

# Lessons from phase III clinical trials on anti-VEGF therapy for cancer

Rakesh K Jain\*, Dan G Duda, Jeffrey W Clark and Jay S Loeffler

## SUMMARY

In randomized phase III trials two anti-vascular endothelial growth factor (VEGF) approaches have yielded survival benefit in patients with metastatic cancer. In one approach, the addition of bevacizumab, a VEGF-specific antibody, to standard chemotherapy improved overall survival in colorectal and lung cancer patients and progression-free survival in breast cancer patients. In the second approach, multitargeted tyrosine kinase inhibitors that block VEGF receptor and other kinases in both endothelial and cancer cells, demonstrated survival benefit in gastrointestinal stromal tumor and renal-cell-carcinoma patients. By contrast, adding bevacizumab to chemotherapy failed to increase survival in patients with previously treated and refractory metastatic breast cancer. Furthermore, addition of vatalanib, a kinase inhibitor developed as a VEGF receptor-selective agent, to chemotherapy did not show a similar benefit in metastatic colorectal cancer patients. These contrasting responses raise critical questions about how these agents work and how to combine them optimally. We summarize three of the many potential mechanisms of action of anti-VEGF agents, and also discuss progress relating to the identification of potential biomarkers for anti-VEGF-agent efficacy in humans.

**KEYWORDS** antiangiogenic, bevacizumab, multitargeted, normalization, anti-vascular endothelial growth factor (anti-VEGF)

## REVIEW CRITERIA

Information on completed phase III trials for antiangiogenic agents (available from the NIH databases) and the publications related to these trials were retrieved from <http://www.clinicaltrials.gov> and <http://www.nci.nih.gov/clinicaltrials>, using the search engines on these sites. PubMed was searched using Entrez for articles published up to 30 August 2005, including electronic early-release publications. Search terms included "cancer", "phase III trial", "biomarker", "angiogenesis", "anti-vascular", "multi-targeted", "tyrosine kinase", "tyrosine kinase inhibitor" and "tumor biomarker". The abstracts of retrieved citations were prioritized by relative content. Full articles were checked for additional material when appropriate. The results of some experiments conveyed to the authors by personal communication have also been included.

*RK Jain is the Andrew Cook Professor of Tumor Biology at Harvard Medical School (HMS), and Director of the Edwin L Steele Laboratory for Tumor Biology at Massachusetts General Hospital (MGH), DG Duda is an Instructor at HMS, and an assistant biologist at MGH, JW Clark is the Medical Director for Clinical Programs at the MGH Cancer Center, and Director for Clinical Trials Support at Dana-Farber/Partners Cancer Care, and JS Loeffler is the Herman & Joan Suit Professor of Radiation Oncology at HMS and Chief at MGH, Boston, MA, USA.*

## Correspondence

\*Department of Radiation Oncology, Harvard Medical School and Massachusetts General Hospital, 100 Blossom St, Cox-7, Boston, MA 02114, USA  
jain@steele.mgh.harvard.edu

Received 18 September 2005 Accepted 17 November 2005

www.nature.com/clinicalpractice  
doi:10.1038/ncponc0403

## INTRODUCTION

The dependence of tumor growth and metastasis on blood vessels makes tumor angiogenesis a rational target for therapy.<sup>1–7</sup> Strategies have been pursued to inhibit neovascularization and/or destroy existing tumor vessels, including direct targeting of endothelial cells, and indirect targeting by inhibiting the release of proangiogenic growth factors by cancer or stromal cells. Unlike preclinical studies in mice, an overall survival benefit with anti-vascular endothelial growth factor (VEGF)-specific agents (e.g. monoclonal antibodies) used as monotherapy has yet to be demonstrated in phase III trials. The addition of a VEGF-specific antibody, bevacizumab (Avastin®; Genentech, Inc., South San Francisco, CA) to current cytotoxic regimens, however, led to improved overall survival (OS) in previously untreated colorectal and lung patients and in previously treated colorectal cancer patients, as well as improved progression-free survival (PFS) in previously untreated breast cancer patients.<sup>8,9</sup> By contrast, adding bevacizumab to cytotoxic therapy did not enhance survival in previously treated metastatic breast cancer patients.<sup>10</sup> Moreover, replacing bevacizumab with VEGF-receptor-selective, multitargeted agents such as vatalanib (Novartis, Basel, Switzerland, a receptor tyrosine kinase inhibitor [TKI]) in the combined regimen did not show similar efficacy in chemotherapy-naïve or previously treated colorectal cancer patients.<sup>11</sup> Nevertheless, monotherapy using multitargeted agents that have a broader spectrum of inhibitory effect on VEGF receptors and a number of growth-factor pathways in cancer cells (sorafenib, Nexavar®, Bayer AG, Leverkusen, Germany and Onyx Pharmaceuticals, Emeryville, CA, and sunitinib, Pfizer, New York, NY) has resulted in significantly prolonged PFS in patients with renal-cell cancer and gastrointestinal stromal tumors (GIST).<sup>12,13</sup> Of interest, several agents that target oncogenic signaling pathways (such as the EGFR/HER2-specific antibodies cetuximab, Erbitux®, ImClone, New York, NY

or trastuzumab, Herceptin®, Genentech) and may indirectly inhibit angiogenesis, have yielded increased OS with chemotherapy in clinical trials and are approved for human use in Europe, the US and elsewhere.

These contrasting results raise important questions about the use of anti-VEGF agents in clinical practice. Will anti-VEGF monotherapy increase survival in randomized phase III trials? How can tumor-vessel regression by combined anti-VEGF treatment—instead of compromising the delivery and efficacy of cytotoxic treatment—prolong OS in previously treated colorectal cancer, chemotherapy-naïve colorectal cancer and lung cancer patients, as well as PFS in breast cancer patients? Why do anti-VEGF agents not prolong survival in certain previously treated cancer patients? Why are some broad-spectrum multitargeted agents, which block signaling from receptors present on both endothelial and cancer cells, effective as monotherapy? What biomarkers of treatment efficacy can be used to guide the optimal use of anti-VEGF agents in cancer patients? In this review, we will summarize the results of recent phase III clinical trials of anti-VEGF agents, address the questions raised above, and suggest new avenues for further investigation (Boxes 1 and 2).

### CLINICAL DEVELOPMENT OF ANTI-VEGF THERAPY

Vascular endothelial growth factor A (VEGF-A, also called VEGF) is a potent proangiogenic growth factor expressed by most cancer-cell types and certain tumor stromal cells.<sup>3,5,14</sup> VEGF stimulates endothelial-cell proliferation, migration and survival, expression of adhesion molecules, and potently induces increased vascular permeability.<sup>2,3</sup> VEGF can also affect new-vessel formation in tumors by acting as a chemoattractant for bone-marrow-derived progenitor/stem cells.<sup>15</sup> VEGF expression can be triggered during early stages of neoplastic transformation by environmental stimuli (e.g. hypoxia or low pH) or by genetic mutations (e.g. in *K-ras*, *p53*, or *HER2/Erbb2*), and persists during progression.<sup>2,16</sup> Proteolytic enzymes produced by cancer cells or stromal cells can also release sequestered VEGF bound to the extracellular matrix.<sup>2</sup> Treatment itself (e.g. radiation or anti-VEGF therapy<sup>17–19</sup>) may increase VEGF production or accumulation, and inhibiting its activity might be important in maintaining antitumor efficacy.

Thus, blocking the action of VEGF appears to be a promising antiangiogenic approach to treating multiple types of solid tumors. Such inhibition can be achieved by direct or indirect targeting of the ligand (VEGF) at the mRNA or protein level, direct targeting of its receptors (VEGFR1, VEGFR2, and neuropilin 1 [NP1]; Table 1), or by blocking downstream signaling pathway components. Each of these strategies is currently being investigated in clinical trials (Figure 1).

Direct treatments for antiangiogenesis include antibodies, soluble receptors, low-molecular-weight TKIs, antisense oligonucleotides, APTAMERS and RNA INTERFERENCE (Table 1). Monoclonal antibodies that block VEGFR1 (IMC-18F1, ImClone Systems, New York, NY) or its specific ligand placental growth factor (PlGF) (TB-403, BioInvent International, Lund, Sweden and ThromboGenics Ltd, Dublin, Ireland) are also under clinical development. VEGF-Trap (Regeneron, Tarrytown, NY), a composite decoy receptor based on VEGFR1 and VEGFR2, fused to an antibody Fc segment, potently blocks VEGF and PlGF action. RNA-interference-based approaches (e.g. ICS-283; Intradigm Corporation, Rockville, MD) that target VEGF are currently under preclinical development. Of interest, aptamer (pegaptanib sodium; Macugen®, Eyetech Pharmaceuticals, Inc., New York, NY), as well as antibody fragment (ranibizumab; Lucentis®, Genentech, South San Francisco, CA) that target VEGF, are FDA-approved for patients with age-related macular degeneration. Finally, because the VEGF pathway acts downstream of most oncogenes, treatment with agents that target these oncogenic pathways can indirectly inhibit VEGF (further information is provided in Supplementary Table 1; see website for details).

Anti-VEGF-specific monotherapy has been shown to have antivasular effects on tumor vessels,<sup>19,20</sup> but has yet to yield an OS benefit in phase III trials. In lung and colorectal carcinoma trials, bevacizumab monotherapy was discontinued because the primary endpoint (OS) was unlikely to be achieved based on the inferior responses compared with the chemotherapy and combination-regimen groups.<sup>8,21</sup>

VEGF blockade by bevacizumab has yielded improved OS or PFS in cancer patients in four phase III trials when combined with standard chemotherapy (Table 2). These results emphasize the potential benefit of targeting

### GLOSSARY

#### APTAMERS

Short strands of synthetic DNA or RNA that change their shape to bind specific sites on target molecules, including DNA promoter or enhancer regions

#### RNA INTERFERENCE

Use of double-stranded RNA to interfere with normal RNA processing, causing rapid degradation of endogenous RNA and precluding translation; a simple method of studying the effect of absence of a gene product

**Box 1** Lessons from phase III clinical trials of anti-VEGF agents.**1 Clinical application of anti-VEGF therapy is more complex than initially thought**

The mechanisms of action of anti-VEGF therapy in cancer patients are far from fully understood. Of the various mechanisms possible based on preclinical data, clinical evidence is available for only three effects: (a) anti-VEGF agents can prune tumor vessels in patients and thus kill a fraction of cancer cells; (b) anti-VEGF agents can normalize tumor vasculature and microenvironment; and (c) anti-VEGF treatment can reduce the number of blood-circulating endothelial cells and progenitor cells.

**2 To date, anti-VEGF specific monotherapy has not been shown to increase survival in cancer patients**

Data from current trials suggest that anti-VEGF monotherapy was less efficient than standard chemotherapy. Monotherapy can yield increased response rates, but tumors may escape agent targeting by alternative proangiogenic pathways. The anti-VEGF combinations that have increased survival have simultaneously targeted the cancer cells either by using bevacizumab with chemotherapy (see below) or broad-spectrum multitargeted small molecule tyrosine kinase inhibitors (TKIs).

**3 Bevacizumab increases survival when added to chemotherapy in first-line treatment of patients with metastatic colorectal cancer, and patients with breast and lung tumors**

Bevacizumab with chemotherapy enhanced overall survival in previously untreated metastatic colorectal cancer and lung cancer patients, and progression-free survival in chemotherapy-naïve metastatic or recurrent breast cancer patients compared with standard chemotherapy alone. These outcomes are consistent with the concept that bevacizumab enhances the efficacy of cytotoxic agents by normalizing tumor vasculature and microenvironment.

**4 In previously treated cancer patients, bevacizumab combined with chemotherapy increased survival in metastatic colorectal patients, but not metastatic breast cancer patients**

The reason for these contrasting results is unknown, but it might be the fact that FOLFOX 4, used in the colorectal cancer trial, is an effective, proven second-line treatment.

**5 VEGF blockade by vatalanib did not enhance survival conferred by FOLFOX 4 in metastatic colorectal cancer patients.**

The discrepancy between the benefits of chemotherapy with anti-VEGF therapy with bevacizumab versus vatalanib is yet to be explained. Bevacizumab targets the ligand VEGF, while vatalanib targets VEGF receptors. Vatalanib affects PDGFR- $\beta$ —present on tumor stromal cells and involved in perivascular cell recruitment—and thus might interfere with vascular normalization. Vatalanib also blocks VEGFR3 and c-Kit but does not directly affect the VEGF receptor NP1—a non-tyrosine kinase receptor present on certain cancer cells and a mediator of cell survival—but only some of its associated tyrosine kinase receptors.

**6 Multitargeted TKIs that target multiple pathways critical for both cancer cell growth and angiogenesis, increase survival in monotherapy**

Broad-spectrum multitargeted TKIs such as sunitinib (which targets VEGFR2, PDGFR- $\beta$ , FLT3 and c-Kit) and sorafenib (an inhibitor of Raf kinase, VEGFR2, PDGFR- $\alpha$  and PDGFR- $\beta$ , FLT3 and c-Kit) affect pathways involved in both endothelial and cancer cell growth and/or survival. They are the first agents to show increased progression-free survival in monotherapy.

**7 Several molecularly targeted therapies—approved or under trial—target directly cancer cells and indirectly tumor angiogenesis**

FDA-approved HER2/EGFR-specific antibodies (e.g. trastuzumab, cetuximab) block EGF/TGF- $\alpha$  signaling and decrease the expression of multiple angiogenic factors. Adding a VEGF-specific blocker such as bevacizumab might further benefit patients and this hypothesis is currently being tested in patients. Alternatively, TKIs under clinical development may be used to block both EGFR and VEGFR.

**8 Anti-VEGF therapies have rare but serious associated toxicities**

Side effects of anti-VEGF agents are moderate compared with other therapies, but the etiology is poorly understood. Major safety concerns have been raised by an increased morbidity, and a number of treatment-related deaths from bowel perforations, thromboembolic events, and hemorrhage. VEGF inhibition can affect the function of organs with fenestrated endothelium (e.g. kidney, thyroid). Many cancer patients receive radiotherapy. Blockade of VEGF in this context might increase the toxicity of radiotherapy.

**9 There is an urgent need for biomarkers to guide anti-VEGF monotherapy and combination therapy**

Robust biomarkers to guide patient selection and protocol design are yet to be clinically validated. Ongoing efforts might identify such surrogate markers (e.g. proteins in tumor tissue or bodily fluids, circulating endothelial cells, tumor interstitial fluid pressure, tumor physiological parameters evaluated by imaging techniques).

**10 Optimized treatment strategies are needed to prevent tumor escape after anti-VEGF therapy, and extend survival beyond 2–5 months**

Several strategies may significantly improve treatment outcome. Patients who may benefit from anti-VEGF treatment need to be identified. For these patients, novel doses and schedules of combination therapies based on tumor biology and validated biomarkers should improve survival beyond that seen with anti-VEGF and cytotoxic therapies. Moreover, the molecular and cellular mechanisms underlying tumor escape from anti-VEGF therapy and relapse should be identified and tumor angiogenesis targeted with alternative available or novel antiangiogenic agents.

and killing both endothelial and neoplastic cells to enhance survival in multiple types of cancer. Impressive results have been achieved by combining anti-VEGF therapy with contemporary cytotoxic agents or by using broad-spectrum multitargeted agents that block VEGF and other growth-factor pathways in both cell types (Figure 1).

**Targeting of cancer and endothelial cells by anti-VEGF therapy and chemotherapy**

Bevacizumab, a humanized VEGF-specific antibody with a reported half-life of 17–21 days,<sup>5</sup> was given to cancer patients in combination with traditional cytotoxic regimens. Some phase I and II trials demonstrated objective responses (including a few complete responses<sup>22</sup>) to this

combined therapy.<sup>23</sup> The first randomized placebo-controlled phase III trial of bevacizumab, however, failed to show increased PFS or OS when bevacizumab was combined with chemotherapy in previously treated metastatic breast cancer patients.<sup>10</sup> In this trial, despite an increased response rate, no survival benefit was seen in patients receiving bevacizumab with capecitabine versus patients receiving capecitabine alone.

The clinical breakthrough for antiangiogenic therapy came from a randomized phase III trial showing a 4.7-month increase in OS (the primary endpoint) when bevacizumab was used with chemotherapy (irinotecan/5-fluorouracil/leucovorin) in previously untreated, metastatic colorectal cancer patients (Table 2).<sup>9</sup> Based on these data, bevacizumab became the first anti-VEGF agent to be approved by the FDA for cancer patients. Three other unpublished randomized phase III trials have shown positive results. One trial investigated the efficacy of bevacizumab with standard chemotherapy (paclitaxel) in patients with chemotherapy-naïve recurrent or metastatic breast cancer and achieved the primary endpoint of increased PFS.<sup>8</sup> In another trial, previously treated patients with advanced colorectal cancer who received bevacizumab in combination with proven second-line therapy (an oxaliplatin–5-fluorouracil–leucovorin regimen, FOLFOX 4) had a 2.1-month increase in OS—the primary endpoint—compared with patients who received FOLFOX 4 alone (Table 3). Whether combining bevacizumab with FOLFOX 4 or other chemotherapy regimens will be the best option for first-line therapy for colorectal cancer is under investigation (see reference 24 for review and discussion). Finally, a trial of patients with previously untreated advanced nonsquamous and non-small-cell lung cancer showed a 2.3-month increase in median survival (the primary endpoint) when bevacizumab was added to standard chemotherapy (paclitaxel and carboplatin).<sup>21</sup> This was the first randomized trial of this agent in combination with chemotherapy that showed a median survival of greater than 1 year in one of the arms, and the first to use a targeted agent and demonstrate a survival advantage in combination with chemotherapy in patients with untreated metastatic lung cancer. The utility and feasibility of using bevacizumab in previously treated lung cancer patients is unknown. Even in renal-cell carcinoma, which is a highly VEGF-dependent malignancy, increase

### Box 2 Potential mechanisms of tumor escape after anti-VEGF therapy.

Beyond achieving proof-of-principle confirmation in clinical trials, there is a need to optimize antiangiogenic therapy to delay tumor relapse beyond a few months. The mechanisms of tumor escape from the anti-VEGF agent might be different—yet not mutually exclusive—during therapy. As tumors progress, they secrete an increasing number of proangiogenic factors and become hypervascular, but the vessels are leaky and the blood flow is spatially and temporally heterogeneous. To achieve an antivascular effect, an antiangiogenic agent should target critical angiogenic pathways in a given tumor; otherwise, tumors might escape this effect soon after the onset of therapy. When efficacious, the effect of antiangiogenic therapy can be threefold: inhibition of new vessel formation and pruning of immature tumor vessels, transient normalization of the remaining vasculature and reduction in the number of blood-circulating endothelial cells and progenitor cells. If the cytotoxic effect of anti-VEGF monotherapy is insufficient for killing of all cancer cells, or the anti-VEGF agent is combined with a cytotoxic agent to which the tumors are refractory, the tumors relapse. Thus, efficacious cytotoxic agents should be added to effective anti-VEGF agent(s). Even if a synergistic effect is achieved by combining the anti-VEGF agent and the cytotoxic agent(s), relapse can occur after combination therapy by at least three mechanisms. First, long-term or high-dose anti-VEGF therapy might lead to a vasculature that is inefficient for drug delivery (in addition to increasing the rate and grade of its side effects). Optimizing the dose and schedule of the anti-VEGF agent might prevent these outcomes. Second, tumors may relapse because they use alternative pathways for neovascularization or as a result of acquired genomic instability by the endothelial cells. These pathways could be targeted with available or novel antiangiogenic agents. Third, cancer cell clones can acquire resistance to the chemotherapeutics used or repopulate between chemotherapy cycles. These clones should be specifically targeted with available or novel therapeutics.

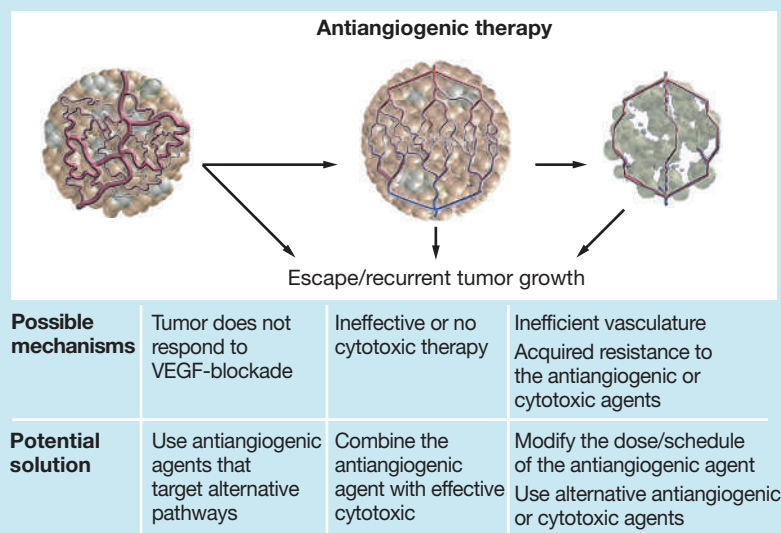


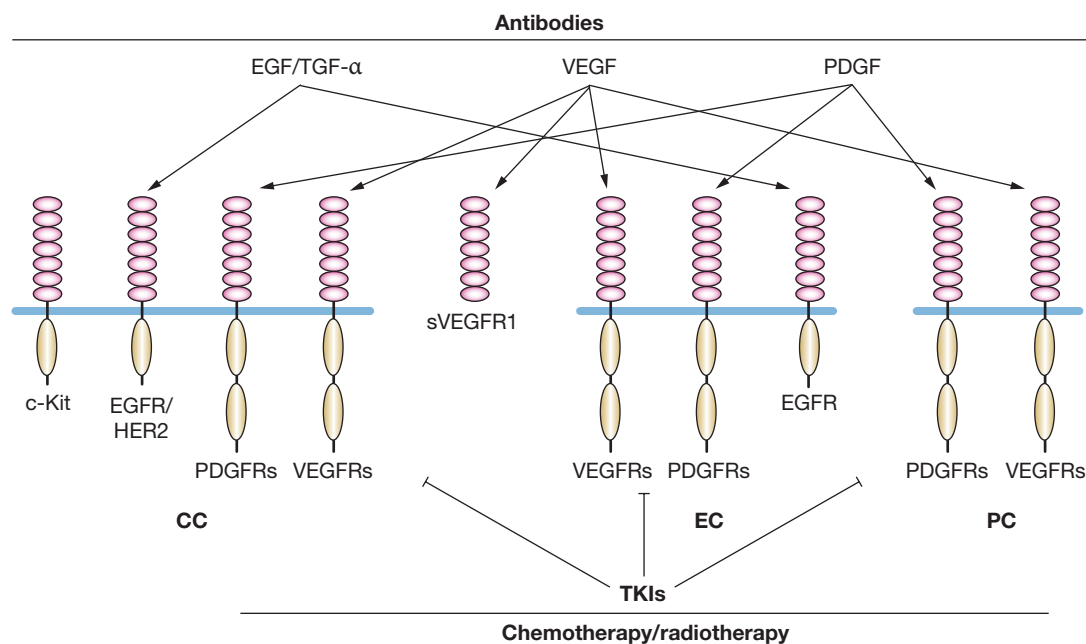
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in OS by bevacizumab monotherapy has yet to be demonstrated in a phase III study. A phase III trial of bevacizumab and interferon- $\alpha$  versus interferon- $\alpha$  alone for renal-cell cancer has been completed, but results are not yet available. Collectively, these phase III trials show that anti-VEGF therapy can increase OS and/or PFS in colorectal, breast and lung cancer patients when combined with cytotoxic agents.

**Table 1** Anti-VEGF agents currently in clinical development.

Phase of development	Drug	Targets	Description
<b>Specific anti-VEGF agents</b>			
Marketed/phase III–IV	Bevacizumab (Avastin®)	VEGF	Monoclonal antibody
Phase I	VEGF Trap	VEGF, PlGF, VEGF-B	Soluble receptor
Phase I	VEGF-AS (Veglin®)	VEGF, VEGF-C, VEGF-D	Antisense oligonucleotide
Phase I	Aplidin® Dehydrodidemnin B	VEGF	Peptide
<b>Multitargeted agents that selectively target VEGF receptors<sup>a</sup></b>			
Phase III	Vatalanib (PTK787/ZK 222584)	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-Kit	Small-molecule tyrosine kinase receptor inhibitor
Phase III	AE-941 (Neovastat®)	VEGF–VEGFR binding MMP2, MMP9	Shark-cartilage component
Phase I–II	AZD2171	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-Kit	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	CEP-7055	VEGFR1, VEGFR2, VEGFR3	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	CHIR258	VEGFR1, VEGFR2, FGFR1, FGFR 3	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	CP-547632	VEGFR2	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	GW786034	VEGFR2	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	IMC-C1121b	VEGFR2	Monoclonal antibody
Phase I–II	OSI-930	VEGFR, c-Kit	Small-molecule tyrosine kinase receptor inhibitor
<b>Broad spectrum multitargeted agents that target VEGF receptor and other kinases present in endothelial and cancer cells<sup>a</sup></b>			
Phase III	Sorafenib (formerly BAY 43-9006)	VEGFR-2, PDGFR-β, FLT3, c-Kit	Small-molecule raf kinase and tyrosine kinase inhibitor
Phase III	Sunitinib (SU11248)	VEGFR2, PDGFR-β, FLT3, c-Kit	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	ZK-CDK	VEGFRs, PDGFR, CDKs	Small-molecule tyrosine kinase inhibitor
Phase I–II	AG013736	VEGFR, PDGFR-β, c-Kit	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	AMG706	VEGFR1, VEGFR2, PDGFR-β, c-Kit	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	KRN-951	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-Kit	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	BMS-582664	VEGFR2, FGFR	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	XL999	FGFR, VEGFRs, PDGFR, FLT3	Small-molecule tyrosine kinase receptor inhibitor
Phase I–II	Zactima® (ZD6474)	VEGFR2, EGFR, RET	Small-molecule tyrosine kinase receptor inhibitor

<sup>a</sup>This classification is based on the available data (evaluated primarily *in vitro*) on the affinity of these tyrosine kinase inhibitors. CDK, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FLT3, fms-related tyrosine kinase 3; MMP, matrix metalloproteinase; PDGFR, platelet-derived growth factor receptor; PlGF, placental growth factor; VEGF, vascular endothelial growth factor. Manufacturers: AE-941 (Neovastat®; Aeterna Zentaris, Inc., Quebec, Canada); AG-013736 (Pfizer, New York, NY); AMG706 (Amgen, Thousand Oaks, CA); Aplidin® Dehydrodidemnin B, Pharma Mar, Madrid, Spain; AZD2171 (AstraZeneca Pharmaceuticals, Cheshire, UK); Bevacizumab (Avastin®; Genentech, Inc., South San Francisco, CA); BMS-582664 (Bristol-Myers Squibb, New York, NY); CEP-7055 (Cephalon, Frazer, PA); CHIR258 (Chiron, Emeryville, CA); CP-547632 (OSI Pharmaceuticals, Melville, NY); GW786034 (GlaxoSmithKline, Research Triangle Park, NC); IMC-C1121b (ImClone Systems, New York, NY); KRN-951 (Kirin Brewery, Takasaki, Japan); OSI-930 (OSI Pharmaceuticals, Melville, NY); Sorafenib (formerly BAY 43-9006, Nexavar, Bayer Aktiengesellschaft, Leverkusen-Bayerwerk, Germany, and Onyx Pharmaceuticals Inc., Emeryville, CA); Sunitinib (SU11248, Pfizer, New York, NY); vatalanib (Novartis, Basel, Switzerland); VEGF-AS (Veglin®; Vasgene Therapeutics, Inc., Sharon Hill, PA); VEGF-Trap (Regeneron, Tarrytown, NY); XL999 (Exelixis, San Francisco, CA); Zactima (ZD6474, AstraZeneca Pharmaceuticals, Cheshire, UK); ZK-CDK (Schering AG, Berlin, Germany).



**Figure 1** Schematic representation of direct targeting of cancer, endothelial and perivascular cells by anti-VEGF agents. Combined and direct targeting of cancer cells and endothelial and perivascular cells has yielded increased survival in phase III trials of anti-VEGF agents. This has been achieved by two approaches. The first combines traditional cytotoxic agents (which may kill any proliferating cell) with the VEGF-specific antibody bevacizumab. VEGF blockade will inhibit its signaling pathways in endothelial cells responsible for cell survival, migration, proliferation and vascular permeability; VEGF blockade might also affect cancer cells, when their survival depends on VEGF (e.g. via NP1). The second approach uses low-molecular-weight tyrosine kinase inhibitors with broad inhibitory spectra (i.e. active against VEGFRs, EGFRs, PDGFRs, c-Kit receptors, and/or downstream soluble kinases such as Raf), which may be present in all these cell populations and on other tumor stromal cells (e.g. immune cells and fibroblasts). Alternatively, combinations of antibodies that block the ligands (e.g. VEGF and EGFR/HER2 or PDGF) might be effective in targeting both cancer and endothelial cells. The intracellular tyrosine kinase domains are shown in yellow and the extracellular ligand binding domains are depicted in small pink spheres.

CC, cancer cells; EC, endothelial cells; EGF, epidermal growth factor; EGFR, EGF receptor; HER2, human epidermal growth factor receptor 2; PC, perivascular cells; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; TKIs, tyrosine kinase inhibitors; TGF- $\alpha$ , transforming growth factor- $\alpha$ ; VEGF, vascular endothelial growth factor; sVEGFR1, soluble VEGF receptor 1.

### Targeting of cancer and endothelial cells by multitargeted anti-VEGF agents with or without chemotherapy

The second approach is to target both cancer cells and endothelial cells with small molecules that inhibit signaling pathways for VEGF and other factors by blocking tyrosine kinase activity with or without chemotherapy. Similar to bevacizumab, VEGFR receptor kinase-selective multitargeted agents have been used in combination with chemotherapeutic agents in phase III clinical trials (Table 3). A randomized phase III study of semaxinib (SU5416; Pharmacia, San Francisco, CA), which primarily targets VEGFR1, VEGFR2 and VEGFR3, and secondarily targets PDGFR- $\beta$  and c-Kit, with 5-fluorouracil/leucovorin and 5-fluorouracil/leucovorin/irinotecan in patients

with metastatic colorectal carcinoma, failed to show, at interim analysis, any survival benefit for the SU5416-containing regimens, resulting in the cessation of further development of this compound.<sup>25</sup> Vatalanib (PTK787/ZK 222584; Novartis, Basel, Switzerland) also primarily targets VEGFR1, VEGFR2, and VEGFR3, and secondarily targets PDGFR- $\beta$  and c-Kit, and can decrease blood flow at doses greater than 750 mg/day.<sup>26</sup> One trial (CONFIRM 1) compared the efficacy of oral vatalanib in combination with FOLFOX 4 versus FOLFOX 4 alone for first-line treatment of metastatic colorectal cancer. The primary endpoint of this trial, improvement in PFS as judged by an independent central review, was not met. A secondary endpoint of the trial, OS, is perhaps just as clinically relevant, and these

**Table 2** Completed Phase III trials for the VEGF-specific antibody bevacizumab with standard contemporary chemotherapy.

Tumor type	Selection criteria				Outcome of trial	Significant toxicity of bevacizumab with chemotherapy	Reference
	Stage	Previous treatment	Regimen	Patients enrolled			
Breast cancer	Metastatic	Anthracycline/taxane/Herceptin® (Genentech, Inc., San Francisco, CA)	BV (15 mg/kg) + capecitabine (3-week cycles)	462	Increased RR (19.8% vs 9.1%; $P=0.001$ ) Comparable PFS (4.86 months vs 4.17 months; HR 0.98) and OS (15.1 vs 14.5 months)	Hypertension, proteinuria, thromboembolic events, bleeding, pulmonary embolism	Miller <i>et al.</i> (2005) <sup>10</sup>
Colorectal cancer	Metastatic	No	BV (5 mg/kg) + IFL (2-week cycles)	813	Increased RR (44.8% vs 34.8%, $P=0.004$ ) Prolonged PFS (10.6 months vs 6.2 months, $P<0.001$ ) and OS (20.3 months vs 15.6 months, $P<0.001$ )	Hypertension, thromboembolic events, deep thrombophlebitis, bleeding, gastrointestinal perforation	Hurwitz <i>et al.</i> (2004) <sup>9</sup>
Lung cancer (nonsquamous, non-small cell)	Metastatic	No	BV (15 mg/kg) + paclitaxel/carboplatin (3-week cycles)	878	Increased RR (27.2% vs 10.0%) Prolonged PFS (6.4 months vs 4.5 months, $P<0.0001$ ) and OS (12.5 months vs 10.2 months, $P<0.007$ )	Hypertension, thrombosis, life-threatening or lethal (eight patients) bleeding	NCI fact sheet <sup>8</sup>
Colorectal cancer	Metastatic	5-FU/Irinotecan	BV (10 mg/kg) + FOLFOX 4 (2-week cycles)	829	Increased RR (21.8% vs 9.2%, $P=0.0001$ ) Prolonged PFS (7.2 months vs 4.8 months, $P<0.0001$ ) and OS (12.9 months vs 10.8 months, $P<0.001$ )	Hypertension, bleeding, bowel perforation	NCI fact sheet <sup>8</sup>
Breast cancer	Recurrent/metastatic	No	BV (10 mg/kg) + paclitaxel (2-week cycles)	722	Increased RR (28 months vs 14 months) Prolonged PFS (10.97 months vs 6.11 months) OS: N/A	Hypertension, proteinuria, bleeding	NCI fact sheet <sup>8</sup>

5-FU, 5-fluorouracil; BV, bevacizumab; IFL, irinotecan, 5-fluorouracil and leucovorin; FOLFOX 4, oxaliplatin, 5-fluorouracil and leucovorin; HR, hazard ratio; N/A, not available yet; OS, overall survival; PFS, progression-free survival; RR, response rate.

data may be available in early 2006.<sup>27</sup> Interim results from a phase III trial of vatalanib in combination with FOLFOX 4 chemotherapy as second-line treatment for metastatic colorectal cancer (CONFIRM 2), however, suggested no significant benefit in OS. Thus, multitargeted TKIs are yet to show survival benefit in phase III trials when combined with chemotherapeutics. Questions about the efficacy of these classes of agents and their scheduling can only be answered with additional pharmacokinetic and correlative clinical studies.

Conversely, monotherapy with other multitargeted broad-spectrum TKIs has shown

efficacy in two randomized phase III trials for tumors with limited treatment options. Sorafenib (BAY 43-9006; Nexavar®, Bayer Aktiengesellschaft, Leverkusen-Bayerwerk, Germany, and Onyx Pharmaceuticals Inc., Emeryville, CA) targets VEGFR2 and VEGFR3, PDGFR-β, Raf, c-Kit and FLT3 (fms-related tyrosine kinase 3), and efficiently inhibits both tumor-cell proliferation and angiogenesis in preclinical models.<sup>28</sup> Interim data from a randomized, placebo-controlled phase III trial (with OS as the primary endpoint) showed that renal-cell-carcinoma patients taking 400 mg sorafenib twice daily (sorafenib's half-life is about 20–27 h)

**Table 3** Completed Phase III trials for multi-targeted agents with VEGF receptor 2 kinase as one of their targets.

Tumor type	Selection criterion	Regimen used	Target	Number of enrolled patients	Outcome of trial	Grade 3 and 4 toxicity	Reference
Colorectal cancer	Untreated metastatic CRC (CONFIRM 1)	Vatalanib or placebo with FOLFOX 4	VEGFR1, VEGFR2, VEGFR3, PDGFR- $\beta$ , and c-Kit	1,168	No significant change in PFS	Hypertension, neutropenia, diarrhea, nausea, neuropathy, vomiting, thrombosis, dizziness, thrombocytopenia, neutropenia, pulmonary embolism	No authors listed (2005) <sup>11</sup>
Colorectal cancer	Previously treated metastatic CRC (CONFIRM 2)	As above	As above	>1,000	As above	As above	As above
Renal cancer	Advanced RCC	Sorafenib (formerly BAY 43-9006)	RAF kinase, VEGFR2, PDGFR- $\alpha$ and PDGFR- $\beta$ , FLT3 and c-Kit	>900	Improved PFS (24 weeks vs 12 weeks, $P < 0.01$ )	Rash, diarrhea, hand-foot syndrome, nausea, fatigue	Branca (2005) <sup>13</sup> Marx (2005) <sup>29</sup>
Gastrointestinal stromal tumor	Previously treated gastrointestinal stromal tumor	Sunitinib	VEGFR2, PDGFR- $\beta$ , FLT3 and c-Kit	312	Prolonged time to progression (6.3 months vs 1.5 months, $P < 0.01$ )	Hypertension, fatigue, diarrhea	Branca (2005) <sup>13</sup> Marx (2005) <sup>29</sup>

CRC, colorectal carcinoma; PDGFR, platelet-derived growth factor receptor; PFS, progression-free survival; RCC, renal-cell carcinoma; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

had a significant improvement in PFS,<sup>12,13</sup> despite a marginal (2%) partial-response rate. Thus, patients on the placebo arm were allowed to cross over to sorafenib after unblinding. A new drug application has been filed with the FDA for sorafenib for use in patients with advanced renal-cell carcinoma. Another recent success was obtained with sunitinib (SU11248; Pfizer, New York, NY) in patients with GIST. In addition to targeting VEGFR2, sunitinib targets c-Kit, PDGFR- $\beta$ , and FLT3. The activated form of the c-Kit receptor is often expressed in GISTs, and is thus a good candidate for treatment with TKIs that inhibit c-Kit activity, such as the FDA-approved agent imatinib mesylate (Gleevec®; Novartis, Basel, Switzerland), which also targets PDGFR- $\alpha$  and PDGFR- $\beta$  and BCR/ABL1. A randomized phase III study assessing sunitinib in the treatment of imatinib-resistant GIST successfully met its predetermined efficacy endpoint, time-to-progression (Table 4).<sup>29</sup> Sunitinib has a half-life of 40 h and was given daily at a dose of 50 mg in 4-week cycles with

2-week breaks to patients randomized 2:1 to patients receiving placebo. A planned interim analysis of the phase III study data led to the recommendation that the study be 'unblinded' to give all enrolled patients access to sunitinib. Sunitinib showed high response rates in refractory metastatic renal cancers in a large phase II trial.<sup>30</sup> An application for FDA approval of sunitinib has also been submitted. Both sorafenib and sunitinib are expected to increase OS in patients in these trials. These contrasting outcomes for multitargeted TKIs when used in monotherapy versus combined with chemotherapy call for further investigations on the importance of each target and their mechanisms of action.

#### Inhibiting tumor angiogenesis indirectly

The use of approved targeted agents—that can indirectly inhibit angiogenesis—in combination with chemotherapy has also shown increased survival in breast cancer patients. In a pivotal phase III trial, trastuzumab (Herceptin®;

**Table 4** Surrogate markers under testing for the evaluation of the efficacy of anti-VEGF agents.

Marker	Parameter evaluated	Comments/limitation	References
<b>Invasive</b>			
Tissue biopsy	Immunohistochemistry: <ul style="list-style-type: none"> <li>▪ protein expression as a marker</li> <li>▪ microvascular density</li> <li>▪ perivascular cell coverage of tumor vessels</li> <li>▪ cell proliferation/apoptosis</li> </ul> Genomic analyses	Not easily available in some tumors	Willett <i>et al.</i> (2004, 2005) <sup>19,20</sup> Ince <i>et al.</i> (2005) <sup>67</sup> Jubb <i>et al.</i> (in press) <sup>68</sup> Rugo <i>et al.</i> (2005) <sup>90</sup>
Interstitial fluid pressure measurement	Tumor interstitial fluid pressure	Difficult accessibility in some tumors	Willett <i>et al.</i> (2004, 2005) <sup>19,20</sup> Boucher <i>et al.</i> (1991, 1997) <sup>70,71</sup> Gutmann <i>et al.</i> (1992) <sup>72</sup> Less <i>et al.</i> (1992) <sup>73</sup> Milosevic <i>et al.</i> (2001) <sup>74</sup> Nathanson and Nelson (1994) <sup>75</sup> Padera <i>et al.</i> (2002) <sup>76</sup> Roh <i>et al.</i> (1991) <sup>77</sup>
Measurements of tissue oxygenation	Tumor oxygen tension	Lack of accessibility in some tumors	Dunst <i>et al.</i> (1998) <sup>78</sup>
Skin wound healing	Wound healing time	Explored both as biomarker of efficacy and indicator of side effects	Mundhenke <i>et al.</i> (2001) <sup>92</sup>
<b>Minimally invasive</b>			
Blood CECs	Concentration of viable CECs	Unclear origin, viability and surface phenotype of CECs	Rafii <i>et al.</i> (2002) <sup>15</sup> Willett <i>et al.</i> (2004, 2005) <sup>19,20</sup> Beaudry <i>et al.</i> (2005) <sup>80</sup> Shaked <i>et al.</i> (2005) <sup>82</sup> Blann <i>et al.</i> (2005) <sup>93</sup>
Blood CPCs	Concentration of CPCs	Low concentration of CPCs in humans, heterogeneous population	As above
Protein level in plasma	Protein concentration (e.g. VEGF, PlGF, TSP1, adhesion molecules)	Inability to detect active protein versus protein bound to the anti-VEGF agent (e.g. VEGF bound to bevacizumab, VEGF or PlGF bound to VEGF Trap)	Bocci <i>et al.</i> (2004) <sup>18</sup> Willett <i>et al.</i> (2005) <sup>19</sup>
Protein level in ascites	Protein concentration	Limited to certain tumors, might be altered by changes in permeability induced by anti-VEGF therapy	Kraft <i>et al.</i> (1999) <sup>94</sup>
Protein level in pleural effusions	Protein concentration	Limited to certain tumors, might be altered by changes in permeability induced by anti-VEGF therapy	Sack <i>et al.</i> (2005) <sup>95</sup>
<b>Non-invasive</b>			
CT imaging	Blood flow and volume, permeability–surface area product mean transit time	Resolution, measurement of composite parameters	Willett <i>et al.</i> (2004, 2005) <sup>19,20</sup> Jennens <i>et al.</i> (2004) <sup>84</sup>
PET imaging	Tracer uptake	Resolution, measurement of composite parameters	Willett <i>et al.</i> (2004, 2005) <sup>19,20</sup> Jennens <i>et al.</i> (2004) <sup>84</sup>
MRI	Blood flow, permeability	Resolution, measurement of composite parameters	Liu <i>et al.</i> (2005) <sup>85</sup> Miller <i>et al.</i> (2005) <sup>86</sup>
Protein level in urine	Urine MMPs, VEGF, etc.	Limited to excreted proteins, depends on factors that might be altered by treatment such as renal function (e.g. proteinuria)	Chan <i>et al.</i> (2004) <sup>87</sup>

CECs, circulating endothelial cells; CPCs, circulating progenitor cells; CT, computer tomography; MMPs, matrix metalloproteinases; MRI, magnetic resonance imaging; PET, positron emission tomography; PlGF, placental growth factor; TSP1, thrombospondin 1; VEGF, vascular endothelial growth factor.

Genentech, Inc., South San Francisco, CA) administered in combination with chemotherapy (anthracycline/cyclophosphamide or paclitaxel) was compared with chemotherapy alone.<sup>31</sup> The

combination was found to produce significant OS benefit in HER2-positive metastatic breast cancer patients. These results led to the approval of trastuzumab for clinical use in the US and

elsewhere. Recently, two phase III trials showed that breast cancer patients with HER2-positive tumors who received trastuzumab in combination with doxorubicin, cyclophosphamide and paclitaxel had a 52% decrease in disease recurrence compared with patients treated with chemotherapy alone.<sup>32,33</sup> This difference was statistically significant. Most of the patients enrolled in these studies had lymph-node-positive disease.<sup>32,33</sup> Whether improved OS can be achieved by combining direct and indirect inhibitors of angiogenesis in cancer patients awaits the outcome of the ongoing phase III trials.

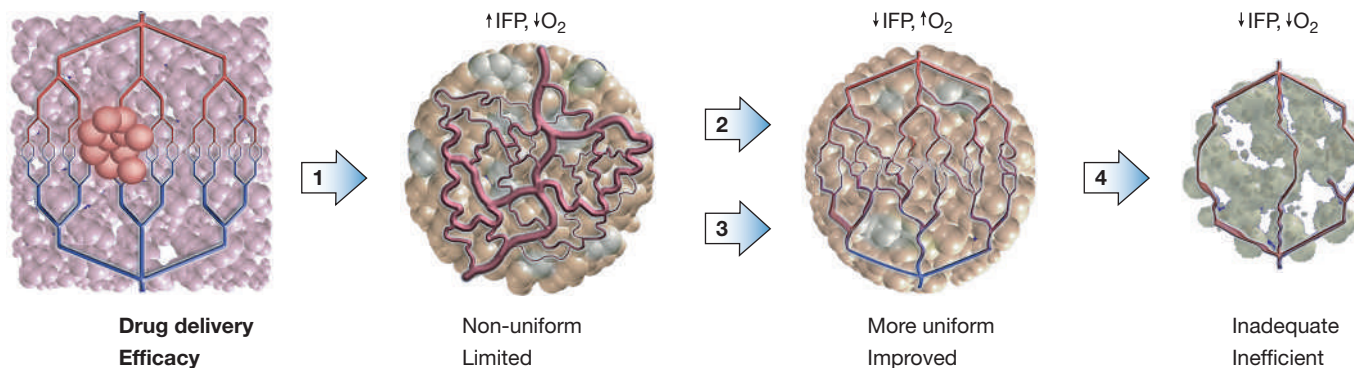
### SAFETY OF ANTI-VEGF AGENTS

Most agents that target VEGF or its receptors have been well tolerated by patients in clinical trials, either as single agents or in combination with standard chemotherapy.<sup>6</sup> The toxicity that occurs most consistently for anti-VEGF agents is hypertension, which may be secondary to the inhibition of nitric oxide release. Hypertension is usually manageable with medical treatment and ongoing therapy (Tables 2 and 3).<sup>26,34–38</sup> Fatigue is a more-common side effect across the multitargeted TKIs than for monoclonal antibodies. Other toxicities occur more commonly with specific agents. For example, serious but rare toxicities of bevacizumab include thromboembolic events (primarily arterial), gastrointestinal perforations (seen with greater frequency in colorectal and ovarian cancer patients), impaired wound healing (including surgical), and life-threatening or fatal hemorrhage.<sup>35</sup> Sorafenib is associated with fatigue, anorexia, diarrhea, rash and hand–foot syndrome.<sup>34,36</sup> Vatalanib has been associated with a higher incidence of fatigue, light-headedness/dizziness, nausea and uncommon thrombotic events.<sup>26,38</sup> Fatigue, nausea, diarrhea, mucositis and hand–foot syndrome have been seen with sunitinib.<sup>37</sup> Bevacizumab and multitargeted TKIs may also potentiate some of the toxicities associated with the chemotherapeutic agents with which they are given, such as neuropathy, asthenia, and bone marrow suppression. Such toxicities tend to be mild and usually do not entail significant dose modifications of the chemotherapy regimen. One important, albeit relatively uncommon, toxicity potentiated by these agents, however, is the increased risk of congestive heart failure; as noted with the use of bevacizumab (increase from 0.5% to 2.2%, mainly in patients who have received prior or concomitant anthracyclines, or prior left chest

wall radiation).<sup>10</sup> As more patients receive these agents for prolonged periods, more unexpected toxicities might occur.<sup>6</sup>

### MECHANISMS OF ACTION OF COMBINATION THERAPIES THAT INCLUDE ANTI-VEGF TARGETING

Effective anti-VEGF agents should block the formation of new tumor vessels and prune the existing ones. Despite the successes of some direct anti-VEGF agents (bevacizumab, sorafenib, sunitinib) and indirect inhibitors of angiogenesis (such as trastuzumab), their mechanism of action in patients is far from fully understood. For example, a recent phase I trial in rectal cancer patients has demonstrated that a single infusion of bevacizumab alone can prune tumor vessels and reduce tumor microvascular density by 40–50%.<sup>19,20</sup> However, to date, no phase III trial of bevacizumab has shown survival benefit in monotherapy arms over chemotherapy. On the other hand, one would expect that vessel pruning should interfere with the delivery of chemotherapeutic agents given to these patients, and thus fail to provide any survival benefit. Indeed, the very first phase III trial for bevacizumab in breast cancer patients failed to show a benefit. The failure of bevacizumab to increase survival in heavily treated breast cancer patients was initially explained by first, the highly refractory and advanced nature of the tumors in the patients enrolled, and second, the increased expression of other angiogenic factors during breast cancer progression caused by chemotherapy, which rendered VEGF less critical for continued tumor growth.<sup>10</sup> These hypotheses were partially supported by the success of a subsequent trial of bevacizumab combined with a different chemotherapeutic agent in treatment-naïve advanced breast cancer patients.<sup>8</sup> But the hypotheses seem to be contradicted by the efficacy of bevacizumab with chemotherapy in heavily treated colorectal cancer patients. Vatalanib does not seem to confer the same survival advantage as bevacizumab in colorectal cancer patients when combined with chemotherapy.<sup>8</sup> These contrasting results raise outstanding questions about the mechanisms of action of these agents alone and in combination. Here, we will discuss only three of the many mechanisms of action postulated for anti-VEGF therapy<sup>6</sup> and the combination with chemotherapy, which can potentially reconcile the differing outcomes in clinical trials.



**Figure 2** Potential mechanisms of action of bevacizumab on tumor vasculature. Owing to high levels of proangiogenic molecules produced locally, such as VEGF, tumors make the transition from *in situ* carcinoma to frank carcinoma (1). At this stage, tumors become hypervascular, but the vessels are leaky and the blood flow is spatially and temporally heterogeneous. This leads to increased interstitial fluid pressure (IFP) and focal hypoxia, creating barriers to delivery and efficacy of therapeutics. The proposed mechanism of action of the VEGF-specific antibody bevacizumab is twofold: inhibition of new-vessel formation and killing of immature tumor vessels (2); and transient normalization of the remaining vasculature by decrease in macromolecular permeability (and thus the IFP) and hypoxia, and improvement of blood perfusion (3). Another effect of bevacizumab may be the direct killing of cancer cells in subsets of tumors in which the cells express VEGF receptors. Regardless of the mechanisms involved, monotherapy with bevacizumab is not curative because it cannot kill all cancer cells, and in the longer term leads to a vasculature that is inefficient for drug delivery (4), and to tumor relapse using alternative pathways for neovascularization. Combinations of bevacizumab with chemotherapeutics have therefore been pursued in phase III trials and have led to a survival benefit in patients with chemosensitive tumors, showing the synergistic effect of the two treatment modalities. Synergy may have been achieved as a result of increased cell killing following tumor vascular normalization: the lowered IFP leads to improved delivery of chemotherapeutics and molecularly targeted agents; the improved oxygenation sensitizes cancer cells to cytotoxic therapeutics and reduces the selection of more-malignant phenotype; and, finally, increased cellular proliferation around normalized vessels might increase the cytotoxicity of chemotherapeutics. Normalization of the vasculature might also benefit the direct killing of cancer cells by bevacizumab, in synergy with the chemotherapeutics. Of interest, cytotoxic therapeutics may kill proliferating endothelial cells, and thus may also normalize the tumor vasculature and improve drug delivery to tumors.<sup>7</sup> Figure adapted with permission from Ref 41 © (2001) Nature Publishing Group.

### Normalization of tumor vasculature and microenvironment

More than a decade ago, Teicher<sup>39</sup> proposed that combining antiangiogenic therapy with cytotoxic treatments would have synergistic effects because it allows targeting of both the malignant cell compartment and the vascular stroma (Figures 1 and 2). Bevacizumab efficiently reduces the number of tumor vessels;<sup>19,20</sup> however, the reduction in the number of tumor vessels achieved by anti-VEGF therapy might antagonize chemotherapy and radiotherapy by impeding the delivery of therapeutics and oxygen (a radio-sensitizer), respectively. Indeed, a number of preclinical studies have demonstrated such antagonism.<sup>7,40</sup>

At the same time, such combinations of cytotoxic and antiangiogenic therapies have been successful in a number of preclinical and clinical studies. To resolve this paradox, in 2001 we proposed that anti-VEGF therapy can 'normalize' tumor vasculature.<sup>41</sup> Such normalization can be defined as the structural and functional changes occurring in the context of

a significant reduction in the number of tumor vessels that allow, at least transiently,<sup>42</sup> an increased and/or more-uniform delivery of drugs and oxygen, ultimately leading to improved outcome.<sup>7,41</sup> While improved oxygenation can increase the efficacy of radiation therapy or of certain chemotherapeutic agents, it might also accelerate tumor growth. Indeed, we have shown that bevacizumab monotherapy can increase cancer-cell proliferation in some rectal cancer patients.<sup>19</sup> These proliferating cells are likely to be more sensitive to chemotherapy. The increase in proliferation, however, occurs in the context of increased apoptosis of cancer cells. Hence, the effects of vessel normalization in some regions of the tumor seem to be overwhelmed by simultaneous vessel regression in other regions. In addition, the inability of tumors to recruit new vessels during antiangiogenic therapy limits the ability of this transient increase in vascular efficiency to expand the tumor mass. Of interest, we have seen no significant changes in tumor fluorodeoxy glucose (FDG)-uptake in rectal cancer patients receiving bevacizumab, despite a

40–50% reduction in vascular density.<sup>19,20</sup> This suggests that the residual ‘normalized’ vasculature is more efficient. Emerging preclinical results from our laboratory and others support the concept of vascular normalization.<sup>42–49</sup> More importantly, the clinical data from a phase I trial of bevacizumab in rectal cancer patients are also consistent with the vascular normalization hypothesis.<sup>19,20</sup> Our ongoing phase II study of bevacizumab and radiochemotherapy in rectal cancer patients will provide additional data on these vascular and cellular parameters.

Vascular normalization can potentially explain why bevacizumab is efficacious in combination with chemotherapy, despite the limited efficacy of bevacizumab as monotherapy.<sup>7,40</sup> The process suggests that bevacizumab increased survival rates in chemotherapy-naïve metastatic breast cancer patients by improving the delivery of chemotherapeutics to chemoresponsive tumors, while such an increase was not seen in chemotherapy-refractory metastatic breast cancer patients, in whom improved delivery of chemotherapeutics might have less of an effect. Vascular stabilization during VEGF blockade potentially also decreased the shedding of metastatic cancer cells from the primary tumors. The alleviation of hypoxia by bevacizumab might make the tumors more chemosensitive and less metastatic. Finally, if during vascular normalization the improved tumor microenvironment led to increased proliferation of the surviving cancer cells, this might have rendered them more sensitive to cytotoxic agents (Figure 2).<sup>7,19,40</sup>

Then why did VEGF blockade by a multitargeted-agent (vatalanib) treatment not show a clear benefit with FOLFOX 4 regimen in metastatic colorectal cancer patients? Besides the simple explanation that it is not as effective an agent at administered doses, vatalanib has a considerably shorter half-life (~6 h) than bevacizumab (~20 days), and the phase III trials for vatalanib used a single daily dose of the drug. Contradicting these data, however, is the fact that pharmacokinetic data suggest that an active dose of vatalanib is maintained in the blood circulation for 24 h, and that it has a rapid and pronounced anti-vascular effect.<sup>50</sup> Another explanation could be the off-target effects (i.e. other than on the VEGF receptor kinases). For example, vatalanib might target PDGFR- $\beta$  on perivascular cells. This action was shown in mice to be beneficial for vascular targeting, since the PDGF-B-PDGFR- $\beta$  axis is known to control

vascular stabilization/maturation by recruitment of supporting perivascular cells. Blocking PDGFR- $\beta$ , however, may interfere with vascular normalization, by blocking perivascular cell recruitment and excessive vessel pruning, and thus prevent the synergistic effect of combined therapy.<sup>51</sup> Thus, the clinical benefit of targeting perivascular cells in addition to endothelial cells with multitargeted TKIs in the context of chemotherapy remains unclear. How anti-VEGF therapy affects the recruitment or response of cells of the immune system in cancer patients is not known.

Given these facts, why did other multitargeted TKIs, with broader inhibitory spectra and longer half-lives, prove efficacious in other tumor types? Our hypothesis is that broad-spectrum multitargeted TKIs (i.e. those that simultaneously target multiple receptor or soluble kinases such as c-Kit, Raf, FLT3, PDGFR- $\alpha$ , etc.) mimic the synergistic effect offered by vascular normalization for combinations of anti-VEGF antibody and chemotherapy more effectively than the combinations of multitargeted VEGF receptor kinases-selective TKIs used with chemotherapeutics. This concept is yet to be proven in the clinic, but is strongly supported by the PFS gain produced by sorafenib in renal-cell carcinoma and by sunitinib in imatinib-resistant GIST patients.<sup>29</sup>

Collectively, these considerations imply that, if we are to optimally use single-targeted or multitargeted anti-VEGF agents, treatment schemes must be tailored for each agent. For example, it is not yet clear whether the addition to chemotherapy of existing multitargeted TKIs—which selectively target VEGF receptor kinases—to chemotherapy will impact outcome (e.g. vatalanib or semaxinib) to an extent comparable with the responses seen for the combination of bevacizumab—which specifically targets VEGF—with chemotherapy. In choosing a multitargeted agent, the ability to define its spectrum and to match it at the molecular level with the disease will be desirable.

Some agents that target cancer cells directly may indirectly block angiogenesis. In a preclinical model of HER2 overexpressing human breast cancer, trastuzumab (a HER2-specific antibody) decreased the expression of several angiogenic factors (including VEGF by cancer cells), while increasing the expression of the endogenous angiogenesis inhibitor thrombospondin 1, and induced changes (i.e. reduction in tumor vascular permeability, vessel diameter and

vascular volume, but not vessel length) consistent with vascular normalization.<sup>52</sup> In this tumor model, however, trastuzumab actually increased VEGF expression in stromal cells. Thus, efficacious targeting of endothelial and cancer cells might be achieved by regimens that combine tumor-cell targeting by anti-epidermal growth factor receptor (EGFR)-specific agents with direct anti-VEGF agents (such as bevacizumab). Alternatively, monotherapy regimens might consist of multitargeted TKIs that concomitantly target the EGFR/HER2 on cancer cells in addition to VEGF receptor kinases. Preliminary data from the combination of cetuximab and bevacizumab, either alone or in combination with the chemotherapeutic agent irinotecan, for patients with irinotecan refractory colorectal cancer, suggest that these combinations are feasible and have potentially promising response rates.<sup>53</sup> Additional trials combining trastuzumab, cetuximab or erlotinib with bevacizumab, as well as trials of Zactima® (ZD6474; AstraZeneca Pharmaceuticals, Cheshire, UK), which is a multitargeted TKI (selective for VEGFR, EGFR and RET), have reached phase II and/or III (in thyroid, breast, colorectal, lung, pancreatic and head-and-neck-cancer patients), and the results will have important implications for the therapy of HER2-positive or EGFR-positive cancers.<sup>54</sup>

#### **Antiangiogenic effects of chemotherapeutic agents and ionizing radiation**

Another mechanism for the beneficial effects seen for combined cytotoxic and anti-VEGF therapy might be the direct killing of proliferating endothelial cells by chemotherapeutics. Whereas none of the successful phase III trials of bevacizumab to date used dose-dense<sup>55,56</sup> or low-dose 'metronomic'<sup>57,58</sup> chemotherapy, this method could augment the antiangiogenic therapy by killing tumor blood vessels. By killing endothelial cells and cancer cells (the major source of proangiogenic factors), chemotherapy might also normalize the tumor vasculature and thus make drugs penetrate tumors more uniformly.<sup>7</sup>

Similar to chemotherapy, certain doses of ionizing radiation can induce apoptosis in tumor endothelial cells in mice.<sup>59</sup> Radiation fractionation and dose scheduling may determine the relative toxicity of radiation to endothelial versus cancer cells.<sup>60</sup> To date, although early-phase trials have demonstrated the feasibility and tolerability of combining bevacizumab with radiotherapy, this combination has not

been evaluated in phase III trials. Given the role of tissue oxygenation in tumor response to radiation, as well as the potential protective role of VEGF against endothelial cell apoptosis in response to radiation, a greater understanding of these issues in conjunction with vascular normalization and anti-VEGF therapies will be critical to development of this approach.

#### **Cytotoxic effects of anti-VEGF therapy**

Treatment with anti-VEGF agents was designed to target tumor endothelial cells and was expected to indirectly kill cancer cells. VEGF inhibition, however, might also have direct cytotoxic effects on cancer cells that express VEGF receptors and which depend on VEGF for survival.<sup>61–63</sup> Many cancer cells express the receptor NP1, which modulates signaling pathways including VEGF. Blockade of this receptor has been reported to mediate apoptosis *in vitro*.<sup>64</sup> The blockade of the ligand (i.e. VEGF) will prevent its binding to NP1, while TKIs have no direct effect on this receptor in cancer cells because NP1 does not have any tyrosine kinase activity,<sup>65</sup> but will affect the activity of some of the NP1-associated receptors (such as VEGFR2).

We have found a significant increase in rectal carcinoma cell apoptosis after bevacizumab treatment alone.<sup>19</sup> The extent to which the increase in apoptosis in these patients was due to direct killing of cancer cells by bevacizumab or due to indirect killing by the reduction of blood vessels is not known. A better understanding of this issue is even more critical for multitargeted agents that affect multiple growth-factor pathways in endothelial and cancer cells.

#### **POTENTIAL BIOMARKERS FOR ANTI-VEGF THERAPY**

Currently, there are no proven biomarkers of efficacy of anti-VEGF therapy. These biomarkers are urgently needed to validate the mechanistic hypotheses, to identify responsive patients and optimal doses, to predict efficacy of regimens that include anti-VEGF agents, and to detect and prevent tumor escape (Box 2). These biomarkers would facilitate the optimization of the dose and schedule of bevacizumab for colorectal, breast and lung cancer, and extrapolation of the existing efficacy data to other tumor types. For example, the efficacy and toxicity of bevacizumab with chemotherapy were not always dose-dependent.<sup>66</sup> Unlike in preclinical studies, the survival benefit

for colorectal cancer patients from the addition of bevacizumab to irinotecan/5-fluorouracil/leucovorin was independent of *K-ras*, *BRAF*, or *p53* mutation status, *p53*, VEGF or thrombospondin 2 expression, or microvascular density evaluated prior to treatment.<sup>67,68</sup>

We have measured significant functional, structural, cellular and molecular changes in tumors in response to VEGF blockade, without a significant reduction of tumor volume.<sup>19,20</sup> How these changes during anti-VEGF treatment relate to PFS or OS, however, is not known. Thus, in the absence of an overt cytotoxic effect of the anti-VEGF-specific agents, other surrogate markers for efficacy must be identified. Significant advances have been made in identifying candidate markers; however, no such marker has been shown to be predictive in the clinical setting.<sup>69</sup> Some of the candidate markers include classic diagnostic or prognostic biomarkers, as well as newly developed, target-based and mechanism-based biomarkers (Table 4).

Biopsy of tumor tissue is a routine but highly invasive diagnostic and prognostic method that has great potential to identify valuable markers for therapeutic efficacy. Where serial biopsies can be obtained, immunostaining for the protein/cell of interest and/or gene profiling using laser capture microdissection of the cellular components of interest might yield invaluable information on the effect of a given anti-VEGF agent. For example, we have evaluated microvascular density,  $\alpha$ -smooth muscle actin and Ang2 expression, as well as tumor-cell apoptosis and proliferation during bevacizumab treatment in rectal cancer patients.<sup>19,20</sup> Interstitial fluid pressure and tissue oxygenation are parameters that reflect vascular function and, by extension, delivery of therapeutics.<sup>70–78</sup> Changes in tumor interstitial fluid pressure<sup>19,20</sup> or tissue oxygen level during treatment might be valuable surrogate markers of the efficacy of anti-VEGF therapy, and can be measured in several tumor types and sampled from areas of accessible tumors.

Less-invasive methods include measuring changes in protein concentration (e.g. growth factors) in bodily fluids as surrogate markers for therapy. For example, blood plasma levels of VEGF were significantly increased by VEGFR2 blockade in mice and were proposed as a surrogate marker for VEGFR2 blockade.<sup>18</sup> In rectal cancer patients, bevacizumab increased plasma levels of VEGF, but also of PlGF, in all patients analyzed.<sup>19</sup> To date, it is unknown whether

commercially available kits detect free VEGF from VEGF bound to bevacizumab. The level of VEGF stored in platelets<sup>79</sup> might also serve as a surrogate marker, calling for evaluation of VEGF both in the serum and in the plasma. We found that bevacizumab decreased the concentration of viable circulating endothelial cells (CECs) and progenitor cells in rectal cancer patients.<sup>19</sup> We also found that the total number of CECs (which includes non-viable/apoptotic CECs) did not decrease, but rather increased, with bevacizumab treatment, consistent with recent preclinical data.<sup>80</sup> This result may reflect the shedding of non-viable tumor endothelial cells following antiangiogenic treatment (DG Duda, unpublished observations), and might become an independent surrogate marker for anti-VEGF treatment and vascular normalization if quantitative methods of detecting tumor-derived CECs can be optimized. In preclinical studies, progenitor cells have been extensively studied both as surrogate markers and for their role in cancer growth and treatment,<sup>15,81,82</sup> but their concentration in whole blood in humans is very low (two orders of magnitude lower than viable CECs<sup>19</sup>). Elucidation of the biology of different subsets of progenitor cells in humans, and development of improved techniques to reliably quantify cells in this concentration range may allow the future use of circulating cells as surrogate markers in the clinic. In addition, this will help us to understand any putative role that circulating endothelial cell populations might play in tumor response or escape from anti-VEGF therapy.<sup>19,20</sup>

Noninvasive techniques have the potential to measure functional parameters and offer surrogate markers for therapy, regardless of tumor type or location.<sup>83</sup> Such techniques include dynamic MRI, CT, and positron emission tomography, and are being pursued in clinical trials by our own team and others.<sup>19,20,50,84–86</sup> One main limitation of these imaging methods is that they measure composite parameters, which depend on both blood flow and permeability. Another limitation relates to the high heterogeneity of blood flow. The spatial distribution of blood flow determines the distribution of chemotherapeutics and oxygen in tumor tissue, and cannot be evaluated with high spatial resolution by the imaging techniques that are currently available. With improvement of these techniques to measure the spatial and temporal changes in tumor blood flow and vascular permeability with higher resolution, it might be possible to assess more precisely the

efficacy of anti-VEGF therapy. Nevertheless, the cost of such investigations might be prohibitive for larger trials. Finally, protein measurements in urine have become increasingly feasible,<sup>87–89</sup> and exploring them during therapy might offer independent surrogate markers for the effect of anti-VEGF agents. Combining and comparing multiple parameters obtained using different assays in phase I–II clinical trials hold great promise for defining the effects of individual anti-VEGF agents and identifying simple and meaningful surrogate markers of efficacy.<sup>19,20,84,90</sup> The best candidates should eventually be validated and used in larger phase III trials and integrated into routine clinical practice.<sup>83,91</sup>

### CONCLUSION

The recent successes of the antiangiogenic agents have raised great hope and have taught us important lessons about the significance of the target, timing and dosage of each agent (Box 1). Anti-VEGF agents are now expected to make a difference in cancer patients with a wide variety of tumor types. With the advent of specific and potent new agents—approved or in the process of being approved—oncologists have a variety of direct and indirect antiangiogenic agents to choose from when designing therapy protocols. Determining whether the regimens used in the successful trials are optimal, however, and whether anti-VEGF agents will work in patients outside the rigorous inclusion criteria used for those trials, will be critical for deciding the standard of care for different malignancies. Establishing the most advantageous combinations will require a better understanding of the mechanisms of action of each anti-VEGF agent and the sensitivity of each tumor type, as well as development of robust biomarkers and imaging techniques to guide patient selection and protocol design. A deeper understanding of the mechanisms of antitumor activity of specific and multitargeted anti-VEGF agents in patients, how they can best be combined with other treatment approaches such as chemotherapy and radiation therapy, and how optimization of these effects can be monitored clinically, should contribute to significantly improved cancer treatment and extend survival of cancer patients in the near future, as well as enhance the prospects of developing curative treatment for different cancers in the more-distant future.

**Supplementary information** is available on the *Nature Clinical Practice Oncology* website.

### KEY POINTS

- Based on the results of the phase III trials completed to date, bevacizumab can increase median survival when combined with standard chemotherapy, but not when used as monotherapy
- Anti-VEGF therapy with bevacizumab can increase overall survival and/or progression-free survival in colorectal, breast and lung cancer patients when combined with cytotoxic agents
- Improved survival has been observed with broad-spectrum multitargeted tyrosine kinase inhibitors (e.g. sorafenib, sunitinib) when used in monotherapy
- In colorectal cancer patients, vatalanib, a VEGF-receptor-selective tyrosine kinase inhibitor, does not confer the same survival advantage as bevacizumab when combined with chemotherapy
- Anti-VEGF therapy can prune and ‘normalize’ tumor vasculature, and decrease the number of circulating endothelial cells and progenitor cells
- There is an urgent need to identify biomarkers to guide anti-VEGF therapy and combination therapies using anti-VEGF agents

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**Acknowledgments**

The authors thank the members of the Steele Lab, especially Y Boucher, E di Tomaso, D Fukumura, K Kozak, S Kozin, L Munn, and T Padera. We also thank T Batchelor, H Chen, V Natarajan, W Novotny, L Rosen, A Ryan, J Samson, C Willett, and J Wood for their helpful comments on this manuscript. The authors' work is supported by grants from the National Cancer Institute.

**Competing interests**

RK Jain declared competing interests; go to the article online for details. The other authors declared they have no competing interests.

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