Antiangiogenesis Treatment Combined with Chemotherapy Produces Chondrosarcoma Necrosis[1]

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ABSTRACT

A combination therapy protocol using a marine chemotherapeutic and an antiangiogenic molecule was tested in a mouse tumor xenograft model for the ability to curtail the growth of a human chondrosarcoma (CHSA). Ecteinascidin-743 (ET-743), a marine-derived chemotherapeutic, was effective at slowing the growth of a primary CHSA. Plasminogen-related protein B, which antagonizes various endothelial cell activities, also elicited a significant inhibition of neoplastic growth, albeit with reduced effectiveness. The combination of the two agents resulted in only a modest further repression of tumor growth over that associated with ET-743 treatment alone, as measured by tumor volume (82% *versus* 76% inhibition, respectively). However, analysis of the extent of tumor necrosis and vascularization of the tumor revealed that the coadministration of the two compounds was clearly more effective, eliciting a 2.5-fold increase in tumor necrosis relative to single-agent treatment. The combination therapy also was most effective at antagonizing tumor-associated microvessel formation, as assessed by CD31 immunostaining, suggesting that combination therapy may hold promise for treating CHSA. Tumor necrosis produced by combination therapy of ET-743 and recombinant plasminogen-related protein B was also significantly greater than that produced by conventional doxorubicin treatment, further corroborating the efficacy of combination therapy.

INTRODUCTION

Chemotherapy is used as a first-line treatment for a wide variety of tumors and, together with surgical intervention and radiation, comprises the traditional troika of cancer therapies. Although chemotherapy is effective in combating the growth and spread of some tumors, the cancer mortality rate remains staggering. In recent years new approaches for battling cancer have been promulgated. In particular, angiogenesis inhibitors have been touted for use as cytostatic cancer therapy (1). The rationale, as set forth by Folkman and others, is that by inhibiting tumor-induced angiogenesis and thus depriving tumors of essential nutrients and oxygen, a counterbalancing between apoptosis and tumor cell proliferation ensues, leading to a "dormant" state in which tumor expansion is stalled, although viable cells would still persist (1). However, in contrast to the data generated from animal studies, the early results of various clinical trials aimed at evaluating angiogenesis inhibitors for treating cancer have been somewhat equivocal, raising questions as to the utility of this class of molecules (2).

Various fragments of the fibrinolytic protein plasminogen, including the kringle-containing angiostatin molecule, act as antiangiogenesis factors, and in animal studies they have been shown to inhibit the growth of primary and metastatic tumors (3, 4). *PRG-B*[4] encodes a putative M_r 9000 protein (PRP-B) that resembles the plasminogen NH_2-terminal activation peptide, a 77-amino acid polypeptide liberated from plasminogen during conversion to the active serine proteinase plasmin (5). Consistent with this hypothesis, the mRNA for *PRG-B* was originally discovered in cultured articular chondrocytes derived from human cartilage, one of the few avascular tissues in the body (5). In contrast to normal cartilage, CHSAs display significant microvascularity that has been correlated with biological aggressiveness (6). Moreover, two features of *PRG-B* may be relevant to its ability to inhibit tumor growth. *PRG-B* is transcriptionally activated in malignant tissues, suggesting a distinct role in regulating neoplastic growth (5, 7). Secondly, PRP-B is a full-length protein, in contrast to many other widely touted angiogenesis inhibitors that represent fragments of larger parent molecules (5). Recent work in our laboratory has established that PRP-B can thwart the growth of tumors and selectively down-regulates endothelial cell metabolism, suggesting that PRP-B is

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[4] The abbreviations used are: *PRG-B*, plasminogen-related gene B; PRP-B, plasminogen-related protein B; CHSA, chondrosarcoma; ET-743, ecteinascidin-743; rPRP-B, recombinant plasminogen-related protein B; TV, tumor volume; PECAM, platelet endothelial cell adhesion molecule.

an antiangiogenic molecule (8).[5] These data potentially implicate PRP-B as a unique agent expressed by cells in the vicinity of a tumor.

Malignant cartilaginous tumors such as CHSA are frequently refractory to conventional chemotherapy and radiation therapy. The fact that human musculoskeletal sarcomas express a number of proangiogenic factors suggests that antiangiogenesis therapy may be efficacious, possibly in combination with other treatment modalities such as chemotherapy (9, 10). Interestingly, a novel marine chemotherapeutic, ET-743, possesses a unique mode of action and appears to have clinical benefit in patients with bone and soft tissue sarcomas refractory to conventional chemotherapy, and *in vitro* studies have demonstrated that sarcoma cell lines are exquisitely sensitive to ET-743 (11–13). By binding to the minor groove of DNA and alkylating the N2 position of guanine, ET-743 produces a bending of the DNA toward the major groove as well as single-strand DNA breaks (14). Thus, ET-743 appears to act differently mechanistically with respect to other DNA minor groove alkylators. Recent reports have demonstrated that ET-743 can interfere with induced but not basal transcription of various genes, possibly by forming covalent adducts that act as impediments to transcription factors or by interfering with assembly of transcription complexes (15). The mechanism of drug resistance to ET-743 has also been explored, and defects in the nucleotide excision pathway have been linked to this phenomenon (14).

On the basis of these observations, we hypothesized that combination therapy with PRP-B as an antiangiogenic protein expressed in cartilage and ET-743 as a potent chemotherapeutic compound for sarcomas might elicit an enhanced antitumor response against high-grade CHSA. We used a xenograft model of a human high-grade CHSA to address this hypothesis.

MATERIALS AND METHODS

Cell Line and Culture Conditions. The human CHSA cell line was established in 1999 from a surgically resected human high-grade CHSA removed from a 62-year-old man with metastatic CHSA who was not previously exposed to chemotherapy or radiation therapy. We have performed reverse transcription-PCR analysis and detected mRNA for type II collagen, a conventional chondrocytic marker, in this cell line (data not shown). Two cytogenetic analyses performed 1 year apart revealed that each of 20 cells was near triploid and that all cells contained identical chromosomal rearrangements.[6] The established human high-grade CHSA cell line was grown in RPMI 1640 (Invitrogen Life Technologies, Inc., Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. The CHSA cells were morphologically stable, displaying a spindle shape with numerous small surface filopodia.

Purification of rPRP-B. The cloning of *PRG-B* and characterization of a recombinant form of the bacterial-derived recombinant polyhistidine-tagged PRP-B (rPRP-B) have been described previously (16). A modification of the purification procedure was used, by first isolating the inclusion bodies fraction from 35 liters of fermented bacteria, denaturing with 6 M urea, and purifying the rPRP-B using CM-Sepharose column chromatography (pH 5) followed by nickel-nitrilo-triacetic acid affinity chromatography. The protein refolding from urea was monitored by hydrophobic reverse phase high-performance liquid chromatography. The endotoxin level was found to be 0.25 endotoxin units/10 μg/ml solution (performed by Charles River Endosafe, Charleston, SC).

Chemicals. Doxorubicin (Sigma, St. Louis, MO) was diluted to 2 mg/ml in PBS before use. ET-743 was supplied by PharmaMar USA (Cambridge, MA) and diluted with PBS to 20 μg/ml. The *in vitro* antiproliferative effect of ET-743 against CHSA cells has been described previously (17).

Animals. Six-week-old male athymic nude mice (nu/nu; body weight, 20–25 g) were obtained from the Massachusetts General Hospital animal facility. The mice were maintained in isolation under specified pathogen-free conditions, and food and water were supplied *ad libitum*.

Human High-Grade CHSA Xenograft. All animal experiments were approved by the Massachusetts General Hospital Animal Care Committee and performed in accordance with institution guidelines. CHSA cells harvested from xenografts were adjusted to a concentration of 10^7 cells/ml in RPMI 1640, and 0.1-ml cell suspensions in serum-free media containing Matrigel were injected s.c. into the right flank of ketamine-anesthesized nude mice. After the tumors reached a TV of 100 mm^3, the mice were randomized into five groups (7 animals/group), and treatments were started. TV was assessed with a slide caliper, using the formula TV (mm^3) = $ab^2/2$, in which a is the length, and b is the width. Control mice received PBS alone, whereas experimental groups received i.v. injection of doxorubicin (at the maximum tolerated dose of 10 mg/kg) on day 0, i.v. injections of ET-743 at a concentration of 0.1 mg/kg on day 0 and 0.05 mg/kg on days 4 and 8, s.c. injections of rPRP-B at 20 mg/kg once daily, or a combination of ET-743 and rPRP-B. All mice were sacrificed when tumor weights reached 10% of total body weight.

Histological Analysis and Immunohistochemistry. Tumor tissues were fixed immediately in 10% buffered formalin phosphate and embedded in paraffin. Sections of each group were stained with H&E, and percentage of necrosis was assessed microscopically. The percentage of tumor necrosis was evaluated by a thorough histological evaluation of the serial coronal slices of the entire tumor stained with H&E. Each slide was examined by light microscopy, and the necrosis was determined to the nearest 5% by averaging two independent visual assessments of two slides per tumor treatment. Vascularization was evaluated by performing CD31-PECAM immunostaining of endothelium with representative paraffin-embedded histological sections, using a goat polyclonal CD31-PECAM antibody (Santa Cruz; diluted 1:500) and the ABC Kit from Vector Laboratories for visualization. The number of CD31-immunostained blood vessels/magnification field present in tissue sections prepared from excised tumors exposed to the different drug treatments was quantified at ×160 magnification, using a Nikon Microphot-FX fluorescence microscope. Each value represents the average of five different fields (±SD).

[5] H. Morioka, T. Morii, T. Vogel, F. J. Hornicek, and L. Weissbach., submitted for publication.
[6] J. A. Fletcher, L. Weissbach, and F. J. Hornicek, unpublished data.

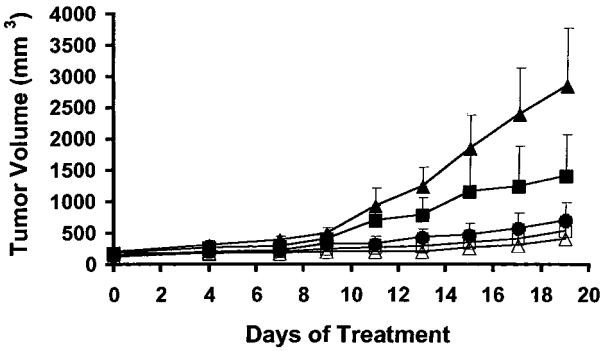

Fig. 1 Growth of a CHSA in mice treated with individual or combination therapies. Experimental procedures were described in "Materials and Methods." Briefly, after the TV reached 100 mm^3, the mice were randomized into five groups (7 animals/group), and treatments were started. ▲, PBS control; ■, rPRP-B treatment; ●, ET-743 treatment; □, combination therapy with rPRP-B and ET-743; △, doxorubicin treatment. TV was assessed with a slide caliper, using the formula TV (mm^3) = $ab^2/2$, in which *a* is the length, and *b* is the width. Control mice received PBS alone, whereas experimental groups received i.v. injection of doxorubicin (at the maximum tolerated dose of 10 mg/kg) on day 0, i.v. injections of ET-743 at a concentration of 0.1 mg/kg on day 0 and 0.05 mg/kg on days 4 and 8, s.c. injections of rPRP-B at 20 mg/kg once daily, or a combination of ET-743 and rPRP-B. The size of tumors and body weights were measured every other day until control tumors reached approximately 10% of body weight on day 19. Values at each point represent the mean of seven determinations (±SD). TV was measured as described in "Materials and Methods," and the relative inhibition was determined as follows: relative inhibition (%) = [(control value – value of mice treated with either rPRP-B, ET-743, doxorubicin, or combination therapy)/TV of control] × 100. All values represent the mean (±SD) for seven mice. The statistical significance was calculated for each treatment relative to control: $P < 0.05$, rPRP-B treatment *versus* control; $P < 0.001$, ET-743, doxorubicin, or combination therapy *versus* control. There was no statistically significant difference when comparing either ET-743 treatment *versus* doxorubicin chemotherapy or ET-743 treatment *versus* combination therapy.

Statistical Analysis. The differences between the mean values were analyzed for significance using the unpaired two-tailed Student's *t* test for independent samples; *P*s < 0.05 were considered to be statistically significant.

RESULTS

Animal Studies. Regression of tumor growth was not observed in any of the treated groups, including the doxorubicin-treated cohort. Doxorubicin is considered a standard treatment for sarcomas, either alone or in combination with ifosfamide (18). However, 10 days after treatments were initiated, TV was observed to be significantly lower in all experimentally treated groups when compared with the control group (Fig. 1). After a lag phase, TVs of control mice given PBS increased rapidly after day 9. In contrast, tumor growth in the drug-treated groups was delayed as compared with the control group. The respective inhibition relative to control of tumor growth as assessed by TV for mice treated with rPRP-B, doxorubicin, ET-743, or a combination of rPRP-B and ET-743 demonstrated that chemotherapeutic treatment had a greater negative impact on tumor growth when compared with antiangiogenic therapy with rPRP-B (Fig. 1; Table 1). Coadministration of rPRP-B with ET-743 appeared to slightly promote the slowing of tumor growth, although this difference was not statistically significant.

Table 1 Relative inhibition rate of rPRP-B, ET-743, doxorubicin, and combination therapy relative to control

See Fig. 1 for experimental details.

Condition	% Inhibition of tumor growth at day 19	
	Volume	Weight
rPRP-B	51	61
ET-743	76	82
Doxorubicin	86	90
rPRP-B + ET-743	82	91

Table 2 The percentage of tumor necrosis for individual and combination treatment

See Fig. 2 for experimental details.

Treatment	% Necrosis
Control	5
rPRP-B	5
Doxorubicin	0
ET-743	10
ET-743 + rPRP-B	25

Determination of tumor weight yielded parallel results, with antiangiogenic treatment (*i.e.*, rPRP-B) eliciting a marked reduction of tumor weight relative to control, although the response was not as potent when compared with the chemotherapeutics doxorubicin and ET-743. The data suggest that rPRP-B and ET-743, when administered individually, can slow CHSA growth in this model. Furthermore, there was no evidence of treatment-related toxicity in any of the mice except for the 10% decrease in body weight for mice treated with doxorubicin.[7]

Histological Analysis. Tumor necrosis induced by combination therapy with rPRP-B and ET-743 was assessed by microscopic examination of H&E-stained tumor specimens. In those animals not exposed to drugs, microscopic examination demonstrated robust tumor growth with many viable cells and patchy regions of necrosis (approximately 5% of the total tumor tissue; Table 2). Fig. 2*A* depicts the absence of necrosis in tumor specimens from doxorubicin-treated animals that was essentially indistinguishable histologically from the control animals, whereas the significant increase in percentage of tumor necrosis elicited by rPRP-B and ET-743 combination therapy is shown in Fig. 2*B*. These areas of necrosis were primarily found in the peripheral portions of the excised tumor specimen. The tumors exposed to ET-743 alone had less tumor necrosis compared with the tumors from animals exposed to combination therapy. The histological data summarized in Table 2 suggest that combination therapy produces more extensive tumor necrosis relative to the individual treatments. Noteworthy is the observation that although any treatment that includes ET-743 or doxorubicin produces a relatively similar inhibition of TV relative to control

[7] H. Morioka, L. Shao, and L. Weissbach, unpublished data.

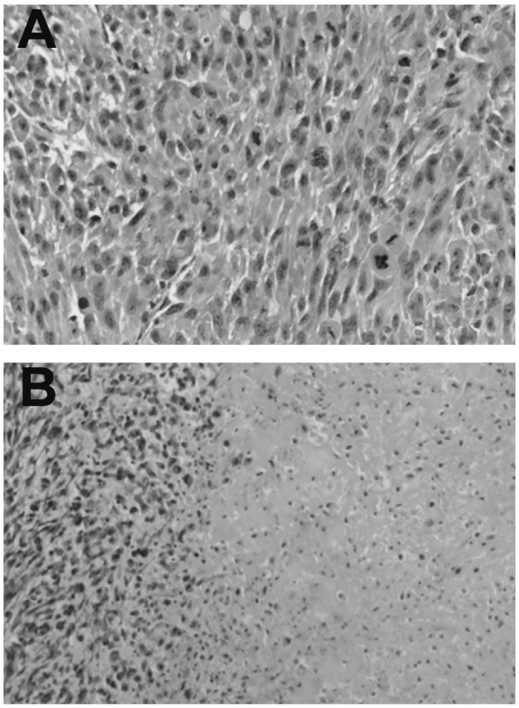

Fig. 2 Histological analysis of tumor sections. Tumor tissues were fixed immediately in 10% buffered formalin phosphate, and tissues were embedded in paraffin. Sections representative of either (*A*) doxorubicin treatment or (*B*) combination treatment (ET-743 and rPRP-B) were stained with H&E and photographed with an Olympus BX-40 microscope (*A*, ×320; *B*, ×160), and percentage of tumor necrosis was determined as stated in "Materials and Methods." Control and rPRP-B-treated tumors had no more than 5% cellular necrosis and were indistinguishable histologically from the doxorubicin-treated group. The ET-743-treated tumors had an intermediate level of cellular necrosis as compared with other treatment groups (values for percentage of necrosis given in Table 2).

(*i.e.*, 76–86%), the corresponding percentage of necrosis is divergent, ranging from 5% to 25% (compare Table 1 *versus* Table 2).

CD31 Immunostaining. To assess the degree of vascularity present in the CHSA after the different treatments, we performed CD31 immunostaining to visualize the blood vessel endothelium. As shown in Fig. 3 *A–C*, all treatments except for the combination therapy exhibited morphologically similar vessels (including doxorubicin treatment),[7] whereas the combination therapy produced relatively short, truncated vessels (Fig. 3*D*). The data presented in Table 3 demonstrate that all treatments yielded a reduction in vessel number, with doxorubicin and ET-743 displaying a more pronounced inhibition of angiogenesis as compared with rPRP-B, a result reminiscent of the inhibition of tumor growth elicited by these compounds. Combination therapy was clearly the most effective at quelling tumor vascularization, suggesting that the ability to repress endothelial cell function is enhanced by simultaneous administration of a chemotherapeutic and antiangiogenic molecule.

DISCUSSION

CHSA is the second most common malignant tumor of bone, yet conventional treatments are ineffective in a substantial number of cases. The reasons for this are unclear, but one explanation may be related to extracellular matrix molecules, such as collagens and proteoglycans, secreted by the CHSA cells that could impede the access of anticancer agents (19). However, our *in vivo* data using a xenograft model suggest that CHSA is accessible to chemotherapeutics such as doxorubicin and ET-743 because of the significant slowing of tumor growth elicited by these compounds, although viable cells clearly remain after treatment (Figs. 1 and 2). Moreover, an agent capable of repressing tumor-induced angiogenesis such as PRP-B can also derail tumor growth, although with reduced effectiveness.

Plasminogen may be considered as a parent molecule of different proteins that play important roles in fibrinolysis and angiogenesis. Our previous studies have shown that rPRP-B can interfere with the growth of the Lewis lung carcinoma in mice (8). However, the fact that angiogenesis inhibitors do not act directly on cancer cells suggests that complete eradication of a tumor is unlikely with antiangiogenesis treatment alone. Therefore, we felt that a combination therapy approach that used rPRP-B in conjunction with a chemotherapeutic agent might be efficacious. In fact, anticancer drugs such as doxorubicin are currently being used for treatments of malignant tumors, including musculoskeletal sarcomas. However, drug resistance and dose-related side effects such as myelosuppression and nausea complicate treatment. In contrast, the use of an angiogenesis-specific inhibitor such as rPRP-B has the advantage over chemotherapy treatment in that toxicity toward normal cells is usually absent. Moreover, angiogenesis inhibitors are less likely to elicit drug resistance because of the genetic stability of endothelial cells. The reduction in vascularization of CHSA elicited by rPRP-B alone, as assessed by CD31 immunostaining (Table 3), supports the contention that this molecule is antiangiogenic, although doxorubicin and ET-743 were significantly more effective at curtailing tumor vascularization (Table 3).

The fact that a reduction in endothelium is observed after combination treatment (Table 3) without concomitant reduction in tumor size relative to ET-743 treatment alone (Table 1) may indicate that ET-743 is indirectly augmenting the antiangiogenic activity of rPRP-B because ET-743 is a rather weak antiangiogenic compound *in vitro*.[7] Thus, the effectiveness of treatment for CHSA may be directly related to the ability of the treatment to minimize tumor-induced angiogenesis, and a two-pronged attack on both the CHSA cells and the endothelial cells may be critical. The fact that doxorubicin treatment alone was not able to produce necrosis supports this contention. The beneficial effect of the combination therapy also contradicts the intuitive rationale that inhibition of tumor-induced vascularization by antiangiogenesis agents might dampen the effects of chemotherapeutics because of the reduced ability of these molecules to access the tumor bed. Additional studies are needed to directly address the ability of low levels of chemotherapeutics to potentiate the curtailment of vascularization mediated by antiangiogenic molecules. Previous work has demonstrated that chemotherapeutics can have a marked antiangiogenesis effect and that CHSA cells secrete both vascular endothelial growth factor and

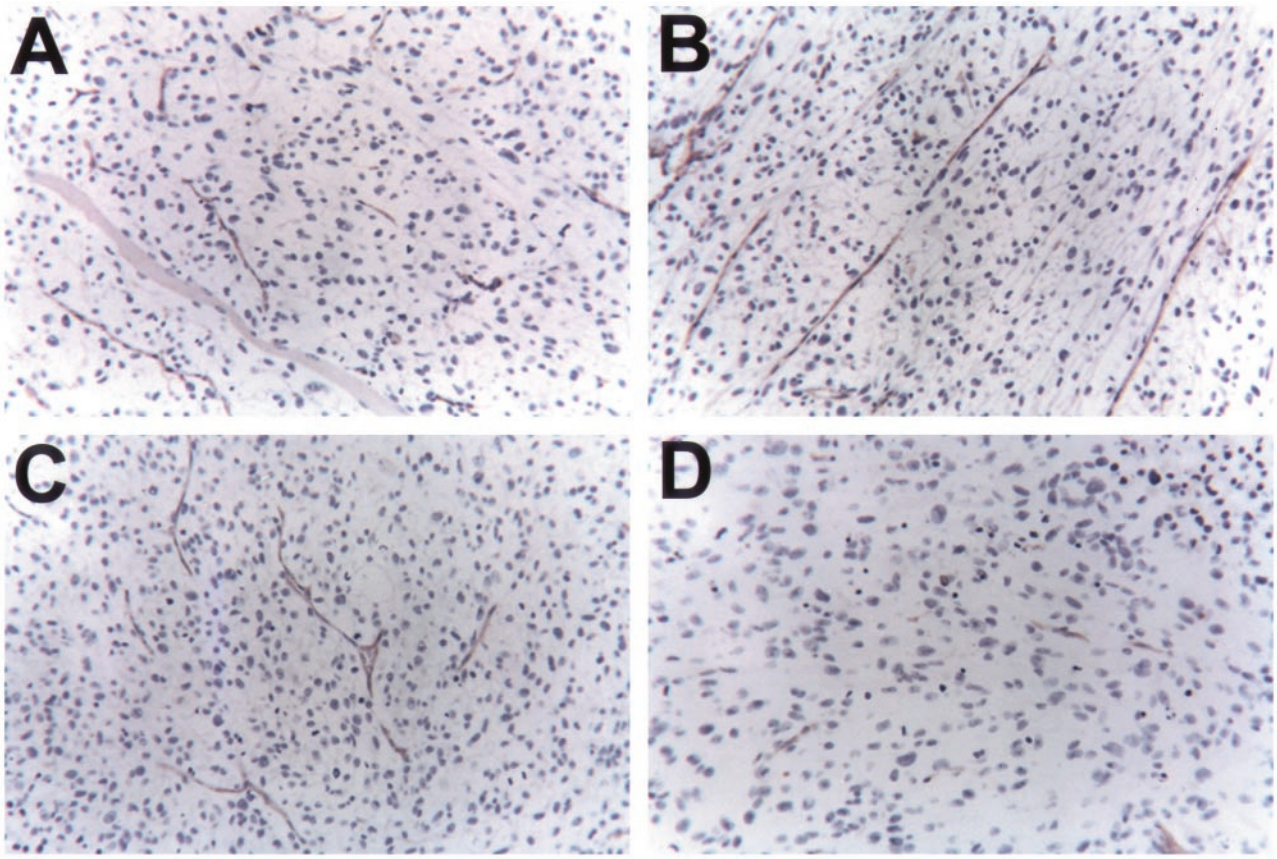

Fig. 3 Immunostaining of tumor-associated endothelium. Paraffin-embedded tumor sections were prepared as described in Fig. 2 and subjected to immunostaining with a goat polyclonal CD31-PECAM antibody. The stained slides were then photographed (×40) using a Nikon Microphot-FX fluorescence microscope. *A*, control; *B*, rPRP-B treatment; *C*, ET-743 treatment; *D*, rPRP-B and ET-743 combination treatment.

Table 3 The number of CD31-immunostained blood vessels/magnification field ($n = 5$ for each condition) for individual and combination treatment

Treatment	No. of vessels ± SD
Control	22.8 ± 2.4
rPRP-B	15 ± 4.5
Doxorubicin	7.8 ± 2.6
ET-743	5.2 ± 1.1
ET-743 + rPRP-B	2.6 ± 1.1

basic fibroblast growth factor to carry out tumor-induced angiogenesis as well as autocrine growth of the CHSA cells (20, 21). These reports lend credence to our attempts at using combination therapy for treating CHSA.

Further evidence for enhanced cytotoxicity by combination treatment was obtained from histological examination, which revealed that combination therapy of rPRP-B and ET-743 caused significantly greater necrosis relative to any individual treatment, although TV measurements did not parallel the necrosis values. These data raise questions as to the proper end points that should be invoked to assess the effects of experimental cancer treatment regimens. These results may have clinical relevance because histological necrosis rather than TV is used as a criterion in clinical situations for determining chemotherapeutic regimens after preoperative response to chemotherapeutic agents (22). In fact, TV may actually increase with increasing tumor necrosis.[8] Furthermore, as shown for various musculoskeleton tumors, the better the response to chemotherapy, as judged by tumor necrosis, the lower the probability of local recurrence after limb-salvage surgery and the higher the 5-year disease-free survival rate (23, 24). The higher rate of necrosis observed with ET-743 treatment alone as compared with doxorubicin administration may be because of a more potent inhibition of tumor-induced angiogenesis, as suggested by the CD31 immunostaining data presented in Table 3. Although not striking in terms of statistical significance, the additional 33% decrease in microvessel density produced by ET-743 treatment relative to doxorubicin treatment may indicate that ET-743 is better at repressing tumor-dependent angiogenesis, thus eliciting more cell death.

[8] T. F. DeLaney, I. J. Spiro, H. D. Suit, M. C. Gebhardt, F. J. Hornicek, H. J. Mankin, A. L. Rosenberg, D. I. Rosenthal, F. Miryousefi, M. Ancukiewicz, and D. C. Harmon, submitted for publication.

The fact that combination treatment was unique in producing significant necrosis suggests that necrosis of tumor tissue will only occur below a threshold level of vessel density. Although a vessel count of ≥7.8 (as observed for rPRP-B and doxorubicin treatment) did not generate necrosis > 5%, ET-743 treatment yielded a vessel count of 5.2 and 10% necrosis, and combination therapy produced a vessel count of 2.6 and 25% necrosis. Therefore, simply inhibiting tumor-induced angiogenesis may not be sufficient for producing tumor cell death; rather, there must be a nearly complete eradication of vascularization in the vicinity of the tumor. However, as others have cautioned, it is not clear whether microvessel density is an accurate surrogate marker for angiogenesis (25).

Positive effects of combining chemotherapy with either angiogenesis inhibitors or vascular targeting agents (which can reduce the extent of preexisting blood vessels) have been reported, reinforcing our data that suggest that dual inhibition of both neoplastic cells and the endothelium may have merit (26–31). However, the use of tumor necrosis as a parameter for assessing cytotoxicity in combination therapies has not been routinely performed in experiments involving xenograft models. Moreover, the effects of a multiple therapy protocol for treating CHSA have not been evaluated. In other tumor models, enhanced tumor cytotoxicity has been demonstrated for combination therapies involving chemotherapy and either radiation or immunotherapy (32–34). The fact that our data show that necrosis was significantly increased in the tumor tissue derived from mice receiving ET-743 in combination with an antiangiogenic agent provides a strong incentive to explore this treatment protocol as an option for CHSA patients.

Conventional chemotherapy regimens for high-grade CHSA generally have a low therapeutic response rate. Therefore, new approaches are required for treating patients with high-grade CHSA to improve clinical outcomes. ET-743 is a marine-derived anticancer agent with a unique mode of action and confirmed clinical activity in patients with soft tissue and bone sarcomas refractory to previous chemotherapy (12). Interestingly, there is no significant difference in the potency of doxorubicin and ET-743 when tested against a high-grade CHSA. Moreover, ET-743 appears less toxic than doxorubicin because a 10% weight loss was observed in mice treated with doxorubicin[7]. However, additional studies are necessary to clarify the antitumor activity of ET-743 for high-grade CHSA. Nonetheless, combination therapy may be more effective at producing tumor necrosis, possibly because of a reduction of tumor-associated vasculature, and holds promise as an improved treatment for CHSA, which currently has no effective adjuvant therapy.

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