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Gastrointestinal Cancer

Overcoming Resistance to Antiangiogenic Therapies

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ABSTRACT

The concept of targeting new blood vessel formation, or angiogenesis, in tumors is an important advancement in cancer therapy, resulting, in part, from the development of such biologic agents as bevacizumab, a monoclonal antibody directed against vascular endothelial growth factor (VEGF)-A. The rationale for antiangiogenic therapy is based on the hypothesis that if tumors are limited in their capacity to obtain a new blood supply, so too is their capacity for growth and metastasis. Additional evidence suggests that pruning and/or "normalization" of irregular tumor vasculature and reduction of hypoxia may facilitate greater access of cytotoxic chemotherapy (CT) to the tumor. Indeed, for metastatic colorectal cancer, bevacizumab in combination with established CT regimens has efficacy superior to that of CT alone. Despite ~2-month longer progression-free and overall survival times than with CT alone, patients still progress, possibly because of alternative angiogenic "escape" pathways that emerge independent of VEGF-A, or are driven by hypoxic stress on the tumor. Other VEGF family members may contribute to resistance, and many factors that contribute to the regulation of tumor angiogenesis function as part of a complex network, existing in different concentrations and spatiotemporal gradients and producing a wide range of biologic responses. Integrating these concepts into the design and evaluation of new antiangiogenic therapies may help overcome resistance mechanisms and allow for greater efficacy over longer treatment periods. ***The Oncologist*** 2012;17:1039–1050

INTRODUCTION

It has been recognized for some time that the growth and spread of primary tumors requires, at least in part, the development of new blood vessels, also known as angiogenesis. If tumors are inhibited in their capacity to form new blood vessels, so too is their potential for continued growth and metastasis. This forms the basis for the use of antiangiogenic therapy in the treatment of cancer [1]. Tumor reliance on angiogenesis, therefore, creates targets for therapeutic inhibition in cancer [2]. Part of the initial rationale for using antiangiogenic therapy in cancer is related to the possibility of "starving" the tumor of oxygen and nutrients, thereby causing it to be inhibited in its ability to grow and spread [3]. However, the antiangiogenic monotherapies in current clinical use have not met the initial expectations [4]. It is becoming increasingly clear that hypoxia itself results in cellular "escape" that actually fosters, rather than inhibits, invasiveness and metastasis [5]. Thus, contrary to the initial hypothesis of "starving" tumors by depriving them of blood flow and oxygenation, an emerging concept is that "normalization" of tumor vasculature and increased tumor oxygenation may prevent the unfavorable switch to a more metastatic phenotype [5, 6]. Improved oxygenation in tumors may also serve to increase the efficacy of cytotoxic therapies, such as radiation [7].

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Table 1. The VEGF family of angiogenic factors and receptors [4, 15, 44]

Ligand	Binds to[a]	Biologic activities
VEGF (VEGF-A)	VEGFR-1, VEGFR-2	Regulates angiogenesis and vascular permeability
VEGF-B	VEGFR-1	Vascular and nonvascular activity
VEGF-C	VEGFR-2, VEGFR-3	Lymphangiogenesis, angiogenesis, and vascular permeability
VEGF-D	VEGFR-2, VEGFR-3	Lymphangiogenesis, angiogenesis, and vascular permeability
PlGF	VEGFR-1	Vascular and nonvascular activity, myeloangiogenesis

[a]NRP coreceptors NRP-1 and NRP-2 may bind some isoforms and contribute to cellular responses.
Abbreviations: NRP, neuropilin; PlGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Vascular endothelial growth factors (VEGFs) belong to a family of cytokines that are known to play an important role in angiogenic processes through both direct and indirect mechanisms—inhibiting the VEGF pathway has become a focal point for antiangiogenic therapy for cancer [2, 8]. VEGF-A, in particular, has received the most attention for angiogenesis inhibition, and bevacizumab, a humanized monoclonal antibody directed against VEGF-A, which prevents its interaction with its receptor, was the first antiangiogenic therapy to be approved for clinical use in cancer (in combination with chemotherapy [CT]) in 2004 [4, 9]. Bevacizumab treatment causes both regression of existing tumor blood vessels and normalization of those that remain [9]. Reducing VEGF signaling has been proposed to provide a "transient normalization window," whereby the effect of antiangiogenic treatment can be expected to improve tumor oxygenation and the effect of cytotoxic therapies such as radiation and CT [7, 10]. The results of several key clinical trials of bevacizumab have shown important improvements in endpoints such as the progression-free survival (PFS) interval, response rate (RR), and overall survival (OS) time when used in combination with cytotoxic CT regimens such as irinotecan, 5-fluorouracil, and leucovorin (IFL) [9, 11–13]. Despite short-term improvements in clinical endpoints such as PFS and OS times, however, progression occurs in most patients on bevacizumab, and the potential mechanisms underlying this apparent resistance to therapy are many, and are not fully understood [9, 14]. In addition, although VEGF-A has been a main target of therapy, it is clear that many ligands, receptors, and cell types play roles in processes as important as angiogenesis, and it is largely unknown which of these mediators and systems is the most relevant for angiogenesis in cancer.

In this review, we describe the use of antiangiogenic therapies for cancer and present hypotheses on the role of angiogenesis in cancer that have, and have not, been borne out in clinical studies, as well as hypotheses remaining to be tested. We also discuss the potential to overcome resistance to antiangiogenic therapy by targeting additional factors and signaling pathways involved in this process.

Regulation of Angiogenesis: A Network of Signaling Molecules and Pathways

It is important to bear in mind that, as discussed further below, angiogenic processes are complex and not determined by a single signaling molecule or pathway functioning in a vacuum; rather, any one factor exerts its biological effects in the context of many others that may be present at a specific time and place. The VEGF family consists of at least five known ligands, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF), and three tyrosine kinase receptors, VEGFR-1, VEGFR-2, and VEGFR-3 (Table 1) [4, 15]. Neuropilins (NRPs), originally discovered as neuronal receptors, may also play a role in modulating angiogenesis by VEGF family members [2, 16]. NRP-1 and NRP-2 interact with VEGFRs and appear to enhance signaling by VEGFR-2; they also bind to VEGF family members, including VEGF-A, VEGF-C, and PlGF [17, 18]. Interaction of NRPs with other growth factors may also modulate angiogenesis, and it has been shown that NRPs are overexpressed in pathological conditions and malignancy [2, 17–19]. There is evidence that anti-NRP antibodies can modulate signaling by VEGFR-2, and anti-NRP antibodies also appear to have at least additive activity in combination with anti-VEGF antibodies, suggesting different mechanisms of action [20]. Additional evidence from mouse models suggest that NRPs function in lymphangiogenesis [21], and thus the exact role of NRPs in regulating angiogenesis requires further study [2, 17].

VEGF-A is the best-studied member of the VEGF family and plays a role in many stages of angiogenesis, including endothelial cell proliferation, migration, invasion, and survival; chemotaxis of bone marrow progenitors; and increasing vascular permeability and vasodilation [4]. The principle actions of VEGF-A (mitogenic, angiogenic, and permeability-increasing effects) are thought to be mediated by its binding to VEGFR-2, although VEGF-A also binds to VEGFR-1 with at least a 10-fold higher affinity [2, 9, 22, 23]. By comparison, VEGF-B and PlGF are less well-characterized members of the VEGF family and bind to VEGFR-1 [15]. Adding to the complexity of VEGF signaling is the finding that VEGF-A, VEGF-B, and PlGF can homo- and heterodimerize (resulting in multiple ligands with differing activities), as can VEGFR-1 and VEGFR-2, leading to a wide range of signaling possibilities [15, 24]. A soluble form of VEGFR-1 (sVEGFR-1) has also been identified, and the ratio of the soluble to membrane-bound forms of VEGFR-1 could play a role in regulating angiogenesis during development; sVEGFR-1 may function as a decoy receptor by limiting the interaction of VEGF-A with VEGFR-2 [16].

The role of VEGF-B in endothelial angiogenesis is not fully understood. As with VEGF-A and PlGF, VEGF-B also binds VEGFR-1, and although it does not appear to be required for blood vessel growth, there is evidence that VEGF-B plays an important role in blood vessel survival in vivo. It has, thus, been termed a blood vessel survival factor, rather than a classical proangiogenic factor [25]. VEGF-B effects are also observed pleiotropically in multiple types of vascular cells [25, 26], and a role in cell adhesion and migration has also been proposed [27]. Results from the Alitalo laboratory have shown a role for VEGF-B in promoting myocardial growth and protecting against ischemia [28], and there is also evidence for a role of VEGF-B in endothelial fatty acid uptake [29].

Four isoforms of PlGF have been identified: PlGF-1, PlGF-2, PlGF-3, and PlGF-4. PlGF binds to and activates only VEGFR-1 and the NRPs [30, 31]. Different isoforms of PlGF may have distinct dimerization, binding, and biological activities [32]. Mice overexpressing PlGF showed greater number and size of blood vessels as well as greater vascular leakiness, and this effect was specific for blood but not lymphatic vessels, suggesting a strong angiogenic effect of PlGF in vivo [33]. There is also evidence that PlGF serves to potentiate the response to VEGF-A via signaling through VEGFR-1, and this role of PlGF appears to be restricted to pathologic conditions, such as cancer [16]. As detailed below, evidence exists that PlGF is involved in tumor angiogenesis and growth, albeit through less well-characterized and distinct mechanisms than VEGF-A [30, 34].

The remaining members of the VEGF family, VEGF-C and VEGF-D, bind to VEGFR-3 as well as VEGFR-2 and play roles in angiogenesis and lymphangiogenesis [15]. Recent results have further implicated the VEGFR-3 pathway in stalk-to-tip conversion in angiogenesis. Depletion of VEGFR-3 in endothelial cells results in hypervascularization in both developmental and tumor angiogenesis [35]. These results suggest an important role for VEGF-C derived from macrophages and VEGFR-3 in the conversion of tip cells to stalk cells, which is important in sprout fusion and the establishment of cell–cell junctions [35].

The VEGF pathway thus includes multiple targets for inhibition with therapeutic agents, including antibodies affecting ligand binding or receptors, ligand traps, and receptor tyrosine kinase inhibitors (TKIs) [36]. However, beyond the VEGF pathway, there are multiple other direct and indirect modulators of angiogenesis that could play roles in tumor angiogenesis and/or escape from angiogenesis inhibition, including interleukin (IL)-12 [37], hepatocyte growth factor (HGF)/c-Met [38, 39], the fibroblast growth factor (FGF) pathway [40, 41], and endogenous inhibitors of angiogenesis such as angiostatin, endostatin, and thrombospondin (TSP)-1 and TSP-2 [42]. Although a detailed discussion of these and other pathways is beyond the scope of the current review, we note these multiple additional angiogenic mechanisms as examples of the many potential targets that should be considered in the design of antiangiogenic therapies.

VEGF and VEGFR in Tumor Angiogenesis

Overexpression of VEGF ligands is one possible mechanism for increasing angiogenesis in tumors, and VEGF overexpression in some tumors can result from deregulated signaling pathways, oncogene overexpression, and other genetic abnormalities [43]. Many different cytokines and growth factors can also indirectly stimulate angiogenesis by increasing VEGF-A expression [44]. Upregulation of VEGF-A mRNA expression has been shown in primary tumors from colorectal cancer (CRC) patients as well as liver metastases, and evidence from an experimental model suggested that inhibition of VEGF-A could effectively prevent liver metastases [45]. Moderate but consistent overexpression of VEGF-A, as assessed by mRNA levels, has also been observed in colon adenocarcinomas, regardless of stage, compared with control tissue [46]. By comparison, VEGF-B mRNA levels were not different in adenomas and adenocarcinomas, and although poorly expressed in the colon, VEGF-C was moderately overexpressed in some adenocarcinomas, compared with controls [46]. Studies assessing mRNA levels or using immunohistochemical methods, however, may be subject to many confounders and provide only a preliminary assessment of tumor VEGF expression at any given time. It should also be noted that VEGF ligands can be produced in the local tumor microenvironment by inflammatory cells and by hypoxic stresses on the tumor [47, 48].

Upregulation of VEGFRs in tumor cells is another possible mechanism of transition to a more invasive and proangiogenic state. VEGFR-1 (which binds VEGF-A, VEGF-B, and PlGF) has been demonstrated to be involved in epithelial to mesenchymal transition (EMT), a process involved in progression to invasive cancer. Also, an autocrine pathway involving VEGF and VEGFR-1 was shown to be necessary for cell survival after EMT [49]. VEGFR-1 additionally mediates migration of VEGFR-1$^+$ myeloid bone marrow precursor cells to peritumor sites, which secrete angiogenic factors, and this is thought to be involved in resistance to therapies targeting VEGF-A, such as bevacizumab [50]. There is also evidence for involvement of VEGFR-1 in tumor "homing" to metastatic sites from a murine model of lung metastasis [51]. Using VEGFR-1 knockout mice, it was shown that tumor-mediated induction of matrix metalloprotease 9 in lung endothelial cells and macrophages was dependent on VEGFR-1 signaling, and this was associated with increased tumor metastasis to the lung [51].

As noted earlier, a hypothesis has been proposed whereby there is a normalization "window" that occurs with therapies targeting the VEGF pathway (specifically, anti–VEGFR-2 antibodies), and vessels can be normalized allowing greater efficacy of cytotoxic therapies, such as radiation, in tumors [7]. Because VEGF-A is believed to mediate its biologic effects via its interaction with VEGFR-2, a similar mechanism may be responsible for the additive or synergistic effects of bevacizumab in combination with CT. This proposed mechanism appears to be dependent on the upregulation of angiopoietin-1 (Ang)-1 and signaling by its receptor, Tie-2, with an accompanying recruitment of pericytes to blood vessels; this results in improved tumor oxygenation and susceptibility to radiother-

apy [7]. These findings bring into question the important issue of timing and dosing with antiangiogenic therapies, that is, when and how should they be used to produce the maximal tumor inhibitory effect [7]. At present, the optimal timing and dosing of antiangiogenic treatment for a given tumor, combination therapy, or patient remains unknown [2].

PlGF

Although redundant for vessel development in healthy adults, PlGF has been postulated as promoting vessel formation in pathological states, and it may contribute to the angiogenic and inflammatory "switch" that occurs with cancer [30]. Gene-targeting experiments in mice suggest that PlGF can serve to potentiate the response to VEGF-A through its interaction with and signaling via VEGFR-1, and this effect was specific for VEGF and not basic FGF (bFGF) [16]. Additionally, there is evidence from mouse models that PlGF produced by both the host and tumor is involved in promoting tumor angiogenesis—when both compartments lacked PlGF, small and poorly vascularized tumors developed [16]. In CRC, the expression of PlGF was significantly higher in tumor than in normal tissues ($p = .001$), and it was significantly higher in stage III–IV than in stage I–II tumors ($p = .011$) [52]. Furthermore, PlGF protein expression was significantly correlated with microvessel density, patient survival, and lymph node metastasis, suggesting a role for PlGF as a correlate of disease progression [52]. Evidence also exists for higher PlGF expression in tumor tissue from CRC than from control tissue and in patients with poor outcomes, compared with those who remained disease free [53]. PlGF also increased prior to progression in patients treated with VEGF-A–targeting therapy, suggesting a possible role in resistance to therapy [54]; however, because this was not a randomized trial, PlGF may also have increased as a function of tumor burden.

Some tumor model systems, however, have shown no apparent effect of anti-PlGF on tumor growth, although this could reflect differences in experimental procedures and models [55]. It is also noteworthy that only the effects of PlGF-2 are examined in mouse models, because this is the only isoform identified in mice, whereas humans express four isoforms [30]. Results from Yao et al. [34] identified an axis involving PlGF and VEGFR-1, whereby expression of VEGFR-1 was necessary for tumor inhibition using anti-PlGF antibody. In addition, no decreases in microvessel density were observed in sensitive cell lines, suggesting that reduction in angiogenesis was not a component of anti-PlGF efficacy in these models [34]. Recent results from our own lab suggest that host-produced factors in the tumor stroma that are regulated by PlGF play important roles in vessel normalization and in the modulation of immune cells in the tumor microenvironment (discussed further below), providing a molecular link between PlGF and additional host factors that can impact the need for tumor metastasis and escape from hypoxic conditions [56].

Taken together, these findings provide evidence for multiple potential roles for PlGF in direct or indirect modulation of tumor angiogenesis, and possibly in mediating escape from angiogenesis inhibition. It remains to be seen whether or not specifically targeting PlGF with inhibitors will be of clinical value as a component of antiangiogenic treatment in cancer.

Targeting the VEGF Pathway in Cancer

The VEGF family of ligands and their receptors (Table 1) provide a range of possible therapeutic interventions that can be directed at reducing the levels of the ligands themselves (such as bevacizumab with VEGF-A) or inhibiting the activity and/or signaling pathways of the VEGFRs. Examples of the latter strategy include TKI drugs such as sunitinib, sorafenib, and BIBF 1120 as well as neutralizing antibodies to VEGFRs. Many of these agents are currently under evaluation in clinical trials [4, 8, 36, 57]. The first of its kind for anti-VEGF therapy, bevacizumab, was demonstrated to have moderate activity in CRC patients when combined with CT [8]. Bevacizumab also was shown to have activity against selected cancers, including renal cell carcinoma in combination with interferon-α, glioblastoma as a single agent, and ovarian cancer as a single agent [8], whereas its efficacy in colon cancer patients is limited to combination therapy with CT [11, 12]. In the pivotal trial of bevacizumab for metastatic CRC (mCRC), a greater OS time, PFS interval, and RR were observed when bevacizumab was added to IFL in patients with previously untreated mCRC [11]. Other notable trials have investigated its use in the second-line mCRC setting and in combination with epidermal growth factor receptor inhibitors. These trials are summarized in Table 2. Both positive and negative results from these and other ongoing trials highlight the fact that the most optimal use of this agent as an antiangiogenic agent for mCRC is still the subject of ongoing investigation (Table 2).

Progression and Antiangiogenic Therapy Failure: Intrinsic and Adaptive Mechanisms

Patients on antiangiogenic therapy, especially those on combination therapy with CT, may survive longer than those treated with CT alone, but they eventually succumb to progressive disease and most clinical trials with bevacizumab show a relatively consistent 2-month longer PFS time [12, 13]. Other strategies for antiangiogenic therapy are frequently characterized by an initial response followed by disease progression and, in some cases, no objective benefit with the therapy at all [14]. Mechanisms of progression following angiogenesis inhibition may thus be either adaptive or intrinsic [14]. "Intrinsic resistance" refers to an innate ability of the tumor to be insensitive to angiogenesis inhibition (e.g., as a result of host or genomic factors) [14], whereas "adaptive resistance" refers to the ability of the tumor to display evasive resistance to angiogenesis inhibition (i.e., the tumor adapts by upregulation of other proangiogenic signaling mechanisms and/or pathways). In such situations, a combination strategy involving the use of angiogenesis inhibitors in conjunction with therapies that target possible resistance mechanisms may, therefore, be a more efficient approach to cancer therapy [14]. An additional component of adaptive resistance could also be related to the development of tumor resistance to the CT used in combination therapy, in which case, any additive benefit of the antiangio-

Table 2. Key clinical findings with bevacizumab for mCRC

Trial name (status)	Description and relevant findings	Reference
E3200 (completed)	•Examined Bev in combination with FOLFOX4 in patients with previously treated mCRC •Compared with FOLFOX4 alone, the addition of Bev led to superior: ○Median survival time, 12.9 mos versus 10.8 mos ($p = .0011$) ○PFS interval, 7.3 mos versus 4.7 mos (HR, 0.61; $p < .0001$) ○RR (via the RECIST), 22.7% versus 8.6% ($p < .0001$) •Grade 3 or 4 AE rates were higher: 75% versus 61%	[13]
NSABP C-08 (completed)	•Investigated the safety and efficacy of adding Bev to mFOLFOX6 versus mFOLFOX6 alone as adjuvant therapy in patients with stage II and III CRC •Addition of Bev to mFOLFOX6 did not result in a higher DFS rate than with mFOLFOX6 alone: 77.4% versus 75.5%; HR, 0.89 ($p = .15$) •No evidence for a rebound effect in patients with recurrence in the group receiving Bev versus those who did not	[95]
CAIRO2 (completed)	•Investigated the use of cetuximab (EGFR inhibitor) with CBC versus the same regimen without cetuximab (i.e., CB) as first-line treatment for mCRC •Median PFS interval was longer in the CB group (without cetuximab): CBC, 9.4 mos versus CB, 10.7 mos ($p = .01$) •Grade 3/4 AEs were also more common with CBC than with CB: 81.7% versus 73.2% ($p = .006$)	[96]
PACCE (stopped)	•Investigated the use of oxaliplatin and irinotecan-based CT with Bev with or without panitumumab (EGFR inhibitor) •Interim analysis revealed significantly poorer PFS and OS outcomes in the group receiving panitumumab •More AEs grade >3 were observed in the panitumumab arm	[97]
TML (ongoing)	•Investigating the efficacy and safety of adding Bev to crossover CT in patients with mCRC and progression under first-line treatment with standard CT + Bev: stratum 1, CT (AIO-IRI, FOLFIRI, CAPIRI or XELIRI) alone or with Bev; stratum 2, CT (FUFOX, FOLFOX, CAPOX, or XELOX) alone or with Bev	[58]
CALGB 80405 (ongoing)	•Randomized phase III trial investigating cetuximab or Bev in combination with mFOLFOX6 or FOLFIRI CT in patients with untreated advanced CRC or mCRC; a third arm will investigate the use of these two biologic agents sequentially, with mFOLFOX6 or FOLFIRI, and with cetuximab initially followed by Bev	[98, 99]

Abbreviations: AEs, adverse events; AIO-IRI, Arbeitsgemeinschaft Internistische Onkologie-Irinotecan; Bev, bevacizumab; CALGB, Cancer and Leukemia Group B; CAIRO2, Capecitabine, Irinotecan, and Oxaliplatin in Advanced Colorectal Cancer 2; CAPIRI, irinotecan and capecitabine; CAPOX or XELOX, oxaliplatin and capecitabine; CB, capecitabine, oxaliplatin, and Bev; CBC, cetuximab, capecitabine, oxaliplatin, and Bev; CT, chemotherapy; DFS, disease-free survival; EGFR, epidermal growth factor receptor; FOLFIRI, leucovorin, 5-fluorouracil, and irinotecan; FOLFOX, oxaliplatin, 5-fluorouracil, and leucovorin; FUFOX, oxaliplatin and 5-fluorouracil; HR, hazard ratio; mCRC, metastatic colorectal cancer; mFOLFOX, modified infusional–bolus FOLFOX; PACCE, Panitumumab Advanced Colorectal Cancer Evaluation; PFS, progression-free survival; RECIST, Response Evaluation Criteria In Solid Tumors; RR, response rate; TML, ML18147 Trial; XELIRI, capecitabine and irinotecan.

genic therapy is negated by insensitivity to the cytotoxic therapy used.

Resistance to bevacizumab combination therapy during treatment for CRC may involve resistance to bevacizumab, the CT with which it is administered, or both [9]. An ongoing study is currently evaluating the use of bevacizumab following progression on bevacizumab in combination with CT [58]. The efficacy of bevacizumab plus capecitabine and oxaliplatin (XELOX) followed by maintenance with the same regimen or with single-agent bevacizumab was also examined in the MACRO trial [59]. The results of that trial showed no significant difference (i.e., noninferiority of single-agent bevacizumab) in the PFS times, overall RR, or OS times between the treatment arms; thus, maintenance with single-agent bevacizumab treatment may be a viable option for some patients following "induction" as combination therapy with CT [59]. Still,

further studies evaluating single-agent bevacizumab after standard CT for mCRC are warranted.

Other Resistance Mechanisms

As noted, multiple pathways in addition to the VEGF pathway contribute to angiogenesis [9]. Mechanisms of resistance to antiangiogenic therapy may involve the FGF signaling pathway, the Notch ligand and receptor, angiopoietins, recruitment of bone marrow stromal cells, and increased pericyte coverage of tumor blood vessels to support vasculature [9]. Resistance to antiangiogenic therapy may also involve recruitment of other lymphangiogenic factors, pathways (e.g., VEGF-C, VEGF-D), and myeloid cells, and the evolution of more resistant tumors (e.g., hypoxia resistance), although at present there is no specifically defined marker for bevacizumab resistance [2, 60].

Two recent studies have reported variations in cytokines and angiogenic factors, including PlGF, in patients treated with a bevacizumab-containing regimen. In a phase II study of bevacizumab plus CT in patients with mCRC, increased expression of proangiogenic cytokines following therapy was observed. Specifically, PlGF was found to be significantly elevated following leucovorin, 5-fluorouracil, and irinotecan (FOLFIRI) plus bevacizumab treatment and also prior to radiographic progression, relative to baseline, in these patients ($p < .001$) [54]. In addition, elevated baseline plasma IL-8 levels were associated with a shorter PFS interval [54], and increases in several other cytokines, compared with baseline prior to progression, were observed, including bFGF and HGF, the myeloid chemoattractant factors, stromal derived factor 1, and macrophage chemoattractant protein [54]. Another study of patients treated with bevacizumab plus CT found consistent and significant increases in both PlGF (32%; $p < .0001$) and VEGF-D (6%; $p = .018$) after progression in a large cohort of patients ($n = 403$) [61]. In a smaller cohort of patients ($n = 42$), increases in VEGF-C were observed prior to (43%; $p = .045$) and at the time of (72%; $p = .004$) progression, and increases in VEGF-D were observed at progression (39%; $p = .04$) following FOLFIRI plus bevacizumab treatment [61]. Evidence also exists for upregulation of angiogenic factors following cytotoxic radioimmunotherapy; depending on the tumor cell type, upregulation of VEGF, VEGFR, Ang-2, Tie-2, and PlGF have been observed [62]. These findings imply a role for upregulation of selected angiogenic factors and pathways associated with resistance to anti-VEGF therapy, and suggest that patients progressing on a selective antiangiogenic therapy may benefit from therapies that are more broadly targeted [54, 62].

Inhibition of Proangiogenic Factors to Overcome Progression on Bevacizumab: Some Agents in Development

A number of therapeutic strategies are currently in development aimed at improving upon antiangiogenic therapy by targeting additional factors and pathways including, but not limited to, VEGF-A–VEGFR-2. Although a detailed description of all antiangiogenic therapies under development is beyond the scope of the current review, we have focused the section below on some of the more novel agents and strategies in development, some of which have moved on to phase III studies and others that are still investigational. Most of the examples below are under investigation for use in mCRC patients, such as bevacizumab, although these agents may be useful for other cancer types (e.g., glioblastoma) as well.

Aflibercept (VEGF Trap)

Aflibercept is a recombinant fusion protein composed of VEGFR-1 and VEGFR-2 ligand-binding components fused to the Fc portion of human IgG_1 [57, 63]. Aflibercept binds specifically to VEGF-A, VEGF-B, PlGF-1, and PlGF-2 and inhibits its activation of VEGFR-1 and VEGFR-2 by these ligands (Table 3) [57, 63]. Additionally, unlike other forms of soluble VEGFR-1 that have poor pharmacokinetic (PK) properties, aflibercept has been engineered to have minimal interactions with the extracellular matrix and, therefore, a better PK profile, and it is also composed of human sequences so as to be minimally immunogenic [64]. In contrast to bevacizumab, which forms multimeric complexes, aflibercept forms stable, 1:1 monomeric inert complexes with VEGF, which allows for an accurate assessment of tumor and host VEGF production in vivo [65]. In preclinical studies, aflibercept inhibited the growth of tumor xenografts [64] and was shown to cause regression of established tumor vasculature in a variety of tumor models [63]. Tumor growth and metastasis in pancreatic cancer cell lines were also inhibited by aflibercept, and a reduction in microvessel density was observed [66]. In an open-label, phase II trial of previously treated patients with mCRC ($n = 51$), aflibercept showed activity both in bevacizumab-naïve patients ($n = 24$; disease control rate, 29%; median PFS time, 2.0 months) and in those with prior bevacizumab therapy ($n = 27$; disease control rate, 30%; median PFS time, 3.4 months) [67]. Results of the recent phase III VELOUR study of aflibercept in combination with FOLFIRI in mCRC patients who failed prior 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX)-based therapy showed a significantly greater OS time (hazard ratio [HR], 0.817; $p = .0032$), PFS interval (HR, 0.758; $p = .00007$), and overall RR (19.8% versus 11.1%; $p = .0001$) than with FOLFIRI alone [68]. Grade 3 and 4 adverse events (AEs) occurring with ≥2% incidence in the aflibercept arm than in the placebo arm were diarrhea, asthenia or fatigue, stomatitis or ulceration, infections, hypertension, gastrointestinal or abdominal pain, neutropenia or neutropenic complications, and proteinuria [68]. AEs leading to treatment discontinuations occurred in 26.6% and 12.1% of patients in the aflibercept and placebo arms, respectively [68]. Findings of the VELOUR study demonstrate a benefit of combining the multitargeted VEGF inhibitor aflibercept with CT in patients with mCRC progressing on a prior oxaliplatin-based therapy. Importantly, ~30% of the patients in the VELOUR trial had received prior bevacizumab. Of note, recent press releases of other trials of aflibercept for non-small cell lung cancer (NSCLC) (VITAL, in combination with docetaxel; ClinicalTrials.gov identifier, NCT00532155) and metastatic pancreatic cancer (Aflibercept [VEGF Trap] Plus Gemcitabine Versus Placebo Plus Gemcitabine for the First-Line Treatment of Metastatic Pancreatic Cancer [VANILLA], in combination with gemcitabine; Clini-

Table 3. Strategies for overcoming resistance to angiogenic therapies: some agents in development

Agent	Target	Inhibitor type	Preclinical data	Clinical data	Reference
Aflibercept	VEGF-A, VEGF-B, PlGF	Composite decoy receptor/ligand trap	Forms stable, inert complexes with VEGF; causes regression of established tumor xenografts in a variety of tumor types	Phase III: mCRC patients with failed prior CT (VELOUR); significantly greater PFS, OS, and RR outcomes in combination with FOLFIRI; 30% of patients had prior bevacizumab; key adverse events include diarrhea, asthenia or fatigue, stomatitis or ulceration, infections, hypertension, gastrointestinal or abdominal pain, neutropenia	[63, 65, 68]
AMG 386	Ang-1, Ang-2	Peptide Fc fusion/peptibody	Suppresses tumor growth in mouse xenograft models; reduces tumor vessels and normalizes remaining vessels	Phase I study showed antitumor activity in combination with CT; phase I and II studies: currently under way in combination with CT; adverse events include diarrhea and hypomagnesemia	[69, 70, 100, 101]
TB 403	PlGF	Humanized monoclonal antibody	Impact on angiogenesis and tumor growth in some tumor models; others show no impact of anti-PlGF; role in metastasis seeding	Phase I: well tolerated in healthy volunteers; additional phase I (single agent) study currently under way in patients with solid tumors; adverse events include mild nasopharyngitis, headache, and neck and joint pain	[30, 55, 72, 77, 102]
Cediranib	VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, c-Kit	Tyrosine kinase inhibitor	Broad spectrum antitumor activity in mouse xenografts; doses were well tolerated; causes significant vascular regression in tumor models	Phase II: recurrent glioblastoma; partial responses in 56.7%; no evidence for rebound angiogenesis; commonly observed toxicities include hypertension, diarrhea, and fatigue	[78, 80–82]
Regorafenib	VEGFR-1, VEGFR-2, VEGFR-3, others	Tyrosine kinase inhibitor	Antiproliferative and antiangiogenic activity in multiple mouse xenograft models	Phase III: mCRC with progression on standard therapy; significant benefit of regorafenib over placebo in OS and PFS outcomes	[85, 87]

Abbreviations: Ang, angiopoietin; CT, chemotherapy; FOLFIRI, leucovorin, 5-fluorouracil, and irinotecan; mCRC, metastatic colorectal cancer; OS, overall survival; PDGFR, platelet-derived growth factor receptor; PlGF, placental growth factor; PFS, progression-free survival; RR, response rate; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; VELOUR, Aflibercept Versus Placebo in Combination with Irinotecan and 5-FU [FOLFIRI] in the Treatment of Patients with Metastatic Colorectal Cancer after Failure of an Oxaliplatin Based Regimen.

calTrials.gov identifier, NCT00574275) have reported discontinuation of the trials for failure to attain the primary endpoint of a longer OS time.

AMG 386

Ang-1 and Ang-2 are endogenous factors involved in the control of angiogenesis through their interaction with a receptor tyrosine kinase, Tie-2 [69]. AMG 386 is a peptide Fc-fusion, or peptibody, that inhibits the interaction of Ang-1 and Ang-2 with their tyrosine kinase receptor, Tie-2 [69]. In the Colo205 mouse tumor xenograft model system, inhibition of Ang-1 and Ang-2 using AMG 386 resulted in greater tumor suppression than was observed by inhibiting either Ang-1 or Ang-2 alone, suggesting that both are required for tumorigenesis [69]. Specifically, Ang-2 inhibition caused both a reduction in number of tumor vessels and normalization of the remaining vessels, whereas inhibition of Ang-1 blocked the latter "undesired" effect of vessel normalization in this model [69]. These findings provide an example of using a single peptibody-based therapy to inhibit two angiogenic factors and achieve effective tumor suppression. In a phase I study, AMG 386 antitumor activity was observed when it was used in combination with commonly used chemotherapeutic regimens (FOLFOX4, carboplatin–paclitaxel, and docetaxel) in patients with advanced solid tumors ($n = 22$), and AEs thought to be related to AMG 386 (mostly diarrhea and hypomagnesemia) were grade <2 [70]. In a preliminary investigation of potential biomarkers for AMG 386, it was notable that increases in PlGF were observed with all combinations, whereas VEGFR-2 levels decreased and VEGF-A and VEGFR-1 levels did not change appreciably with treatment [70]. A phase II clinical trial is currently under way comparing the efficacy and safety of AMG 386 with placebo in combination with FOLFIRI as second-line therapy in patients with mCRC [71].

RO5323441 (TB 403)

As discussed, there is increasing evidence for a role for PlGF in tumor growth and metastasis, and whether or not PlGF inhibi-

tion may also be beneficial in overcoming resistance to agents such as bevacizumab is an area of active investigation. TB 403 is a humanized monoclonal antibody that targets PlGF, and the efficacy and safety of this agent are just beginning to be explored [72]. In healthy male subjects ($n = 16$), administration of TB 403 was associated with mild nasopharyngitis, headache, and neck and joint pain, compared with placebo, and the highest dose used in the study, 5 mg/kg, was well tolerated, with all subjects studied completing the study, no serious AEs, and no AEs leading to withdrawal [72]. A dose-finding study of TB 403 was also completed in patients with treatment-refractory mCRC or ovarian cancer [73]. Inhibiting PlGF with agents such as TB 403 has the potential to inhibit a range of myeloangiogenic pathways, including impairing myeloid cell–mediated establishment of premetastatic niches and impeding macrophage recruitment [50]. Anti-PlGF is currently under investigation in phase I trials, and a phase I study of TB 403 in patients with solid tumors was recently completed [74]. Nonetheless, because the exact role of PlGF is less well understood, side effects will need to be studied over the long term [50]. It is also unclear at present if targeting PlGF will be of benefit in reducing tumor angiogenesis per se, rather than tumor growth. A recent study assessing the impacts of anti-PlGF and anti-VEGFR–1 tyrosine kinase signaling showed no impact on angiogenesis or tumor growth in a range of tumor cell lines tested [55]. Despite this, and as previously noted, other results suggest important roles for PlGF in vessel normalization in hepatocellular carcinoma [75], in seeding and bone marrow angiogenesis in chronic myeloid leukemia [76], and in the establishment of tumors in the bone microenvironment prior to metastasis [77]. Thus, the true contribution of anti-PlGF antibodies as a component of antiangiogenic therapy remains to be fully elucidated.

Cediranib (AZD2171)

Cediranib provides an example whereby targeted agents may prove to be particularly useful for selected types of tumors. Cediranib is a TKI with activity against VEGFRs (particularly VEGFR-2) as well as platelet-derived growth factor receptors (PDGFRs) and c-Kit, and studies show that this agent appears to be effective in normalizing blood vessels and alleviating edema in glioblastoma patients, a major cause of morbidity [78–80]. VEGFR-2, PDGFR-a, and PDGFR-b are present in recurrent glioblastoma patients, and these tumors respond to cediranib treatment [78]. The normalization of blood vessels with cediranib may also be beneficial in combination therapy with cytotoxic agents, allowing for greater access to the tumor in glioblastoma patients [79]. In a phase II study, 25.8% of recurrent glioblastoma patients were alive and progression free after 6 months with single-agent cediranib, with radiographic partial responses in 56.7% using three-dimensional (3D) magnetic resonance imaging assessment [81]. Toxicities with cediranib were largely manageable, with hypertension, diarrhea, and fatigue being most commonly observed [81]. Interestingly, unlike after chemoradiation therapy, there was evidence for a lack of "rebound" angiogenesis in patients with recurrent glioblastoma progressing after cediranib therapy [82]. This is evidenced by the finding that, after anti-VEGF therapy with cediranib, there was a loss of cellularity in the center of the tumor and blood vessels had a normal molecular expression and morphology with no evidence of a second wave of angiogenesis [82]. The findings suggest a more invasive phenotype of recurrent glioblastoma following anti-VEGF therapy, but not a switch to alternative angiogenesis pathways [82]. In that study, there was also evidence for c-Met and PDGF-C as possible mediators of resistance to cediranib [82]. Currently under way is a placebo-controlled trial of cediranib in combination with either FOLFOX or XELOX in patients with previously untreated mCRC; estimated completion of that trial is mid-2013 [83]. In another trial of first-line treatment for mCRC, cediranib combined with FOLFOX is under investigation in comparison with bevacizumab plus FOLFOX [84].

Regorafenib (BAY 73-4506)

Another notable drug in development of the TKI class is regorafenib. This TKI inhibits VEGFR-1, VEGFR-2, and VEGFR-3 as well as Tie-2, PDGFR-b, c-Kit, FGFR-1, RET, B-raf, and p38 mitogen-activated protein kinase [85]. The drug also appears to be more pharmacologically potent in its inhibition of VEGFR-2, PDGFR, c-Kit, and FGFR-1 than the structurally related TKI sorafenib, although the possible clinical relevance of this potency with respect to angiogenesis inhibition remains to be determined [85]. The drug has been demonstrated to have both antiproliferative and antiangiogenic activities across multiple tumor xenograft models [85]. As a first- or second-line therapy, regorafenib, in combination with mFOLFOX6 or FOLFIRI also demonstrated activity and acceptable tolerability in a small phase Ib study of patients with CRC [86]. The results of the phase III CORRECT trial (formal preplanned interim analysis), which investigated the use of regorafenib or placebo along with best supportive care in patients with mCRC ($n = 760$) after failure of standard therapy, were recently reported [85, 87]. Significant benefits in terms of the OS time (6.4 months versus 5.0 months; HR, 0.77; one-sided $p = .005$) as well as the PFS interval (HR, 0.49; one-sided $p < .000001$) were observed with regorafenib relative to placebo [87]. Given the results, the study was unblinded so as to allow placebo patients to cross over to regorafenib. Notable AEs of grade ≥3 included hand–foot skin reaction, fatigue, diarrhea, hyperbilirubinemia, and hypertension [87].

Emerging Concepts in Angiogenesis: Rethinking the "Single-Agent" Paradigm

It is clear that the process of angiogenesis, as with many other essential processes, is regulated by a complex system of positively and negatively interacting factors that exist in an interconnected network, with feedback regulatory loops at multiple levels. These factors include the VEGF family (VEGF-A, VEGF-B, PlGF, and their alternately spliced isoforms, homo- and heterodimers), VEGFR-1, VEGFR-2, VEGFR-3, and NRPs, as well as many other proangiogenic growth factors and cytokines and endogenous inhibitors of angiogenesis. A similarly complex network, with multiple regulatory molecules and receptors, is observed in neuronal systems [88]. Accord-

ingly, if any single component of the network is disrupted (e.g., through targeted inhibition of VEGF-A), there are accompanying perturbations in other signaling molecules and/or receptors in the network, much the same way that perturbations in a single neurotransmitter can impact multiple others [88]. This is best exemplified in the angiogenesis pathway by the finding that targeted inhibition of the VEGF-A ligand (e.g., with bevacizumab) [54] and inhibition of receptor kinase signaling (e.g., with sunitinib or cediranib) [81, 89] are met with changes in other components of the pathway (e.g., increases in PlGF, decreases in sVEGFR-2), which in turn mediate their own effects, possibly leading to resistance to the therapy.

In neuronal systems, there has been a growing recognition of the fact that neuroregulatory molecules exist as a "cocktail" of factors released from multiple cell types and at varying spatiotemporal concentrations in the extracellular milieu [88]. Whereas the conventional approach to designing therapeutic agents for these complex pathways has been realistically targeted to one factor at a time, the biologic impact of inhibiting that one factor can be dramatically different in relation to the other factors that may exist in the "cocktail" at any given time or place in the system [88].

The angiogenesis pathway can, therefore, be viewed in a similar way as the neuromodulatory network; that is, a dynamic process involving multiple factors, each of which exerts multiple actions and with each action being exerted by multiple factors. Like the neuronal network, this would allow for greater scope and flexibility in the angiogenic response on the basis of environmental change [88]. Recently, a model system was described that attempts to delineate the role of the VEGF pathway components VEGF-A and sVEGFR-1 in vessel morphogenesis [90]. Those investigators showed that local secretion of sVEGFR-1 by endothelial cells serves to reduce the VEGF level, decreasing its availability for binding to VEGFR-2 [90]. This also causes an increase in the gradient of VEGF–VEGFR-2 complexes on the surface of the vessel sprout, which could alter the perception of directionality cues by the endothelial cell. The proximity of neighboring sprouts may further impact the gradient of available VEGF, its binding to VEGFRs, and directionality cues [90]. These findings begin to provide insight into some of the mechanisms whereby this complex network of angiogenesis modulators functions in the local control of angiogenesis in tumors [90]. A limitation of the model, however, is its two-dimensional design, as opposed to the more realistic 3D situation. The model also does not take into account the contributions of coreceptor molecules such as NRPs, which will be important to integrate into future models in order to better understand their correlation with angiogenesis [90].

In summary, despite their widespread use in a number of cancer types, the mechanism of action of antiangiogenic agents is still not well understood. Although it has been proposed that anti-VEGF therapy "normalizes" the tumor vasculature, reducing intratumoral pressure and allowing better delivery of therapeutic agents to the tumor, thereby maximizing antitumor activity [7, 10], the exact mechanism by which bevacizumab enhances the efficacy of CT is unknown. Indeed, a recently reported study in patients with NSCLC has challenged this hypothesis because it was found that, within 5 hours of bevacizumab therapy, the perfusion and net influx rate of docetaxel decreased, with the effect persisting up to 4 days [91]. It is also unknown whether the vascular endothelial cells or the tumor itself are the target of the antiangiogenic therapy. This might explain why, despite significant effort, no biomarkers that predict response to antiangiogenic agents have been found. Of several putative biomarkers for bevacizumab, plasma VEGF-A is a candidate, although it remains unknown whether or not it will be useful as a possible predictive or prognostic marker. In this regard, we note recent work investigating the use of circulating VEGF levels as a beneficial biomarker in phase III trials of bevacizumab for mCRC, lung cancer, and renal cancer (>1,800 patients) [92]. Those authors found that a higher circulating VEGF level was associated with poorer PFS and OS outcomes, regardless of bevacizumab treatment, indicating its utility as a prognostic—but not predictive—biomarker [92]. In contrast, recently reported results from the Avastin in Gastric Cancer (AVAGAST) trial, which investigated the use of bevacizumab with CT for gastric cancer, identified baseline VEGF-A levels and NRP-1 as potential predictors of bevacizumab efficacy [93]. In that study, patients with high plasma VEGF-A levels at baseline had a better OS outcome, as did patients with low NRP-1 expression at baseline. However, for both biomarkers, significance was only demonstrated in the non-Asian geographic subgroup [93]. Further study is needed to identify and validate these and other antiangiogenic biomarkers for different tumor types as well as different patient populations.

Targeting tumor angiogenesis has an important place in cancer therapy, and agents that target VEGF have been proven to be useful primarily as combination therapies with CT. Nonetheless, patients eventually progress on antiangiogenic therapies, and treatments do not necessarily lead to lower rates of recurrence and metastatic disease. As with other cancer therapies, in the face of long-term VEGF inhibition, tumors eventually develop mechanisms to evade its antivascular effects. In reality, despite encouraging preclinical and clinical findings, the effective inhibition of VEGF with agents such as bevacizumab has translated into meaningful but modest OS improvements [11–13, 94]. The recruitment of multiple additional angiogenic factors may lead to resistance to VEGF-A–targeting therapies, and as agents that target these pathways continue to be developed, it remains to be seen from emerging clinical data whether or not targeting these factors and pathways (e.g., with agents such as aflibercept or regorafenib) may improve outcomes (Table 4). Results of the VELOUR and CORRECT trials have been most notable in this regard. Additional challenges of antiangiogenic therapy include identifying the optimal dosage, schedule, and combination of agents to use [9, 30, 94]. Lastly, it may be beneficial in the future to view the angiogenesis system not as overly redundant but rather as a complex network of modulators that is capable of adapting to and facilitating a wide range of biologic responses in the tumor microenvironment. Integrating factors such as the distribution of proangiogenic and antiangiogenic influences for a given tumor type and stage over time may help to better design more tar-

Table 4. Angiogenesis and cancer: preclinical hypotheses and clinical data

Preclinical hypothesis	Mediator/therapy	Clinical data
Targeting angiogenesis may lead to tumor regression and reduced invasiveness, thereby improving outcomes	VEGF/Bev	Bev shows efficacy both as a single agent and in combination with CT; improvement in OS is modest; however, patients eventually progress; aflibercept shows efficacy in combination with CT for mCRC
Combining antiangiogenic therapies with chemotherapy may lead to synergistic antitumor activity	VEGF/Bev + CT	Bev shows efficacy in combination with multiple CT regimens over the short term; however, long-term follow-up of some studies shows no benefit on DFS or OS outcomes
Targeting multiple angiogenesis pathways may lead to better antitumor efficacy	VEGF/Bev, TKIs, VEGFR antibodies	Currently under investigation; optimal doses, schedules, durations of treatment, and combinations are unclear; may be tumor specific
Targeting PlGF may be of benefit in some cancers	PlGF/anti-PlGF antibodies, TB 403	Currently under investigation; exact role of PlGF in angiogenesis is not clear
Targeting multiple VEGF ligands with ligand traps may improve antiangiogenic/antitumor efficacy and reduce resistance	VEGF, PlGF, VEGF-B/aflibercept (VEGF Trap)	Efficacy and safety established in combination with FOLFIRI in second-line management of patients with mCRC
Targeting other angiogenesis mediators may help to overcome resistance to Bev	Ang-1, endostatin, FGF, PlGF, PDGF, TSP-2, VEGF-B, VEGF-C, VEGF-D, EGF, HGF, IL-12/TKIs, antibodies, ligand traps, other targeted therapies	Currently under investigation

Abbreviations: Ang-1, angiopoietin-1; Bev, bevacizumab; CT, chemotherapy; DFS, disease-free survival; EGF, endothelial growth factor; FGF, fibroblast growth factor; FOLFIRI, leucovorin, 5-fluorouracil, and irinotecan; HGF, hepatocyte growth factor; IL-12, interleukin 12; mCRC, metastatic colorectal cancer; OS, overall survival; PDGF, platelet-derived growth factor; PlGF, placental growth factor; TKI, tyrosine kinase inhibitor; TSP-2, thrombospondin 2; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

geted, effective, and durable angiogenesis therapies for cancer. As a future perspective, one means of integrating the many factors that regulate angiogenesis and the possible molecular mechanisms to target them would be to develop a standard, nonxenograft, transgenic tumor model whereby different antiangiogenic therapies could be evaluated and assessed, perhaps using fluorescent endothelial cell markers, in order to determine their activity and potency.

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