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Angiogenesis and Vascular Targeting in Ewing Sarcoma: A Review of Preclinical and Clinical Data

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Abstract

Ewing sarcoma is the second most common type of bone cancer in children and young adults. In recent years, the mechanisms by which these tumors develop and maintain their vascular supply have been elucidated. Additional work has demonstrated that inhibition of angiogenic pathways or disruption of established vasculature can attenuate the growth of Ewing sarcoma mouse xenografts. Early clinical data suggest that these results may also extend to patients with Ewing sarcoma treated with anti-angiogenic or anti-vascular therapies. This review summarizes the available data supporting this approach.

Keywords

Angiogenesis; Ewing sarcoma; VEGF; bevacizumab; thrombospondin

Introduction

Ewing sarcoma is the second most common primary bone cancer affecting children and young adults. Despite improvements in outcome for patients with localized tumors[1, 2], treatment for those who have metastatic disease remains unsatisfactory, with long-term overall survival (OS) below 30%, even with significant dose intensification of cytotoxic therapies.[3, 4] Patients with relapsed disease also fare poorly, particularly if disease recurs early.[5, 6] Improvements in outcome for patients with metastatic or relapsed disease will likely require novel therapeutic approaches.

One innovative strategy that has gained interest in recent years has been the use of angiogenesis inhibition and vascular disruption for patients with solid tumors. We performed an extensive review of the available medical literature related to angiogenesis and vascular supply in Ewing sarcoma. We utilized the US National Library of Medicine's PubMed (www.pubmed.gov) search function to find relevant primary articles based on key search terms including VEGF, PDGF, thrombospondin, angiogenesis, angiogenic, microvessel, endothelial, vascular, vasculogenesis, bevacizumab, and metronomic. These search terms were searched together with "Ewing", "Ewing's", or "bone cancer". The "Related Articles" function of PubMed and references of relevant articles were utilized to expand the search and identify additional relevant articles.

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This review summarizes the results of this search and will begin with a description of the mechanisms by which Ewing tumors develop and maintain their vascular supply. This will be followed by a discussion of the relationship between biomarkers of angiogenesis and features of Ewing sarcoma. The preclinical data supporting an anti-angiogenic or anti-vascular strategy in this disease will then be examined The review will conclude with an assessment of the few clinical studies to date that have treated patients with Ewing sarcoma using these approaches.

Mechanisms of Vascular Supply in Ewing Sarcoma

As with all solid tumors, Ewing sarcoma tumors require a viable vascular supply in order for tumor cells to grow beyond the limits of oxygen and nutrient diffusion into tissues. These tumors utilize three main strategies to develop and maintain their vascular supply: angiogenesis; vasculogenesis; and tumor cell vascular mimicry.7 These processes are summarized in the Figure. Vascular endothelial growth factor (VEGF) appears to play a crucial role in these processes across a wide range of tumor types.8 It is commonly understood that the tumor cells produce one or more VEGF isoforms which stimulate both proliferation and survival of the associated tumor endothelium via its receptors, VEGFR-1 and VEGFR-2. Endothelial cells in Ewing sarcoma tumors demonstrate a high proliferative rate, indicating active angiogenesis in these tumors.9 Several lines of evidence have highlighted the importance of VEGF in driving this process in Ewing sarcoma. For example, Ewing sarcoma cells deficient in expression of one VEGF isoform, $VEGF_{165}$, produce smaller tumors with reduced microvessel density.10 Re-expression of $VEGF_{165}$ in these cells restores microvessel density.

However, $VEGF_{165}$ also appears to be important in the recruitment of bone marrow derived cells into the vascular network of Ewing sarcoma, a process known as vasculogenesis. These bone marrow derived cells can differentiate into both tumor-associated endothelial cells and pericytes.11, 12 Studies have demonstrated that only CD34-positive bone marrow derived cells contribute to this process.12 $VEGF_{165}$-deficient Ewing sarcoma cells recruited significantly fewer bone marrow derived cells into xenograft tumors compared to $VEGF_{165}$-expressing cells.10, 13 Forced expression of another VEGF isoform, $VEGF_{189}$, into $VEGF_{165}$-deficient cells did not enhance bone marrow cell recruitment, indicating a specific role for $VEGF_{165}$ in driving this process. Inhibiting VEGF receptor-2 attenuates the recruitment of bone marrow cells into growing tumors.14 While VEGF, and specifically $VEGF_{165}$, stimulates vasculogenesis, other cytokines such as stromal cell-derived factor 1 (SDF-1) may also promote bone marrow derived cell recruitment into tumors and provide an alternate pathway supporting vascular development.15

Given the critical role of VEGF in both angiogenic and vasculogenic processes supporting tumor growth, interesting data have emerged supporting the hypothesis that the VEGF pathway may actually be a target of the EWS/ETS fusion oncoproteins characteristic of Ewing sarcoma. In one study, plasmids expressing EWS/FLI or EWS/ETV were transfected into RK13 cells and levels of VEGF expression recorded.16 Transfection of either EWS/FLI or EWS/ETV resulted in an increase in activation of the VEGF promoter. Follow-up experiments demonstrated that this effect does not appear to be due to direct DNA binding by either EWS/FLI or EWS/ETV and does not require the presence of the Hypoxia Response Element of the VEGF promoter.16

Another vascular network system in Ewing sarcoma may not be driven by VEGF. Ewing sarcoma cells appear capable of directly lining vascular spaces in a process known as vascular mimicry. In one study, pools of blood, known as blood lakes, were found in 92% of human Ewing sarcoma tumor samples.9 The cells lining these blood lakes were evaluated by

immunohistochemistry (IHC) and found to be CD31and CD34 negative, but CD99 positive. Their appearance by electron microscopy was also characteristic of tumor and not endothelial cell origin. Further experiments demonstrated that these tumor cells expressed TFPI-1/2, VE-cadherin, and EphA2, proteins previously shown to be important in vascular mimicry. Follow-up preclinical studies showed that the Ewing sarcoma cell lines could form vascular structures to a variable extent when grown in a collagen matrix, however the formation of these structures was neither enhanced by VEGF nor inhibited by VEGF blocking antibody.9 Those cell lines with a greater propensity for forming vascular structures showed greater expression of genes identified in other malignancies as markers of vascular mimicry, including integrin α3, VE-cadherin, TFPI-1, EphA2, laminin5γ2, Tie-1, neuropilin, and endoglin. Perfusion and intravital microscopy studies in Ewing xenografts have demonstrated that these tumor cell-lined blood lakes appear to be functional, and in continuity with the systemic circulation.

Several parallel pathways have recently been implicated in the modulation of angiogenesis and vasculogenesis in Ewing sarcoma. The insulin-like growth factor (IGF) pathway, for example, may positively regulate VEGF expression. One study has demonstrated that treatment of Ewing sarcoma cells *in vitro* with IGF-1 increases expression of VEGF mRNA and protein.17 Moreover, treatment of Ewing sarcoma cells with either a monoclonal antibody directed against the IGF-1 receptor or transfection with antisense directed against the IGF-1 receptor resulted in significant attenuation in VEGF production.17 Additional work has shown that treatment of Ewing sarcoma cells or mouse xenografts with a small molecule inhibitor of the IGF-1 receptor attenuated VEGF production and tumor vascular mimicry.18 On the other hand, Ewing sarcoma cells may also stimulate angiogenesis through down-regulation of the endogenous anti-angiogenic protein, thrombospondin. In one study, NIH3T3 cells transfected with the Ewing sarcoma specific fusion oncogenes *EWS/FLI1*, *EWS/ERG*, and *EWS/ETV1* showed reduced expression of thrombospondin 2.19 This effect may be mediated by binding of the fusion oncoprotein to the thrombospondin promoter. In Ewing sarcoma cell lines, thrombospondin 1 expression is repressed. With shRNA treatment intended to block the expression of *EWS/FLI1*, thrombospondin 1 expression is restored in these cells.19 These studies indicate that Ewing sarcoma cells rely on multiple mechanisms to initiate and maintain tumor vascular supply.

Finally, some consideration must be given to the tumor microenvironment that supports the vascular structures. Migration and proper application of pericytes and vascular mural cells fortifying endothelial tubes in developing tissues or tumors has been shown to selectively protect vessels against apoptosis when VEGF levels decline. PDGF-B secreted by the endothelium and signaling via PDGFR-β has been identified as a key mediator of this process.20, 21 Bone microvascular endothelial cells have been shown themselves to expressPDGFR-β *in vitro* and ligand-binding induces rapid phosphorylation and subsequent activation of Aktand ERK1/2 associated with an increase in endothelial cell division and survival.22 Indeed, functional PDGFR-β expression has also been demonstrated in Ewing sarcoma cell lines and Ewing sarcoma primary tumor specimens suggesting an important role for this signaling pathway in tumor development. However, the ligands PDGF-B or PDGF-D do not appear to be derived from the tumor cells and may derive in fact, from the vascular endothelium.23 Finally, in experimental Ewing sarcoma tumors which recur after exposure to VEGF blockade, vessels are characterized by significant increases in diameter and proliferation of vascular mural cells with increased expression of factors that promote endothelial integrity (angiopoietin-1; Ang-1) and PDGF-B.24 Thus, the PDGF pathway is likely to contribute to vascular stability and tumor proliferation in Ewing sarcoma.

Markers of Angiogenesis in Patients with Ewing Sarcoma

In order to consider the potential utility of anti-angiogenic and vascular targeting therapies, it would be helpful to understand the clinical impact of angiogenesis biomarkers in patients with Ewing sarcoma. Unfortunately, these data are relatively scarce and difficult to interpret. At least three groups have investigated tumor microvessel density (MVD) as a prognostic factor in Ewing sarcoma. One group demonstrated a significant association between elevated (MVD) and poor event-free and overall survival in 29 patients with Ewing sarcoma.25 In contrast, two other groups have reported that tumor MVD does not correlate with metastatic status, disease-free survival, or overall survival.26, 27

Other studies have focused on tumor and blood levels of VEGF, again with conflicting results. One group assessed VEGF expression by IHC in 40 patient tumor samples and found that patients with tumor VEGF expression above the median had improved relapse-free and OS compared to those with low VEGF expression.26 Another group studying 31 patient tumors concluded that those patients with tumors lacking VEGF expression had superior OS compared to patients with tumors staining positively for VEGF.16 As in most oncologic processes, mean serum VEGF levels in patients with Ewing sarcoma appear to be higher than those in healthy controls.28, 29 No study has evaluated the impact of serum VEGF levels on outcome specifically in patients with Ewing sarcoma. However, one study investigated this issue in a cohort of children with a range of solid tumors, including 5 patients with Ewing sarcoma.30 In this analysis, patients with higher serum VEGF levels had inferior outcomes compared to patients with lower serum VEGF levels.

Other biomarkers of angiogenesis have not been systematically studied in patients with Ewing sarcoma. These markers include soluble VEGF receptor, thrombospondin, placental growth factor, and circulating endothelial cells.31 Larger studies which specifically include patients with Ewing sarcoma will be required to further assess the utility of angiogenic biomarkers for prognostic and therapeutic guidance.

Preclinical Efficacy of Anti-Angiogenic and Vascular Targeting Strategies in Ewing Sarcoma

Several anti-angiogenic and vascular targeting strategies have been evaluated in preclinical models of Ewing sarcoma (Table 1). Most of these strategies have focused on blocking VEGF production or VEGF signaling activity via ligand neutralization or receptor inhibition. One group utilized small interfering RNA directed against VEGF to attenuate VEGF expression in Ewing sarcoma cell lines, and used these modified cells for subsequent *in vivo* studies.32 Attenuation of VEGF expression in this manner slowed the establishment of xenograft tumors, diminished their growth, and extended the length of time before mice died from tumor. Tumors from VEGF-attenuated cells showed diminished microvessel density and diminished osteolysis compared to control cells.32

Bevacizumab is a monoclonal antibody directed against all human VEGF isoforms. While often used to study effects in mouse xenografts, it should be recognized that bevacizumab does not neutralize murine VEGF and thus leaves unopposed any local production of VEGF by host stroma. DC101 is a monoclonal antibody directed against murine VEGF receptor 2. VEGF-Trap (aflibercept) is a recombinant soluble VEGF decoy receptor that binds VEGF with a tenfold higher affinity than bevacizumab. VEGF-Trap has the additional properties of being active across species and of effectively neutralizing placental growth factor, which may play a role in vascular remodeling. In mouse Ewing sarcoma xenografts, treatment with DC101 resulted in significantly smaller tumors with reduced microvessel density compared to mice treated with immunoglobulin control.14 Bevacizumab and VEGF-Trap have also

been evaluated preclinically and have been shown to retard the growth of Ewing sarcoma mouse xenografts and even transiently regress established tumors and lung metastases when compared to vehicle controls.[33, 34] However, regressed tumors uniformly recur and xenograft tumors derived from one cell line appeared to be more sensitive to these therapies than those derived from another cell line. These observations suggest that anti-VEGF monotherapy is unlikely to be clinically effective and that tumor heterogeneity has the potential to influence clinical response.

Several small molecule inhibitors of the VEGF receptor each with additional "off-target" but potentially advantageous tyrosine kinase inhibitory activity have also been studied for their effects on Ewing sarcoma growth. Two early prototypes of these compounds, SU5416 and SU6668, attenuated tumor growth in Ewing sarcoma mouse xenografts.[34] The Pediatric Preclinical Testing Program has now evaluated AZD2171 (cedirinib), an inhibitor of VEGF receptors 1–3, c-kit and PDGFR-β, as well as the even broader spectrum sunitinib, which inhibits VEGF receptors 1–3, c-kit and PDGFR-β, RET and Flt-3.[35, 36] Both compounds resulted in little *in vitro* activity against a panel of Ewing sarcoma cell lines. In contrast, treatment with either compound *in vivo* resulted in significant prolongation of time to event (e.g tumor of the size requiring sacrifice) in mouse xenograft models. This pattern of *in vivo* activity and lack of *in vitro* activity supports an anti-angiogenic effect from these agents, rather than a direct tumor cell inhibitory effect. Interestingly, sunitinib was administered following the usual human dosing schedule of daily for 4 weeks followed by a 2-week rest period. The EW-5 Ewing sarcoma xenograft showed essentially complete inhibition of tumor growth during the 4-week treatment period followed by an abrupt increase in tumor size after discontinuation of treatment.[36] The similarity of the findings between AZD2171, a relatively pure VEGF receptor tyrosine kinase antagonist, and sunitinib, with its broader inhibitory effects, suggests that the majority of anti-tumor activity observed with sunitinib was due to inhibition of VEGF receptor signaling. However, given that both compounds also inhibit c-kit, the contribution of c-kit inhibition to the observed anti-tumor activity observed in these experiments is unclear.

In addition to strategies directed specifically at VEGF, other groups have attempted to increase the expression of endogenous anti-angiogenic proteins. One group transfected Ewing sarcoma cells to over-express murine thrombospondin. Mouse xenografts derived from these transfected cells showed attenuated tumor growth *in vivo* with diminished microvessel density when compared to tumors derived from control cells.[19] Another group investigated the endogenous anti-angiogenic isoform of VEGF known as $VEGF_{165b}$ in Ewing sarcoma.[37] Tumors derived from Ewing sarcoma cells engineered to over-express $VEGF_{165b}$ also showed attenuated growth. These results suggest that augmentation of endogenous anti-angiogenic pathways may have a role against Ewing sarcoma.

The majority of xenograft experiments described above examined tumor suppression, or the ability of an agent to prevent tumor growth shortly after tumor inoculation. However, patients often present with bulky tumors with established vasculature. Another strategy reported recently has been to utilize a group of compounds known as vascular-disrupting agents, which typically disrupt existing blood vessels by binding to tubulin. In one study, researchers evaluated compound OXi4503/CA1P in preclinical models of Ewing sarcoma.[38] Mouse xenografts demonstrated attenuated tumor growth in response to treatment with OXi4503/CA1P compared to vehicle control. Treatment with this agent resulted in increased areas of necrosis and reduced microvessel density compared to control. This compound did not reduce the proliferation rate of tumor cells or induce tumor cell apoptosis in these models, indicating that the effect was due to the effect on tumor vasculature. Treatment with OXi4503/CA1P and doxorubicin resulted in synergistic reductions in tumor growth,

suggesting that this class of drugs may be best used in combination with chemotherapy for this disease.

Anti-angiogenic and Anti-vascular Therapies for Patients with Ewing Sarcoma

Preclinical studies have evaluated a range of different strategies and compounds to inhibit angiogenesis in laboratory models of Ewing sarcoma. Substantially fewer studies have been performed using these approaches in patients with Ewing sarcoma (Table 2). In most of these studies, patients with Ewing sarcoma have been treated within the context of phase I dose escalation trials evaluating new agents in patients with refractory solid tumors. Thus, the clinical evaluation of anti-angiogenic agents for Ewing sarcoma is at a very early stage of development. While suggestive of a potential role in this disease, these clinical results are nevertheless anecdotal and require more systematic evaluation in studies that specifically aim to evaluate the activity of antiangiogenic and antivascular stategies in patients with Ewing sarcoma.

The first anti-angiogenic agent to earn US Food and Drug Administration (FDA) approval was bevacizumab. A Children's Oncology Group (COG) phase I study investigated bevacizumab in children with refractory solid tumors.39 This study included 5 patients with relapsed or refractory Ewing sarcoma who were treated with bevacizumab every 2 weeks. No objective responses were reported. One patient had stable disease for 4 months and two patients had stable disease for 9 months of therapy. Another study treated four patients with Ewing sarcoma with bevacizumab and liposomal doxorubicin.40 Two of these patients had stable disease for at least 10 months. These results, while not definitive, suggest that bevacizumab may have a clinical role in inhibiting the growth of Ewing sarcoma.

In part based on these results, and in part based on clinical trial successes in adult colon breast and non-small cell lung carcinoma, bevacizumab is currently being evaluated in two ongoing trials for patients with Ewing sarcoma in combination with chemotherapy. This strategy is supported by growing evidence that VEGF blockade can transiently "normalize" tumor vasculature.41, 42 Nascent tumor vasculature is disorganized and hyperpermeable, with poorly applied pericytes and the absence of normal draining lymphatics. These abnormalities result in both increased interstitial fluid pressure (IFP), which impedes drug delivery, and poor intratumoral perfusion with resultant hypoxia and acidosis, which can impair cytotoxic drug efficacy. By restoring balance between angiogenic cytokines in the tumor microenvironment, it is believed that VEGF neutralization can induce vascular pruning and remodeling which transiently improves perfusion and reestablishes a gradient favoring drug transit into tumor parenchyma. The COG has initiated a randomized phase II trial of retrieval chemotherapy with or without the addition of bevacizumab for patients with first relapse of Ewing sarcoma. Patients on this study receive two cycles of therapy and undergo disease restaging. Patients with progressive disease are taken off study and patients with at least stable disease are eligible to receive an additional 10 cycles of this therapy. The primary outcome is progression-free survival and approximately 75 patients are expected to enroll. The European consortium, Innovative Therapies for Children with Cancer (ITCC) is pursuing a similar randomized phase II design of standard chemotherapy with and without bevacizumab for newly diagnosed patients with metastatic Ewing sarcoma. In addition to these ongoing studies, the St. Jude Children's Research Hospital is planning a trial combining conventional chemotherapy with bevacizumab for patients with newly diagnosed Ewing sarcoma. The results of these trials are eagerly anticipated, but phase III randomized trials with larger numbers of patients will still be required if definitive efficacy is to be demonstrated.

Clinical evaluation of oral multi-targeted tyrosine kinase inhibitors in patients with Ewing sarcoma has been more limited. The COG is conducting phase I studies of sunitinib43, VEGF-Trap (aflibercept), and sorafenib44, which targets Raf kinase in addition to VEGFR and PDGFR. The Pediatric Oncology Branch of the NCI is conducting a phase I trial of AZD2171 (cedirinib). The Dana-Farber Cancer Institute is conducting a phase II study of sorafenib in adult patients with refractory sarcomas. All these trials include patients with Ewing sarcoma but they are ongoing and response data are not currently available.

Additionally, an anti-angiogenic strategy known as metronomic chemotherapy that involves the application of daily low-dose continuous chemotherapy has been used in patients with Ewing sarcoma. With this low-dose approach, apoptosis is induced in the less frequently dividing endothelial cell rather than the tumor cell and the release of bone marrow derived endothelial precursors is suppressed 45. At least three small studies of metronomic chemotherapy have included patients with Ewing sarcoma, with some indication of disease stabilization with this approach (Table 2).46–48 The COG has completed a pilot feasibility study of the addition of celecoxib and thrice-weekly vinblastine along with a conventional chemotherapy backbone in 36 patients with newly diagnosed metastatic Ewing sarcoma. The results of this study are anticipated shortly.

Finally, two other classes of biologic agents with anti-angiogenic properties are under initial investigation in patients with Ewing sarcoma. First, several monoclonal antibodies directed against the IGF-1R are being evaluated and early studies have demonstrated activity in patients with refractory Ewing sarcoma.49, 50 Second, rapamycin and related mTOR inhibitors have recently been studied for the treatment of a range of malignancies. These agents have anti-angiogenic properties which may contribute to their antineoplastic effects. 51 One rapalogue, deforolimus, has shown particular promise in Ewing sarcoma. In phase I, one patient with Ewing sarcoma had a confirmed partial response52 and a phase II study of this agent included 50 patients with bone sarcoma and reported a 30% clinical benefit rate. 53 Given that inhibition of the IGF-1R or mTOR pathways may directly inhibit the growth of Ewing sarcoma cells,54, 55 the contribution of the anti-angiogenic effects of these agents to the activity in Ewing sarcoma remains unclear.

Conclusions

Patients with metastatic and relapsed Ewing sarcoma require novel approaches to improve their outcomes. Ewing sarcoma tumors have multiple mechanisms by which they develop and maintain their vascular supply. These mechanisms allow these tumors to continue to grow in size and to metastasize. Numerous and robust preclinical studies have demonstrated that targeting these mechanisms provides a valid approach to inhibiting the growth of these tumors. Early clinical data suggest that anti-angiogenic and anti-vascular strategies may be beneficial in the treatment of patients with Ewing sarcoma. Additional studies are urgently needed to expand these findings, to further evaluate the role of angiogenesis biomarkers, and to determine how to optimize these strategies for use in the management of patients with Ewing sarcoma.

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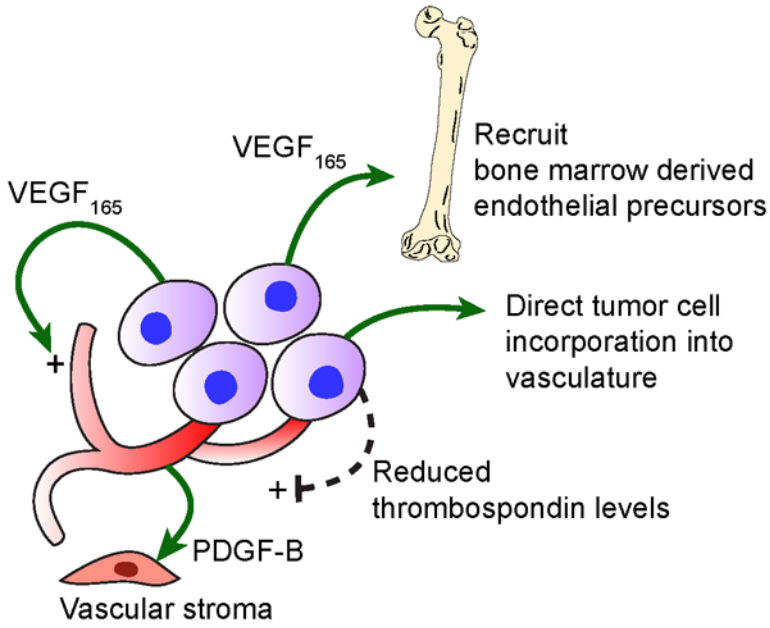

Figure 1.

Schematic diagram indicating mechanisms that promote vascular development in Ewing sarcoma. $VEGF_{165}$ directly stimulates tumor angiogenesis and also recruits bone marrow derived endothelial precursors. Tumor cells can be incorporated directly into the tumor vasculature. Down-regulation of thrombospondin by Ewing sarcoma cells also promotes angiogenesis. Endothelial-derived PDGF-B supports the vascular stroma surrounding the tumor vasculature.

Table 1

Anti-angiogenic strategies evaluated in preclinical studies of Ewing sarcoma.

Strategy	Specific Example	Preclinical Model	Ref
Reducing VEGF Expression	si-RNA against *VEGF*	TC71 xenograft	32
Binding VEGF	Bevacizumab	RD-ES and A673 xenografts	34
	VEGF Trap	RD-ES, A673, and SK-NEP xenografts	33, 34
Inhibiting VEGF Receptor(s)	Anti-VEGFR-2 monoclonal antibody	TC71 xenograft	14
	Cedirinib (AZD2171)	PPTP panel	35
	Sunitinib (SU11248)	PPTP panel	36
	SU5416	RD-ES and A673 xenografts	34
	SU6668	RD-ES and A673 xenografts	34
Augmenting Endogenous Anti-Angiogenic Pathways	Increasing thrombospondin expression	TC71 and TC667 xenografts	19
	Increasing $VEGF_{165b}$ expression	TC71 xenograft	37

si-RNA = small interfering RNA; PPTP panel = panel of Ewing sarcoma cell lines and xenografts used by the Pediatric Preclinical Testing program

Table 2

Clinical experience with agents with anti-angiogenic effects in patients with Ewing sarcoma.

Drug(s)	Number of Patients with Ewing sarcoma	Outcomes	Reference(s)
Bevacizumab	5	3 patients with SD for at least 4 months	39
Bevacizumab/Doxorubicin	4	2 patients with SD for at least 10 months	40
Sunitinib	2	Not reported	43
Sorafenib	2	Not reported	44
Metronomic Chemotherapy	1	SD for 7 months	46
	1	SD for 15 weeks	47
	2	Not reported	48
	36	Not reported	Not reported

SD = stable disease; PR = partial response; CR = complete response