Combined Vascular Endothelial Growth Factor Receptor/Epidermal Growth Factor Receptor Blockade with Chemotherapy for Treatment of Local, Uterine, and Metastatic Soft Tissue Sarcoma

Wenhong Ren, Borys Korchin, Guy Lahat, Caimiao Wei, Svetlana Bolshakov, Theresa Nguyen, William Merritt, Adam Dicker, Alexander Lazar, Anil Sood, Raphael E. Pollock, and Dina Lev

Abstract

Purpose: Soft tissue sarcoma (STS) is a rare heterogeneous malignancy. Overall survival has been stagnant for decades, primarily because systemic therapies are ineffective versus metastases, the leading cause of STS lethality. Consequently, we examined whether tyrosine kinase receptors active in STS growth signaling might be blockable and whether multireceptor blockade might synergize with low-dose STS chemotherapy by therapeutically affecting STS cells and their associated microenvironment.

Experimental Design: Vandetanib (AstraZenca), a tyrosine kinase inhibitor of vascular endothelial growth factor receptor 2 and epidermal growth factor receptor, was evaluated alone and with chemotherapy in vitro and in vivo in three human STS nude mouse xenograft models of different STS locations (muscle, uterus, lung), stages (primary, metastatic), and subtypes (leiomyosarcoma, fibrosarcoma, uterine sarcoma: luciferase-expressing MES-SA human uterine sarcoma cells surgically implanted into uterine muscularis with bioluminescence tumor growth assessment; developed by us).

Results: In vitro, human STS cells were sensitive to vandetanib. Vandetanib alone and with chemotherapy statistically significantly inhibited leiomyosarcoma local growth and fibrosarcoma lung metastasis. Direct injection of MES-SA into nude mice uterine muscularis resulted in high tumor take (88%), whereas s.c. injection resulted in no growth, suggesting microenvironmental tumor growth modulation. Vandetanib alone and with chemotherapy statistically significantly inhibited uterine sarcoma growth. In all models, vandetanib induced increased apoptosis, decreased tumor cell proliferation, and decreased angiogenesis.

Conclusions: Vandetanib has antitumor effects against human STS subtypes in vitro and in vivo, where it also affects the tumor-associated microenvironment. Given the urgent need for better systemic approaches to STS, clinical trials evaluating vandetanib, perhaps with low-dose chemotherapy, seem warranted.

Available soft tissue sarcoma (STS) chemotherapies have modest response rates with significant toxicities (1). Consequently, new STS therapies must be developed to improve the current STS 5-year survival rates of <50%. However, new STS treatment development and trials validation are hampered by their rarity and remarkable clinical heterogeneity. More than 50 separate STS histologic subtypes are recognized; these possess markedly diverse clinical courses and outcomes, perhaps reflecting the complex and variable array of molecular derangements underlying these STS biological distinctions (2). In the era of targeted therapeutics, exploiting any relevant unifying molecular abnormalities as might exist in STS has appeal. Analogous to solid malignancies, STS consist of both tumor and tumor-associated normal cells; STS growth, migration, and dissemination depend on cross-talk between these two compartments. STS are generally highly vascular and angiogenic, resulting in increased metastatic potential (3). Amidst diversity, these common STS properties could provide the basis for novel, clinically relevant therapeutics, targeting both STS cells and associated endothelial cells.

Tyrosine kinase receptors, such as vascular endothelial growth factor receptor 2 (VEGFR2) and epidermal growth factor receptor (EGFR) are promising targets for cancer therapy (4). Previously, we showed that VEGFR2 blockade combined with conventional chemotherapy inhibits STS growth and metastasis in a human STS xenograft animal model (5, 6); these effects were secondary to tumor-associated endothelial cell growth inhibition and chemosensitization rather than direct
Translational Relevance

There is a crucial need for improved therapeutic approaches for the treatment of STS. This goal is impeded by the rarity and heterogeneity of this cohort of malignancies. We have shown that combined vascular endothelial growth factor receptor and epidermal growth factor receptor blockade results in significant antitumor activity against several human STS subtypes in vitro and in vivo. This antitumor effect is secondary to the effect on both tumor cells and the tumor-associated microenvironment. Concomitant use with doxorubicin, the most active single STS agent, resulted in additional STS cytotoxicity with lowered doxorubicin doses. In light of these results, this novel combination merits evaluation in clinical trials for patients burdened by these debilitating diseases.

effects on STS cells per se. We have also shown that EGFR blockade combined with low-dose chemotherapy results in STS growth inhibition in vitro and in vivo (7).

In light of these initial findings, we investigated the effect of dual VEGFR2/EGFR inhibition combined with low-dose chemotherapy on STS growth and metastasis. Vandetanib (ZD6474, Zactima) is an orally given small molecule inhibitor of VEGFR2 and EGFR tyrosine kinase activity. By targeting these two pathways, vandetanib has the potential benefit of inhibiting both genetically stable STS-associated endothelial cells and also directly inhibiting genetically unstable STS cells. EGFR inhibitors also exert antiangiogenic effects by reducing tumor expression of VEGF and other angiogenic factors (8) and by inhibiting tumor-associated endothelial cell EGFR expression (9, 10). Here, we present results of combined vandetanib and chemotherapy in three in vivo xenograft models representing different human STS histologic subtypes and stage; demonstrating marked anti-STS efficacy.

Materials and Methods

Cell culture and reagents. Human SKLMS1 (leiomyosarcoma), HT1080 (fibrosarcoma), and MES-SA (uterine sarcoma) STS cell lines and human umbilical vascular endothelial cells were obtained from American Type Culture Collection. Uterine microvascular endothelial cells were obtained from Lonza. The dual EGFR/VEGFR2 inhibitor vandetanib was provided by AstraZeneca (Pharmaceuticals). Doxorubicin (Ben Venue Lab) was obtained from UTMDACC Pharmacy. rEGF (R&D Systems) was used for EGFR stimulation.

Commercially available antibodies were used for immunoblot detection of EGFR, phosphorylated EGFR, VEGFR2, phosphorylated VEGFR2, Akt, phosphorylated Akt S473, phosphorylated extracellular signal-regulated kinase (ERK), ERK (Cell Signaling), and β-actin (Santa Cruz Biotechnology). Antibodies used for immunohistochemical staining included anti-phosphorylated EGFR (Invitrogen), anti-EGFR (Zymed Laboratories), anti-VEGFR-2 and anti–phosphorylated VEGFR (Cell Signaling), and anti-CD31 and anti–proliferating cell nuclear antigen (Dako Cytomation). Dead-end fluorometric TUNEL system (Promega) was used for TUNEL staining. Secondary antibodies included horseradish peroxidase–conjugated (Universal kit HRP, Biocare Medical) and fluorescent secondary antibodies (antiaabbit Alexa488 and antimouse Alexa 594, Jackson Immuno Research). Other reagents included CytoQ FC receptor block (Innovex Bioscience), Hoechst 33342 (Polysciences, Inc.), and propyl gallate (ACROS Organics).

Western immunoblotting. Western immunoblotting was done, as previously described (7).

Measurement of cell proliferation. Cell growth assays were done using CellTiter96 aqueous nonradioactive cell proliferation assay kit (Promega), as per manufacturer’s instructions.

In vivo leiomyosarcoma animal model. All animal procedures and care were approved by the UTMDACC Institutional Animal Care and Use Committee. Animals received humane care as per the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. Trypan blue staining–confirmed viable SKLMS1 STS cells (1 × 10⁶/0.1 mL HBSS/mouse) were injected into the flank of 6-wk-old female nude/nude mice (National Cancer Institute/NIH; n = 40). Tumors were measured using calipers twice weekly, and tumor volume was calculated as V = L × W × π/6 (V, volume; L, length; and W, width). When average tumor volumes reached 100 mm³, mice were assigned to four treatment groups (seven to eight mice per group): (a) control (vehicle only), (b) doxorubicin (1.2 mg/kg/biweekly, i.p.), (c) vandetanib (50 mg/kg/d, gavage), and (d) doxorubicin + vandetanib (given concurrently on days when both drugs were given).

Dose and schedule of vandetanib treatments were determined based on previous studies (11, 12). Vandetanib, at a range of 12.5 to 100 mg/kg, was well tolerated and active in histologically diverse human xenograft models. Antitumor efficacy was observed in mice when the drug was given continuously and concomitantly with chemotherapy rather than sequentially (13). The vandetanib dose used in our preclinical studies results in plasma drug levels in mice within the range of plasma drug levels seen in human clinical trials (14). Low-dose chemotherapy schedules were used as before, with minimal toxicities (6).

Mice were followed for tumor size and body weight and sacrificed when control group tumors reached an average of 1.5 cm in largest dimension. Tumors were resected, weighed, and frozen or fixed in formalin and paraffin-embedded for immunohistochemical studies.

In vivo fibrosarcoma experimental lung metastasis model. Trypan blue staining–confirmed viable HT1080 cells (fibrosarcoma) stably transfected to express luciferase (HT1080/GL; 1 × 10⁶/0.1 mL HBSS/mouse) were injected i.v. into the tail vein of 6-wk-old female nude/nude mice (National Cancer Institute/NIH; n = 40). Lung metastases development and growth were evaluated using bioluminescent imaging (BLI) technology, wherein the level of luciferase expression was assessed as a sensitive biomarker of in vivo tumor growth. For BLI, a bioluminescent IVIS imaging System (Xenogen Corp.) was used; animals were in an acrylic chamber anesthetized with 2% isofluorane/oxygen mixture and injected i.p. with 15 mg/mL α-luciferin potassium salt in PBS (150 mg/kg) and then placed onto a warmed stage inside a light-tight chamber. Imaging was done 12 min after the injection of α-luciferin. To maintain sedation, animals received isofluorane continuously during the procedure. The light emitted from luciferase-expressing tumor cells was camera detected, then digitized and displayed as a pseudocolor image onto a grayscale animal image.

When BLI suggested established lung metastases, mice were assigned to four treatment groups (seven to eight mice per group) as above: (a) control (vehicle only), (b) doxorubicin (1.2 mg/kg/biweekly, i.p.), (c) vandetanib (50 mg/kg/d, gavage), and (d) doxorubicin + vandetanib.

Mice were followed for BLI readout and body weight and sacrificed when control group tumors exhibited high luciferase readout. During necropsy, lungs were excised, visible metastasis was counted, and the lungs were fixed in formalin and paraffin-embedded for immunohistochemical studies.

In vivo uterine sarcoma model. Evaluating vandetanib therapeutic effects in uterine sarcoma required creating a reproducible orthotopic human uterine sarcoma xenograft mouse model. MES-SA cells stably transfected to express luciferase (MES-SA/GL) were used. Mice (n = 16) were anesthetized; a surgical incision in the flank-enabled direct
Fig. 1. Combined VEGFR2/EGFR blockade with doxorubicin inhibits STS and endothelial cell growth. A, human leiomyosarcoma and fibrosarcoma cells (SKLMS1 and HT1080), but not uterine sarcoma cells (MES-SA), express the EGFR. None of the STS cells tested express the VEGFR2, which is highly expressed in human umbilical vascular endothelial cells. B, vandetanib (V; for 96 h) inhibits STS cell growth in a dose-dependent manner (MTS assay); a correlation between EGFR expression level and growth inhibition is observed. C, vandetanib inhibits EGF-induced EGFR phosphorylation and downstream ERK and AKT signaling in EGFR-expressing STS cells (SKLMS1; left); a decrease in AKT and ERK phosphorylation is also noticed in MES-SA cells (not shown to express the EGFR) after vandetanib treatment, suggesting that the drug possibly inhibits other targets upstream to these signaling pathways (right). D, cell growth assays demonstrating superior STS and endothelial cell growth inhibition after combined treatment with vandetanib (5 μmol/L) and doxorubicin (0.25 μmol/L for STS cells, and 0.01 μmol/L for endothelial cells) for 48 h.
visualization and isolation of the uterus. 1 × 10⁶ cells were injected into the uterine muscularis under direct vision, thereby avoiding tumor cell spillage into the abdominal or intrauterine cavities. Tumor growth was followed by BLI. Upon tumor establishment, mice (seven to eight per group) were allocated to the following groups: (a) control (vehicles only), (b) doxorubicin (1.2 mg/kg/biweekly, i.p.), (c) vandetanib (50 mg/kg/d, gavage), and (d) doxorubicin + vandetanib.

Mice were followed by BLI and for body weight and sacrificed when control group tumors exhibited high luciferase readout. Tumors were resected, weighed, and frozen or fixed in formalin and paraffin embedded for immunohistochemical studies.

**Immunohistochemical analysis.** Immunohistochemistry was done, as previously described (6). Staining distribution (percentage of positive-stained tumor cells) and intensity (0, no staining; 1, low; 2, high), as well as CD31 counts (in 10× HPF), were evaluated and scored by two independent reviewers (B.K. and A.L.).

**Statistical analysis.** Cell culture based assays (as well as Western immunoblotting analysis) were repeated at least thrice, and mean ± SD was calculated. Tumor volume was first transformed using the natural logarithm scale to better fit the assumptions of linear mixed models. STS xenograft weights and volumes were analyzed using a mixed model with fixed effects of doxorubicin (no versus treated), ZD6474 (no versus treated), and the interaction between doxorubicin and ZD6474 (interaction testing for synergism between two drugs). Linear mixed models were used to assess temporal tumor volume change. This model included fixed effects of doxorubicin (no versus treated), vandetanib (no versus treated), time, and two-way interactions between three factors. The first order autoregressive (AR1) variance-covariance was used to incorporate the correlation between assessments within animals based on Akaike’s Information Criterion and Schwarz’s Bayesian Criterion. Comparisons between treatment groups within each time point were made within the linear mixed models. Exact logistic regression model was used to assess the differences in the proportions of mice having more than 20 lung metastases between treatment groups. Model assumptions were examined. All statistical models were done using SAS (version 9.1).

**Results**

STS cells are sensitive to vandetanib. Based on our previous findings (6, 7), we hypothesized that combining EGFR and VEGFR2 blockade with chemotherapy might yield enhanced anti-STS effects *in vivo*, possibly affecting both STS cells and the STS-associated microenvironment. Because STS are markedly heterogeneous, we evaluated whether this treatment approach was effective against STS of different histology, location, and stage. Accordingly, we selected three histologic subtype human STS cell lines: SKLMS1 (leiomyosarcoma), HT1080 (fibrosarcoma), and MES-SA (uterine sarcoma). Vandetanib inhibits VEGFR2 and EGFR phosphorylation (15, 16); using Western immunoblotting, we evaluated the expression of these tyrosine kinase targets in the selected cell lines (Fig. 1A). EGFR expression was shown in SKLMS1 and HT1080 cells, but not in MES-SA. VEGFR2 was detected in none of the STS cell lines tested.

Next, we evaluated the effect of vandetanib on STS cell growth *in vitro* (Fig. 1B). Both SKLMS1 and HT1080 were sensitive to vandetanib (IC₅₀ 2.4 ± 0.2 μmol/L/96 h and 3.8 ± 0.4 μmol/L/96 h, respectively). Interestingly, MES-SA cells, although not demonstrably expressing EGFR or VEGFR2,
exhibited vandetanib-mediated (albeit lesser) growth inhibition ($IC_{50}$, 6.2 ± 0.6 μmol/L/96 h). Previous studies suggest that besides being a potent inhibitor of VEGFR2 ($IC_{50}$ 40 nmol/L) and EGFR ($IC_{50}$ 500 nmol/L), vandetanib may also inhibit other tyrosine kinases (i.e., Flt-1, Flt-4, RET, PDGFRβ, Tie2, and FGFR1) at doses <10 μmol/L (11). Therefore, it is possible that vandetanib inhibition of MES-SA was due to blockade of other kinases critical for MES-SA proliferation.

Fig. 3. Combining vandetanib (orally, 50 mg/kg/d) with low-dose doxorubicin (i.p., 1.2 mg/kg/biweekly) results in significant inhibition of human fibrosarcoma (HT1080) experimental lung metastases growth in nude mice. Nude mice injected with HT1080 cells ($1 \times 10^6$ per mouse) through tail vein were followed using BLI. Treatment was initiated once BLI first suggested lung metastases establishment (day 0; bar on the left shows levels of luciferase readout). Sequential BLI imaging of two representative mice of each treatment arm are shown demonstrating the increase in luciferase readout in control group mice and the significantly decreased readouts in treated mice, most pronounced in combination therapy group. H&E staining shows the reduction in lung metastatic deposits (arrows) in vandetanib-treated groups, especially in the combination arm.
Vandetanib inhibits EGF-induced downstream signaling in STS cells. We evaluated vandetanib effects on EGFR signaling in STS cells expressing this receptor (Fig. 1C). EGF stimulation resulted in EGFR phosphorylation with increased downstream AKT and ERK activation. Vandetanib treatment blocked EGFR phosphorylation in a dose-dependent manner, resulting in AKT and ERK inhibition. We further evaluated the effect of vandetanib on the AKT and ERK pathways using MES-SA cells, which do not express EGFR but are sensitive to vandetanib, as shown above. Interestingly, activated AKT was observed in MES-SA cells under serum-free conditions. Whereas EGF stimulation did not affect AKT and ERK phosphorylation (Fig. 1C), vandetanib treatment resulted in a decrease in phosphorylated AKT and phosphorylated ERK. A higher vandetanib dose than that used for SKLMS1 and HT1080 cells was needed to achieve complete MES-SA AKT inhibition. Based on previous studies (14), vandetanib cannot effectively directly inhibit phosphorylation of AKT and ERK (IC50, >100 μmol/L and >20 μmol/L, respectively). Thus, the observed decrease in phosphorylation of these signaling molecules may support the hypothesis that vandetanib inhibits another target that activates these pathways in MES-SA.

Combined vandetanib and low-dose chemotherapy results in tumor and endothelial cell growth inhibition. Before testing the vandetanib/low-dose chemotherapy regimen in vivo, we evaluated the effect of this combination on tumor and endothelial cell proliferation in vitro. STS cell lines were treated for 48 hours with vandetanib (5 μmol/L) alone, doxorubicin (0.25 μmol/L) alone, or with both agents combined (Fig. 1D). For all three cell lines, combined therapy caused superior growth inhibition than either drug alone. Similarly, normal human endothelial cells (human umbilical vascular endothelial cells and uterine microvascular endothelial cells) were treated for 48 hours with vandetanib (5 μmol/L), doxorubicin (0.01 μmol/L), or the combination of both agents (Fig. 1D). As with STS cells, combination therapy resulted in the strongest normal endothelial cell growth inhibition.

Vandetanib alone and in combination with doxorubicin inhibits leiomyosarcoma growth in vivo. The described experiments suggested that vandetanib inhibits growth of several STS cell

Fig. 4. An orthotopic human uterine sarcoma animal model. A, injection of human uterine sarcoma cells (MES-SA; 1 x 10^6 per mouse) was done surgically (a-d), and mice were followed for tumor growth using BLI (e-h); large uterine tumors were identified (i). B, human uterine sarcoma cells grow to a significantly larger size (right) and weight (left) when injected orthotopically (intrauterine) compared with subcutaneously (s.c.).
lines to varying degrees, and this is enhanced when vandetanib is combined with low-dose chemotherapy. Furthermore, the vandetanib and chemotherapy combination significantly inhibits normal endothelial cell growth. To evaluate whether anti-STs effects can be achieved by such combinations in vivo, we used three human STs xenograft animal models incorporating different STs histologies, locations, and stages.

First, we investigated the effect of vandetanib and chemotherapy on human leiomyosarcoma growth in nude mice. A four-armed study (seven to eight mice per arm) compared the effects of low-dose doxorubicin, vandetanib, and their combination on human STs growth. Therapy was initiated after tumor establishment (100 mm³), thereby more closely mimicking a clinical therapeutic trial rather than a less relevant prophylactic regimen. Treatment regimen was highly tolerated; no significant weight loss was observed. Treatment with low-dose doxorubicin alone did not significantly affect SKLMS1 xenograft growth (Fig. 2A); average tumor volume (analyzed as log values) was similar to that of control mice at each follow up time point. Vandetanib alone induced significant tumor growth inhibition compared with control or doxorubicin alone treatments (P < 0.0001). Combined low-dose doxorubicin and vandetanib was markedly inhibitory compared with control, doxorubicin alone, or vandetanib alone (P < 0.01). Linear mixed model showed that the antitumor activity induced by the combination of vandetanib and doxorubicin was synergistic (P = 0.0327). Moreover, treatment with vandetanib significantly reduced tumor weight compared with control (P < 0.0001), whereas low-dose doxorubicin did not (P = 0.18). However, combination therapy showed no significant effect on tumor weight when compared with vandetanib alone. Average tumor weights at study termination were 1.73 g (SD 0.76), 1.46 g (SD 0.82), 0.70 g (SD 0.40), and 0.37 (SD 0.30) in control, doxorubicin, vandetanib, and combination therapy groups, respectively.

Tumor sections containing viable cells from each treatment group were selected for immunohistochemical studies. To first confirm that vandetanib blocked EGFR and VEGFR phosphorylation in vivo, immunostaining for phosphorylated EGFR and phosphorylated VEGFR2 was done. Figure 2B shows the marked inhibition of EGFR activation in vandetanib-treated groups without demonstrable effect on total EGFR expression levels. Furthermore, significant decreases in phosphorylated VEGFR was also detectable in the vandetanib groups; phosphorylated VEGF expression was limited to the endothelial structures as shown by double staining (CD31/pVEGFR; Fig. 2B). No significant decrease in total VEGFR expression was identified on remaining blood vessels; however, decreased large blood vessels (shown by CD31 positivity) were observed, confirming that vandetanib functions as an antiangiogenic agent.

Next, we evaluated the effect of the different therapies on STs cell proliferation and apoptosis (Fig. 2B). Scoring of immunohistochemical preparations for proliferating cell nuclear antigen (a nuclear marker for proliferation) and TUNEL assay staining (marker for apoptosis) revealed the following: control group, 70 ± 5 and 7 ± 2; doxorubicin group, 61 ± 11 and 8 ± 2;
vandetanib group, 18 ± 5 and 16 ± 5; and combination group, 12 ± 6 and 20 ± 4, respectively. We conclude that vandetanib treatment alone and in combination with chemotherapy resulted in significantly decreased tumor cell proliferation and increased apoptosis (no statistically significant effect on proliferating cell nuclear antigen and TUNEL expression was identified when comparing combination therapy to vandetanib alone). Taken together, these data suggest that EGFR/VEGFR2 blockade combined with low-dose conventional chemotherapy affects tumor cells, as well as the tumor-associated microenvironment, thereby inducing significant leiomyosarcoma growth inhibition, an observation of potential clinical utility.

**Vandetanib alone and in combination with doxorubicin blocks fibrosarcoma metastasis in vivo.** Most metastatic STS are markedly chemoresistant, and initially, chemoresponsive STS frequently become chemoresistant during treatment; consequently, metastases (especially pulmonary) are the main cause of STS-specific mortality (17). To evaluate whether vandetanib alone and/or in combination with chemotherapy results in pulmonary metastastic outgrowth inhibition, we used an experimental fibrosarcoma lung metastasis model. HT1080 cells transfected to stably express luciferase (HT1080/GL) were injected into the nude mouse tail vein. Mice exhibiting similar luciferase readout (confirming the presence of initial established lung metastasis; Fig. 3) were selected and randomized to four treatment arms (seven to eight mice per group) as above: (a) PBS alone, (b) doxorubicin, (c) vandetanib, and (d) vandetanib and doxorubicin. Mice were followed weekly with BLI and then sacrificed after 3 weeks of treatment when control mice exhibited a high luciferase readout suggesting extensive lung metastasis (Fig. 3); lungs were harvested and visible metastases counted. All control mice exhibited more than 20 lung metastases. All doxorubicin-treated mice exhibited metastases, of which three had more than 20 metastases and the remaining four showed 6 to 10 metastases. Two of the vandetanib-treated mice exhibited more than 20 metastases, four had 4 to 10 lesions, and in one mouse no lung metastases were visible. None of the mice treated with vandetanib/chemotherapy combination exhibited more than 10 metastases, three had no demonstrable metastases, and five exhibited 4 to 10 metastases. Odds ratios of having more than 20 metastases for the doxorubicin-alone treatment versus control, vandetanib-alone treatment versus control, and combination of doxorubicin and vandetanib treatment versus control were 0.102 (P = 0.051), 0.05 (P = 0.007), and 0.013 (P = 0.002), respectively. Macroscopic findings were also confirmed on H&E staining (Fig. 3), demonstrating large lung tumor deposits in control and doxorubicin groups and smaller, microscopic lesions in vandetanib and combination groups. Taken together, these results suggest that vandetanib alone and especially in combination with chemotherapy inhibits the growth of fibrosarcoma lung metastases.

**An orthotopic human uterine sarcoma animal model.** Uterine sarcoma is a rare tumor accounting for 3% of all uterine malignancies (18). The prognosis for uterine sarcoma is grave due to poor response to systemic chemotherapy and high rates of distant metastasis; therefore, the need for new and effective therapies is urgent. Based on our hypothesis that vandetanib combined with chemotherapy can affect multiple STS subtypes by targeting both tumor and tumor-associated microenvironment cells, we examined this possibility in uterine sarcoma. To conduct this experiment, we created (to the best of our knowledge, the first described) a reproducible orthotopic human uterine sarcoma mouse model. The human uterine sarcoma-originating MES-SA cell line was transfected to stably express luciferase. MES-SA/GL cells (1 × 10^6 in 0.1 ml HBSS) were carefully injected into the nude mouse uterine muscularis via a left flank incision which enabled direct visualization and isolation of the uterus (Fig. 4A), thereby avoiding tumor cell spillage into the abdominal or intrauterine cavities. Subsequent tumor growth was followed by BLI. Most mice developed uterine tumors (14 of 16; 88% tumor-take; P < 0.001); luciferase expression was detected within 7 to 10 days of tumor cell injection and increased gradually thereafter (Fig. 4A). Mice were sacrificed 6 weeks after injection. Large tumors confined to the uterus (mean tumor weight, 1.8 ± 0.4 g) were identified in all luciferase-expressing mice. No mice developed lung metastases; extrauterine intraabdominal lesions were identified in two mice, whereas these could be metastases they may have developed due to tumor cell spillage.

To further evaluate if the microenvironment plays a role in the growth of human STS cells, we compared MES-SA cell growth in the uterus to their subcutaneous growth (Fig. 4B). MES-SA cells exhibited significantly increased uterine versus subcutaneous tumor take (eight of eight versus four of nine, respectively). Mean tumor volumes and weights of uterine-growing MES-SA cells were significantly higher than those growing subcutaneously (1,010 ± 436 mm^3 and 1.79 ± 0.5 g versus 10.75 ± 12.2 mm^3 and 0.1 ± 0.1 g, respectively; P < 0.0001). These studies show the importance of the orthotopic organ microenvironment in STS cell growth and provide a much-needed model for development of novel therapeutics.

**Vandetanib alone and in combination with doxorubicin block uterine sarcoma growth in vivo.** Using our animal model of uterine sarcoma, we conducted the four-armed therapeutic study described above, comparing the effect of low-dose doxorubicin, vandetanib, and combination of both agents on uterine sarcoma growth. Therapy was initiated after BLI readout suggested tumor establishment (Fig. 5A). Mice in all groups were followed by BLI and evaluated for toxicity; treatment was terminated when control (vehicle only) tumors highly expressed luciferase, implying development of large tumors (Fig. 5A). Average natural log–transformed tumor volume (mm^3) and weight (g) at the termination of the experiment was 6.57 (SD 0.12) and 1.46 (SD 0.12), 5.29 (SD 0.29) and 0.49 (SD 0.11), 3.66 (SD 0.22) and 0.24 (SD 0.07), and 2.77 (SD 0.69) and 0.13 (SD 0.05) for control, doxorubicin, vandetanib, and combination groups, respectively (Fig. 5B). Linear mixed models revealed that tumor volume and weight were significantly lower in any treatment arm compared with control (P < 0.0001). Moreover, the effect of vandetanib was significantly higher than that of doxorubicin (P < 0.0001), and the effect of combination was superior to that of either doxorubicin (P < 0.0001) or vandetanib (P = 0.0002) alone. The effect of combination therapy was found to be additive regarding tumor volume, with no statistically significant interaction between drugs shown (P = 0.2164), whereas synergism between the drugs was suggested when tumor weights were analyzed (F test for interaction P < 0.0001).

Next, we evaluated representative tumor sections from the four treatment arms (two per group) using immunohistochemistry. Double immunostaining for phosphorylated
VEGFR2/CD31 showed that phosphorylated VEGFR2 is expressed on MES-SA tumor vasculature and that it is effectively inhibited in tumors treated with vandetanib (Fig. 5C). Staining results for CD31, proliferating cell nuclear antigen, and TUNEL were similar to those for SKLMS1 tumors above (Fig. 2B). Vandetanib alone and in combination with chemotherapy induced a decrease in large blood vessels. Interestingly, small collapsed CD31-positive vessels were detected in vandetanib-treated tumors, especially in the combination group. Additionally, an increase in apoptosis (TUNEL scores were $9 \pm 2$, $8 \pm 3$, $18 \pm 5$, and $18 \pm 4$ for control, doxorubicin, vandetanib, and combination-treated tumors, respectively) and a decrease in proliferation (proliferating cell nuclear antigen scores were $69 \pm 3$, $67 \pm 10$, $23 \pm 6$, and $18 \pm 3$ for control, doxorubicin, vandetanib, and combination-treated tumors, respectively) were observed.

Taken together, these results suggest that EGFR/VEGFR2 inhibition, especially when combined with low-dose doxorubicin, significantly inhibits local and metastatic growth of different STS subtypes, orthotopic locations, and STS stages. It is possible that the effect of this therapeutic regimen on both tumor cells, as well as tumor-associated endothelial cells, underlies this broad (and clinically relevant) therapeutic potential.

**Discussion**

There is a crucial need for better STS therapeutic strategies; however, a major impediment to achieving this goal is that STS are a particularly diverse malignant cohort. When using molecularly targeted therapy, one plausible approach might be to first identify the molecular “addiction” of a specific STS subtype; if such exists, then evaluate the implication of inhibiting that particular target/pathway. This strategy has led to remarkable strides in treatment of gastrointestinal stromal tumor, an STS subtype where activating c-Kit mutations are the major tumorigenesis and progression driver (19), whose inhibition results in significantly improved outcomes (20). However, the rarity of STS makes this approach to discovery and therapeutic efficacy validation of a novel agent extremely challenging. Another possibility, as presented in this study, is to identify and target pathways or tumor-related processes that are dysregulated in a broad range of STS. Successful treatment of STS, especially those harboring complex karyotypes, might necessitate inhibition of multiple targets (21–23). Furthermore, optimal therapeutic results may emerge from targeting both STS cells and STS-associated normal cells. The findings presented here support these hypotheses, demonstrating that a regimen consisting of vandetanib (a small molecule multi-targeted tyrosine kinase inhibitor) combined with low-dose chemotheraphy significantly inhibited STS growth in three in vitro models involving different STS histologic subtypes and different tumor-microenvironmental interface conditions.

Targeting the STS microenvironment, particularly the vascular compartment, holds genuine promise because STS commonly grow to large, angiogenesis-dependent sizes (24). Much evidence suggests that sarcomas, regardless of subtype, over-express VEGF (25); VEGF expression was found to correlate with stage, grade, and prognosis (26) and was suggested to play a major role in STS metastatic progression (27). Antiangiogenic therapy provides important potential advantages: a broad spectrum of activity against many STS subtypes; decreased therapeutic resistance as endothelial cells are relatively genetically stable; improved drug access to targets, especially in large, hypoxic STS, in which the tumor per se is not easily accessible; and (hopefully) modest toxicities.

Previously, we showed that VEGFR2 blockade caused inhibition of local and metastatic rhabdomyosarcoma and leiomyosarcoma growth in vivo (6). Others have shown similar effects in other STS subtypes, including neurogenic sarcoma (28), Ewing sarcoma (29), and angiosarcoma (30). VEGF/VEGFR2 inhibitors have been evaluated for human STS treatment. SU5416 (a potent tyrosine kinase inhibitor of VEGFR2) was tested in a phase-I trial, resulting in one partial and five stable disease responses (31). However, a subsequent phase-II trial failed to show significant effects (32). Another phase-II trial evaluated bevacizumab (a recombinant anti-VEGF monoclonal antibody) combined with doxorubicin for metastatic STS patients. A documented time to progression of 8 months was encouraging; however, overall response rates did not differ from doxorubicin alone historical controls (33). Ongoing studies are currently investigating recently developed antiangiogenic agents in treatment of metastatic STS (34). It is possible that combined targeting of both angiogenesis and direct STS cell inhibition will yield results superior to those described above; the findings of the current study support this possibility.

Vandetanib is a potent antiangiogenic agent via VEGFR2 signaling blockade in endothelial cells, but it can also possibly directly inhibit STS cell growth by blocking the EGFR pathway (12). Several studies have reported increased EGFR expression in several STS subtypes (35–37), although studies focusing on the STS subtypes presented in our study are generally lacking: the largest contemporary series showed positive EGFR staining in 60% of 281 human adult STS. We have recently shown that EGFR is highly expressed in many STS cell lines and human STS specimens (7). Most importantly, we showed that ligand stimulation of EGFR results in receptor phosphorylation, down-stream target activation, and increased proliferation of STS cells, thus suggesting a functional role for STS-expressed EGFR. We also showed that EGFR blockade alone and (even more so) in combination with chemotherapy exhibited anti-STS effects in vitro and in vivo. Results presented here suggest that EGFR/VEGFR2 inhibition combined with low-dose chemotherapy might be even more effective in blocking STS growth and metastasis. Furthermore, whereas vandetanib potently inhibits VEGFR2 and EGFR, similar to other ATP competitive small molecule tyrosine kinase inhibitors, with increasing doses vandetanib can block the catalytic pocket of additional tyrosine kinases. Our studies suggest that, within relevant therapeutic dose ranges, vandetanib can affect the growth of STS cells which do not express the EGFR or VEGFR2, both in vitro and in vivo, possibly expanding the therapeutic applicability of this drug to an even wider range of STS.

Paget first proposed that certain tissues (the soil) may provide a better environment than others for the growth of specific tumors (the seed; ref. 38). The crucial role of the microenvironment in STS development is shown in our model of uterine sarcoma. We showed that uterine STS cell growth in the uterine muscularis is enhanced compared with the growth of these cells injected s.c. To the best of our knowledge this is the first reproducible orthotopic uterine sarcoma xenograft...
model described to date and may be useful in developing meaningful interventions, as we have also shown here. Orthotopic animal models for drug therapy efficacy studies are crucially important, as nonorthotopic models do not adequately address cross-talk between specific organ environments and tumor cells (39). Furthermore, tumor gene expression is markedly altered when implanted s.c. versus orthotopically (40).

Results of our studies support the continued evaluation of vandetanib or other such tyrosine kinase inhibitors in combination with low-dose chemotherapy for human STS, especially because phase I clinical trials show that vandetanib is well tolerated and observed toxicities are mild and readily controlled with dose adjustment or appropriate therapy (41). If a vandetanib/chemotherapy regimen can be used successfully versus human STS, it will hopefully result in significantly increased response rate in a wide range of STS subtypes, as well as reduction of doxorubicin-related side effects, a critical and major limitation of STS therapy. However, successful animal studies do not invariably translate into human applicability; to maximize success, STS clinical trials must incorporate appropriate patient selection, drug scheduling, and therapy combinations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Jill Hood and Anderson Ryan (AstraZeneca Pharmaceuticals) for kindly providing vandetanib, Paul Cuevas for the expert assistance in the preparation and submission of this manuscript, and Kim Vu for her aid in figure preparation.
Combined Vascular Endothelial Growth Factor Receptor/Epidermal Growth Factor Receptor Blockade with Chemotherapy for Treatment of Local, Uterine, and Metastatic Soft Tissue Sarcoma

Wenhong Ren, Borys Korchin, Guy Lahat, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/17/5466

Cited articles
This article cites 40 articles, 20 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/17/5466.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
/content/14/17/5466.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.