Multimodal targeting of tumor vasculature and cancer stem-like cells in sarcomas with VEGF-A inhibition, HIF-1α inhibition, and hypoxia-activated chemotherapy

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ABSTRACT

Vascular endothelial growth factor A (VEGF-A) inhibition with pazopanib is an approved therapy for sarcomas, but likely results in compensatory pathways such as upregulation of hypoxia inducible factor 1α (HIF-1α). In addition, cancer stem-like cells can preferentially reside in hypoxic regions of tumors and be resistant to standard chemotherapies. In this study, we hypothesized that the combination of VEGF-A inhibition, HIF-1α inhibition, and hypoxia-activated chemotherapy with evofosfamide would be an effective multimodal strategy. Multimodal therapy was examined in one genetically engineered and two xenograft mouse models of sarcoma. In all three models, multimodal therapy showed greater efficacy than any single agent therapy or bimodality therapy in blocking tumor growth. Even after cessation of therapy, tumors treated with multimodal therapy remained relatively dormant for up to 2 months. Compared to the next best bimodality therapy, multimodal therapy caused 2.8–3.3 fold more DNA damage, 1.5–2.7 fold more overall apoptosis, and 2.3–3.6 fold more endothelial cell-specific apoptosis. Multimodal therapy also decreased microvessel density and HIF-1α activity by 85–90% and 79–89%, respectively, compared to controls. Sarcomas treated with multimodal therapy had 95–96% depletion of CD133(+) cancer stem-like cells compared to control tumors. Sarcoma cells grown as spheroids to enrich for CD133(+) cancer stem-like cells were more sensitive than monolayer cells to multimodal therapy in terms of DNA damage and apoptosis, especially under hypoxic conditions. Thus multimodal therapy of sarcomas with VEGF-A inhibition, HIF-1α inhibition, and hypoxia-activated chemotherapy effectively blocks sarcoma growth through inhibition of tumor vasculature and cancer stem-like cells.

INTRODUCTION

Sarcomas arise in nearly 20,000 people in the United States annually, occur in individuals of all ages, and are fatal for about 40% of patients due to either locoregional recurrence or distant metastasis [1]. The treatment of primary tumors typically includes aggressive surgical resection and radiation therapy, but local recurrence remains a significant problem for treating tumors in difficult locations such as the head and neck, paraspinal region, retroperitoneum, and pelvis [2]. Furthermore, up to 50% of patients with large, high-grade sarcomas develop distant metastases, most frequently to the lung. The efficacy of chemotherapy in treating local and
distant recurrence is modest at best, and for the minority of patients who respond, nearly all eventually develop chemotherapy resistance [3].

Common to most solid tumors are areas of hypoxia and the requirement to generate new tumor blood vessels [4]. Tumors respond to hypoxic stress through several mechanisms including the stabilization of hypoxia inducible factor 1α (HIF-1α) [5]. HIF-1α is then transported to the nucleus where it binds hypoxia response element (HRE) DNA sequences and activates the expression of at least 150 target genes, including genes eliciting changes in tumor angiogenesis (e.g. vascular endothelial growth factor A or VEGF-A) [4] and invasion (e.g. c-MET) [6]. Several studies from our laboratories and others highlight the importance of HIF-1α in sarcoma progression and metastasis. Expression of HIF-1α and 25 other hypoxia-related genes in human sarcomas is highly elevated compared to normal tissues [7], and sarcoma hypoxia is associated with a higher risk of recurrence and decreased overall survival [8-10]. Gene expression profiling of 177 sarcomas identified a signature of hypoxia-related genes that predicted metastasis [11].

Vascular endothelial growth factor A (VEGF-A) is one of the most important factors promoting tumor angiogenesis [12]. Expression of VEGF-A in sarcomas correlates with extent of disease and survival [13]. Circulating levels of VEGF-A are elevated in sarcoma patients compared to controls [14], and inhibition of VEGF-A or its receptors can effectively suppress tumor angiogenesis in mouse models of sarcoma [7]. In patients with advanced sarcomas, pazopanib, an orally available tyrosine kinase inhibitor of VEGF-A receptors 1-3 (VEGFR-1-3), was shown in a phase III randomized trial to increase progression-free survival over placebo by nearly 3 months [15]. However in certain sarcomas, VEGF-A pathway inhibition can increase intratumoral hypoxia and HIF-1α activity, which in turn leads to increased tumor formation and metastasis via upregulation of effectors such as PLOD2 and FOXM1 [16-18].

The cancer stem cell theory postulates that cancers harbor a subset of cells that share characteristics of normal stem cells, with a capacity for self-renewal and differentiation [19]. Numerous studies have demonstrated that putative cancer stem cells (CSCs) are more resistant to chemotherapy than non-CSCs [20] and are a source of distant metastases [21]. Methods to identify CSCs include tumor initiation in immunodeficient mice, spheroid colony formation in vitro, and expression of certain cell surface markers. While one may argue the existence of CSCs, solid tumors such as sarcomas clearly are composed of heterogeneous populations of tumor cells with varying properties. Given there are now over 50 distinct histologic subtypes of sarcoma, many have also suggested that sarcomas originate from multipotent cells such as mesenchymal stem cells [22]. CSCs may be more reliant on HIF-1α activity [23]. A few studies have found that CSCs preferentially reside in hypoxic regions of tumors [24]. The most commonly identified cell surface marker for sarcoma CSCs is CD133 [25-27]. Given CSCs may be largely responsible for chemotherapy resistance and metastasis, determining novel ways to target these cells may result in new and effective treatment regimens.

Evofosfamide is a 2-nitroimidazole-linked prodrug of a brominated version of isophosphoramide mustard (Br-IPM) [28, 29]. This molecule is relatively inert under normoxic conditions but when reduced under hypoxic conditions leads to the release of Br-IPM, which then acts as a DNA-alkylating chemotherapeutic. Evofosfamide enhanced the activity of a wide range of chemotherapy drugs in numerous xenograft models including HT1080 fibrosarcomas xenografts [30] and was found to be a promising agent in combination with doxorubicin for patients with metastatic sarcoma [31]. However, a phase III randomized trial of doxorubicin with or without evofosfamide in patients with soft tissue sarcoma (NCT01440088) failed to reach its primary efficacy endpoint with the addition of evofosfamide to doxorubicin. In this study, we hypothesized that the combination of VEGF-A inhibition and HIF-1α inhibition would attenuate tumor vasculature and enhance the effects of hypoxia-activated chemotherapy on sarcoma stem-like cells.

RESULTS

Multimodal therapy with metronomic doxorubicin in KP mice

VEGF-A inhibition in tumors can lead to inhibition of tumor vasculature, increase intratumoral hypoxia, and upregulation of HIF-1α [32, 33]. Lee et al. found after screening 3,120 drugs from the Johns Hopkins Drug Library that doxorubicin at low doses is a potent inhibitor of HIF-1α by blocking HIF-1α binding to DNA [34]. We used DC101, an anti-VEGFR-2 antibody, to block the primary receptor of VEGF-A, metronomic doxorubicin to block HIF-1α binding to DNA, and the hypoxia-activated chemotherapeutic evofosfamide (a.k.a. multimodal therapy) in the LSL-KrasG12D+/Trp53fl/fl genetically engineered mouse model of sarcoma, which we have previously described [35]. In this “KP” mouse model, intramuscular delivery of an adenovirus expressing Cre recombinase into the extremity of these mice results in activation of oncogenic K-ras and loss of both Trp53 alleles. More than 90% of mice then develop sarcomas at the site of injection after a median of 80 days. The sarcomas in these KP mice closely resemble human undifferentiated pleomorphic sarcomas according to the genetic and histologic analyses [16]. When tumors reached
50-100 mm³, mice were randomized to 8 treatment groups. After 14 days of treatment, single modality therapy with DC101, evofosfamide, or doxorubicin inhibited tumor growth by 44%, 12%, and 41%, respectively. Bimodality therapies inhibited tumor growth by 50-61%, and multimodal treatment with all three agents inhibited tumor growth by 83% (Figure 1A).

Tumors from each treatment group were harvested at the end of the treatment period and analyzed by immunohistochemistry and immunofluorescence. When tumors were examined for proliferation using PCNA staining, all therapies including multimodal therapy caused at most a 10% reduction in proliferation (Figure 1B, 1C). When tumors were examined for overall apoptosis using TUNEL staining, multimodal therapy resulted in significantly more apoptosis (41.4 cells per 5 fields) than any other single modality (15.4-18.6 cells per 5 fields) or bimodality treatment (17.8-19.2 cells per 5 fields). Multimodal therapy led to an 8-fold increase in endothelial cell-specific apoptosis and a 90% decrease in microvessel density compared to the control tumors. Levels of nuclear HIF-1α expression (used as a measure of HIF-1α activity) were 89% lower in tumors treated with multimodal therapy compared to control tumors. Thus multimodal therapy with VEGF-A pathway inhibition, HIF-1α inhibition, and hypoxia-activated chemotherapy effectively blocks sarcoma growth though induction of apoptosis, loss of tumor vasculature, and inhibition of HIF-1α.

To better understand levels of hypoxia in KP mouse sarcomas, we treated KP tumors when they reached 50 mm³ in size with DC101 or control IgG and examined tumors at 200, 500, and 1000 mm³ in size (Suppl. Figure S1A). Mice were injected with Hypoxyprobe and tumors were then harvested and examined. When controlling for tumor size, DC101 treatment did not appear to alter levels of hypoxia as assessed by pimonidazole staining (Suppl. Figure S1B) and did not appear to alter microvessel density (Suppl. Figure S1C). Thus multimodal therapy is effective even though DC101 by itself does not increase intratumoral hypoxia.

**Multimodal therapy with HIF-1α shRNA in HT1080 xenografts**

While doxorubicin is a potent inhibitor of HIF-1α, doxorubicin likely has off-target effects even at low doses. We thus created HT1080 fibrosarcoma cell lines transduced with HIF-1α shRNA or scrambled control shRNA. We confirmed effective knockdown of HIF-1α in HT1080 cells by Western blot analysis (Figure 2A), and then used these HT1080 cell lines to create xenografts in athymic nude mice. Once tumors reached 50-100 mm³, mice were additionally treated with DC101 and/or evofosfamide. Single agent therapy with DC101, HIF-1α shRNA, or evofosfamide reduced HT1080 xenograft growth at 14 days compared to control tumors by 36%, 24%, and 16%, respectively (Figure 2B). Bimodality therapies inhibited tumor growth by 41-55%. Multimodal therapy was again more effective than any single agent or bimodality therapy, blocking tumor growth by 79%.

Following 14 days of therapy, mice were injected with Hypoxyprobe and tumors were then harvested and examined. Total apoptosis, endothelial cell-specific apoptosis, and non-endothelial cell apoptosis were assessed by immunofluorescence using cleaved caspase 3 and CD31 antibodies. Total apoptosis following multimodal therapy was 16-fold greater than control tumors and 2.6-fold greater than the best bimodality therapy (Figure 2C and Suppl. Figure S2). When apoptosis was assessed in CD31-positive endothelial cells and CD31-negative cells, the increase in apoptosis following multimodal therapy was relatively equal in endothelial and non-endothelial cells. Tumors treated with multimodal therapy also had the greatest reduction in microvessel density (Figure 2C, 2D). The degree of hypoxia was assessed in tumor xenografts by quantifying pimonidazole staining. Tumors treated with multimodal therapy had the smallest areas of hypoxia as well as the least number of cells with nuclear HIF-1α expression (Figure 2C, 2D).

**Multimodal therapy effects on DNA damage**

We next sought to (1) confirm that metronomic doxorubicin has similar effects on HT1080 xenografts as HIF-1α knockdown, (2) examine multimodal therapy in a third sarcoma mouse model, SK-LMS-1 human leiomyosarcoma xenografts, and (3) examine the effects of multimodal therapy on DNA damage. HT1080 and SK-LMS-1 xenografts were generated and treatment was started when tumors reached 50-100 mm³. Similar to our prior studies, we found that multimodal therapy with DC101, metronomic doxorubicin, and evofosfamide effectively halted tumor growth better than any single agent or any bimodality therapy (Figure 3A, 3B). Again, there was a more than additive effect of multimodal therapy in inducing global apoptosis and EC apoptosis and reducing microvessel density and nuclear HIF-1α expression (Suppl. Figures S3 and S4). After the 14 day treatment period, some xenografts treated with multimodal therapy were not harvested but rather followed to determine the subsequent tumor growth off therapy. For HT1080 and SK-LMS-1 xenografts, tumors were all less than 250 mm³ even at 60 days and 30 days, respectively (Figure 3A, 3B).

Given evofosfamide under hypoxia is converted into a DNA damaging agent, we next assessed DNA damage in all three mouse sarcoma models following therapy using γH2AX immunofluorescence. γH2AX levels increase in response to DNA double-strand breaks [36]. We also co-stained with cleaved caspase 3 to assess for apoptosis and with CD31 to mark endothelial cells.
Figure 1: DC101, evofosfamide, and low dose doxorubicin multimodal treatment of KP sarcomas. A. Tumor volume of KP sarcomas. Groups were treated by intraperitoneal injection of control IgG 20 mg/kg 3 times per week, DC101 20 mg/kg 3 times per week, evofosfamide (Evo) 50 mg/kg 5 times per week, and/or low dose doxorubicin (Dox) 1 mg/kg 3 times per week. Photos B., and graphs C. of PCNA immunohistochemistry for proliferation, TUNEL immunofluorescence for apoptosis, CD31 immunohistochemistry for microvessel density, CD31 and TUNEL immunofluorescence for endothelial cell (EC)-specific apoptosis, and nuclear HIF-1α immunohistochemistry for HIF-1α activity. Bars represent standard deviation. **p < 0.05 compared to all other groups.
Figure 2: DC101, evofosfamide, and HIF-1α shRNA treatment of HT1080 sarcomas. A. Western blot of HT1080 cells stably transduced with HIF-1α shRNA (sh.HIF-1α) or scrambled control shRNA (sh.Scr) and grown in 21% oxygen or 1% oxygen. β-actin serves as loading control. B. Growth of HT1080 cells stably transduced with sh.HIF-1α or sh.Scr in athymic nude mice. Groups were treated with control IgG 20 mg/kg 3 times per week, DC101 20 mg/kg 3 times per week, and/or evofosfamide (Evo) 50 mg/kg 5 times per week. C. Photos of H&E staining, cleaved caspase 3 IF, cleaved caspase 3 and CD31 IF, CD31 IHC, pimonidazole IHC, and HIF-1α IHC. D. Graphs of microvessel density, pimondizale area, and nuclear HIF-1α expression. Bars represent standard deviation. **p < 0.05 compared to all other groups.
Figure 3: Multimodal treatment of HT1080 and SK-LMS-1 xenografts. Growth of HT1080 human fibrosarcoma A. and SK-LMS-1 human leiomyosarcoma B. xenografts in athymic nude mice. Groups were treated by intraperitoneal injection of control IgG 20 mg/kg 3 times per week, DC101 20 mg/kg 3 times per week, evofosfamide (Evo) 50 mg/kg 5 times per week, and/or low dose doxorubicin (Dox) 1 mg/kg 3 times per week for 2 weeks. Photos C. and graphs D. of tumors for γH2AX and cleaved caspase 3 (CCR3) immunofluorescence. Scale bar 20 µm. Bars represent standard deviation. **p < 0.05 compared to all other groups.
In all three mouse sarcoma models, multimodal therapy led to dramatic increases in γH2AX-positive and cleaved caspase 3-positive cells (Figure 3C, 3D). This increase in DNA damage was seen in both non-endothelial tumor cells and well as endothelial cells.

**In vitro studies of multimodal therapy on tumor endothelial cells and sarcoma cells**

We next examined the effect of multimodal therapy on tumor endothelial cells and sarcoma cells *in vitro*. We isolated endothelial cells from HT1080 xenografts using magnetic bead separation with CD31 antibody as previously described [37]. Multimodal therapy reduced tumor endothelial cell proliferation under normoxia and hypoxia by 64% and 78%, respectively (Figure 4A). Similar results were seen in human umbilical vein endothelial cells (HUVEC) (Suppl. Figure S5A). HT1080 and SK-LMS-1 sarcoma cell proliferation was inhibited by multimodality therapy by 20-27% in normoxia and 52-62% in hypoxia (Figure 4A, Suppl. Figure S4A).

DNA damage in endothelial cells and in sarcoma cell lines was assessed by examining levels of γH2AX expression. γH2AX expression in tumor endothelial cells and HUVEC after multimodal therapy was 18-fold greater than control and 6.6 to 7.5-fold greater than any bimodality therapy when treatment was given under hypoxia (Figure 4B, Suppl. Figure S5B). The effects under normoxia were much less pronounced. In sarcoma cell lines, γH2AX expression after multimodal therapy in hypoxia was 9.4 to 10.8-fold higher than control (Figure 4B and Suppl. Figure S5B). We then determined if DNA damage led to apoptosis by measuring expression of cleaved caspase 3. In hypoxia, cleaved caspase 3 expression in tumor endothelial cells was 12 to 14.6-fold greater following multimodal therapy compared to bimodality therapies (Figure 4C, Suppl. Figure S5C). The effects on apoptosis were also much less pronounced under normoxic conditions. Cleaved caspase 3 expression in sarcoma cell lines was 14 to 19-fold higher after multimodal therapy in hypoxia compared to controls (Figure 4C, Suppl. Figure S5C).

**Effects on sarcoma cancer stem-like cells**

Given multimodal therapy results in prolonged periods of tumor dormancy even after cessation of therapy (Figure 3A), we hypothesized that multimodal therapy has synergistic inhibitory effects of sarcoma cancer stem-like cells (CSCs). MS4515 is a mouse undifferentiated pleomorphic sarcoma cell line derived from a genetically engineered mouse model similar to the previously described KP model [35]. We examined levels of HIF-1α in MS4515 cells as well as HT1080 and SK-LMS-1 cells grown as monolayers or as spheroids. Growth in spheroid formation conditions enriches for cancer stem-like cells [38]. In all three sarcoma cell lines, levels of HIF-1α were higher in cells grown as spheroids compared to cells grown as monolayers (Figure 5A). When cells were exposed to 1% hypoxia, HIF-1α levels in spheroids increased even further. We next examined these cells lines for 4 putative mesenchymal stem cells markers to identify a marker of CSCs and found only CD133 to be consistently elevated in all sarcoma cell lines when grown as spheroids (Suppl. Figure S6). This is consistent with prior studies [25-27]. Expression of the self-renewal transcription factors Nanog, Oct-4, and Sox2 were significantly increased in CD133(+) cells compared to CD133(-) cells from sarcoma cell lines (Figure 5B).

We then examined the number of CD133(+) cells in tumors from our prior mouse tumor studies by immunofluorescence and immunohistochemistry (Figure 5C). CD133(+) cells were significantly depleted following multimodal treatment of HT1080, SK-LMS-1, and KP tumors (Figure 5D). HT1080 flank xenografts from HT1080 cell stably transduced with HIF-1α shRNA or control shRNA and treated with DC101 and/or evofosfamide were stained by immunofluorescence for pimonidazole and CD133. In control tumors, CD133(+) cells were more concentrated in hypoxic regions of tumors compared to non-hypoxic regions (Figure 5E, 5F). Multimodal therapy was effective in eradicating CD133(+) cells in both hypoxic and nonhypoxic regions. Thus destruction of CSCs may be another mechanism by which multimodal therapy inhibits sarcomas.

We next compared the effects of multimodal therapy on HT1080 monolayer cells compared to spheroid cells *in vitro*. Spheroid cells proliferated in 1% hypoxia significantly better than monolayer cells, but under hypoxia, spheroid cells were highly susceptible to multimodal therapy (Figure 6A). We measured levels of DNA damage and apoptosis using immunofluorescence for γH2AX and cleaved caspase 3, respectively. Multimodal therapy led to dramatic increases in both DNA damage and apoptosis when administered under hypoxic conditions (Figure 6B, 6C). Thus sarcoma CSCs are more susceptible to multimodal therapy under hypoxic conditions compared to unselected sarcoma cells.

**DISCUSSION**

In this paper, we describe a three-pronged approach to the treatment of sarcomas that includes VEGF-A inhibition with DC101 which targets the primary VEGF-A receptor, HIF-1α inhibition with low dose doxorubicin or with shRNA, and hypoxia-activated chemotherapy with evofosfamide. We found in three different mouse models of sarcoma that this multimodal therapy is highly effective in blocking tumor growth, with more than additive increases in DNA damage, total apoptosis, and endothelial cell specific apoptosis, and more than additive decreases in microvessel density, HIF-1α activity, and CD133(+) CSCs.
Figure 4: Multimodal therapy effects on proliferation, DNA damage, and apoptosis *in vitro*. Proliferation assay A, γH2AX immunofluorescence expression B, and cleaved caspase 3 immunofluorescence expression C, in HT1080 tumor endothelial cells (EC) and HT1080 fibrosarcoma cells after VEGF withdrawal (No VEGF), evofosfamide (Evo, 10 µM), and/or low dose doxorubicin (Dox, 0.005 µM). All experiments done in normoxia and hypoxia. Bars represent standard deviation. *p < 0.05 compared to control. **p < 0.05 compared to all other groups.
Figure 5: Effects of multimodal therapy on sarcoma cancer stem-like cells. A. Western blot analysis of sarcoma cell lines grown as monolayers and as spheroids in 21% and 1% oxygen for HIF-1α. β-actin serves as loading control. B. Western blot of CD133(+) and CD133(-) sarcoma cells for self-renewal proteins. β-actin serves as loading control. C. CD133 immunofluorescence (IF) and immunohistochemistry (IHC) of sarcomas in three mouse tumor models. Scale bar 20 µm. D. CD133 expression in HT1080, SK-LMS-1, and KP tumors. Photos E. and graphs F. of CD133 expression in pimonidazole-positive and pimonidazole-negative regions of HT1080 tumors. Scale bar 20 µm. Bars represent standard deviation. **p < 0.05 compared to all other groups.
Figure 6: Multimodal therapy effects on sarcoma monolayer and spheroid cell proliferation, DNA damage, and apoptosis. Proliferation assay A, γH2AX expression B, and cleaved caspase 3 expression C, in HT1080 and SK-LMS-1 monolayer cells and spheroid cells after VEGF withdrawal (No VEGF), evofosfamide (Evo, 10 µM), and/or low dose doxorubicin (Dox, 0.005 µM). All experiments done in 21% and 1% O₂. Bars represent standard deviation. **p < 0.05 compared to all other groups.
Mechanisms of resistance to anti-angiogenic therapies include: upregulation of alternative pro-angiogenic signals, protection of the tumor vasculature either by recruiting pro-angiogenic inflammatory cells or by increasing protective pericyte coverage, accentuated invasiveness of tumor cells into local tissue to co-opt normal vasculature, and increased metastatic seeding and tumor cell growth in lymph nodes and distant organs [39]. Upregulation of HIF-1α and HIF-1α target genes in response to hypoxia may contribute to all these resistance mechanisms. Casanovas and colleagues found that VEGFR-2 inhibition of RIP1-Tag2 mouse pancreatic endocrine tumors led to an increase in intratumoral hypoxia along with increased tumor invasiveness and liver metastases [32, 40], and Ebos et al. found that sunitinib (which targets VEGF and other pathways) increased liver and lung metastases for both experimental and spontaneous metastases [33]. Given the heterogeneity and genetic instability of tumors, combination of different therapies that target primary pathways as well as resistance pathways is a rational approach. This study demonstrates that destruction of hypoxic regions within tumors using evofosfamide complements anti-VEGF-A therapy and anti-HIF-1α therapy in sarcomas.

As noted earlier, evofosfamide is a 2-nitroimidazole-linked prodrug of a brominated version of isophosphoramide mustard (Br-IPM) [28]. The 2-nitroimidazole moiety of evofosfamide is a substrate for intracellular 1-electron reductases and, when reduced in deeply hypoxic conditions, releases Br-IPM. In vitro cytotoxicity and clonogenic assays employing human cancer cell lines show that evofosfamide has little cytotoxic activity under normoxic conditions and greatly enhanced cytotoxic potency under hypoxic conditions. The activated prodrug works as a DNA cross-linking agent and is able to kill both non-dividing and dividing cells. Evofosfamide enhances the activity of a wide range of chemotherapy drugs in numerous xenograft models including HT1080 xenografts [29, 30] and has been found to be a promising agent in combination with doxorubicin for patients with metastatic sarcoma [31]. Saggar et al. found in two xenograft models that evofosfamide combined with chemotherapy increases DNA damage and apoptosis throughout the tumor [41]. Evofosfamide was found to be more effective in cell lines deficient in homologous recombination secondary to mutations in BRCA1 or BRCA2 [42, 43]. A phase III randomized trial of doxorubicin with or without evofosfamide in patients with soft tissue sarcoma (NCT01440088) failed to reach its primary efficacy endpoint. Perhaps one reason for this negative trial is that tumors required more hypoxia to achieve more activation of evofosfamide. There are currently several clinical trials examining the combination of anti-angiogenic agents and evofosfamide given anti-angiogenic agents can increase intratumoral hypoxia, and the results of this study support this strategy.

There are several limitations to this study. First, sarcomas are a heterogeneous group of tumors comprised of over 50 histologic subtypes, and thus the results of this study may not be applicable to all sarcoma subtypes. However, we examined one genetically engineered mouse model of sarcoma and two xenograft models of sarcoma and found generally consistent results. Moreover, one predominant effect of multimodal therapy appears to be on the tumor vasculature. Another predominant effect was depletion of CSCs which preferentially reside in hypoxic regions of tumors. Thus cancer cell autonomous variations in sarcoma subtypes may be less important. Second, in this study we examined proliferation, DNA damage, and apoptosis in cancer cells and endothelial cells but did not examine alternative mechanisms or effects on other cells in the tumor microenvironment. Such studies are currently ongoing but beyond the scope of this study. Third, the tumors treated in our in vivo studies were relatively small and thus may not be entirely comparable to tumors in patients in terms of relative size or the microenvironment. Finally, this study lacks clinical correlation in patient samples. We are currently collecting tumor samples from sarcoma patients who underwent chemotherapy to determine if levels of CD133 or HIF-1α correlate with response to therapy.

In conclusion, most solid tumors including sarcomas possess multiple growth pathways that promote initiation, growth, and metastasis. These pathways are often redundant such that inhibition of one pathway leads to compensation via an alternative pathway. Thus strategic combinations of various therapeutics have been a mainstay of cancer therapy. VEGF-A is an important driver of tumor angiogenesis in sarcomas, and VEGF-A inhibition alone has modest effects in patients with metastatic sarcoma [15]. HIF-1α is an important driver of tumorigenesis and metastasis in sarcomas [18], and is also a compensatory pathway following VEGF-A inhibition [45]. One approach to inhibit HIF-1α is to inhibit the protein directly along with targeting hypoxic regions of tumors with hypoxia-activated chemotherapy. The multimodal approach used in this study of VEGF-A inhibition, HIF-1α inhibition, and hypoxia-activated chemotherapy appears to also effectively deplete sarcomas of CSCs. Given doxorubicin (which blocks HIF-1α binding to target DNA) is one of the commonly used chemotherapies for sarcomas and pazopanib (which blocks VEGF-A receptors) is FDA-approved for sarcomas, one can readily envision a clinical trial of doxorubicin combined with VEGF-A inhibition and hypoxia-activated chemotherapy for patients with advanced sarcomas. Of note, we have previously demonstrated that VEGF-A inhibition and evofosfamide increase the efficacy of radiation in mouse models of sarcomas [37]. The findings in this article support the use of this multimodal strategy in clinical trials of patients with advanced or metastatic sarcomas.
MATERIALS AND METHODS

Cell lines and reagents

HT1080 human fibrosarcoma cells and SK-LMS-1 human leiomyosarcoma cells were obtained from the America Type Culture Collection (ATCC, Manassas, VA, USA). MS4515 mouse undifferentiated pleomorphic sarcoma cell lines were derived from a genetically engineered mouse models which we have previously described [35]. All sarcoma cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/ml streptomycin, and L-Glutamine 2 mM. Human cancer cell lines were actively passaged for less than 6 months from the time that they were received from ATCC, and United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines were followed [46]. Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Basel, Switzerland) and used within 8 passages. All endothelial cells were grown in EGM-2-MV media (Lonza).

The anti-VEGFR-2 antibody DC101 was purchased from Bio X Cell. IgG antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin was purchased from Teva Pharmaceuticals (Tikva, Israel). Evofosfamide was obtained from Threshold Pharmaceuticals (South San Francisco, CA, USA).

Silencing of HIF1-α was achieved via lentiviral transduction of human HIF1-α shRNA (SC- 35561-V; Santa Cruz Biotechnology). A scramble shRNA control (SC-108080) was also used. Maximal knockdown of HIF1-α occurred 72 to 96 hours after transduction.

Mouse studies

All mouse protocols were approved by the Institutional Animal Care and Use Committee. Hind limb tumors were generated in LSL-KrasG12D/+/Trp53fl/fl mice with conditional mutations in oncogenic Kras and the Trp53 tumor suppressor gene as previously described [35]. To generate subcutaneous flank tumor, 1 x 10^6 HT1080 or SK-LMS-1 cells were resuspended in 100 µl of Hank’s balanced salt solution (HBSS) and injected subcutaneously into the right flank of athymic, nude, 6-8 week old male BALB/c nu/nu mice (Taconic, Hudson, NY) following isoflurane anesthesia. Mice were assigned into treatment groups (5-6 mice per group) when tumors reached 50-100 mm^3 in volume, designated as day 0. DC101 (20 mg/kg) or isotype control IgG (20 mg/kg) was given 3 times a week by intraperitoneal injection. Evofosfamide 50 mg/kg was delivered daily by intraperitoneal injection 5 days per week. When mice were treated with combination therapies, DC101 or control IgG was delivered first and evofosfamide and/or doxorubicin were delivered within 2 hrs of DC101 administration [47]. Tumor volume (TV) was calculated by using the following formula: TV = length x (width)^2 x 0.52.

Immunohistochemistry and immunofluorescence

Mice were euthanized and tumors were harvested immediately and placed into 10% formalin for 2-3 days at 4 °C. At least 3 tumors were analyzed for each treatment group. Tumors were then rinsed and stored in saline and sent to our pathology core facility. At least 4 sections were analyzed from each tumor. Formalin-fixed, paraffin-embedded sections were deparaffinized by xylene and rehydrated. Immunohistochemistry was performed with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer’s protocol. For antigen retrieval, the sections were placed in citrate buffer (pH 6.0) and heated in a microwave oven for 10 min. For immunoperoxidase labeling, endogenous peroxidase was blocked by 0.3% H$_2$O$_2$ in absolute methanol for 15 min at room temperature (RT). The sections were then incubated overnight at 4°C with primary antibody and washed with PBS containing 0.05% Triton X-100. Incubation with corresponding secondary antibody and the peroxidase-antiperoxidase (PAP) complex were carried out for 30 min at room temperature. Immunoreactive sites were visualized by 3,3’-DAB. Afterward, the slices were counterstained by hematoxylin. Antibodies used were anti-TUNEL (ApopoTag Peroxidase kit, EMD Millipore, Temecula, CA, USA), anti-HIF-1α (NB100-105, Novus Biologicals, Littleton, CO, USA), anti-CA9 (NB100-417, Novus), anti-PCNA (sc-56, Santa Cruz Biotechnology, Dallas, TX), and anti-CD133 (MBS46020, San Diego, CA)

CD31 immunohistochemical localization and analysis of microvessel density were performed as previously described [48]. For detection of EC apoptosis, TUNEL and CD31 immunofluorescence was performed as previously described [45]. Four tumors from each treatment group were analyzed for total apoptosis, endothelial cell-specific apoptosis, microvessel density, HIF-1α expression, and CA9 expression. Hypoxia in tumors was measured using the Hypoxprobe™-1 kit (HPI, Burlington, MA) following manufacturer’s instructions.

For examination of cells for γH2AX, CD31, CD133 and cleaved caspase 3, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were incubated with the appropriate primary antibody in a solution of PBS with 1% BSA and 0.1% Triton X-100 at 4°C overnight. Antibodies used were as follows: human anti-γH2AX (mouse polyclonal antibody, 1:100, 05-636, EMD Millpore, Billerica, MA), anti-cleaved caspase 3 (rabbit polyclonal antibody, 1:100, #9661, Cell Signaling Technology, Beverly, MA), anti-
CD31 (rat monoclonal antibody, 1:100, DIA-310, Dianova, Hamburg, Germany) and anti-CD133 (rabbit polyclonal antibody, 1:100, MBS46020,MyBioSource, USA). Cells were then stained with anti-rat Alexa Fluor 488, anti-rabbit Alexa Flour 594, and anti-mouse Alexa Flour 647 (Life Technologies, Norwalk, CT, USA). After 2 hours, nuclei were counterstained using DAPI (Sigma-Aldrich). Stained cells were visualized with an inverted confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA). Image processing was performed in Imaris 7.6 software (Bitplane, Zurich, Switzerland).

Tumor endothelial cell isolation

Tumor endothelial cell were harvested from HT1080 xenografts. When tumors reached 100-150 mm³ in size, they were removed, minced, and digested in 25 ml of pre-warmed collagenase I (2 mg/ml, EMD Millipore) in PBS for 45 min at 37 ºC. Cells were strained through a 100 µM strainer, and collagenase activity was quenched with 10 ml of isolation medium [DMEM (high glucose 4500mg/L) + 20 % FCS+ Pen/Strep]. Cells were resuspended in 5 ml of cold sterile PBS with 0.1% BSA followed by centrifugation at 400 x g for 8 min. The cloudy interface containing endothelial cells was removed and washed with sterile PBS with 0.5% BSA. Cells were then resuspended in 400 ml PBS with 0.5% BSA. Anti-mouse CD31 1 µg/ml(ab28364, Abcam, Cambridge, MA) 2.5 µl was added to the cell suspension and incubated for 20 min at RT. After using Dynal beads coated with CD31 antibody (110.35, Life Technologies), the cells were plated in a gelatin-coated T-75 flask. After 1-2 weeks (or if the attached cell clones are observed in the dish), cells were used for experiments between passages 2 and 6 as previously described [49].

Proliferation assay

To assay for proliferation, 3000 cells were plated onto 24-well plates and maintained in media overnight. A colorimetric MTT assay was used to assess cell number by optical density after 3 days as previously described [50]. Hypoxia was created by placing cells into Heracell™ 150i Tri-Gas Incubator (Thermo Scientific, Waltham, MA, USA) with 1% oxygen, 94% nitrogen, 5% CO₂. Each experiment was performed at least three times. Data reflect the mean of six samples.

Western blot analysis

For Western blot analysis of HIF-1α, cells were incubated in 21% oxygen or 1% oxygen for 24 hrs. Samples were collected in RIPA buffer (Sigma-Aldrich) containing Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA), and protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Western blot analysis was performed for HIF-1α using the following antibodies: HIF-1α (C-Term Polyclonal Antibody (10006421, Cayman Chemical, Ann Arbor, MI, USA), anti-CD31 (rat monoclonal antibody, DIA-310, Dianova), CD133 (MBS46020, USA), Nanog (#8822, Cell Signaling Technology), Oct-4 (#83932, Cell Signaling Technology), Sox-2 (#2748, Cell Signaling Technology), CD271 (orb224643, Biocompare), TNAP (sc-23430, Santa Cruz Biotechnology, INC.), CD44 (#3570, Cell Signaling Technology) and β-actin (A5441, Sigma-Aldrich).

Statistical analysis

Statistical analyses were performed using Microsoft Office Excel 2010 software. P values were calculated using Student’s t-test. For comparisons between more than 2 groups, treatment groups were compared to the control group using one-way ANOVA with Bonferroni adjustment for multiple comparisons. P-values < 0.05 were considered significant.

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CONFLICTS OF INTEREST

C.P.H. is an employee of Threshold Pharmaceuticals

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