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Gemcitabine-Mediated Radiosensitization of Human Soft Tissue Sarcoma[1]

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Abstract

BACKGROUND/PURPOSE: Local and systemic control of soft tissue sarcoma (STS) remains a clinical challenge, particularly for retroperitoneal, deep truncal, or advanced extremity disease. 2′,2′-Difluoro-2′-deoxycytidine (gemcitabine) is a potent radiosensitizer in many tumor types, but it has not been studied in human STS. The purpose of this study was to determine the radiosensitizing potential of gemcitabine in preclinical models of human STS. *MATERIALS AND METHODS:* The *in vitro* radiosensitizing activity of gemcitabine was assessed with clonogenic survival assay on three human STS cell lines: SK-LMS-1 (leiomyosarcoma), SW-872 (liposarcoma), and HT-1080 (fibrosarcoma). Cell cycle distribution was determined using dual-channel flow cytometry. The *in vivo* radiosensitizing activity of gemcitabine was assessed with subcutaneous SK-LMS-1 nude mice xenografts. Tumor-bearing mice were treated with concurrent weekly gemcitabine and fractionated daily radiotherapy (RT) (2 Gy daily) for 3 weeks (a total dose of 30 Gy). *RESULTS:* The 50% inhibitory concentration (IC_{50}) of gemcitabine for the human STS cell lines ranged from 10 to 1000 nM. Significant *in vitro* radiosensitization was demonstrated in all three human STS cell lines using gemcitabine concentrations at and below the IC_{50}. Maximal radiosensitization was associated with accumulation of cells in early S-phase. SK-LMS-1 xenografts displayed significant tumor growth delay with combined gemcitabine and RT compared to either treatment alone. Treatment related toxicity was greatest in the gemcitabine plus RT arm, but remained at an acceptable level. *CONCLUSIONS:* Gemcitabine is a potent radiosensitizer in preclinical models of human STS. Clinical trials combining gemcitabine and RT in human STS are warranted.

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Introduction

Soft tissue sarcomas (STSs) are a heterogeneous group of rare neoplasms that are thought to arise from mesenchymal tissues [1]. Whereas the majority of STS develop in the extremity, these tumors can arise in any anatomic site, including the retroperitoneum, deep trunk, and head and neck [2]. In sites such as the extremity, surgical resection and radiotherapy have resulted in local control rates exceeding 90% [3–5]. In contrast, in the retroperitoneum, the deep trunk, and in advanced extremity disease, complete surgical resection with wide margins is difficult to achieve because of anatomic constraints. In these sites, local control is suboptimal, and local recurrence is a major determinant of morbidity and mortality [6–9]. As such, there is a need for novel combined modality treatment strategies to enhance local control in STS.

2′,2′-Difluoro-2′-deoxycytidine (dFdCyd or gemcitabine) is a deoxycytidine analog that has been shown to be a strong radiosensitizer in a number of tumor types, including pancreas, head and neck squamous cell carcinoma, non–small cell lung cancer, and colon carcinoma cells [10–13]. Gemcitabine is transported into the cell followed by phosphorylation by deoxycytidine kinase to its active di- and triphosphorylated forms [14,15]. Gemcitabine diphosphate inhibits ribonucleotide reductase, leading to decreases in deoxynucleotide pools, and gemcitabine triphosphate competitively incorporates into the DNA resulting in the interruption of DNA chain elongation. Whereas gemcitabine-mediated radiosensitization appears to be associated with S-phase accumulation [16] and depletion of deoxyadenosine

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triphosphate (dATP) pools [10,13], the precise mechanism of this potent radiosensitization remains unknown.

The laboratory observations of the synergism between gemcitabine and radiation have been translated into clinical trials in pancreas [17,18], head and neck [19], and breast cancer [20]. The purpose of this study was to examine the radiosensitizing activity of gemcitabine in preclinical models of human STS. If successful, these studies would provide a preclinical foundation for clinical trials combining gemcitabine and radiation therapy for STS in anatomic sites where local control rates are suboptimal.

Materials and Methods

Cell Culture

HT-1080 fibrosarcoma, SW-872 liposarcoma, and SK-LMS-1 leiomyosarcoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in minimum essential medium (HT-1080) or in Dulbecco's modified Eagle's medium (SW-872 and SK-LMS-1) containing 10% fetal bovine serum. Cells were passaged twice weekly and routinely tested for *Mycoplasma*.

Clonogenic Survival Assay

Cells were plated in 100-mm^2 dishes and allowed to adhere for at least 24 hours before treatment. Gemcitabine (Eli Lilly, Indianapolis, IN) was dissolved in PBS and stored at −20°C until use. Irradiation was carried out using a 250-kV orthovoltage unit (Philips, Hamburg, Germany) at a dose rate of approximately 2 Gy/min. Dosimetry was carried out using an ionization chamber connected to an electrometer system that is directly traceable to a National Institute of Standards and Technology calibration.

Cytotoxicity was assessed after a 24-hour exposure to gemcitabine. Radiosensitization was assessed with a 24-hour exposure to gemcitabine followed immediately by irradiation. After treatment, floating cells were collected and added to the trypsinized adherent cells. This combination of cells was then washed with PBS, counted on a Coulter Counter (Hialeah, FL), and replated at clonal density. After 5 to 10 days of incubation, colonies were fixed with 7:1 methanol/acetic acid and stained with 0.5% crystal violet. Only colonies with greater than 50 cells were counted. The plating efficiency of all three cell lines was ~30%.

Cytotoxicity of gemcitabine was determined from the inhibitory concentrations (ICs) derived from sigmoid curves fit to the results of three independent experiments. Radiosensitization by gemcitabine was determined by fitting radiation survival curves to each drug concentration using the linear quadratic equation. The mean inactivation dose [21] (equal to the linear area under the cell survival curve) was calculated for each condition, and dose enhancement ratios (DERs) were determined by dividing the mean inactivation dose of control cells by the mean inactivation dose of treated cells. DERs greater than 1 indicate radiosensitization.

Cell Cycle Analysis

Bromodeoxyuridine (BrdU) flow cytometry was performed as previously described [22]. Briefly, after a 24-hour exposure to gemcitabine, cells were pulsed with 30 μM BrdU for 15 minutes, harvested, counted, fixed in ice-cold 70% ethanol, and stored at 4°C until analysis at which time they were treated with an anti–BrdU mouse antibody (Pharmingen, San Diego, CA), then a fluorescein isothiocyanate–conjugated goat anti–mouse secondary antibody (Sigma, St. Louis, MO), and finally resuspended in a solution containing 18 μg/ml propidium iodide (PI) with 40 μg/ml RNase A. Sample sizes were approximately 1×10^6 cells, and floaters were included with adherent cells. Trout erythrocyte nuclei (BioSure, Grass Valley, CA) were used as an internal standard in all experiments. Samples were analyzed by counting 10,000 events on a Becton Dickinson FACScan flow cytometer (San Jose, CA). Cell cycle data were analyzed using WEASEL software (version 2.3.1) from The Walter and Eliza Hall Institute, Melbourne, Australia.

Apoptosis

Apoptosis was determined by analyzing the sub-G_1 DNA content using PI flow cytometry. Cells were harvested, washed, fixed in ice-cold 70% ethanol, and stored at 4°C until the time of analysis when they were washed again and resuspended in a solution containing 18 μg/ml PI and 40 μg/ml RNase. Cells were analyzed using a Becton Dickinson FACScan flow cytometer as described above.

In Vivo *Tumor Growth Assays*

Exponentially growing SK-LMS-1 cells (5×10^6) were resuspended in PBS (200 μl) and injected subcutaneously into the flanks of 6- to 8-week-old athymic male nude mice (Charles River Laboratories Inc., Wilmington, MA). Treatment commenced approximately 14 days after inoculation, once tumors reached an average volume of 150 mm^3. Mice were randomized into four treatment arms (control, gemcitabine alone, radiation alone, and gemcitabine + radiation) with eight mice per arm. Gemcitabine was dissolved in PBS and administered through intraperitoneal (i.p.) injection on days 1, 8, and 15, at a dose of 150 mg/kg, given 6 hours before radiotherapy (RT). Radiotherapy was administered in 2-Gy daily fractions commencing on day 1 with five fractions per week for 3 weeks (a total dose of 30 Gy). Mice were anesthetized with i.p. injections of ketamine (80 mg/kg) and xylazine (4 mg/kg) and were placed prone in custom-made holders. Cerrobend (Cerro Metal Products, Co., Bellefonte, PA) shielding was placed over the entire mouse minus the tumor, creating a conformal treatment field. After RT, mice were kept warm with heating pads until they fully recovered from anesthesia. Animals were handled according to the University of Michigan Laboratory Animals Maintenance Manual.

Tumor volumes (V) were estimated from the formula of a spheroid: $V = (\pi/6) \times D_1 \times (D_2)^2$, where D_1 and D_2 are the longer and shorter perpendicular diameters, respectively. Tumor growth was expressed as the relative change in volume compared to day 1. Growth delay of a treatment arm ($GD_{treatment}$) was defined as the time it took the treated group to reach five times the initial volume, minus the time it took the control group to reach the same relative volume. The growth delay enhancement ratio (GDER) was calculated as follows: $GDER = (GD_{Gem + RT} - GD_{Gem\ alone})/GD_{RT\ alone}$. GDER greater than 1 implies synergism between gemcitabine and RT. All animals were euthanized when tumor volumes reached 2 cm^3, and an additional animal was sacrificed from each group on the last day of treatment (day 19), except those of the control group which were euthanized on day 15 because of tumor size. Tumors were harvested, formalin-fixed, paraffin-embedded, and stained with hematoxylin and eosion for histologic analysis.

Results

In Vitro *Radiosensitization*

The sensitivity of all three cell lines to gemcitabine alone was first established to determine the appropriate range of doses to evaluate

the radiosensitizing effects of gemcitabine. The 50% inhibitory concentration (IC_{50}) after a 24-hour exposure to drug alone ranged widely for all three cell lines (Table 1). The HT-1080 cells were the most sensitive with an IC_{50} of 11 ± 1 nM. The SW-872 cells were slightly more resistant with an IC_{50} of 45 ± 14 nM. The most resistant cell line was SK-LMS-1 cells with an IC_{50} of 1070 ± 90 nM. To evaluate the radiosensitizing properties of gemcitabine, we used doses ranging from mildly to moderately cytotoxic (IC_{10}–IC_{80}). All three cell lines demonstrated radiosensitization at doses at or below the IC_{50} (Figure 1 and Table 2). Additionally, maximal radiosensitization occurred with more cytotoxic doses of gemcitabine.

To begin to understand the mechanism of radiosensitization, we examined the cell cycle distribution after exposure to gemcitabine. Previous studies have demonstrated an association between S-phase accumulation and radiosensitization [16]. In all three cell lines, after the 24-hour treatment with gemcitabine, there was an arrest in early S-phase, which correlated with doses of gemcitabine where radiosensitization occurred (Figure 2). The most cytotoxic and radiosensitizing concentrations of gemcitabine yielded a more than two-fold increase in accumulation of cells in early S-phase compared with control-treated cells.

To address the potential role of apoptosis as a factor of gemcitabine-mediated radiosensitization, cells were treated with gemcitabine and/or radiation, and harvested immediately and 6 hours after treatment. There was no increase in the apoptotic fraction (data not shown) as assessed by measurement of sub-G_1 content, suggesting that apoptosis is not likely a factor involved in the radiosensitization in these three STS cell lines.

In Vivo *Radiosensitization*

To evaluate *in vivo* radiosensitization, SK-LMS-1 nude mouse xenografts were used. Of the three cell lines tested, we selected SK-LMS-1 because they were the most resistant to gemcitabine alone and gemcitabine-mediated radiosensitization. The dose of gemcitabine was determined from previous experiments (data not shown), which found the dose of 150 mg/kg per week to be relatively nontoxic as measured by weight loss, and produced a slight growth delay compared with controls. The treatment schedule of weekly gemcitabine and daily fractionated radiation delivery was designed to mimic a schedule that would be used in a clinical setting. The group treated with gemcitabine and RT experienced the most significant growth delay compared with either the RT-alone or the gemcitabine-alone groups (Figure 3). The GDER for this combined chemoradiotherapy group was 1.89 ± 0.45 (P = .024), indicating strong synergism between gemcitabine and RT. In fact, one xenograft in the combined treatment group responded completely, and this mouse remains without evidence of the disease 19 weeks after treatment.

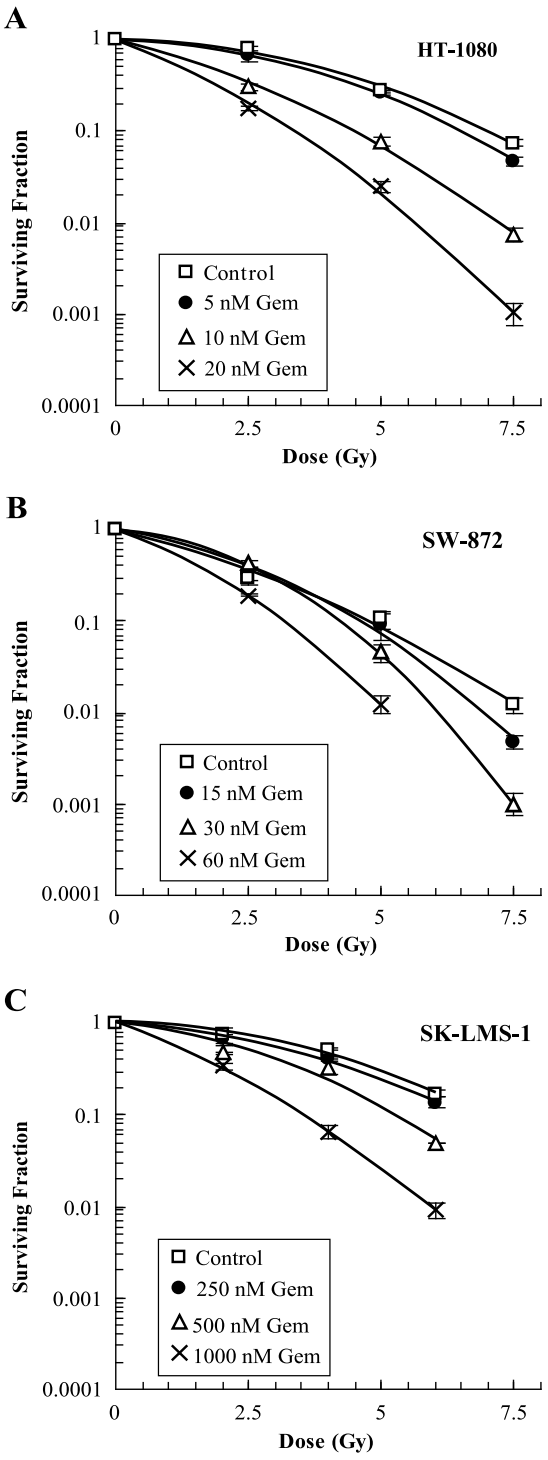

Figure 1. Clonogenic survival of gemcitabine and radiation. HT-1080 (A), SW-872 (B), and SK-LMS-1 (C) cells were exposed to gemcitabine for 24 hours before irradiation. Experiments were repeated at least three times with representative data shown.

Table 1. Cytotoxicity of Gemcitabine.

Cell Line	Gemcitabine Dose (nM)		
	IC_{10}	IC_{50}	IC_{90}
HT-1080	5.4	11	24
SW-872	8.2	45	240
SK-LMS-1	620	1070	1860

IC_{10}, IC_{50}, and IC_{90} indicate 10%, 50%, and 90% inhibitory concentrations.

Treatment-related toxicity, as measured by percent body weight loss, was greatest in the gemcitabine + RT group, but remained at an acceptable level (<10% loss in body weight). In this group, the maximum weight loss (8% of initial weight) occurred on day 12 of treatment, and all animals had returned to their starting weight 6 days after the end of treatment (day 25). One animal in the gemcitabine-alone

Table 2. *In Vitro* Radiosensitization of Gemcitabine.

Gemcitabine Dose (nM)	Radiation Enhancement Ratio
HT-1080	
5	1.22 ± 0.06
10	1.73 ± 0.09
20	2.32 ± 0.18
SW-872	
15	1.27 ± 0.11
30	1.55 ± 0.36
60	2.15 ± 0.43
SK-LMS-1	
250	1.06 ± 0.18
500	1.11 ± 0.17
1000	1.72 ± 0.36

treatment group died of unknown causes 5 days after the second dose of gemcitabine.

To improve the understanding of the response to treatment, tumors were harvested from each treatment group on the last day of treatment (Figure 4). Control, gemcitabine-alone, and radiation-alone xenografts remained very mitotically active despite treatment. In contrast, the combined gemcitabine and radiation xenograft was without mitotic figures and demonstrated enlarged cells, elongated pleomorphic nuclei, along with significant fibrotic changes in the surrounding stroma. These histologic findings correspond with the significant growth delay response demonstrated in this treatment group.

Discussion

In this study, gemcitabine was found to be a potent radiosensitizer of STS cell lines. Treatment with gemcitabine under modestly cytotoxic conditions radiosensitized cells *in vitro*. As observed in previous studies, maximal radiosensitization was associated with accumulation of cells in early S-phase [16]. Gemcitabine also proved to be an excellent radiation sensitizer *in vivo* and significantly prolonged the tumor growth delay produced by radiation, which was associated with histologic changes of tumor response. Given the difficulty in attaining local control in large extremity, truncal, and retroperitoneal sarcomas with current therapies, these findings suggest that it may be worthwhile to investigate the role of concurrent gemcitabine and radiation therapy for these more difficult sarcoma cases.

Radiation therapy plays an important role in the treatment of STS. Combined wide local excision and radiation therapy results in equivalent rates of survival compared to radical amputation [23]. Randomized studies confirm that adjuvant radiation therapy improves local control after wide excision in extremity STS [3,5]. Durable local control rates for extremity STS typically exceed 90% [3–5]; however, local control rates are significantly worse for certain locally advanced extremity STS cases and for STS located in unfavorable anatomic locations, such as the retroperitoneum, pelvis, or deep trunk [6–9]. Strategies to improve the therapeutic efficacy of radiation therapy for STS would be particularly valuable for these cases.

Gemcitabine is a deoxycytidine analog with significant systemic activity in a variety of solid tumors, including STS. Several phase II

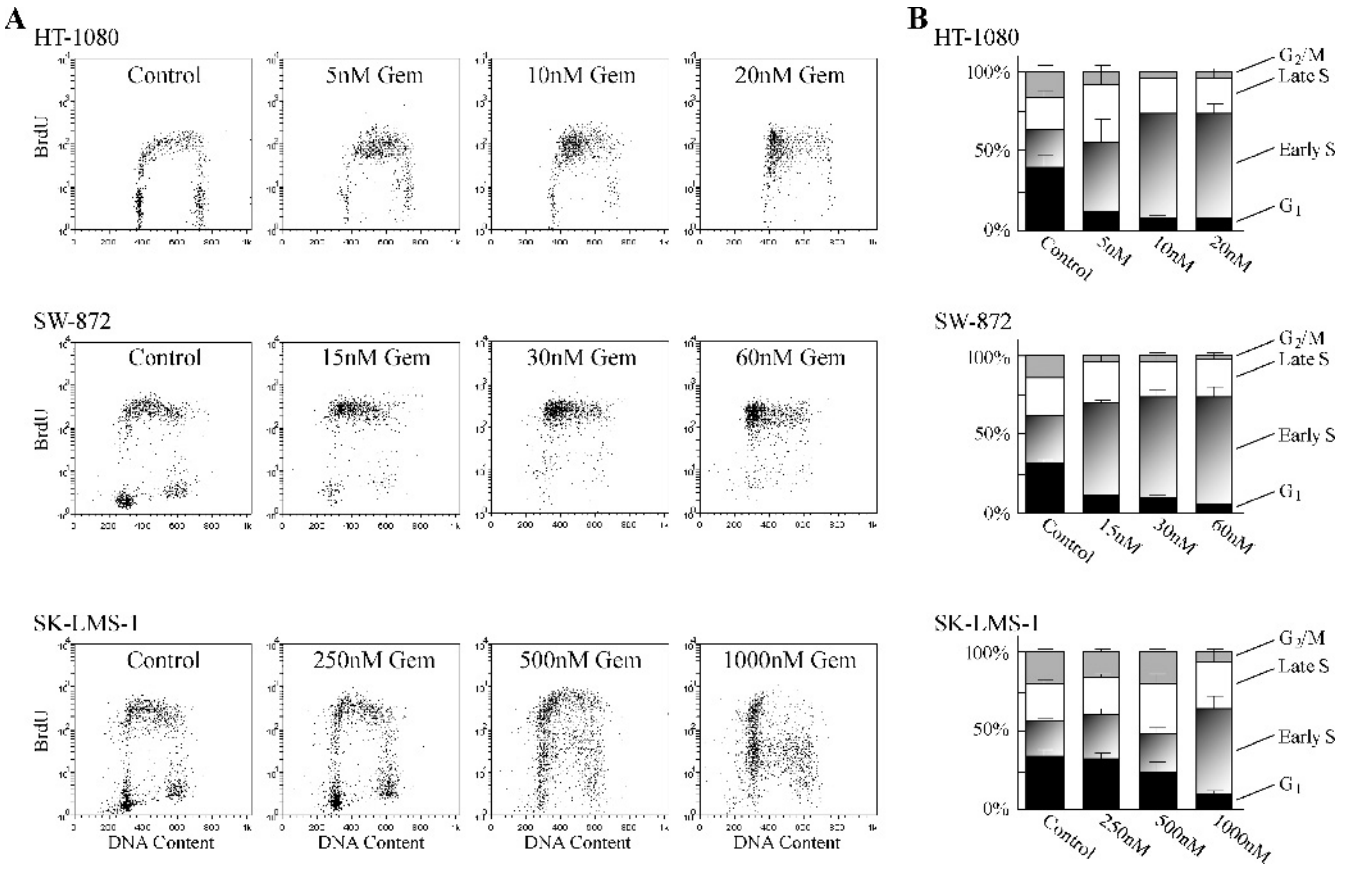

Figure 2. Cell cycle arrest with gemcitabine. Cells were treated with 24 hours of gemcitabine. BrdU incorporation and PI staining were analyzed with flow cytometry: (A) representative data for each cell line; (B) mean ± SEM for at least two independent experiments.

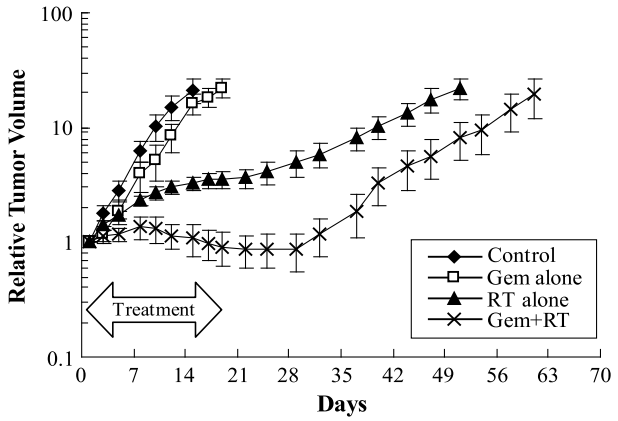

Figure 3. Effect of gemcitabine and radiation on SK-LMS-1 xenograft tumors in nude mice. SK-LMS-1 nude mouse xenografts were randomized to four treatment arms with seven mice per arm. Mice received weekly i.p. gemcitabine (150 mg/kg) on days 1, 8, and 15. Radiation included 2-Gy daily fractions on days 1 to 5, 8 to 12, and 15 to 19, for a total of 30 Gy. Relative volume represents tumor volume compared with day 1 of treatment.

clinical studies with gemcitabine as a single agent have been conducted in patients with advanced STS [24–33]. Response rates (measured as freedom from disease progression) in these studies have ranged from 3% to 53%. Chemotherapy doublets coupling gemcitabine with other agents have recently shown promise in STS. A recently completed large randomized phase II trial comparing gemcitabine to gemcitabine–docetaxel demonstrated superior progression-free survival (6.2 *vs* 3.0 months) and overall survival (17.9 *vs* 11.5 months) in the gemcitabine–docetaxel arm [26]. The role of concurrent gemcitabine–docetaxel with radiation therapy remains to be evaluated.

In addition to its activity as a single agent, gemcitabine is known to be a potent radiosensitizer in a variety of cell types, but the majority of data are in carcinoma models and little data exists for sarcoma. *In vitro*, gemcitabine enhances the cell-killing effects of ionizing radiation with DERs ranging from 1.3 to 1.8 in pancreas, head and neck squamous cell, and breast carcinoma cell lines [10–13]. These preclinical studies provided the foundation for successful clinical trials in pancreas, head and neck, bladder, and breast cancer [18–20,34].

Despite significant preclinical findings and numerous clinical trials, the mechanism by which gemcitabine enhances the effect of radiation is not completely understood. Other antimetabolites, such as 5-bromo-2′deoxyuridine (bromodeoxyuridine) and 5-fluoro-2′-deoxyuridine (fluorodeoxyuridine), are thought to increase cytotoxicity to radiation by increasing the number of radiation-induced double-strand breaks or inhibiting their repair [35,36]. However, studies have demonstrated that neither of these mechanisms factor into gemcitabine radiosensitization [37,38]. Other investigators have looked at the effect of p53 status. Whereas mutant p53 cancer cells often have altered sensitivities to chemotherapy and radiation, gemcitabine-mediated radiosensitization appears to be independent of p53 status [39,40].

Researchers from our institution have recently postulated a novel radiosensitizing mechanism. Previous studies have shown that gemcitabine, in its diphosphorylated form, inhibits ribonucleotide reductase [41], causing a decrease in deoxynucleotide triphosphate pools, particularly in dATP [13]. The decrease in dATP correlates with cells accumulating in early S-phase and radiosensitization [13,38]. Further research has demonstrated that these gemcitabine-induced alterations in deoxynucleotide triphosphate pools correlate with increased rates

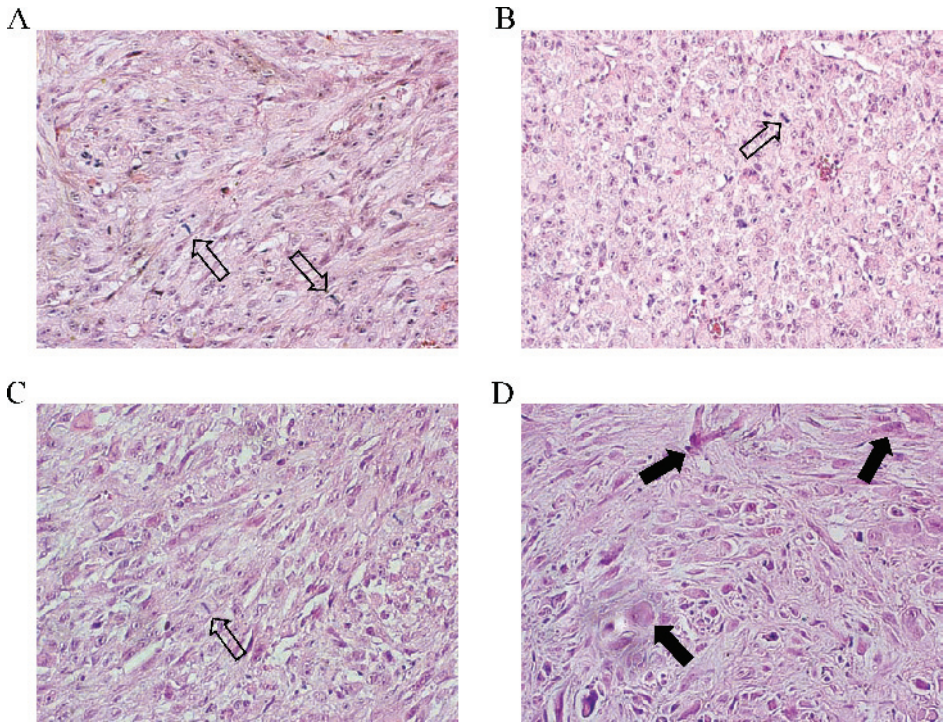

Figure 4. Treatment effect of gemcitabine and/or radiation. On the last day of treatment (day 19), one mouse was sacrificed from each treatment arm. Control (A), gemcitabine-alone (B), RT-alone (C), and gemcitabine + RT (D) tumors were processed for routine hematoxylin–eosin staining. Open arrows indicate mitotic figures, and closed arrows represent pleomorphic nuclei.

of single-base substitutions, and it is thought that these DNA errors contribute to the increased sensitivity to radiation [42]. This mechanism is supported by the finding that cells with deficient mismatch repair both acquire more DNA errors, *and* have increased sensitivity to gemcitabine and radiation compared to cells with functional mismatch repair [42,43].

In this study, we used three cell lines representing some of the most common human STS histologies: fibrosarcoma, leiomyosarcoma, and liposarcoma. These cell lines have significantly different sensitivity to gemcitabine alone, as demonstrated by the wide range of IC_{50} concentrations, yet all demonstrated radiosensitization under moderately cytotoxic doses of gemcitabine. This is also consistent with radiosensitization of other tumor types, where even doses of gemcitabine significantly below the commonly used schedule were still able to increase apparent local tumor control [19]. Taken together, the data reported in this study provide compelling evidence that gemcitabine has similar radiosensitizing activity in sarcoma cells to that observed in carcinoma cells. This provides a valuable preclinical foundation for the clinical application of concurrent radiosensitizing gemcitabine with radiation therapy for STS.

Recently, researchers at M.D. Anderson Cancer Center have reported promising preliminary results of a phase I clinical trial examining preoperative gemcitabine with radiation therapy for STSs of the extremity or trunk [44]. Given the already high rates of local control achieved with conventional surgery and radiation therapy for resectable extremity STSs, this strategy may be most useful for locally advanced or unresectable extremity or trunk sarcomas. However, the most significant local control challenge in STSs is for the disease located within the retroperitoneum or deep trunk [45]. Delivery of gemcitabine with preoperative radiation therapy to retroperitoneal sarcomas may be an attractive strategy to improve local control in these cases. Certainly, there are significant clinical challenges in the delivery of gemcitabine and radiotherapy to large volumes in the abdomen, as was demonstrated in early trials of concurrent gemcitabine and radiation therapy for pancreatic cancer [46]. However, a number of studies have indicated that by using conformal radiation techniques with small treatment margins thus minimizing the volume of stomach, duodenum, and small bowel irradiated, it is safe and feasible to treat abdominal malignancies with concurrent gemcitabine and radiation therapy [17,18,47]. We are currently developing a phase I clinical protocol to determine the maximal dose of gemcitabine that can be safely administered with concurrent preoperative radiation therapy for resectable retroperitoneal and deep truncal STSs.

Acknowledgments

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References

[1] Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, and Thun MJ (2006). Cancer statistics, 2006. *CA Cancer J Clin* **56**, 106–130.

[2] Lawrence W Jr, Donegan WL, Natarajan N, Mettlin C, Beart R, and Winchester D (1987). Adult soft tissue sarcomas. A pattern of care survey of the American College of Surgeons. *Ann Surg* **205**, 349–359.

[3] Pisters PW, Harrison LB, Leung DH, Woodruff JM, Casper ES, and Brennan MF (1996). Long-term results of a prospective randomized trial of adjuvant brachytherapy in soft tissue sarcoma. *J Clin Oncol* **14**, 859–868.

[4] Potter DA, Kinsella T, Glatstein E, Wesley R, White DE, Seipp CA, Chang AE, Lack EE, Costa J, and Rosenberg SA (1986). High-grade soft tissue sarcomas of the extremities. *Cancer* **58**, 190–205.

[5] Yang JC, Chang AE, Baker AR, Sindelar WF, Danforth DN, Topalian SL, DeLaney T, Glatstein E, Steinberg SM, Merino MJ, et al. (1998). Randomized prospective study of the benefit of adjuvant radiation therapy in the treatment of soft tissue sarcomas of the extremity. *J Clin Oncol* **16**, 197–203.

[6] Catton CN, O'Sullivan B, Kotwall C, Cummings B, Hao Y, and Fornasier V (1994). Outcome and prognosis in retroperitoneal soft tissue sarcoma. *Int J Radiat Oncol Biol Phys* **29**, 1005–1010.

[7] Kilkenny JW III, Bland KI, and Copeland EM III (1996). Retroperitoneal sarcoma: the University of Florida experience. *J Am Coll Surg* **182**, 329–339.

[8] Stoeckle E, Coindre JM, Bonvalot S, Kantor G, Terrier P, Bonichon F, and Nguyen Bui B (2001). Prognostic factors in retroperitoneal sarcoma: a multivariate analysis of a series of 165 patients of the French Cancer Center Federation Sarcoma Group. *Cancer* **92**, 359–368.

[9] Youssef E, Fontanesi J, Mott M, Kraut M, Lucas D, Mekhael H, and Ben-Josef E (2002). Long-term outcome of combined modality therapy in retroperitoneal and deep-trunk soft-tissue sarcoma: analysis of prognostic factors. *Int J Radiat Oncol Biol Phys* **54**, 514–519.

[10] Lawrence TS, Chang EY, Hahn TM, Hertel LW, and Shewach DS (1996). Radiosensitization of pancreatic cancer cells by 2′,2′-difluoro-2′-deoxycytidine. *Int J Radiat Oncol Biol Phys* **34**, 867–872.

[11] Lawrence TS, Davis MA, Hough A, and Rehemtulla A (2001). The role of apoptosis in 2′,2′-difluoro-2′-deoxycytidine (gemcitabine)–mediated radiosensitization. *Clin Cancer Res* **7**, 314–319.

[12] Lawrence TS, Eisbruch A, and Shewach DS (1997). Gemcitabine-mediated radiosensitization. *Semin Oncol* **24**, S7-24–S7-28.

[13] Shewach DS, Hahn TM, Chang E, Hertel LW, and Lawrence TS (1994). Metabolism of 2′,2′-difluoro-2′-deoxycytidine and radiation sensitization of human colon carcinoma cells. *Cancer Res* **54**, 3218–3223.

[14] Huang P, Chubb S, Hertel LW, Grindey GB, and Plunkett W (1991). Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res* **51**, 6110–6117.

[15] Ruiz van Haperen VW, Veerman G, Vermorken JB, and Peters GJ (1993). 2′,2′-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. *Biochem Pharmacol* **46**, 762–766.

[16] Ostruszka LJ and Shewach DS (2000). The role of cell cycle progression in radiosensitization by 2′,2′-difluoro-2′-deoxycytidine. *Cancer Res* **60**, 6080–6088.

[17] McGinn CJ, Talamonti MS, Small W, Freedman GM, Berlin J, Kinsella TJ, Philip PA, Zalupski MM, Mulcahy MF, Meropol NJ, et al. (2004). A phase II trial of full-dose gemcitabine with concurrent radiation therapy in patients with resectable or unresectable non-metastatic pancreatic cancer [Abstract 96]. *ASCO Program/Proceedings Gastrointestinal Cancers Symposium*, January 22–24, 2004; San Francisco, California.

[18] McGinn CJ, Zalupski MM, Shureiqi I, Robertson JM, Eckhauser FE, Smith DC, Brown D, Hejna G, Strawderman M, Normolle D, et al. (2001). Phase I trial of radiation dose escalation with concurrent weekly full-dose gemcitabine in patients with advanced pancreatic cancer. *J Clin Oncol* **19**, 4202–4208.

[19] Eisbruch A, Shewach DS, Bradford CR, Littles JF, Teknos TN, Chepeha DB, Marentette LJ, Terrell JE, Hogikyan ND, Dawson LA, et al. (2001). Radiation concurrent with gemcitabine for locally advanced head and neck cancer: a phase I trial and intracellular drug incorporation study. *J Clin Oncol* **19**, 792–799.

[20] Suh WW, Schott AF, Hayman JA, Schipper MJ, Shewach DS, and Pierce LJ (2004). A phase I dose escalation trial of gemcitabine with radiotherapy for breast cancer in the treatment of unresectable chest wall recurrences. *Breast J* **10**, 204–210.

[21] Fertil B, Dertinger H, Courdi A, and Malaise EP (1984). Mean inactivation dose: a useful concept for intercomparison of human cell survival curves. *Radiat Res* **99**, 73–84.

[22] Hoy CA, Seamer LC, and Schimke RT (1989). Thermal denaturation of DNA for immunochemical staining of incorporated bromodeoxyuridine (BrdUrd): critical factors that affect the amount of fluorescence and the shape of BrdUrd/DNA histogram. *Cytometry* **10**, 718–725.

[23] Rosenberg SA, Tepper J, Glatstein E, Costa J, Baker A, Brennan M, DeMoss EV, Seipp C, Sindelar WF, Sugarbaker P, et al. (1982). The treatment of soft-tissue sarcomas of the extremities: prospective randomized evaluations of (1) limb-sparing surgery plus radiation therapy compared with amputation and (2) the role of adjuvant chemotherapy. *Ann Surg* **196**, 305–315.

[24] Hartmann JT, Oechsle K, Huober J, Jakob A, Azemar M, Horger M, Kanz L, and Bokemeyer C (2006). An open label, non-comparative phase II study of gemcitabine as salvage treatment for patients with pretreated adult type soft tissue sarcoma. *Invest New Drugs* **24**, 249–253.

[25] Look KY, Sandler A, Blessing JA, Lucci JA III, and Rose PG (2004). Phase II trial of gemcitabine as second-line chemotherapy of uterine leiomyosarcoma: a Gynecologic Oncology Group (GOG) Study. *Gynecol Oncol* **92**, 644–647.

[26] Maki RG, Wathen JK, Patel SR, Priebat DA, Okuno SH, Samuels B, Fanucchi M, Harmon DC, Schuetze SM, Reinke D, et al. (2007). Randomized phase II study of gemcitabine and docetaxel compared with gemcitabine alone in patients with metastatic soft tissue sarcomas: results of sarcoma alliance for research through collaboration study 002 [corrected]. *J Clin Oncol* **25**, 2755–2763.

[27] Merimsky O, Meller I, Flusser G, Kollender Y, Issakov J, Weil-Ben-Arush M, Fenig E, Neuman G, Sapir D, Ariad S, et al. (2000). Gemcitabine in soft tissue or bone sarcoma resistant to standard chemotherapy: a phase II study. *Cancer Chemother Pharmacol* **45**, 177–181.

[28] Okuno S, Edmonson J, Mahoney M, Buckner JC, Frytak S, and Galanis E (2002). Phase II trial of gemcitabine in advanced sarcomas. *Cancer* **94**, 3225–3229.

[29] Okuno S, Ryan LM, Edmonson JH, Priebat DA, and Blum RH (2003). Phase II trial of gemcitabine in patients with advanced sarcomas (E1797): a trial of the Eastern Cooperative Oncology Group. *Cancer* **97**, 1969–1973.

[30] Patel SR, Gandhi V, Jenkins J, Papadopolous N, Burgess MA, Plager C, Plunkett W, and Benjamin RS (2001). Phase II clinical investigation of gemcitabine in advanced soft tissue sarcomas and window evaluation of dose rate on gemcitabine triphosphate accumulation. *J Clin Oncol* **19**, 3483–3489.

[31] Spath-Schwalbe E, Genvresse I, Koschuth A, Dietzmann A, Grunewald R, and Possinger K (2000). Phase II trial of gemcitabine in patients with pretreated advanced soft tissue sarcomas. *Anticancer Drugs* **11**, 325–329.

[32] Svancarova L, Blay JY, Judson IR, van Hoesel QG, van Oosterom AT, le Cesne A, Keizer HJ, Hermans C, van Glabbeke M, Verweij J, et al. (2002). Gemcitabine in advanced adult soft-tissue sarcomas. A phase II study of the EORTC Soft Tissue and Bone Sarcoma Group. *Eur J Cancer* **38**, 556–559.

[33] Von Burton G, Rankin C, Zalupski MM, Mills GM, Borden EC, and Karen A (2006). Phase II trial of gemcitabine as first line chemotherapy in patients with metastatic or unresectable soft tissue sarcoma. *Am J Clin Oncol* **29**, 59–61.

[34] Kent E, Sandler H, Montie J, Lee C, Herman J, Esper P, Fardig J, and Smith DC (2004). Combined-modality therapy with gemcitabine and radiotherapy as a bladder preservation strategy: results of a phase I trial. *J Clin Oncol* **22**, 2540–2545.

[35] Bruso CE, Shewach DS, and Lawrence TS (1990). Fluorodeoxyuridine-induced radiosensitization and inhibition of DNA double strand break repair in human colon cancer cells. *Int J Radiat Oncol Biol Phys* **19**, 1411–1417.

[36] Iliakis G, Kurtzman S, Pantelias G, and Okayasu R (1989). Mechanism of radiosensitization by halogenated pyrimidines: effect of BrdU on radiation induction of DNA and chromosome damage and its correlation with cell killing. *Radiat Res* **119**, 286–304.

[37] Gregoire V, Beauduin M, Bruniaux M, De Coster B, Octave Prignot M, and Scalliet P (1998). Radiosensitization of mouse sarcoma cells by fludarabine (F-ara-A) or gemcitabine (dFdC), two nucleoside analogues, is not mediated by an increased induction or a repair inhibition of DNA double-strand breaks as measured by pulsed-field gel electrophoresis. *Int J Radiat Biol* **73**, 511–520.

[38] Lawrence TS, Chang EY, Hahn TM, and Shewach DS (1997). Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2′,2′-difluoro-2′-deoxycytidine (gemcitabine). *Clin Cancer Res* **3**, 777–782.

[39] Chen M, Hough AM, and Lawrence TS (2000). The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization. *Cancer Chemother Pharmacol* **45**, 369–374.

[40] Pauwels B, Korst AE, Andriessen V, Baay MF, Pattyn GG, Lambrechts HA, Pooter CM, Lardon F, and Vermorken JB (2005). Unraveling the mechanism of radiosensitization by gemcitabine: the role of TP53. *Radiat Res* **164**, 642–650.

[41] Baker CH, Banzon J, Bollinger JM, Stubbe J, Samano V, Robins MJ, Lippert B, Jarvi E, and Resvick R (1991). 2′-Deoxy-2′-methylenecytidine and 2′-deoxy-2′,2′-difluorocytidine 5′-diphosphates: potent mechanism-based inhibitors of ribonucleotide reductase. *J Med Chem* **34**, 1879–1884.

[42] Flanagan SA, Robinson BW, Krokosky CM, and Shewach DS (2007). Mismatched nucleotides as the lesions responsible for radiosensitization with gemcitabine: a new paradigm for antimetabolite radiosensitizers. *Mol Cancer Ther* **6**, 1858–1868.

[43] Robinson BW, Im MM, Ljungman M, Praz F, and Shewach DS (2003). Enhanced radiosensitization with gemcitabine in mismatch repair–deficient HCT116 cells. *Cancer Res* **63**, 6935–6941.

[44] Pisters PW, Ballo MT, Bekele PF, Feig P, Lin JN, Cormier RS, and Benjamin SR (2004). Phase I trial using toxicity severity weights for dose finding of gemcitabine combined with radiation therapy and subsequent surgery for patients with extremity and trunk soft tissue sarcomas. *J Clin Oncol* **22**(14S), 9008.

[45] Feng M, Murphy JD, Griffith KA, Baker LH, Sondak VK, Lucas DR, McGinn CJ, and Ray ME (2007). Long-term outcomes after radiation therapy for retroperitoneal and deep truncal sarcoma. *Int J Radiat Oncol Biol Phys* **69**, 103–110.

[46] Crane CH, Wolff RA, Abbruzzese JL, Evans DB, Milas L, Mason K, Charnsangavej C, Pisters PW, Lee JE, Lenzi R, et al. (2001). Combining gemcitabine with radiation in pancreatic cancer: understanding important variables influencing the therapeutic index. *Semin Oncol* **28**, 25–33.

[47] Murphy JD, Adusumilli S, Griffith KA, Ray ME, Zalupski MM, Lawrence TS, and Ben-Josef E (2007). Full-dose gemcitabine and concurrent radiotherapy for unresectable pancreatic cancer. *Int J Radiat Oncol Biol Phys* **68**, 801–808.