

# Understanding and targeting resistance to anti-angiogenic therapies

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**Abstract:** Therapies targeting tumor angiogenesis are used in a variety of malignancies, however not all patients benefit from treatment and impact on tumor control may be transient and modest. Mechanisms of resistance to anti-angiogenic therapies can be broadly categorized into VEGF-axis dependent alterations, non-VEGF pathways, and stromal cell interactions. Complimentary combinations of agents that inhibit alternative mechanisms of blood vessel formation may optimize inhibition of angiogenesis and improve clinical benefit for patients. The purpose of this review is to detail the preclinical evidence for mechanisms of angiogenic resistance and provide an overview of novel therapeutic approaches exploiting these pathways.

**Key Words:** Cancer; angiogenesis; resistance; VEGF; vascular endothelial growth factor; colorectal cancer



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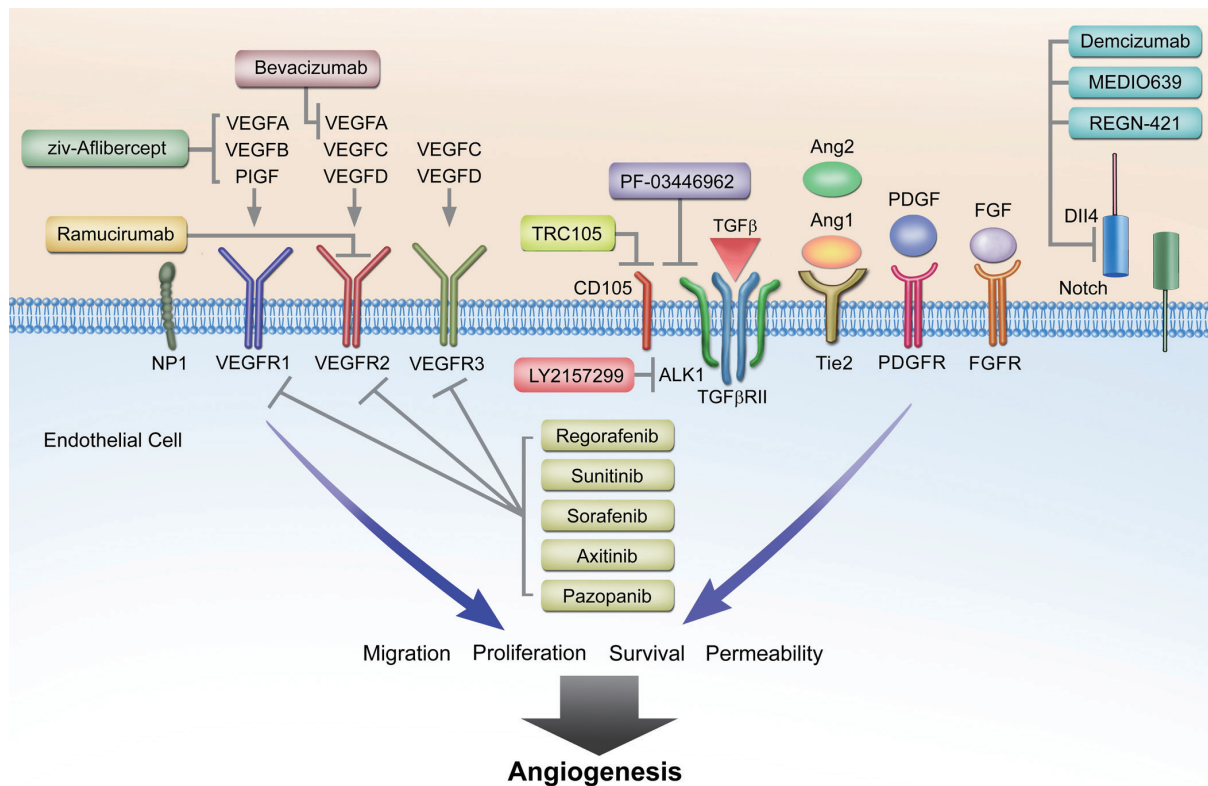
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## Introduction

Therapies targeting angiogenesis are an integral modality of modern anti-tumor treatment for a number of malignancies, in particular metastatic colorectal cancer (CRC). Broadly, tumor angiogenesis relies on a highly complex program of endothelial cell migration and proliferation, growth factor signaling, extracellular matrix remodeling, and stromal cell interaction. The primary proangiogenic driver of this process is VEGF, also known as VEGF-A. The VEGF family includes 5 ligands, VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor (PlGF), three receptors, VEGFR1 (fms-like tyrosine kinase 1/Flt-1), VEGFR2 (Flk-1/KDR), and VEGFR3 (Flt-4), and 2 co-receptors neuropilin 1 and 2 (NRP1/2). All of these receptors and co-receptors are expressed on endothelial cell, although they may also be present on other cells. VEGFR1 binds to VEGFA, VEGFB, and PlGF, while ligands for VEGFR2 include VEGFA as well as VEGFC and VEGD. VEGFR2 is widely considered the primary receptor mediating angiogenesis; and VEGFR1 and VEGFR3 are classically involved in monocyte chemotaxis, hematopoietic stem cell survival, and lymphangiogenesis, respectively (1). Currently, the most common approaches to inhibition of the VEGF axis include: binding of VEGF ligands (i.e., using a monoclonal antibody or soluble receptor), small molecular inhibition of receptor tyrosine kinase

(RTK) and downstream targets, and steric blockade of the VEGFRs (using a monoclonal antibody). FDA approved agents with anti-VEGF properties include bevacizumab, ziv-aflibercept, and multiple small molecule RTK inhibitors (i.e., sorafenib, sunitinib, pazopanib, axitinib, cabozantinib, and regorafenib). Bevacizumab, ziv-aflibercept, and regorafenib are all approved for use in metastatic CRC.

Over the past three decades, a number of additional complementary angiogenic pathways have been described (2,3). These pathways rely on key proteins such as hypoxia inducible factor (HIF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), angiopoietin (Ang), and Notch, along with various inflammatory mediators of angiogenesis. Attention has shifted in recent years to non-VEGF mechanisms of blood vessel formation in the context of understanding resistance to anti-angiogenic therapies. For example in the setting of bevacizumab, not all patients derive clinical benefit from treatment, and duration of response can be highly varied. Furthermore, clinical gains in overall survival have been quite modest in several different malignancies including breast and non-small cell lung cancer (NSCLC). Alterations in critical angiogenic pathways likely provide an explanation for the heterogeneity in clinical outcomes with VEGF-axis directed therapies. Angiogenic resistance mechanisms can be generally categorized into VEGF-



**Figure 1** VEGF-axis dependent and non-VEGF mediated mechanisms of resistance to anti-angiogenic therapies. Non-VEGF axis receptors include TGF- $\beta$  receptor, Tie2, PDGFR, FGFR, and Dll4-Notch. General mechanisms of action for ziv-Aflibercept, bevacizumab, ramucirumab, and VEGFR activity of RTK inhibitors are depicted. Novel targeted therapies include TRC105, PF-03446962, LY2157299, demcizumab, MEDIO639, and RGN-421 inhibiting TGF- $\beta$  and Dll4-Notch signaling

axis dependent alterations, non-VEGF pathways, and stromal cell interactions (*Figure 1*). These broad categories are not mutually exclusive, and given the coordination of both physiological and pathological angiogenesis, multiple factors and pathways are likely to be relevant in any given patient. The purpose of this review is to detail the preclinical evidence for mechanisms of angiogenic resistance and provide an overview of novel therapeutic approaches exploiting these pathways.

### VEGF-axis dependent resistance mechanisms

VEGFA is upregulated in most malignancies and in response to the administration of anti-angiogenic therapies. In the setting of VEGFA inhibition by bevacizumab; however, PIGF, VEGFB, VEGFC, and VEGFD may contribute to VEGFR signaling and ultimately tumor angiogenesis. In patients with mCRC receiving FOLFIRI with bevacizumab, plasma levels of PIGF, VEGFC and VEGFD were elevated prior to or at time of disease progression (4,5). The role of PIGF in tumor blood vessel formation remains controversial

with much evidence published showing both pro- and anti-angiogenic effects in preclinical studies (6,7). Likewise, while VEGFB is upregulated in multiple malignancies including colorectal cancer, the precise function of VEGFB in tumor angiogenesis remains undefined, but may involve promoting tumor cell migration (8). Ziv-aflibercept (Zaltrap, VEGF-Trap) is composed of the extracellular domains of VEGFR1 and VEGFR2 linked to an IgG1 backbone. Ziv-aflibercept binds not only VEGFA but also VEGFB and PIGF. At this point, it is not known if binding VEGFB and PIGF are important to the efficacy or toxicity of ziv-aflibercept. In patients with refractory mCRC with prior oxaliplatin-based therapy, ziv-aflibercept with FOLFIRI resulted in improved overall survival (HR 0.82; 13.5 *vs.* 12.1 months) compared to chemotherapy alone (9). However, a recent study of ziv-aflibercept with docetaxel in patients with metastatic non-small cell lung cancer failed to meet its primary end point with overall survival (10,11).

In the phase III MAX trial of patients with metastatic colorectal cancer receiving capecitabine and mitomycin with or without bevacizumab, levels of VEGF-D measured

by IHC in formalin fixed paraffin embedded tumor samples predicted for benefit from bevacizumab (12). Parent VEGF-D binds to VEGFR-3, however proteolytically processed VEGF-D binds with high affinity to VEGFR2 promoting angiogenesis, and potentially bypassing VEGF-A inhibition (13). VEGFR2 blockade by ramucirumab, for example, would theoretically be sufficient to block processed VEGFD activity on VEGFR2, but not activation of VEGFR3 signaling. Likewise, VEGFC is strongly implicated in angiogenesis and tumor progression in preclinical models, and has also been implicated in resistance to anti-VEGFA directed therapies (14). Importantly, while VEGFR3 is classically described as contributing to lymphangiogenesis, it is expressed on tumor endothelial cells and is involved in angiogenic sprouting and endothelial cell proliferation. *In vivo* stimulation of VEGFR3 is capable of sustaining angiogenesis in the setting of VEGFR2 inhibition; and combined VEGFR2 and VEGFR3 blockade demonstrates additive inhibitory effects on tumor growth (15). Taken together, approaches utilizing novel combinations to account for the proangiogenic effects of VEGFC and VEGFD on both VEGFR2 and VEGFR3 should be considered with future anti-angiogenic regimens.

### Non-VEGF modulators of angiogenesis

The FGF family of growth factors is an important and potent mediator of tumor angiogenesis (16). In some model systems, FGF2 or bFGF has even greater proangiogenic effect than VEGFA, and acts synergistically with VEGFA to induce angiogenesis via endothelial cell proliferation, survival, and migration (17). Importantly, combinations of anti-VEGF and anti-FGF agents also act synergistically to inhibit angiogenesis and tumor growth (18). The interplay between FGF and VEGF signaling is likely mediated through multiple mechanisms including upregulation of NRP1 and hypoxia-inducible factor 1 (HIF1) resulting in increased VEGF signaling (19,20). Preclinical models demonstrate that FGF2 levels increase with VEGF-axis inhibition, and FGF blockade reduces tumor growth in anti-VEGF resistant *in vivo* models (21,22). Kopetz *et al.* showed that plasma FGF levels, along with PDGF, increased prior to disease progression in patients with metastatic colorectal cancer receiving FOLFIRI with bevacizumab (4). Similar temporal changes in circulating FGF2 levels in response to VEGF axis inhibition and disease progression have been documented in glioblastoma patients as well (23). Based on the results by Kopetz *et al.* and others, PDGF may also contribute along with FGF to the proangiogenic milieu implicated in VEGF resistance. PDGF is known to be involved in pericyte recruitment and tumor vessel

coverage, as well as endothelial cell function (24). Additionally, VEGFA and FGF2 signaling results in upregulation of PDGF and PDGFR expression on endothelial cells (25), while combined VEGFR2 and PDGF inhibition is sufficient to overcome anti-VEGF resistance *in vivo* using murine tumor xenografts (26).

PDGFR activity is common in most currently approved RTK inhibitors, however a growing number of novel agents in early phase trials demonstrate activity against FGFR in addition to VEGFR. Brivanib (BMS-582664) has been evaluated in combination with cetuximab in patients with metastatic colorectal cancer; despite improvement in PFS, however OS was unchanged compared to cetuximab alone (27). Dovitinib as well is undergoing phase III evaluation in metastatic renal cancer, and several phase II studies in colorectal cancer and other malignancies are actively recruiting patients (NCT01676714). Several other RTK inhibitors with FGFR activity are also being evaluated including AZD4547 and Nintedanib in phase I and II trials, however no results in colorectal cancer patient populations have been reported. Combined VEGFR and PDGFR blockade using sunitinib has been evaluated recently in metastatic CRC patients. Unfortunately, combination sunitinib with or without FOLFIRI failed to improve PFS (28).

Delta-like ligand 4 (Dll4), a ligand for Notch, is expressed on arterial endothelial cells surfaces and upregulated in multiple malignancies. Together, Dll4 and Notch have been implicated in anti-angiogenic resistance, specifically with VEGFA targeted therapies (29,30). Dll4 and Notch are upregulated by VEGFA, and under physiologic conditions act as a negative feedback mechanism for vessel sprouting and angiogenesis (30). Paradoxically, inhibition of Dll4 in tumor models results hypervascularity with abnormal vessels, reduced perfusion and improved tumor growth inhibition (31,32). Interestingly, upregulation of Dll4 induced bevacizumab resistance, and was in turn overcome by Notch inhibition with dibenzazepine, a  $\gamma$ -secretase inhibitor (33) (which in inhibits Notch signaling). *In vivo* inhibition of Dll4 in pancreatic and ovarian tumor xenografts results in potent growth inhibition (34,35). Hu *et al.* also demonstrated that tissue Dll4 levels were predictive of clinical outcomes and response to anti-VEGF treatment in patients with ovarian cancer. Furthermore, Dll4 downregulation with siRNA in combination with anti-VEGF therapy resulted in greater tumor growth inhibition than with each agent alone (35).

Multiple phase I and II studies are ongoing evaluating novel Dll4 inhibitors. Demcizumab (OMP-21M18), a monoclonal antibody targeting Dll4, is now being evaluated in phase II clinical trials. The phase I results have not yet been reported, but phase II studies in combination with

chemotherapies are currently enrolling for pancreatic cancer, metastatic colorectal cancer, and NSCLC patients (NCT01189942, NCT01189929, NCT01189968). Promising preclinical results showing promotion of hypervascularity with mural cell coverage have been demonstrated for MEDI0639, consistent with Dll4-Notch disruption (36). Phase I studies in patients with advanced solid malignancies are ongoing as well for MEDI0639 and REGN-421. The efficacy of  $\gamma$ -secretase inhibition is also being tested, given promising phase I results with R04929097 and MK-0752 (37,38).

The Angiopoietin (Ang)-Tie axis plays an integral role in tumor blood vessel development as well. Both Ang1 and Ang2 are upregulated in numerous malignancies including non-small cell lung, gastric, and colorectal carcinomas (39). However, each ligand has differential effects on the Tie2 signaling, which is typically localized to activated tumor endothelium. Ang1 binds Tie2 resulting in decreased vascular permeability and promotion of vessel maturation and stabilization. Ang2, on the other hand, antagonizes Ang1 and induces neovascularization by destabilizing endothelial cell-pericyte junctions and promotes endothelial cell survival, migration, and proliferation (40). Accordingly, it is well established that higher ratios of Ang2 to Ang1 levels predict worse clinical outcomes (41-43). While some controversy exists regarding the effect of Ang-Tie on tumor angiogenesis in specific models, the effect of, at least, Ang2 signaling appears to be highly dependent on other proangiogenic cytokines being present, such as VEGFA (39,40,44). Indeed, ectopic Ang2 expression interferes with VEGFR2 blockade, and combined inhibition of Ang2 and VEGFA produces greater reduction in angiogenesis in preclinical models (45-47).

A number of novel agents targeting the Ang-Tie axis are currently in clinical development (48). Most notably, Regorafenib, a multi-target RTK inhibitor with VEGFR1-3 and Tie2 activity, demonstrated efficacy in the 3<sup>rd</sup> line setting for both metastatic colorectal cancer and gastrointestinal stromal tumor (49,50). Trebananib (AMG386) is a peptide-Fc fusion protein that inhibits the interaction between Ang1/2 and Tie2, and has demonstrated tolerability but mixed efficacy in phase II trials (51-54). Several phase III studies ongoing are evaluating combination trebananib with paclitaxel, carboplatin, or pegylated liposomal doxorubicin. CovX-060 (PF04856884) is an Ang-2 specific peptide linked to IgG, which demonstrated safety in phase I trials and is being evaluated in patients with metastatic renal cell carcinoma (NCT00982657) (55,56). REGN-910 and MEDI-3617 are both Ang2 specific monoclonal antibodies, which are currently in phase I development (NCT01248949, NCT01688960, NCT01271972).

As cell proliferation and tumor growth outstrips blood vessel supply of oxygen, tumor cell hypoxia becomes a key driver of angiogenesis. Upregulation of the transcription factor, HIF-1, is central in the cellular response to reduced oxygen tension, and has multiple downstream effects including promotion of VEGFA, VEGFRs, PIGF, Ang1/2, and PDGF (57). Furthermore, HIF-1 activation is intimately involved in promoting cell survival, endothelial cell migration, anaerobic metabolism, and metastasis; and elevated tissue levels correlate with worse prognosis in a number of malignancies (58,59). Indeed, in colorectal cancer patients, HIF-1 levels are an independent predictor of poor survival (60). Extensive preclinical evidence for both direct and indirect strategies to inhibit of HIF-1 activation has been published (61). Approaches to indirect inhibition of HIF-1 have focused on blockade of factors mediating response to hypoxia including PI3-kinase, insulin-like growth factor, and mTOR (57). EZN-2968, an antisense oligonucleotide, which blocks HIF-1 alpha mRNA and is currently in a phase I development (NCT01120288). Combinations of VEGF and mTOR inhibitors have to date been unsuccessful, including in colorectal cancer (62-65).

TGF- $\beta$  is another regulator of endothelial cell function and angiogenesis. TGF- $\beta$  is a ligand for type II TGF- $\beta$  receptors and CD105 (endoglin), which form heterotetrameric complexes with type I receptors, resulting in an intracellular signaling cascade via phosphorylation Smad proteins 1/5/8 (66). Activin receptor-like kinase 1 (ALK-1) is a type I TGF- $\beta$  transmembrane receptor with restricted expression to proliferating endothelial cells and is critical to TGF- $\beta$  mediated angiogenesis (66). ALK-1 is present on the vascular endothelium and circulating endothelial cells in numerous malignancies including, breast, prostate, renal cell, and colorectal carcinomas (67). Following phosphorylation of Smads 1/5/8, upregulation occurs of multiple genes including Id1, stimulating endothelial cell proliferation, migration, and tubule formation and culminating in the activation phase of angiogenesis (68). CD105 is also expressed on proliferating endothelial cell surfaces and modulates angiogenesis through TGF- $\beta$  signaling via ALK-1 and SMAD proteins (69). In addition to being heavily overexpressed on tumor vessel endothelium (70), increased CD105 expression correlates inversely with clinical outcome in a variety of malignancies, including colorectal cancer (71). Intriguingly, VEGF blockade increases CD105 expression in multiple preclinical models, suggesting a role for CD105 in circumventing anti-VEGF therapy (72). The intimate role TGF- $\beta$  with ALK1 and CD105 in endothelial cell function, highlights a promising avenue of exploitation for targeted inhibition of tumor angiogenesis.



Several TGF- $\beta$  inhibitors are currently being developed in clinical trials. PF-03446962 is a IgG2 monoclonal antibody with potent specificity against human ALK-1 (67). Interestingly, melanoma xenografts with acquired overexpression of human VEGF-A and RTK-inhibitor resistance, demonstrate increased tumor growth inhibition when bevacizumab is used in conjunction with PF-03446962, thus suggesting ALK-1 signaling is involved in bevacizumab resistance. Phase I trials are currently ongoing evaluating the safety profile of PF-03446962 in patients with all type of cancers (NCT00557856, NCT01337050). TRC105 is a novel IgG monoclonal antibody directed at CD105, and has been well tolerated in the initial phase I study (73). Several phase I/II studies are ongoing using TRC105 in combination with RTK inhibitors and bevacizumab (NCT01332721, NCT01306058). Finally, LY2157299 is a small molecular inhibitor with a pyrazole structure and specificity for TGF- $\beta$  type I receptors (74). LY2157299 safety has been reported in two clinical trials in the past several years (75,76). Multiple phase II studies are ongoing in patients with advanced pancreatic cancer, recurrent glioma, and hepatocellular carcinoma (Table 1).

### Stromal dependent mechanisms of resistance

The contribution of various cells in the tumor stroma to angiogenesis is well established (77). Bone marrow derived cells (BMDCs) are comprised of both endothelial and pericyte progenitors, as well as proangiogenic, tumor infiltrating immune cells (78). Circulating, Flk1+ (VEGFR2+) endothelial progenitors in particular deposit into the vessel lumen at sites of active angiogenesis *in vivo* and contribute to vessel formation (79,80). Disruption of endothelial cell progenitor function results in decreased angiogenesis of tumor xenografts and increased animal survival (81). Furthermore, anti-VEGFR2 monoclonal antibodies reduce endothelial progenitor cells in murine models and inhibit tumor growth (82). Additionally, resistance to tumor vascular disrupting agents is mediated by endothelial progenitors and can be overcome by VEGFR and PDGFR inhibition (83). Increased levels of circulating VEGFR2+ BMDC progenitors are associated with worse overall survival compared to low levels in patients with advanced cancer (84). Accordingly, suppression of endothelial cell progenitors may be one of the underlying mechanisms of anti-VEGF therapies.

A number of distinct immune cells are also recruited to the tumor microenvironment by secreted cytokines (including G-CSF, PlGF, stromal derived factor 1 $\alpha$ ) and release proangiogenic factors which influence resistance to anti-VEGF therapies (77,85,86). For example, tumor

infiltration by CD11b+Gr1+ myeloid cells is associated with anti-VEGF resistance; recruitment of these cells confers resistance by release of the proangiogenic factor Bv8 (87,88). Tie2 expressing monocytes or macrophages are physically associated with tumor vessels as well in a number of malignancies, and promote angiogenesis via paracrine release of factors including VEGFA (89,90). Ang2 secreted by tumor cells alters the genetic phenotype of Tie2+ monocytes/macrophages and increases expression of multiple proangiogenic genes (91). Indeed, inhibition of Ang2 signaling is effective at decreasing tumor growth and angiogenesis by impeding upregulation of Tie2 and association of Tie2+ cells with blood vessels (92). Future strategies aimed at mediating the inflammatory cytokines driving recruitment of various BMDCs and blunting proangiogenic signals are a promising avenue of research.

### Biomarkers

Given the prominent use of anti-angiogenic agents in colorectal cancer and other malignancies today, predictive biomarkers are urgently needed in order to maximize clinical benefit, decreased unnecessary drug toxicity, and improve costs of cancer care. Biomarkers to predict benefit and guide use of therapies targeting angiogenesis can be measured at baseline (pretreatment) or by relative change during treatment. While baseline measurement may reflect preexisting, intrinsic mechanisms of resistance, angiome changes during treatment may offer insight into acquired or upregulated pathways of angiogenesis. Thus far, biomarkers for both have remained elusive for a multitude of reasons (93). Aside from the complexity of tumor angiogenesis, a major consideration is that robust blood and tissue based biomarker programs were not often embedded into large randomized trials, where such work is best done. In addition, many targets are of low abundance and are highly processed, and reagents for many targets are often limited.

Plasma VEGFA levels in a variety of malignancies, including colorectal cancer, have well-established prognostic value. However plasma VEGF levels have generally not been predictive of benefit with anti-angiogenic therapy (94). Recently, a VEGF assay that is preferential for small VEGF isoforms was reported to predict for benefit from bevacizumab in metastatic breast, pancreatic, and gastric cancers. However these results were of only borderline statistical significance and this assay was not predictive of benefit in colorectal, non-small cell lung, or renal cell cancers. The reason for these differences is not yet known, but may relate to differences in biology or in differences in sample handling across these trials (95). Biomarker analyses from other phase III trial with bevacizumab have plasma

<b>Table 1</b> Selected ongoing trials involving non-VEGF mediated pathways of angiogenesis				
Target	Drug	Malignancy	Phase	Trial
Angiopoietin/tie	AMG 386	Ovarian	III	NCT01281254
		Ovarian	III	NCT01493505
		Ovarian	III	NCT01204749
	REGN-910	Solid tumors	I	NCT01271972
		Solid tumors	I	NCT01688960
	MEDI-3617	Solid tumors	I	NCT01248949
Dll4-notch	Demcizumab (OMP-21M18)	metastatic CRC	II	NCT01189942
		Pancreatic	II	NCT01189929
		NSCLC	I	NCT01189968
	MEDI0639	Solid tumors	I	NCT01577745
	REGN-421	Solid tumors	I	NCT00871559
	RO4929097	NSCLC	II	NCT01193868
		Breast	I	NCT01071564
		Sarcoma	I/II	NCT01154452
	MK-0752	Solid tumors	I	NCT01295632
		Pancreatic	I/II	NCT01098344
FGF (and VEGFR)	Brivanib	HCC	III	NCT00858871
		Metastatic CRC	I	NCT01046864
	Nintedanib	Metastatic CRC	II	NCT01362361
		Endometrial	II	NCT01225887
		Thyroid	II	NCT01788982
		Ovarian	I	NCT01314105
	AZD4547	Gastric	II	NCT01457846
		NSCLC	I/II	NCT01824901
		Solid tumors	I	NCT00979134
	Dovitinib	Metastatic RCC	III	NCT01223027
		Glioblastoma	II	NCT01753713
		Gastric	II	NCT01719549
		Metastatic breast	II	NCT01262027
Castrate Resistant Prostate		II	NCT01741116	
TGF- $\beta$	PF-03446962	Urothelial	II	NCT01620970
		Solid tumors	I	NCT00557856
		Solid tumors	I	NCT01337050
	TRC105	Metastatic breast I/II		NCT01326481
		Glioblastoma	I/II	NCT01648348
		Metastatic RCC	II	NCT01727089
	LY2157299	Solid tumor	I	NCT01332721
		Glioblastoma	II	NCT01582269
		Pancreatic	I/II	NCT01373164
HCC	II	NCT01246986		
HIF	EZN-2968	Solid tumors	I	NCT01120288

VEGF-D, SDF1, and Ang2 in pancreatic cancer and tumor tissue VEGFD measured by IHC in colorectal cancer (12,96). Phase III trials with pazopanib and bevacizumab have implicated high IL6 levels as a predictor of benefit from these agents in renal cell cancer (97).

Intriguingly, anti-angiogenic VEGFA isoforms have been described, although this field remains controversial (98). VEGF<sub>xxx</sub>b isoforms have anti-angiogenic properties and bind to bevacizumab; bevacizumab binding to these isoforms would theoretically deplete both bevacizumab and the anti-angiogenic VEGF<sub>xxx</sub>b ligands. Conversely low levels of VEGF<sub>xxx</sub>b would be predicted to describe a more VEGFA dependent and bevacizumab sensitive state. Interestingly, in an analysis of a subset of patients with tumor available from the 2<sup>nd</sup> line E3200 study (FOLFOX +/- bevacizumab) low ratios of VEGF<sub>165</sub>b:VEGF<sub>total</sub> measured in tissue samples of mCRC patients correlated with clinical benefit from addition of bevacizumab compared to chemotherapy alone; and no benefit from bevacizumab was seen when the VEGF<sub>165</sub>b:VEGF<sub>total</sub> ratio was elevated (99).

Genetic polymorphisms in VEGFA and VEGFR1 may influence angiogenic potential involving the tumor vasculature and response to anti-angiogenic therapies. Accordingly, multiple studies have investigated VEGF genotypes and clinical outcomes. Schneider *et al.* reported that the VEGF-2578 AA genotype was associated with both an overall survival benefit and less toxicity in the phase III E2100 trial of paclitaxel with or without bevacizumab in metastatic breast cancer (100). Furthermore, certain single nucleotide polymorphisms (SNPs) correlated with clinical outcomes in the AVITA and AVOREN trials, using bevacizumab in patients with metastatic pancreatic cancer and metastatic RCC, respectively. VEGFR1 SNP rs9582036 was associated with progression free survival in both trials, and overall survival for patients AC and CC genotypes. No genetic interaction was seen in placebo groups (93). Circulating endothelial cells and tumor vessel imaging with dynamic contrast-enhanced magnetic resonance imaging are also other emerging areas of biomarker research (93,101,102). While exploratory angiogenic biomarker analysis should continue in early phase studies, future phase III trials should have prospective angiogenic marker analysis incorporated into the study design to aid and expedite validation and clinical implementation. For example, the ongoing phase III MERiDIAN trial is evaluating paclitaxel with or without bevacizumab in patients with metastatic breast cancer, stratified by pretreatment plasma VEGF level (101).

### Future directions

A wealth of evidence has been published in the past decade

collectively affirming that VEGF-axis directed therapies confer clinical benefit along the continuum of care for patients with metastatic CRC (11,50,103). Within the past year, novel approaches to targeting angiogenesis have also yielded benefit in phase III trials with regorafenib and ziv-aflibercept. While the clinical effect of anti-VEGF targeted therapies may be well established in this population, not all patients experience benefit. Furthermore, patients inevitably progress while on anti-angiogenic treatment, and the ultimate improvement in overall survival can be modest. There are numerous complementary angiogenic pathways, which may be deregulated or circumvent the mechanism of action for current targeted agents. Alternative mechanisms of tumor vessel formation may explain the various clinical phenotypes of initial treatment nonresponse or inducible resistance to anti-angiogenesis therapies.

Rational combinations of anti-angiogenic agents are needed to overcome resistance mechanisms and exploit alternative pathways of tumor blood vessel formation. Both “vertical” (targeting multiple levels of the same pathway) and “horizontal” strategies (covering multiple different angiogenic pathways) have been attempted in several different tumor types and reviewed recently (104). Although several of these combinations have demonstrated encouraging anti-tumor activity, the unfavorable side effect profiles have proven to be difficult to overcome. Future strategies involving non-overlapping toxicity profiles of anti-angiogenic agents and dosing adjustments based on pharmacokinetic/pharmacodynamics data should be employed to optimize tolerability and balance anti-tumor effect. Lastly, routine incorporation of predictive biomarkers is imperative to tailor patient selection and increase therapeutic efficacy of novel drug combinations.

### Conclusions

Mechanisms of resistance to anti-angiogenic therapies can broadly be categorized by involvement of the VEGF-axis, stromal cell interaction, and non-VEGF pathways. These mechanisms rely on a number of distinct but interrelated paracrine signaling factors and intracellular cascades. Clinical approaches targeting multiple pathways involving VEGFC, VEGFD, Tie2-Ang2, Dll4-Notch, and TGF- $\beta$  may have greater benefit than monotherapies blocking VEGFA or VEGFR2 signaling alone, for example. Numerous clinical trials are ongoing to evaluate targeted therapies with specificity for these resistance mechanisms. Incorporation of biomarkers in future clinical trials will be critical to the development of next generation anti-angiogenic regimens.

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