TRAIL and Doxorubicin Combination Induces Proapoptotic and Antiangiogenic Effects in Soft Tissue Sarcoma In vivo

Suizhao Wang1,6, Wenhong Ren1,6, Jeffery Liu1,6, Guy Lahat1,6, Keila Torres1,6, Gonzalo Lopez2,6, Alexander J. Lazar3,6, Andrea Hayes-Jordan1,4, Kebin Liu7, Jim Bankson5, John D. Hazle5, and Dina Lev2,6

Abstract

**Purpose:** Novel therapeutic approaches for complex karyotype soft tissue sarcoma (STS) are crucially needed. Consequently, we assessed the efficacy of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), in combination with chemotherapy, on local and metastatic growth of human STS xenografts in vivo.

**Experimental Design:** TRAIL was evaluated alone and combined with low-dose doxorubicin in two human STS severe combined immunodeficient mouse xenograft models using fibrosarcoma (HT1080; wild-type p53) and leiomyosarcoma (SKLMS1; mutated p53), testing for effects on local growth, metastasis, and overall survival. Magnetic resonance imaging was used to evaluate local growth and bioluminescence was used to longitudinally assess lung metastases. Tissues were evaluated through immunohistochemistry and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining for treatment effects on tumor cell proliferation, apoptosis, angiogenesis, angiogenic factors, and TRAIL receptor expression. Quantitative real-time polymerase chain reaction (QRTPCR) angiogenesis array was used to assess therapy-induced gene expression changes.

**Results:** TRAIL/doxorubicin combination induced marked STS local and metastatic growth inhibition in a p53-independent manner. Significantly increased ($P < 0.001$) host survival was also demonstrable. Combined therapy induced significant apoptosis, decreased tumor cell proliferation, and increased TRAIL receptor (DR4 and DR5) expression in all treated tumors. Moreover, decreased microvessel density was observed, possibly secondary to increased expression of the antiangiogenic factor CXCL10 and decreased proangiogenic interleukin-8 cytokine in response to TRAIL/doxorubicin combination, as was also observed in vitro.

**Conclusions:** Given the urgent need for better systemic approaches to STS, clinical trials evaluating TRAIL in combination with low-dose chemotherapy are potentially warranted. *Clin Cancer Res; 16(9): 2591–604. ©2010 AACR.*

Complex karyotype soft tissue sarcoma (STS; e.g., leiomyosarcoma and unclassified pleomorphic sarcoma) pose a significant therapeutic challenge (1). Surgical resection combined with radiotherapy is the optimal approach for localized STS management (2). However, STS exhibit a marked propensity for local and systemic failure, frequently manifesting therapeutic resistance. Doxorubicin, the single most active anti-STS chemotherapeutic agent, has a disappointing 30% overall response rate. After initial chemoresponsiveness, breakthrough tumor progression and local and/or distant recurrence are frequently observed (3, 4), contributing to a 50% 5-year STS overall survival rate that has remained stagnant for ~50 years. Accordingly, more effective therapeutic approaches to complex karyotype STS are critically needed.

One of the hallmarks of STS and other malignancies is their pronounced resistance to apoptosis, resulting in cell survival even when confronted by multiple stress stimuli. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL/Apo2L), a member of the tumor necrosis factor superfamily, activates the extrinsic pathway of apoptosis through interaction with death receptors (5). Five receptors are known to bind TRAIL, two of which (DR4 and DR5) initiate an apoptotic cascade upon TRAIL binding. Interestingly, TRAIL has been shown to selectively induce apoptosis in a variety of transformed and cancer cell lines in vitro and in vivo without adversely affecting normal cells.
Translational Relevance

The relative rarity and molecular diversity of complex karyotype soft tissue sarcoma (STS) has hampered progress in the management of this cohort of devastating malignancies. Novel therapeutic approaches are crucially needed. Studies here show that combining tumor necrosis factor–related apoptosis-inducing ligand with chemotherapy yields marked anti-STS effects in human STS xenografts that model local and metastatic growth in vivo. These effects are p53 mutation status independent and are a result of both direct tumor cell apoptosis and abrogation of STS-induced angiogenesis. These data offer new insights into the molecular effects of TRAIL/chemotherapy combinations and support further study of this approach in STS clinical contexts.

Although other death receptor ligands such as tumor necrosis factor α and FasL cause septic shock and hepatotoxicity in vivo, TRAIL is tolerated well in mice and nonhuman primates (9). These novel TRAIL properties have resulted in the consideration of recombinant TRAIL and agonistic anti-TRAIL receptor antibodies in clinical trials for human cancer (10).

Preclinical studies evaluating TRAIL effects in sarcoma are limited and focus mainly on simple karyotype fusion gene STS (i.e., Rhabdomyosarcoma, Ewing Sarcoma, and osteosarcoma; refs. 11–13). Varying responses have been recorded; in general, sarcoma cell lines and freshly prepared primary cultures were relatively TRAIL resistant (14, 15). The mechanism of TRAIL resistance is not well understood and may involve multiple TRAIL-induced apoptotic pathway components. For example, alteration of TRAIL receptors through genetic and epigenetic changes can lead to enhanced TRAIL resistance (12, 16, 17). Similarly, expression of molecules that can interfere with caspase-8 activation, such as FLIP, may confer TRAIL resistance (18–20). Moreover, overexpression of antiapoptotic molecules such as BCL2 and survivin or decreased expression/function of proapoptotic mediators (e.g., BAX) have also been implicated (21).

Although the exact mechanisms remain under investigation, the observed resistance of human cancers to TRAIL in vivo has prompted searches for combination therapies with superior efficacy. Several chemotherapeutic and biological agents have been evaluated for their capacity to sensitize tumor cells to TRAIL-mediated apoptosis (22–24). Recent investigations suggest that combining TRAIL with clinically relevant anti-STS chemotherapies (e.g., doxorubicin) might overcome TRAIL resistance, resulting in significantly augmented apoptotic cell death in vitro (16, 17, 24). However, the effect of this therapeutic approach on STS local and metastatic growth in vivo has not been determined. The goal of studies presented here was to bridge this knowledge gap by evaluating the effect of combined TRAIL/doxorubicin on the growth of human fibrosarcoma and leiomyosarcoma xenografts in immuno-compromised mice. Results show that combined therapy significantly inhibits local and metastatic STS growth, whereas no major effect was elicited by either of the compounds administered alone. Anti-STS effects were due to enhanced tumor cell apoptosis and disrupted tumor-associated angiogenesis. Taken together, our study strongly supports combining TRAIL and chemotherapy as a novel therapeutic approach for complex karyotype STS.

Materials and Methods

Cells lines and reagents. Human STS cell lines HT1080 (fibrosarcoma; wild-type p53) and SKLMS1 (leiomyosarcoma; mutated p53) were obtained from the American Type Culture Collection. Authentication of cell lines was conducted immediately before their use for the current studies using short tandem repeat DNA fingerprinting conducted at the MD Anderson Cancer Center Cell Line Core facility. HT1080 cells were transfected to stably express luciferase (HT.GL). These cells were cultured in DMEM supplemented with 10% FCS (Life Technologies, Inc.). Doxorubicin (Ben Venue Lab) was obtained from the University of Texas MD Anderson Cancer Center Pharmacy. Recombinant human TRAIL was produced as previously described (9). In brief, cDNA of the extracellular domain of TRAIL corresponding to amino acids 114 to 281 was subcloned into the pET17/b (Novagen) bacterial expression vector and expressed in the BL21(DE3)pLysE (Novagen) bacterial host. Following induction of TRAIL expression using isopropyl-β-thio-galactosidase (1 mmol/L), bacterial pellets were harvested and TRAIL was purified following the passage through a nickel column (Ni-NTA) followed by a size exclusion column (Amershams). TRAIL activity was confirmed by treating TC71 cells (TRAIL-sensitive Ewing sarcoma cells) with the compound and evaluating apoptosis rate by propidium iodide staining/fluorescence-activated cell sorting analysis as described below.

Commercially available antibodies were used for immunohistochemical detection of proliferating cell nuclear antigen (PCNA; DAKO), DR4 (Active Motif), DR5 (IMAGE- NEX), Ki67 (Thermo Scientific), CD31 (BD Bioscience), interleukin-8 (IL-8; Invitrogen), CXCL10 (R & D Systems), vascular endothelial growth factor (VEGF; R&D Systems), neutrophils (anti-neutrophil antibody NIMP-R14; Abcam), and macrophages (F4-80; Serotec). Dead End Fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) System (Promega) was used for TUNEL staining. Secondary antibodies included horseradish peroxidase–conjugated (Universal kit HRP; Biocare Medical) and fluorescent secondary antibodies (anti-rabbit Alexa488, anti-mouse Alexa 594; Jackson Immuno Research). Other reagents included CytoQ FC Receptor block (Innovex Bioscience), Hoechst 33342 (Polysciences, Inc.), and propyl gallate (ACROS Organics).
**Cell growth assay.** MTS assays were conducted using CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega), per manufacturer’s instructions. Absorbance was measured at a wavelength of 490 nm and the absorbance values of treated cells are presented as a percentage of the absorbance of nontreated cells.

**Apoptosis assays.** Cell cycle analysis was conducted to determine the sub-G1 fraction. In brief, STS cell monolayers were treated with relevant agents for time periods as indicated. Fixed cells were treated with 50 μg/mL RNase and stained with 50 μg/mL propidium iodide for 30 minutes. Cells were analyzed in a FACSCalibur and data were analyzed with CellQuest and the Flowjo software or ModFitLT v3.1 software (Verity Software House). A caspase-3 apoptosis assay was also used. DIVD-NucView 488 caspase-3 assay kit for live cells (cat #30029) was purchased from Biotium, Inc. Apoptotic cells were detected per manufacturer’s instructions.

**qRT-PCR.** Five micrograms of total RNA were used for cDNA synthesis by RT III kit (Invitrogen) according to the manufacturers’ instructions. Real-time quantitative PCR was done using the PCR Master Mix (Promega) and relevant primers (sequences are available per request). Gene expression was analyzed using a Mastercycler Eppendorf replex (Eppendorf). The levels of gene expression were normalized using glyceraldehyde-3-phosphate dehydrogenase levels based on the comparative threshold cycle method.

**ELISA.** CXCL10 and IL-8 levels were measured in STS cell conditioned media after indicated treatments using ELISA. The assay was constructed and preformed following manufacturer’s instructions (R&D).

**In vivo therapeutic experiments.** All animal procedures and care were approved by the MD Anderson Cancer Center Institutional Animal Care and Usage Committee. Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." Animal models were used as previously described (25, 26). Trypan blue staining—confirmed viable STS cells (SKLMS1 and HT1080 1 × 10^6/0.1 mL HBSS/mouse) were injected s.c. into the flank of 6-week-old female severe combined immunodeficient (SCID) mice (n = 40/experiment) and growth was measured twice weekly; after establishment of palpable lesions, mice (~5 mm in average size; 12 days after HT1080 injection and 16 days after SKLMS1 injection) were assigned to four treatment groups (10/group): (a) control (vehicles only), (b) doxorubicin (1.2 mg/kg biweekly i.p.; low-dose schedule, based on our previous experience results in minimal side effects; refs. 25, 26), (c) TRAIL [10 mg/kg; 5-day per week i.p.; based on previous studies using TRAIL for other tumor types (9, 27), this dose is lower than the maximum tolerated dose in mice (150-200 mg/kg) and was selected to minimize side effects when combined with chemotherapy], and (d) doxorubicin plus TRAIL. Treatment with doxorubicin was initiated on day 1 whereas TRAIL was given starting day 2 of treatment. Mice were followed for tumor size [by tumor measurement and magnetic resonance imaging (MRI) described below], well being, and body weight and were sacrificed when control group tumors reached an average of 1.5 cm in their largest dimension. Tumors were resected, weighed, and frozen or fixed in formalin and paraffin embedded for immunohistochemistry (IHC) studies.

An experimental lung metastasis STS model was used to evaluate metastases growth and survival. HT1080 stably transduced to express green fluorescent protein-luciferase cells (HT1080GL; 1 × 10^6/0.1 mL HBSS/mouse) were injected into the tail vein of female SCID mice. Lung metastases development and growth were evaluated using bioluminescent imaging (BLI) technology as previously described (26). When BLI suggested established lung metastases (14 days after injection), mice were assigned to treatment groups as per above. 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 IHC studies. Similarly, in a second set of experiments, mice were evaluated for survival.

**Magnetic resonance imaging.** Animals were anesthetized with 5% isoflurane in oxygen and maintained using 0.5% to 2% isoflurane in oxygen. A linear volume resonator with 35 mm inner diameter was used for signal excitation and detection. At baseline, animals were scanned using a 4.7T Biospec small animal MRI system (Bruker Biospin MRI). T1-weighted FLASH [echo time (TE) = 3.5 ms; repetition time (TR) = 11 ms; 30° excitation] and three-plane rapid acquisition with relaxation enhancement [RARE; TE = 48 ms; TR = 2,000 ms; echo train length (ETL) = 8] acquisitions were used to confirm animal placement. A T2-weighted coronal RARE sequence [TE = 65 ms; TR = 3,000 ms; ETL = 12; field of view (FOV) = 4 cm × 3 cm on a 256 × 192 image matrix; two averages; 1 mm slice thickness with 0.25 mm skip] was used to localize tumor and axial T1-weighted spin-echo (TE = 8.4 ms; TR = 900 ms) and T2-weighted RARE images (TE = 80 ms; TR = 3,000 ms; ETL = 12; five averages) with matching slice prescriptions (FOV = 3 cm × 2.5 cm on a 256 × 192 image matrix; 0.75 mm slice thickness) were used for tumor visualization. Scheduling conflicts at the end of the treatment necessitated MRI scanning using a 7T Biospec System (Bruker Biospin MRI). T1-weighted FLASH and three-plane RARE acquisitions were again used during animal placement and a T2-weighted coronal RARE sequence (TE = 65 ms; TR = 5,000 ms; ETL = 12; FOV = 4 cm × 3 cm on a 256 × 192 image matrix; 0.75 mm slice thickness with 0.25 mm skip) was used for tumor localization. Axial T1-weighted spin-echo (TE = 11.5 ms; TR = 900 ms; two averages) and T2-weighted RARE images (TE = 57 ms; TR = 3,500 ms; ETL = 12; four averages) with matching slice prescriptions (FOV = 4 cm × 3 cm on a 256 × 192 image matrix; 0.75 mm slice thickness with 0.25 mm skip) were used for tumor visualization.

**IHC and TUNEL assays.** Immunohistochemical assays and TUNEL staining were done as previously described (25, 26). To detect invading neutrophils and macrophages, frozen section slides were examined as follows:
1:1,000 and 1:600 primary antibody dilutions were used, respectively. Staining distribution and intensity was evaluated and scored by at least two (including SW, WR, and KT) independent reviewers blinded to the therapeutic group to which each slide belonged. PCNA scoring was determined as the average of percent-positive immunoreactive cells evaluated by counting tumor cells in five high-power fields (×400). For TUNEL scoring, the average number of positive nuclei was calculated in five high-power microscopic fields (×400) selected from a central region in viable tumor areas avoiding areas containing necrosis. Five of the most vascularized areas within a tumor (“hotspots”) identified based on CD31 positivity were chosen at low magnification and vessels were counted in a representative high-magnification (×400) field in each of these areas. Blood vessel density was calculated as the summation of all counts divided by five. The number of invading neutrophils and macrophages were quantitated by counting positively staining cells in five high-power fields (×400).

**Angiogenesis RT² profiler PCR array.** Total RNA was isolated from frozen xenograft tumor specimens of control and doxorubicin/TRAIL combination–treated mice using the Trizol Reagent (Invitrogen). After DNase treatment, RNA was further cleaned up using the Qiagen RNeasy Mini kit (Qiagen). cDNA was synthesized by RT² First Strand kit (SABiosciences) following the company’s instructions. Gene expression profiling using the Angiogenesis RT² Profiler PCR Array (SABiosciences) was conducted. This platform is designed to profile the expression of 84 key genes in angiogenesis (for a comprehensive list of genes included in this array see http://www.sabiosciences.com). qRTPCR was conducted using Mastercycler Eppendorf reagents (Eppendorf AG) based on the array manufacturers’ instructions. Relative gene expression was determined using the ∆∆CT method. Data were further analyzed by the PCR Array Data Analysis Web Portal (http://www.SABiosciences.com/pcrarraydataanalysis.php).

**Statistical analysis.** Cell culture–based assays were repeated at least thrice and mean ± SD was calculated. Cell lines were examined separately. For qRT-PCR arrays, experiments were repeated twice for both STS models using different tumor samples. For outcomes that were measured at a single time point, two-sample t tests were used to assess the differences. Differences in xenograft growth (tumor/metastases) in vivo were assessed using a two-tailed Student’s t test. Kaplan-Meier curves were constructed to determine the overall survival time. Significance was set at P ≤ 0.05. All statistical analyses were carried out using the SPSS software.

**Results**

**TRAIL/doxorubicin combination induces STS cell apoptosis in vitro.** Two cell lines were selected for investigation: human leiomyosarcoma (SKLMS1) and fibrosarcoma (HT1080). Both reproducibly grow as tumors in immunocompromised mice histologically recapitulating counterpart human lesions. SKLMS1 harbors a mutated p53 (a common mutation in complex karyotype STS) and HT1080 harbors a NRAS mutation but is p53 wild-type (http://www.sanger.ac.uk/genetics/CGP/cosmic). We first investigated the effect of low-dose doxorubicin/TRAIL combination on *in vitro* cell growth. The effect of TRAIL alone (25 ng/mL/24 hours), doxorubicin alone (0.1 μmol/L/48 hour), or their combination (doxorubicin for 24 hours followed by the addition of TRAIL for another 24 hours) on cell growth was evaluated using MTS assays. Only minimal growth inhibition was observed in either cell line after treatment with TRAIL or doxorubicin alone at doses and time points as indicated above (Fig. 1A).

In contrast, the combination of both agents resulted in a significant growth reduction (P < 0.005). These findings were further strengthened by the significant increase (P < 0.05) in sub-G1 cell population and enhanced caspase-3 activity observed after the treatment with combination therapy (Fig. 1B and C). Taken together, these results indicate that combining doxorubicin and TRAIL results in superior growth inhibition and apoptosis in STS cells compared with either compound alone and that this effect is independent of p53 mutation status, further supporting the *in vivo* evaluation of this novel therapeutic regimen. Interestingly, the superior effect of combined therapy was not observed when drugs were delivered together or when TRAIL was administered before doxorubicin (data not shown), possibly suggesting that doxorubicin sensitizes STS cells to TRAIL and providing a rationale for *in vivo* studies scheduling.

**TRAIL/doxorubicin combination inhibits the growth of human leiomyosarcoma in vivo.** First, we investigated the effect of TRAIL and doxorubicin on human leiomyosarcoma growth in SCID mice. Treatments were highly tolerated; no significant weight loss was observed. Treatment with low-dose doxorubicin alone resulted in a small, nonstatistically significant decrease in tumor growth; the average tumor volume of doxorubicin-treated tumors at study termination was 1,453 mm³ ± 481 mm³ compared 1,738 mm³ ± 533 mm³ for controls (P = 0.33; Fig. 2A).

Similarly, TRAIL alone resulted in only a slight decrease in tumor growth compared with controls (1,535 mm³ ± 742 mm³; P = 0.57). In contrast, combined low-dose doxorubicin and TRAIL was markedly inhibitory (tumor volume, 685 mm³ ± 216 mm³) compared with control, doxorubicin alone, or TRAIL alone (P < 0.01). Mouse monitoring (n = 3/group) using MRI (Bruker Biospin MRI) further confirmed the above results (Fig. 2B). A scan conducted on the therapy start date showed similar average tumor volumes in all four mouse cohorts. In contrast, a significant difference in tumor volume was identified at the end of the study; tumors of the combination group were significantly smaller (456 mm³ ± 144 mm³; P < 0.05) than those in control, doxorubicin alone, or TRAIL alone groups (1,692 mm³ ± 204 mm³, 1,374 mm³ ± 330 mm³, and 1,292 mm³ ± 318 mm³, respectively).

Tumor sections containing viable cells from each treatment group were selected to evaluate the effect of the...
different therapies on STS cell proliferation and apoptosis (Fig. 2C). Scoring of IHC staining for PCNA (a nuclear marker for proliferation) showed a statistically significant reduction in proliferation of leiomyosarcoma cells in the combination-treated versus control-, doxorubicin-, or TRAIL-treated groups (80% ± 11% versus 69% ± 2%, 73% ± 15%, 27% ± 13%, respectively; $P < 0.05$). Similarly, TUNEL assay staining (marker for apoptosis) analysis...
Fig. 2. Doxorubicin (Dox; 1.2 mg/kg/biweekly) followed by TRAIL (10 mg/kg, 5 days/week) decreases leiomyosarcoma (SKLMS1) growth in vivo. A, tumor growth curves per treatment group (10 mice/group). B, representative MRI images of STS xenografts at time of treatment initiation and by the end of the study. Graphs depict average tumor volume as calculated from MRI images (n = 3/group; *, $P < 0.05$). White columns, tumor volume at treatment initiation; black columns, tumor volume at study termination. C, PCNA IHC and TUNEL staining (red, nuclei; green, TUNEL) of SKLMS1 xenograft specimens (selected for presence of viable tumor shown in H&E). D, DR4 (green) and DR5 (red) immunofluorescent staining of SKLMS1 xenografts (blue, nuclei).
revealed that combined doxorubicin and TRAIL induced a statistically significant increase in leiomyosarcoma apoptosis (control: 9 ± 5; doxorubicin: 13 ± 7; TRAIL: 18 ± 9; and combination: 99 ± 23 TUNEL-positive cells, respectively; *P* < 0.001). Previous studies suggested that a possible mechanism responsible for the synergistic effect of TRAIL combined with chemotherapy in vitro might be secondary to the induction of TRAIL receptor(s) expression (12, 28); to evaluate if this process occurs in leiomyosarcoma in vivo, IHC for DR4 and DR5 was done (Fig. 2D). Control-treated tumors expressed only minimal levels of DR4, whereas a demonstrable increase in its expression was noticed in all treated specimens: a higher level was seen in doxorubicin-treated samples than in TRAIL-treated samples and was most pronounced in combination therapy group. Similarly, only low DR5 expression was seen in control tumors. However, in contrast to DR4 expression, only a minimal increase in DR5 expression was noticed in TRAIL-treated tumors and a moderate increase was seen after doxorubicin treatment alone, whereas combination therapy resulted in a marked increase in expression intensity and distribution of this TRAIL receptor. It is possible that increased TRAIL receptor expression, especially DR5, is at least partially responsible for the enhanced antitumorogenic effect of combined TRAIL/doxorubicin.

**TRAIL/doxorubicin combination inhibits the local and metastatic growth of human fibrosarcoma in vivo and prolongs survival.** Next, we evaluated the effect of combined TRAIL/doxorubicin on another human STS histologic subtype: HT1080 xenografts growing in SCID mice. As depicted in Fig. 3A, treatment with doxorubicin or TRAIL alone did not significantly affect HT1080 growth compared with control-treated mice (P = 0.73 and 0.65, respectively). However, combined treatment resulted in significant tumor growth inhibition compared with the other three experimental arms (P < 0.01). Moreover, average tumor weights at termination of the study were similar in control-, doxorubicin-, and TRAIL-alone–treated mice (1.27 g ± 0.54 g, 1.19 g ± 0.45 g, and 1.119 g ± 0.43 g, respectively), whereas combination therapy significantly reduced tumor weight (0.30 g ± 0.29) compared with all other therapeutic regimens (P < 0.001).

Similar to above, Ki-67 staining and TUNEL assay scoring revealed that combined doxorubicin/TRAIL combination resulted in significantly decreased tumor cell proliferation (P < 0.05) and increased apoptosis (P < 0.05; control group: 65% ± 13% and 3% ± 2%; doxorubicin group: 47% ± 15% and 5% ± 4%; TRAIL group: 50% ± 12% and 11% ± 5%; and combination group: 14% ± 7% and 72 ± 15%, respectively; Fig. 3B). The baseline DR4 and DR5 expression levels in control HT1080 tumors were higher than those of SKLMS1 tumors (Fig. 3B). An increase in DR4 expression was observed in all treatment cohorts most pronounced in doxorubicin and TRAIL/doxorubicin treatment groups. Similarly, an increase in DR5 was seen in doxorubicin–treated tumors and to the highest extent in combination-treated samples. This pattern of TRAIL receptor expression was similar in both of the STS histologic subtype animal models evaluated.

Metastases (especially pulmonary) are the main cause of STS-specific mortality (29). To evaluate whether combining doxorubicin/TRAIL resulted in pulmonary metastatic outgrowth inhibition, we used an experimental fibrosarcoma lung metastasis model. No major difference in luciferase readout was observed between doxorubicin or TRAIL alone–treated mice compared with controls (Fig. 4A). In contrast, combined TRAIL/doxorubicin resulted in decreased luciferase readout with fewer and smaller lung metastases observed on the lung surface. Macroscopic findings were also confirmed on H&E staining, showing large lung tumor deposits in control, doxorubicin, and TRAIL groups and smaller, microscopic lesions in the combination group. Lung weights were significantly lower in combined versus control, doxorubicin, or TRAIL treatment groups (0.38 g ± 0.12 g versus 0.97 g ± 0.14 g, 0.91 g ± 0.13 g, and 0.83 g ± 0.10 g, respectively; P < 0.005; Fig. 4B).

Lastly, we evaluated the effect of combined TRAIL/doxorubicin on the survival of mice harboring lung metastases. An experiment as above was conducted and mice (n = 6-7/group) were followed for survival. The median survival time of control-, doxorubicin-, and TRAIL-treated mice was 20, 21, and 20 days, respectively, compared with 34 days for mice treated with TRAIL and doxorubicin. A KM plot is shown in Fig. 4C, showing a statistically significant prolongation in overall survival of mice treated with combined TRAIL/doxorubicin (P = 0.001). TRAIL/doxorubicin combination elicits antiangiogenic effects in STS.

STS are highly vascular and angiogenic, perhaps accounting for their capacity to grow to large size and avidly metastasize (30). Therefore, we evaluated if the combined therapeutic approach affected STS microvessel density (Fig. 5A). Treatment with doxorubicin or TRAIL alone resulted in a statistically nonsignificant reduction in the number of CD31-positive vessels compared with controls (SKLMS1: 85 ± 23 and 90 ± 27 versus 110 ± 32, respectively; HT1080: 71 ± 21 and 63 ± 14 versus 80 ± 39, respectively). In contrast, combination therapy resulted in a marked reduction in CD-31–positive vessels (21 ± 11 and 32 ± 15 in SKLMS1 and HT1080, respectively; P < 0.05). Interestingly, no TUNEL staining was identified in CD-31–positive cells upon Immunofluorescence double staining in any one of the treatment cohorts (Fig. 5A). These results suggest that the observed decrease in blood vessel number in response to combined therapy is not secondary to endothelial cell apoptosis and possibly represents de novo inhibition of angiogenesis.

Tumor-associated angiogenesis is a complex process involving many proangiogenic and antiangiogenic factors. Next, we sought to evaluate the effect of TRAIL/doxorubicin combination on the expression of angiogenic factors in vivo. RNA extracted from control- and combination-treated tumors was subjected to an angiogenesis RT2 Profiler RT PCR array. This array only recognizes human RNA; therefore, results represent gene expression changes in STS cells and not in the murine-originating tumor-associated stroma. Interestingly, expression changes in only two genes of those included on the array (n = 84) were found...
Fig. 3. Doxorubicin (Dox) and TRAIL combination decreases fibrosarcoma (HT1080) growth in vivo. A, tumor growth curves per treatment group (left; n = 10 mice/group, P < 0.01) and tumor pictures (top right). Box plots (bottom right) depict average tumor weights at termination of the study (P < 0.001). B, Ki-67 immunofluorescence (blue, nuclei; green, Ki-67) and TUNEL staining (blue, nuclei; green, TUNEL) of HT1080 xenograft specimens (selected for presence of viable tumor shown in H&E). C, DR4 (green) and DR5 (red) immunofluorescent staining of HT1080 xenografts (blue, nuclei).
to occur reproducibly in both STS models; a marked increase in the level of the antiangiogenic factor CXCL10 (7.4 ± 5.5-fold and 9.9 ± 3.0-fold in SKLMS1 and HT1080, respectively) and a significant decrease in the expression of the angiogenic factor IL-8 (9.5 ± 2.3-fold and 8.2 ± 2.5-fold in SKLMS1 and HT1080, respectively) was observed in the TRAIL/doxorubicin-treated tumors compared with control-treated tumors (*, *P < 0.05; Fig. 5B).

qRTPCR was used to evaluate the mRNA expression of CXCL10 and IL-8 in an independent tumor sample cohort of control-, TRAIL-, doxorubicin-, and combined TRAIL/doxorubicin SKLMS1 and HT1080–treated xenografts (Fig. 5C). A significant increase (*P < 0.005) in CXCL10 mRNA expression was observed in combination-treated tumors compared with controls; no significant change was noted in TRAIL- or doxorubicin alone–treated tumors. Similarly, a statistically significant (*P < 0.005) decrease in IL-8 mRNA expression was observed in combination therapy tumors, but not in tumors treated with either compound alone. Treatment-induced effects on CXCL10 and IL-8 protein were further confirmed through IHC (Fig. 5D). The functional effect of decreased in IL-8, one of the most important chemotactic factors for neutrophils, was further reflected by a statistically significant decrease in the number of tumor-infiltrating neutrophils identified in combination-treated samples (*P < 0.05; Fig. 5D). Similarly, an increase in macrophage infiltration was observed in TRAIL/doxorubicin-treated specimens possibly reflecting the enhanced activity of CXCL10 in these tumors and the recruitment of myeloid derived cells with antitumorigenic capacities (M1 macrophages; *P < 0.05; Fig. 5D). Previously published data suggested a TRAIL-induced reduction in VEGF-A expression as a potential mechanism for TRAIL antiangiogenic effects in glioblastoma (31). No effect of TRAIL/doxorubicin on VEGF-A level in STS specimens was shown in the gene expression arrays, qRTPCR, and IHC (data not shown).

Lastly, we evaluated whether the in vivo effect of doxorubicin/TRAIL on CXCL10 and IL-8 expression could be recapitulated in culture. SKLMS1 and HT1080 cells were treated with doxorubicin (0.1 μmol/L), TRAIL (25 ng/mL), or their combination with doxorubicin administered before TRAIL as described; RNA was extracted and conditioned media were collected. As shown in Fig. 6A, combined therapy resulted in a significant increase in CXCL10 mRNA expression
Fig. 5. Doxorubicin (Dox) and TRAIL combination inhibits STS-associated angiogenesis. A, CD31 immunohistochecistry and CD31/TUNEL double immunoflorescence (red, CD31; green, TUNEL) show decrease in blood vessel density in combination treatment samples but no evidence of endothelial cell apoptosis. B, angiogenesis reverse transcription-PCR array showed a significant increase in CXCL10 mRNA expression and a marked decrease in IL-8 mRNA levels in combination treated tumor samples of both STS xenograft models. C, effects of treatment on CXCL10 and IL-8 mRNA expression was further confirmed through reverse transcription-PCR using an independent cohort of tumor samples (*, \( P < 0.05 \)). No significant gene expression changes were noticed in samples treated with either agent alone. D, effect of treatment on IL-8 (red) and CXCL10 (red) protein expression in vivo was evaluated by immunoflorescence (blue, nuclei). An increase in tumor-infiltrating macrophages and a decrease in tumor-infiltrating neutrophils was also observed in TRAIL/doxorubicin-treated tumors. Columns, mean of three repeated experiments; bars, SD.
and a reduction in IL-8 mRNA expression compared with controls or either drug alone ($P < 0.05$). Similarly, ELISA confirmed the respective changes in protein expression levels of these cytokines (Fig. 6B). Although the studies above do not preclude possible effects of TRAIL/doxorubicin on other angiogenesis-related factors, a possible role for CXCL10 induction and IL-8 decrease in the antiangiogenic effects resulting from this therapeutic regimen is suggested in STS.

**Discussion**

A potential role for TRAIL as a novel anticancer agent has emerged due to its potent and possibly tumor selective proapoptotic effects. Several phase I clinical trials evaluated the effects of TRAIL agonist monoclonal antibodies in patients with advanced solid cancers, including sarcoma (32, 33). Although no objective responses were recorded (as might be anticipated in phase I trials), prolonged disease stabilization was documented in several sarcoma patients. For example, Plummer et al. (33) recently reported a study using lexatumumab in which 12 sarcoma patients participated. Their results identified three sarcoma patients (extraosseous osteosarcoma, retroperitoneal liposarcoma, and chondrosarcoma), all with documented progressive disease on standard chemotherapy, in whom lexatumumab resulted in prolonged disease stabilization and minimal side effects. Together, these clinical studies suggest that TRAIL agonist effects are not specific sarcoma

![Fig. 6. Doxorubicin (Dox) and TRAIL combination modulates angiogenesis-related factor expression in STS cells.](image)

A, STS cells grown in culture were treated with doxorubicin (0.1 μmol/L/24 hours), TRAIL (25 μg/mL/12 hours), or their combination. qRT-PCR showed a significant increase in CXCL10 mRNA levels (*, $P < 0.05$) and a decrease in IL-8 mRNA expression (*, $P < 0.05$); B, conditioned medium was collected from STS cells treated as above and was subjected to ELISA. A significant increase in CXCL10 protein expression (*, $P < 0.05$) and a decrease in IL-8 protein levels (*, $P < 0.05$) were observed. Columns, mean of three repeated experiments; bars, SD.
histologic subtype selective. However, their apparent limited clinical effect when used as single antisarcoma agents calls for the identification of more effective combinatorial therapeutic approaches.

Studies here show that the combination of doxorubicin (the most commonly used anti-STS chemotherapeutic agent) and TRAIL, administered in this sequential order, elicits potent local and metastatic growth-inhibitory effects in xenograft models of human STS, whereas no significant effect was observed with either agent alone. These data further expand previously published findings suggesting that chemotherapy might enhance TRAIL-mediated apoptosis in sarcoma cells in vitro (34–37). Importantly, our findings show that the doxorubicin/TRAIL combination effect is independent of p53 mutation status: significant antitumor effects were observed in STS harboring either wild-type or mutated p53. This observation is of potential clinical relevance in STS because p53 dysregulation is very common and STS harboring p53 mutations are thought to be more resistant to current therapeutic strategies (38).

The molecular mechanisms resulting in combined doxorubicin and TRAIL proapoptotic synergistic effects are not well defined. Although the sensitivity of cells to TRAIL does not seem to be a simple function of TRAIL death receptor expression level, the augmentation of TRAIL-induced apoptosis by chemotherapeutic drugs has been suggested to be at least partly the result of drug-induced upregulation of death receptors (14, 34, 36). Concordantly, our studies showed increased DR4 and DR5 expression in STS specimens treated with combined doxorubicin/TRAIL. Chemotherapy effects on TRAIL downstream signaling and the modulation of proapoptotic and antiapoptotic effector expression has also been suggested (27). For example, chemotherapy-induced decrease in cFLIP expression was identified as a potential mechanism of osteosarcoma cell sensitization to TRAIL (9, 36). Alternatively, decreased X-IAP expression was also observed (39). Further exploration of pertinent contributory mechanisms will facilitate combined doxorubicin and TRAIL therapy evaluation in human clinical trials.

In addition to the proapoptotic effects induced by the doxorubicin/TRAIL combination, studies here identified a significant reduction in the number of STS-associated blood vessels in response to therapy, albeit without evidence of endothelial cell apoptosis. STS are markedly proangiogenic and highly dependent on their vasculature for local growth and metastasis (30). Thus, antiangiogenic therapies affecting STS-associated vasculature hold major promise and several are under evaluation in human STS clinical trials (40–42). Interestingly, although TRAIL is commonly thought to have no or minimal effects on normal cells, recent studies have found unanticipated TRAIL proangiogenic–enhanced endothelial cell proliferation, migration, and cytoskeletal reorganization in vitro and in vivo to an extent comparable with VEGF (43, 44). In contrast, other reports suggest a direct proapoptotic effect of TRAIL on endothelial cells (45). Moreover, decreased VEGF secretion, migration, and microvessel formation was shown after the TRAIL treatment of human umbilical vascular endothelial cells (31). The discrepancy between these studies might be a result of the different TRAIL concentrations used; whereas the former studies evaluated the effect of high TRAIL doses, the latter studies used markedly lower TRAIL concentrations (~10-fold). Studies presented here show that within the STS microenvironment, treatment with TRAIL alone resulted in decreased microvessel density although to a statistically nonsignificant level, suggesting that at these concentrations TRAIL does not enhance angiogenesis but instead elicits an antiangiogenic effect that is even more pronounced with combined TRAIL and doxorubicin. As in other solid tumors, STS-associated angiogenesis is a complex process regulated by numerous proangiogenic and antiangiogenic factors.

The lack of TRAIL-induced endothelial cell apoptosis observed in STS xenografts raises the possibility that the antiangiogenic effects are secondary to the modulation of STS cell angiogenic factor expression. A previous report suggested that TRAIL inhibits angiogenesis in human glioblastoma through decreased tumor cell VEGF expression (31). No change in VEGF expression was observed in human STS xenografts after treatment with TRAIL alone or in combination with doxorubicin, suggesting cancer- and even cell type–dependent TRAIL-induced effects. However, a significant increase in the expression of the antiangiogenic cytokine CXCL10 (46) and a decrease in the levels of the proangiogenic cytokine IL-8 (47) were observed in STS in vivo and in vitro after combination therapy but not with either compound alone. Furthermore, treatment-induced changes in these cytokines can potentially affect tumor growth by affecting additional tumor microenvironment constituents such as neutrophils and macrophages in favor of tumor inhibition. The molecular mechanisms resulting in these treatment effects and the potential role of these cytokines in TRAIL/doxorubicin angiogenesis blockade are currently being investigated. Lastly, as these are secreted cytokines, evaluating their circulating blood levels as markers of treatment response is highly feasible; such analysis should be considered for inclusion in further preclinical studies and in human TRAIL/doxorubicin clinical trials. Taken together, our studies suggest that doxorubicin/TRAIL combination has significant anti-STS effects resulting in both tumor cell apoptosis and antiangiogenic effect. Consequently, the merits of this therapeutic regimen should be potentially studied in STS clinical contexts.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Suizhao Wang, Wenhong Ren, Jeffery Liu, et al.

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