: self-sufficiency in growth signals; insensitivity to growth-inhibitory (antigrowth) factors; evasion of programmed cell death (apoptosis); limitless replicative potential; sustained angiogenesis; and tissue evasion and metastasis [1].

Angiogenesis, the growth of new vessels from pre-existing vasculature, is a critical step in tumor progression [2]. New blood vessels are required to support the growth of a tumor beyond the size of about 1–2 mm^3, to supply oxygen and nutrients to proliferating tumor cells and for metastasis formation [3, 4]. Research in angiogenesis inhibition as a therapeutic strategy against cancer started around 1971, when Folkman postulated that tumor growth is dependent on angiogenesis [5]. In the past two decades, inhibitors of angiogenesis have been developed for clinical use [6]. Most notable angiogenesis inhibitors target the vascular endothelial growth factor (VEGF) signaling pathway, such as the monoclonal antibody bevacizumab (Avastin, Genentech/Roche) and two kinase inhibitors sunitinib (SU11248, Sutent, Pfizer) and sorafenib (BAY43-9006, Nexavar, Bayer). Bevacizumab was the first angiogenesis inhibitor that was clinically approved, initially for treatment of colorectal cancer and recently also for breast cancer and lung cancer. The small-molecule tyrosine kinase inhibitors sunitinib and sorafenib target the VEGF

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receptor (VEGFR), primarily VEGFR-2, and have shown clinical efficacy in diverse cancer types [7, 8]. Both drugs have shown benefit in patients with renal cell cancer [9, 10]. In addition, sunitinib has been approved for treatment of gastro-intestinal stromal tumors (GISTs). Sorafenib inhibits Raf serine kinase as well and has been approved for treatment of hepatocellular cancer as well [11]. Numerous clinical trials are ongoing with these and other angiogenesis inhibitors in various cancer types.

Two major problems have been noticed during the clinical development of angiogenesis inhibitors. In both preclinical and clinical settings, resistance to angiogenesis inhibitors occurs. In some patients, treatment with an angiogenesis inhibitor results in an initial response, followed by tumor progression (acquired resistance). In other patients, intrinsic resistance is being observed [12]. Secondly, in contrast to initial expectations, significant clinical toxicities are observed during anti-angiogenic treatment. These toxicities include severe bleeding, disturbed wound healing, gastro-intestinal perforation, hypertension, and fatigue [13].

Insight into the underlying mechanisms of resistance and toxicities of angiogenesis inhibitors will help to further improve treatment strategies of angiogenesis inhibition. This review highlights important tyrosine kinases and their mediated signaling pathways in angiogenesis. We describe the molecular structure and classification of tyrosine kinase inhibitors, their mechanism of action, and their inhibitory activity against specific kinase signaling pathways. In addition, we provide insight into what extent selective targeting of angiogenic kinases by tyrosine kinase inhibitors may contribute to the clinically observed anti-tumor activity, resistance, and toxicity.

Tyrosine kinases

Kinases, also called phosphotransferases, are enzymes that transfer a phosphate group from high-energy donor molecules, for example adenosine triphosphate (ATP), to specific substrates. Protein kinases phosphorylate proteins, resulting in functional changes of target proteins. Of the 518 protein kinases encoded in the human genome [14], 90 kinases belong to the group of tyrosine kinases. Six other groups have been identified whose kinases primarily phosphorylate serine and threonine residues (Fig. 1). The tyrosine kinase group consists of approximately 30 families, for example the VEGFR family and the fibroblast growth factor receptor (FGFR) family. Apart from classification in families, tyrosine kinases can also be classified in receptor tyrosine kinases and non-receptor (cytoplasmic) tyrosine kinases. Receptor tyrosine kinases are essential for the transduction of extracellular signals into the cell, while non-receptor tyrosine kinases accomplish intracellular communication. A receptor tyrosine kinase monomer consists of an N-terminal extracellular ligand-binding domain, a transmembrane domain, and a C-terminal intracellular domain with tyrosine kinase activity (Fig. 2). The kinase domain has a bi-lobar structure, with an ATP-binding cleft located between the N- and C-terminal lobes. The ATP-binding site can be divided into three subregions: the adenine region, the sugar region, and the phosphate-binding region [15]. The C-terminal lobe of kinases contains an activation loop and is marked by a specific amino acid combination at the start of the loop. This combination exists of the amino acids aspartic acid, phenylalanine, and glycine, abbreviated as D, F, and G, respectively, and is therefore called 'DFG motif'. The activation loop can adopt numerous conformations. In the 'out' conformation, the activation loop creates a hydrophobic pocket, nearby the ATP-binding cleft (Fig. 2). This hydrophobic pocket is important for a subgroup of tyrosine kinase inhibitors, as described later.

Ligand binding to the extracellular domain of the receptor promotes receptor dimerization, resulting in autophosphorylation of specific tyrosine residues of the cytoplasmic kinase domain [16]. Besides these phosphorylation sites for regulation of their own kinase activity, other phosphorylation sites of kinases are being used to control protein interactions. The activated receptor recruits interacting proteins that bind to certain phosphorylation sites [17]. Recruited and phosphorylated signaling proteins are subsequently able to phosphorylate other proteins. Activation of (multiple) signaling pathways eventually leads to biological responses [18]. Biological responses include cell activation, proliferation, differentiation, migration, survival, and vascular permeability. We provide here more insight into signaling pathways and biological responses of cells involved in angiogenesis, but every cell uses signaling pathways for their survival, proliferation, and other activities.

Tumor angiogenesis

In normal physiological circumstances, angiogenesis is well controlled by pro- and anti-angiogenic factors and is only promoted during the menstrual cycle, pregnancy, and during wound healing and repair [19]. Though, in cancer, this balance of pro- and anti-angiogenic factors is disturbed, resulting in the so-called 'angiogenic switch'. Tumor cells secrete a number of pro-angiogenic factors that stimulate the proliferation and migration of endothelial cells, resulting in the outgrowth of new capillaries into the tumor. VEGF signaling through its receptor is the major inducer of angiogenesis [20]. Therefore, special attention
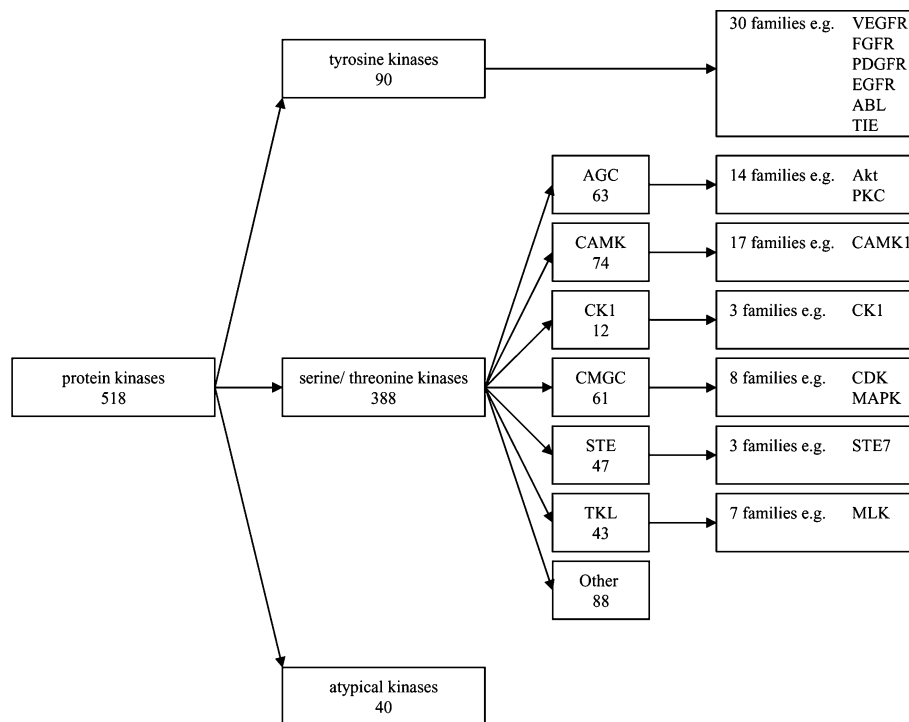
Fig. 1 Classification of protein kinases of the human kinome. Protein kinases can be divided into tyrosine kinases and serine/threonine kinases. Tyrosine kinases can be subdivided into approximately 30 families, which mediate a variety of biological responses. The kinases in six other groups mostly phosphorylate serine/threonine residues. These groups include *AGC*-containing protein kinase A (PKA)/ protein kinase G (PKG)/protein kinase C (PKC) families; *CAMK* calcium/calmodulin-dependent kinase; *CK1* casein kinase 1; *CMGC*-containing cyclin-dependent kinase (CDK)/mitogen-activated protein kinase (MAPK)/glycogen synthase kinase (GSK)/CDK-like kinase (CLK) families; *STE* homologues of yeast sterile 7, sterile 11, sterile 20 kinases; *TKL* tyrosine kinase-like kinase. Each of these groups can also be classified into families, of which at least one example per group is shown. *ABL* Abelson kinase; *Akt* Akt/protein kinase B (PKB); *EGFR* epidermal growth factor receptor; *FGFR* fibroblast growth factor receptor; *MLK* mixed-lineage kinase; *PDGFR* platelet-derived growth factor receptor; *TIE* tyrosine kinase with immuno-globulin-like and EGF-like domain; *VEGFR* vascular endothelial growth factor receptor
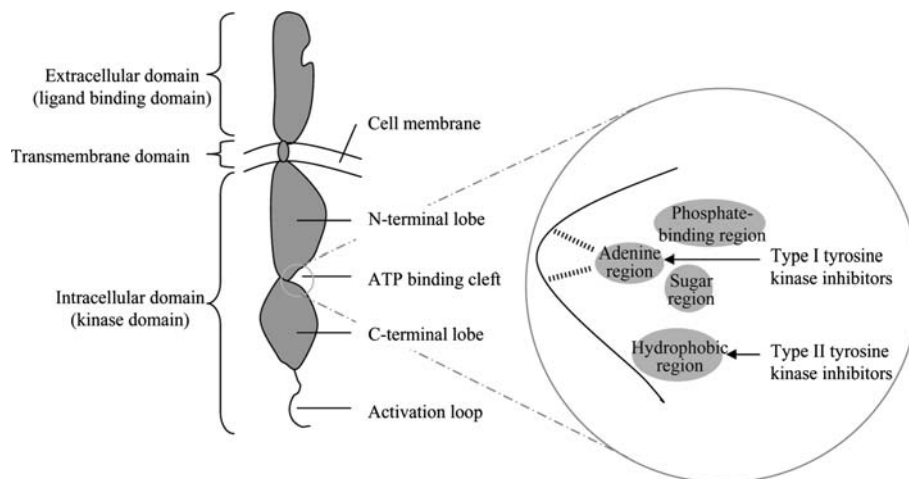

Fig. 2 Structure of a receptor tyrosine kinase. The extracellular domain of a receptor tyrosine kinase can bind specific ligands such as growth factors, while the intracellular domain achieves (auto)phosphorylation of the kinase. The extra- and intracellular domain are parted by the transmembrane region that is anchored in the cell membrane. The ATP-binding cleft is located between the two lobes of the intracellular domain. A schematic representation of the ATP-binding cleft, with its different regions, is shown on the *right side* of the figure. The binding regions of type I and type II tyrosine kinase inhibitors are indicated

has been paid on inhibition of this receptor tyrosine kinase to block formation of new blood vessels in cancer [6]. Anti-angiogenic tyrosine kinase inhibitors that have shown clinical activity in phase I/II clinical trials are listed in Table 1.

Tyrosine kinases and growth factors involved in angiogenesis

The tyrosine kinase VEGFR is a crucial mediator in angiogenesis. The VEGFR family comprises three related receptor tyrosine kinases, known as VEGFR-1, -2, and -3, which mediate the angiogenic effect of VEGF ligands [21]. The VEGF family encoded in the mammalian genome includes five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGFs are important stimulators of proliferation and migration of endothelial cells. VEGF-A (commonly referred to as VEGF) is the major mediator of tumor angiogenesis and signals through VEGFR-2, the major VEGF signaling receptor [20]. A second important growth factor involved in angiogenesis is the platelet-derived growth factor (PDGF). The PDGF family consists of at least four members: PDGF-A, PDGF-B, PDGF-C, and PDGF-D, which bind to two different receptors, known as PDGFR-α and -β [22]. PDGFs facilitate recruitment of pericytes and smooth muscle cells and are important for maturation and stability of the vasculature [23]. Also, basic fibroblast growth factor (bFGF), known as FGF2 as well, has important angiogenic properties. The 18 members of FGF family can be divided into six subfamilies and bind to seven main FGF receptors. FGF2 induces angiogenesis by stimulating migration and proliferation of endothelial cells. Furthermore, it supports proliferation of smooth muscle cells and fibroblasts [24].

Tyrosine kinase signaling in angiogenesis

Stimulation of VEGFRs and other tyrosine kinase receptors causes massive activation of signaling pathways in endothelial cells. Signaling molecules downstream of receptor tyrosine kinases not only include tyrosine kinases, but involves other signaling proteins as well including serine/threonine kinases and G-proteins. Important signaling molecules recruited to tyrosine kinase receptors comprise proteins with a Src homology 2 (SH2) domain. Association of a phosphorylated tyrosine kinase receptor with a SH2 domain-containing protein results in the phosphorylation and activation of this effector protein. In addition, the

Table 1 Anti-angiogenic tyrosine kinase inhibitors in clinical development

Agent	Target	Clinical activity and/or study	Phase of development	Refs
Sunitinib (SU11248; Sutent)	VEGFR-1, -2, -3, PDGFR, KIT, FLT3, CSF-1R, RET	Kidney, breast, prostate, lung, liver, ovarian, colorectal, thyroid, head and neck, gastric, bladder, cervical and pancreatic cancer, GIST, melanoma, glioblastoma, myeloma, lymphoma	Approved for kidney cancer and GIST, phase II or III for other cancers	[7, 9]
Sorafenib (BAY439006; Nexavar)	VEGFR-2, -3, PDGFR, Raf, KIT	Kidney, liver, breast, prostate, lung, ovarian, colorectal, thyroid, head and neck, gastric and pancreatic cancer, GIST, melanoma, glioblastoma, lymphoma, leukemia	Approved for kidney and liver cancer, phase II or III for other cancers	[8, 11]
Pazopanib (GW786034; Votrient)	VEGFR-1, -2, -3, PDGFR, KIT	Kidney, breast, lung, cervical, liver, thyroid, prostate and colorectal cancer, melanoma, glioblastoma	Approved for kidney cancer, phase II or III for other cancers	[99, 100]
Vandetanib (ZD6474; Zactima)	VEGFR-2, EGFR, KIT, RET	Lung, kidney, thyroid, head and neck, prostate, ovarian, breast and colorectal cancer, glioma, neuroblastoma	Phase II or III	[53, 101, 102]
Axitinib (AG013736)	VEGFR-1, -2, -3, PDGFR-β, KIT	Kidney, lung, thyroid, pancreatic, colorectal and breast cancer, melanoma	Phase II or III	[103, 104]
Cediranib (AZD2171; Recentin)	VEGFR-1, -2, -3, PDGFR-β, KIT	Kidney, breast, lung, liver, ovarian, head and neck, prostate and colorectal cancer, GIST, glioblastoma, melanoma	Phase II	[105, 106]
Vatalanib (PTK787; ZK222584)	VEGFR-1, -2, -3, PDGFR-β, KIT	Prostate, colorectal, kidney and pancreatic cancer, melanoma, lymphoma, leukemia	Phase II or III	[107, 108]
Motesanib (AMG706)	VEGFR-1, -2, -3, PDGFR, KIT, RET	Lung, thyroid, gallbladder, breast and colorectal cancer, GIST	Phase II or III	[109, 110]

CSF-1R colony stimulating factor-1 receptor, *EGFR* epidermal growth factor receptor, *FLT3* fms-related tyrosine kinase 3, *GIST* gastro-intestinal stromal tumor, *PDGFR* platelet-derived growth factor receptor, *VEGFR* vascular endothelial growth factor receptor

binding of this SH2 domain-containing protein to the receptor serves as a docking site for other signaling molecules. Phospholipase C-γ (PLCγ) is an SH2 domain-containing protein that is frequently involved in signaling by VEGFRs. PLCγ phosphorylates protein kinase C (PKC) [25], which subsequently phosphorylates a range of kinases. Phosphorylation of MEK (mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinase (ERK) kinase) by PKC stimulates the p42/44 MAPK pathway [26]. Phosphorylated MAPK, a serine/threonine kinase, activates various transcription factors and is known to regulate cell proliferation (Fig. 3a).

Another signaling molecule involved in the MAPK cascade is the growth factor receptor-bound protein 2 (Grb2) [27]. Grb2 contains SH2 and SH3 domains and is able to activate the G-protein Ras via association with the ATP/ADP exchange factor mammalian Son-of-sevenless (Sos) [28]. The Ras protein can bind to and phosphorylate Raf, which in turn can activate the MEK/MAPK pathway. The Ras/Raf pathway is a classical pathway in activation of MAPK and is involved in signaling of many tyrosine kinase receptors, for example, the epidermal growth factor receptor (EGFR). However, activation of the Ras/Raf pathway plays a minor role in VEGFR signaling [29].

An adapter molecule that is important in VEGFR-mediated signaling is the SH2 and β-cells (Shb) protein. Interaction of Shb with a specific phosphorylation site of VEGFR-2 activates phosphatidylinositol 3′-kinase (PI3K). PI3K and its downstream activated serine/threonine kinase Akt/protein kinase B (PKB) are involved in several important processes of angiogenesis, including endothelial cell migration, proliferation, and survival, as shown in Fig. 3b. Activation of Akt/PKB requires generation of phosphatidylinositol 3,4,5-triphosphate (PIP3) by PI3K-mediated phosphorylation of phosphatidylinositol 4,5-biphosphate (PIP2) [30]. Akt/PKB stimulates proliferation and survival by the activation or inhibition of a variety of substrates [31]. Akt/PKB phosphorylates and inhibits the pro-apoptotic protein BAD (Bcl-2 associated death promoter) as well as GSK3 (glycogen synthase kinase 3). Akt/PKB is also able to activate the mammalian target of rapamycin (mTOR) and its downstream p70S6K which are regulators of cell proliferation and survival [32]. In addition, Akt/PKB enhances cellular proliferation through activation of nuclear factor-κB (NF-κB) [33]. Furthermore, Akt/PKB is able to stimulate vasodilation, vascular remodeling, and angiogenesis, through phosphorylation of endothelial nitric oxide (NO) syntheses (eNOS) [34]. Last, but not least, the PI3K pathway seems to be involved in endothelial cell migration [35]. Other signaling molecules that are involved in (endothelial) cell migration are shown in Fig. 3a and include the T-cell specific adaptor (TSAd) protein, p38 MAPK, and the focal adhesion kinase (FAK) [36]. TSAd binds to other phosphorylation site of the VEGFR-2 than PLCγ and Shb. Activated TSAd forms complexes with Src and regulates cell migration and vascular permeability [37]. Interaction of p38 MAPK with a phosphorylation site of the VEGFR-2 mediates actin reorganization and cell migration [38]. Activated FAK is capable of controlling diverse aspects of cell migration,
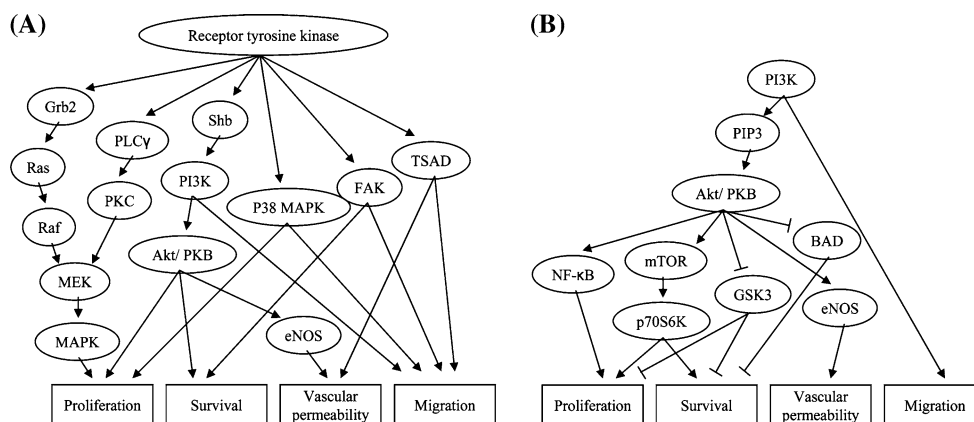

Fig. 3 Signal transduction pathways and biological processes mediated by receptor tyrosine kinases focused on angiogenesis. a A selection of pathways activated by receptor tyrosine kinase involved in angiogenesis is shown. Pathway activation, for example by VEGFR or PDGFR, can result in a variety of angiogenic processes, such as cell proliferation, migration, survival, and vascular permeability. b The phosphatidylinositol 3′-kinase (PI3K) pathway is an important downstream pathway of diverse receptor tyrosine kinases and is involved in various cellular processes in angiogenesis. Akt/protein kinase B (PKB) is activated downstream of PI3K. BAD Bcl-2-associated death promoter; eNOS endothelial nitric oxide synthase; FAK focal adhesion kinase; Grb2 growth factor receptor-bound protein 2; GSK3 glycogen synthase kinase 3; MAPK mitogen-activated protein kinase; MEK MAPK and extracellular-signal-regulated kinase (ERK) kinase; mTOR mammalian target of rapamycin; NF-κB nuclear factor-κB; PIP3 phosphatidylinositol 3,4,5-triphosphate; PKC protein kinase C; PLCγ phospholipase C-γ; p70S6K p70S6 kinase; Shb SH2 and β-cells; TSAd T-cell specific adaptor

including regulation of the cytoskeleton and influences structures of cell adhesion sites [39]. FAK has also been shown to maintain survival signals in endothelial cells [40].

Tyrosine kinase inhibitors

Metabolism of tyrosine kinase inhibitors

Tyrosine kinase inhibitors are small molecules and are in contrast to monoclonal antibodies able to pass through the cell membrane [41]. Monoclonal antibodies can only act on molecules expressed on the cell surface or on secreted molecules. Small-molecule inhibitors are largely hydrophobic and can easily enter the cell where they can interact with the intracellular domain of receptors and intracellular signaling molecules. As a result, small-molecules kinase inhibitors are able to block the activation of various downstream signaling pathways intracellularly. Tyrosine kinase inhibitors can be taken orally, if necessary in a salt form of the inhibitor. For example, sunitinib is taken as sunitinib malate (the malate salt of sunitinib) [42], while sorafinib is taken as tosylate sorafenib (the tosylate salt of sorafenib) [43]. Tyrosine kinase inhibitors are being administered to patients at a fixed dose once or twice daily, because the variability in pharmacokinetics of these agents is not significantly affected by weight [44]. Some of the tyrosine kinase inhibitors are metabolized by the liver primarily by cytochrome-P enzymes. For example, sunitinib is metabolized primarily by the cytochrome P450 enzyme CYP3A4 [42]. The parent compound and active metabolite have similar biochemical activity and potency. The primary metabolite is further metabolized by CYP3A4 to its secondary inactive metabolite [45]. Also, other enzymes are involved in the metabolism of sunitinib. Van Erp et al. [46] investigated polymorphisms genotyped in the pharmacokinetic and pharmacodynamic pathways of sunitinib and studied their association with sunitinib-induced toxicities. Half-life times of sunitinib and its primary metabolite are approximately 40–60 h and 80–110 h, respectively [42]. Elimination of sunitinib is primarily via feces [45].

Sorafenib undergoes oxidative metabolism, mediated by CYP3A4, as well as glucuronidation, mediated by UGT1A9 [43]. Several metabolites of sorafenib have been identified, of which the main circulating metabolite, the pyridine N-oxide, shown potency similar to that of sorafenib in vitro. Sorafenib elimination half-time is between 25 and 48 h, and it is secreted in feces as well as in urine [43].

Modes of tyrosine kinase inhibitor binding

Most small-molecule kinase inhibitors discovered to date compete with ATP. The chemical structure of ATP is
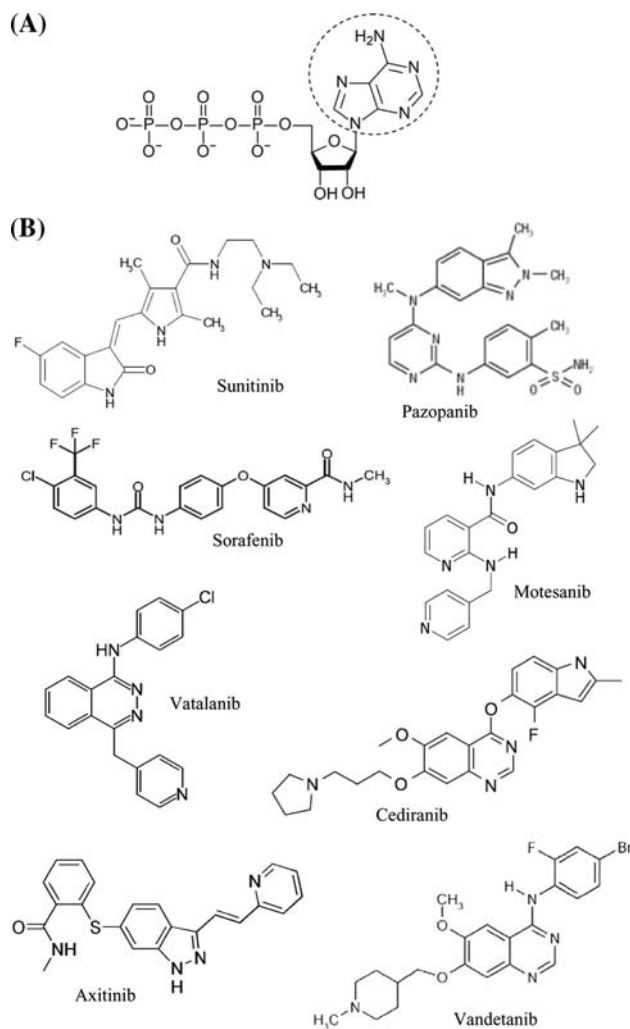

Fig. 4 Chemical structures of ATP and anti-angiogenic tyrosine kinase inhibitors. a Chemical structure of ATP. ATP consists of an adenine ring, a ribose sugar, and three phosphate groups. The adenine ring, which forms hydrogen bonds with the ATP-binding site of its target kinase, is encircled in this figure. b Chemical structures of the anti-angiogenic tyrosine kinase inhibitors sunitinib, sorafenib, pazopanib, vandetanib, axitinib, cediranib, vatalanib, and motesanib. The targets of these inhibitors, their clinical activity, and their phase of development are listed in Table 1

shown in Fig. 4a. ATP consists of adenosine, composed of an adenine ring and a ribose sugar, and three phosphate groups. Binding of ATP to a kinase is characterized by the formation of hydrogen bonds from the adenine ring to the ATP-binding cleft of the kinase. Kinase inhibitors can target (nearby) the ATP-binding site of a kinase. The ATP-binding site is common to all protein kinases, and selectivity of kinase inhibitors is engineered by the chemical structure which is not similar to the ATP structure. The chemical structures of the anti-angiogenic tyrosine kinase inhibitor listed in Table 1 are shown in Fig. 4b. Elements of some compounds can be compared to elements of ATP. For example, the adenine ring of ATP, which forms

hydrogen bonds to the kinase, is in more or lesser similarity seen in pazopanib, vatalanib, and axitinib.

Tyrosine kinase inhibitors can be subdivided in categories. Here, we classify tyrosine kinase inhibitors in three different types and focus on anti-angiogenic tyrosine kinase inhibitors. Type I kinase inhibitors recognize the active conformation of a kinase. They bind to the ATP-binding site by presenting one to three hydrogen bonds which mimic the hydrogen bonds normally formed by ATP [47]. An example of a type I tyrosine kinase inhibitor targeting the VEGF pathway is sunitinib. Sunitinib demonstrated competitive inhibition to ATP against VEGFR-2 (Flk-1) and PDGFR-β [48]. Sunitinib is furthermore a well-known inhibitor of VEGFR-1 and -3, PDGFR-α, KIT, fms-related tyrosine kinase 3 (FLT3), colony stimulating factor-1 receptor (CSF-1R), and RET [7]. It is expected to compete with ATP by presenting several hydrogen bonds to the ATP-binding site [47, 49].

In contrast to type I kinase inhibitors, type II kinase inhibitors recognize the inactive conformation of a kinase. Type II inhibitors indirectly compete with ATP by occupying the hydrophobic pocket which is directly adjacent to the ATP-binding site. This hydrophobic pocket is created by the DFG-out conformation of the activation loop. This unique DFG-out conformation is also known as the allosteric site, and type II inhibitors can modulate kinase activity in an allosteric way. Some type II inhibitors are able to form a hydrogen bond directly to the ATP-binding site, while this is not necessary for functionality [15]. Sorafenib is a type II kinase inhibitor [50] and blocks the phosphorylation of VEGFR, PDGFR, Raf, and KIT by using a hydrophobic pocket to (indirectly) compete with ATP. It binds to its target kinases in an inactive conformation [49].

A third class of kinase inhibitors is known as 'covalent' inhibitors. These inhibitors have been developed to covalently bind to cysteines at specific sites of the kinase. Sulfur (S), present in the cysteine residue, is an electron-rich atom, which reacts with an electrophilic group of the inhibitor. As a result, the inhibitor and the cysteine residue irreversibly bind by sharing electrons. This allows the inhibitor to block binding of ATP to the kinase and prevents activation of the kinase [51]. Examples of covalent tyrosine kinase inhibitors are quinazoline-based inhibitors [52] such as vandetanib (ZD6474, Zactima, AstraZeneca), which in addition to targeting VEGFR, inhibits EGFR [53]. This inhibitor is an anilquinazoline derivate, and inhibits activation of kinases by covalently bind to a cysteine group of the kinase.

Binding regions of type I and type II inhibitors in ATP-binding cleft of the kinase are indicated in Fig. 2. Because covalent inhibitors bind a cysteine residue which can be variably located in the kinase domain, the binding site of this inhibitor type is not illustrated.

Selectivity of tyrosine kinases inhibitors

Many anti-angiogenic tyrosine kinase inhibitors are so-called multi-targeted kinase inhibitors. These agents target a number of different kinases, which are involved in several signaling pathways. It is reasonable to expect that inhibitors of multiple kinases possess a broader efficacy than a single-targeted inhibitor. For example, the VEGF pathway and PDGF pathway both play important roles in angiogenesis. For inhibition of angiogenesis, it is expected that a multi-targeted kinase inhibitor that blocks VEGFR signaling as well as PDGFR signaling will be more effective than an inhibitor that targets only one of these pathways.

On the other hand, inhibitors should be highly selective to minimize treatment-induced toxicities [54]. Toxicities observed in treatment with tyrosine kinase inhibitors are diverse, varying from more general complications like diarrhea and nausea to specific toxicities like hand foot syndrome. Most common toxicities of the anti-angiogenic tyrosine kinase inhibitors sunitinib and sorafenib include hypertension, bleeding, fatigue, diarrhea, nausea and/or vomiting, hand foot syndrome, and myelosuppression [54, 55]. Other toxicities with a low frequency include hypothyroidism, impaired kidney function, and reversible posterior leukoencephalopathy syndrome. In addition, during sunitinib treatment, hemoglobin levels and erythrocyte numbers transiently increase [56]. The anti-angiogenic monoclonal antibody bevacizumab blocks the VEGF ligand and inhibits angiogenesis [57]. Most commonly reported toxicities induced by bevacizumab are hypertension, proteinuria, bleeding, fatigue, and gastro-intestinal symptoms [54, 58, 59]. Since these toxicities highly overlap with toxicities induced by anti-angiogenic tyrosine kinase inhibitors, these toxicities are expected to be related to the targeted (VEGF) pathway. Molecular mechanisms involved in toxicities of targeting the VEGF pathway are discussed by Verheul and Pinedo [13], and by Kamba and McDonald [59].

Tyrosine kinases have a high degree of similarity in the kinase domain. The ATP-binding site is most similar, because of its need to bind ATP for its activity. These binding sites are highly conserved across the kinome. Type I inhibitors invariably occupy the adenine region in the ATP-binding site, and for that reason, it is difficult to develop highly selective type I inhibitors. Inhibitors against less conserved regions of a kinase can be more selective [33]. Selective kinase targeting can be easier achieved within type II inhibitors, because more variability is seen among kinases in their inactive conformation [60]. Covalent inhibitors should be very selective due to their irreversible mechanism of binding to a cysteine residue of the

target kinase. Unexpected targets of covalent inhibitors could result in serious toxicities.

Selectivity of kinase inhibitors is hard to predict when only based on structure and sequence. Specificity profiles of kinase inhibitors can be analyzed by assessment of binding affinities. Fabian and colleagues reported inhibitor–kinase interaction maps for a number of kinase inhibitors, including sunitinib, vatalanib, and vandetanib [61]. The dissociation constant (K_d) was used to describe the binding affinity; the smaller the dissociation constant, the higher the affinity of the inhibitor to a kinase. Selectivity varied largely among agents that are presumed to target the same kinase. Vatalanib specifically binds VEGFR-2, while sunitinib binds many additional kinases. The binding affinities of different inhibitors vary substantially with dissociation constants of less than 1nM to some target kinases, to 1–10 μM to 'non-target' kinases. Although some inhibitors show (low) affinity to a large proportion of kinases, these assays measure *binding affinity*, and no conclusions can be drawn on *activity* of the inhibitor. It is important to realize that a low binding affinity of a tyrosine kinase inhibitor to a certain kinase may have a crucial impact on cell signaling, while the same inhibitor with a high binding affinity to another kinase may have no significant intracellular downstream effect. Not only inhibition of a specific tyrosine kinase, but also the expression level of a kinase and the number of potential parallel pathways (other kinases) determine the downstream biological efficacy of a tyrosine kinase inhibitor. It is important to get more insight into the relative importance of targeted kinases and the alternative activity routes of kinases in cancer. Several approaches to determine kinase activities in tumor samples are being explored in preclinical studies and in the clinical setting [62–64]. We expect that high throughput analyses of kinome activity profiles in tumors from patients can be used to select specific kinase inhibitors for treatment of patients, so-called personalized medicine.

Drug resistance

Drug resistance in patients treated with anti-angiogenic therapies is an important clinical problem [65]. Tumors may acquire resistance during anti-angiogenic treatment or show intrinsic resistance. The majority of patients transiently benefits from anti-angiogenic therapy, before a tumor recovers and starts to grow again and forms metastases. A small fraction of patients fails to show even initial clinical benefit [12].

One possible mechanism involved in treatment resistance might be the excess of signaling pathways which are involved in angiogenesis. Although VEGF-mediated signaling is the predominant stimulator of angiogenesis in cancer, parallel angiogenic pathways also drive tumor growth. Activation of these pathways may overcome inhibition by anti-angiogenic tyrosine kinase inhibitors. For example, Delta-like 4 (Dll4)-mediated Notch signaling represents an important pathway in angiogenesis, and inhibition of this pathway results in excessive, non-productive angiogenesis and in reduced tumor growth [66]. It has been suggested that Dll4/Notch signaling might be involved in resistance to anti-VEGF therapy [67], and that this pathway might be responsible for the escape from anti-angiogenic therapy. Also, the Tie receptors, together with their two major ligands, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), are alternative pathways to induce biological responses involved in angiogenesis, such as vessel maturation [68]. The PI3K/Akt pathway is an example of a downstream signaling pathway of VEGFR, which can also be activated by angiopoietin-Tie signaling [29]. Inhibition of VEGFR-mediated pathways might not be sufficient to completely inhibit signaling pathways involved in angiogenesis, and as a result, tumors are able to grow and progress despite inhibition of the VEGF pathway.

VEGF was discovered as an endothelial cell mitogen and functions as an angiogenesis stimulator [69]. This suggested that VEGF acts by binding to receptors present on endothelial cells. De Vries et al. [70] determined fms-like tyrosine kinase (Flt-1) as a receptor for VEGF, nowadays also known as VEGFR-1. Fetal liver kinase-1 (Flk-1), the mouse homologue of kinase insert domain-containing receptor (KDR), was shown to be a second functional VEGF receptor and was demonstrated to play a role in angiogenesis [71–73]. Quinn et al. [72] demonstrated that Flk-1 in the mouse embryo exclusively is expresses in the vascular endothelium and the umbilical cord stroma. Nowadays, stimulation of VEGFR on (tumor) endothelium by VEGF is well known. However, VEGFRs may also be present on tumor cells, as has been shown by several studies listed by Hicklin and Ellis [21]. For that reason, it could be hypothesized that VEGF is also able to stimulate tumor cells expressing VEGFRs. Inhibitors of VEGFR may not only restrain tumor growth by the inhibition of tumor angiogenesis, but exert additional inhibitory effects on tumor cells [74]. We have recently found that the anti-angiogenic tyrosine kinase inhibitor sunitinib inhibits tumor cell proliferation and clonogenic capacity directly [75]. Therefore, acquired resistance may also be a consequence of alternative signaling of tumor cells including the production of alternative angiogenic growth factors.

Resistance to kinase inhibitors as result of a mutation in the target kinase in tumor cells is a well-known mechanism and is described for inhibitors such as gefitinib and erlotinib. These two inhibitors target the epidermal growth factor receptor (EGFR) and are used for treatment of patients with non-small cell lung cancer and several other

types of cancer. Somatic activating mutations in the EGFR have been associated with sensitivity to these agents [76, 77]. Despite clinical responses to these inhibitors, most patients acquire resistance during treatment. One mechanism of acquired resistance is a specific secondary mutation in the EGFR. In the presence of this secondary mutation, the kinase inhibitors are unable to inhibit phosphorylation of the target kinase [78].

Initially, resistance to anti-angiogenic tyrosine kinase inhibitors was not expected, because these agents were supposed to target endothelial and other stroma cells which are genetically stable and therefore unlikely to develop mutations. However, now, we know that anti-angiogenic tyrosine kinase inhibitors may also inhibit tumor cells directly, mutations in target receptors are more likely to occur and should be explored as possible mechanisms of resistance. Recently, several studies reported mutations in target kinases that correlate with resistance to sunitinib in imatinib-resistant gastro-intestinal stromal tumors (GISTs) [79–82]. Heinrich et al. [79] determined mutational status of KIT and PDGFR-α in tumors of patients with metastatic, imatinib-resistant or intolerant GISTs. They reported that primary and secondary mutations in these kinases influence sunitinib activity. Clinical benefit and objective response rates with sunitinib were higher in patients with primary KIT exon 9 mutations than with exon 11 mutations. In vitro, sunitinib activity against KIT double mutants was dependent on location of the second mutation. The PDGFR-α D842V mutant conferred resistance to imatinib as well as to sunitinib in in vitro experiments. Nishida et al. analyzed KIT mutations in patients with imatinib-resistant GISTs, who had been treated with sunitinib [80]. They reported that the pre-imatinib sample had KIT mutations in exon 9 or exon 11 ($n = 8$), and most imatinib-resistant tumors carried a secondary mutation. Most patients with a secondary mutation in exon 13 or 14 (the ATP-binding domain) obtained clinical benefits from sunitinib, while most tumors with a secondary mutation in exon 17 (the activation loop) showed resistance to the drug. All secondary (and tertiary) mutations were located on the same allele as the primary mutation, so-called *cis*-mutations. Also, Guo et al. [81] investigated mutations in KIT conferring sunitinib resistance in GIST. They found that secondary mutations in the KIT activation domain are associated with sunitinib resistance after initial response to the drug. Gajiwala et al. [82] investigated the molecular basis of resistance to sunitinib in GIST. They reported that the KIT mutants D816H and D816V undergo a change in conformational equilibrium. The conversion from the inactivated kinase conformation to the active conformation results in a drug-insensitive active form and causes loss of inhibition.

Toxicities of tyrosine kinase inhibitors

Toxicities of anti-angiogenic tyrosine kinase inhibitors might be due to inhibitors with multiple so-called 'off-targets'. However, selective inhibitors may also induce toxicities, because their target kinases are not differentially expressed by endothelial cells. Angiogenesis inhibitors are intended to target activated tumor endothelium. Initially, these agents were not expected to target normal vasculature, because most blood vessels remain quiescent during adulthood [19]. However, under normal physiological circumstances, growth factor signaling in endothelial cells seems important for their survival and maintenance of vascular integrity. Inhibitors of angiogenesis are capable of affecting signaling pathways in endothelial cells and might elicit toxicities as a result of decreased endothelial cell renewal capacity [83].

Apart from kinase inhibitors with 'off-targets' activities, downstream signaling pathways of target kinases may also be involved in the development of toxicities. As shown earlier, one kinase is able to activate several downstream signaling pathways. By inhibition of a kinase with a tyrosine kinase inhibitor, a whole spectrum of signaling pathways can be deactivated. This might result in a reduction in specific biological outcomes which are not intended to adjust. It has been shown that specific kinases are involved in the normal physiology of certain organs such as kidneys and the thyroid gland. It has been suggested that specific toxicities, like nephrotic syndrome and fatigue, might be related to interference of these inhibitors with the normal function of these organs [84, 85]. Furthermore, bleeding complications (including subungual bleedings) and wound healing problems may be caused by a disturbance of the close interaction of platelets with the vasculature [13]. In preliminary experiments, we have found that platelet function is disturbed by anti-angiogenic kinase inhibitors (Walraven et al., preliminary results). Another factor involved in toxicity might be an altered pharmacodynamic effect of sunitinib treatment due to certain gene variances as recently reported by Van Erp et al. [46]. These investigators analyzed in a group of 219 patients treated with single agent sunitinib a total of 31 single nucleotide polymorphisms (SNPs) in 12 candidate genes, together with several non-genetic variants and found a correlation between sunitinib-induced leucopenia and SNP-variants. We expect that genetic analyses will be included in routine screening before start of treatment with kinase inhibitors to predict for treatment-related toxicity. Based on this type of analyses, dose adjustments or alternative kinase inhibitor treatment options can be considered.

Optimal treatment strategy: multi-targeted inhibitors and combining inhibitors

Resistance is less likely to arise if multiple regulatory molecules are being targeted at the same time [86]. Multi-targeted kinase inhibitors are able to target various signaling molecules. This may inhibit multiple parallel downstream signaling pathways or enhance inhibition of one specific shared signaling pathway downstream of several signaling molecules. Moreover, a drug with multiple so-called 'off-target' effects binds less precisely to its target kinases and is for that reason less sensitive for dislodging due to a mutation of the target kinase [87].

A decreased incidence of resistance or a delay in its development may also be achieved by combining agents [88]. Anti-angiogenic tyrosine kinase inhibitors can variously be combined. First, anti-angiogenic tyrosine kinase inhibitors can be combined with other anti-angiogenic agents. Tyrosine kinase inhibitors could be combined to simultaneously inhibit multiple-linked signaling pathways. Besides 'horizontal' inhibition of signaling pathways, inhibition in a 'vertical' strategy could enhance therapy efficacy; drugs are combined to inhibit a cascade of signaling molecules [74, 89, 90]. By inhibition in a 'vertical' strategy, feedback loops in the network of signaling pathways may be circumvented. Feedback loops are important regulators of signaling pathway [90] and might be involved in unexpected and undesirable outcomes of targeting therapies. For example, feedback loops might be involved in the (absence of) response to sorafenib therapy in melanomas. Although sorafenib is developed as a Raf inhibitor, some discussion is going on whether sorafenib does certainly inhibit Raf. Activating mutations of B-Raf are present in approximately 70% of the human melanomas [91, 92]. These activating B-Raf mutations may result in an increased activity of the downstream protein MAPK. In human melanoma cell lines, it is shown that apoptosis is increased when B-Raf expression is down-regulated using RNA interference, suggesting that B-Raf is a therapeutic target in melanomas [93]. However, in a phase II clinical trial, it is found that sorafenib has little or no anti-tumor effect in patients with advanced melanoma [94]. In addition, the addition of sorafenib to carboplatin and paclitaxel (CP) did not improve therapy outcomes compared to CP and placebo [95]. It is possible that sorafenib is not strong enough to inhibit (all) the activated mutant B-Raf, although it shows an IC50 of 38nM to the oncogenic B-Raf V600E [8]. Another possibility is that several feedback loops downstream of B-Raf compensate for the B-Raf inhibition. Downstream proteins of B-Raf, for example MAPK, might still be activated by feedback loops, while B-Raf itself is inhibited. Targeting a cascade of signaling molecules might avoid downstream responses to be still activated through triggering of feedback loops.

Targeting a cascade of signaling molecules could also be helpful when it is unknown which proteins are important in the development of resistance to targeted therapy. Kinases with a high expression level are not necessary 'key' kinases of signaling pathways. Kinases with a low expression level can have a high activity and may be crucial for a signaling pathway. Defining key kinases in a signaling pathway might improve the use of targeted therapies, but it is hard to define which protein is most important. By targeting several proteins in the same signaling pathway, this problem may partly be avoided. As shown earlier, downstream signaling pathways of receptor tyrosine kinases include not only tyrosine kinase but also serine/threonine kinases. For that reason, combining tyrosine kinase inhibitors with serine/threonine inhibitors might be a worthwhile strategy, as well as combining anti-angiogenic tyrosine kinase inhibitors with the anti-VEGF monoclonal antibody bevacizumab.

A second strategy to combine anti-angiogenic agents comprises the combination with other anti-cancer therapeutics, for example conventional cytotoxic chemotherapy. It is hypothesized that anti-angiogenic agents can induce vessel normalization of the structurally and functionally abnormal tumor vasculature [96]. This may result in improved delivery of cytotoxic drugs and oxygen to the tumor and could enhance the efficacy of chemotherapy or radiation therapy [97]. In addition, it is suggested that chemotherapy and radiation therapy may also directly damage endothelial cells, and thus may enhance anti-angiogenic effects [21, 97]. Furthermore, combining anti-angiogenic agents with chemotherapy potentially delays the development of resistance to anti-angiogenic drugs as well as to chemotherapy [96, 98]. The monoclonal antibody bevacizumab is in combination with chemotherapy approved for several (metastatic) cancers. Currently, many clinical trials are ongoing to study the combination of anti-angiogenic tyrosine kinase inhibitors with chemotherapy or radiation therapy.

Of course, one should realize that combination of different inhibitors or generating broader targeting inhibitors will induce more toxicity as well. Therefore, analyses of the tumor kinome profile (genetically or with real activity analyses) from each single patient by high throughput analyses may provide specific kinome profiles that should be targeted at once. We expect that combination of currently available or newly designed specific inhibitors may be prescribed based on these kinome profiles as a standard way of personalized treatment.

Conclusions and perspectives

Targeting angiogenesis to inhibit tumor growth has been developed as a new anti-cancer treatment strategy in the past few decades. Anti-angiogenic therapies show clinical efficacy in diverse cancer types. Since many different regulatory factors and signaling pathways are involved in angiogenesis, therapies targeting angiogenesis are quite susceptible for causing toxicity and drug resistance. With respect to the excess of signaling pathways downstream of certain kinases, selective tyrosine kinase inhibitors have some advantages in order to minimize the induction of toxicities. On the other hand, multi-targeted kinase inhibitors, or a combination of inhibitors, may target additional angiogenic pathways and may carry out a broader efficacy and may avoid resistance. It is important to get more insight into the signaling pathways that are modified by the use of (anti-angiogenic) kinase inhibitors. A better understanding of patient-specific kinomes including alterations in signaling pathways may circumvent treatment-induced resistance and toxicities of anti-angiogenic agents leading to improved clinical benefit.

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