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Redox active metals and H_2O_2 mediate the increased efficacy of pharmacological ascorbate in combination with gemcitabine or radiation in pre-clinical sarcoma models☆

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ABSTRACT

Soft tissue sarcomas are a histologically heterogeneous group of rare mesenchymal cancers for which treatment options leading to increased overall survival have not improved in over two decades. The current study shows that pharmacological ascorbate (systemic high dose vitamin C achieving ≥ 20 mM plasma levels) is a potentially efficacious and easily integrable addition to current standard of care treatment strategies in preclinical models of fibrosarcoma and liposarcoma both *in vitro* and *in vivo*. Furthermore, enhanced ascorbate-mediated toxicity and DNA damage in these sarcoma models were found to be dependent upon H_2O_2 and intracellular labile iron. Together, these data support the hypothesis that pharmacological ascorbate may represent an easily implementable and non-toxic addition to conventional sarcoma therapies based on taking advantage of fundamental differences in cancer cell oxidative metabolism.

1. Introduction

Soft tissue sarcomas are a rare group of heterogeneous cancers of mesenchymal origin (> 150 histological subtypes) that represent < 1% of all newly diagnosed cancers in the United States [1,2]. Similar to most solid tumors, sarcoma treatment is dependent upon the stage of the disease. Patients presenting with loco-regional disease frequently receive neoadjuvant radiation therapy followed by definitive surgical resection. Sarcoma patients presenting with metastatic disease typically receive chemotherapy regimens, such as gemcitabine. Gemcitabine is a nucleoside metabolic inhibitor that damages cells actively undergoing DNA synthesis and blocking the progression of cells through the G_1/S-phase boundary as well as inhibiting DNA repair [3]. Gemcitabine has demonstrated anti-tumor effects against a variety of malignancies and has activity as a single agent in soft tissue sarcomas as well as bone sarcomas [4–7].

Despite the development and clinical utilization of new targeted chemotherapeutic agents, improved radiation targeting and normal tissue sparing approaches, as well as surgical techniques, only minimal increases in sarcoma patient overall survival have been demonstrated in the last two decades [8]. There remains a great need for more effective and non-toxic therapeutic approaches designed to enhance locoregional disease control as well as overall survival in sarcoma patients treated with radio-chemotherapy.

Pharmacological ascorbate, high dose intravenous vitamin C resulting in plasma levels ≥ 20 mM, has recently re-emerged as a potential non-toxic adjuvant to standard of care radio-chemotherapy-based cancer therapies in a variety of disease sites [9–12]. The anti-cancer action of pharmacological ascorbate has been proposed to involve the redox-active metal-mediated or auto-oxidation of ascorbate leading to the formation of $O_2^{\bullet-}$ and H_2O_2 [12–15]. Furthermore, pre-clinical and clinical studies in non-small cell lung cancer (NSCLC), pancreatic ductal adenocarcinoma, ovarian cancer, and glioblastoma multiforme (GBM) have demonstrated that ascorbate selectively

Abbreviations: $AscH^-$, ascorbate monoanion (dominant form at biological pH); LIP, labile iron pool; MFI, mean fluorescence intensity; PO-1, PeroxyOrange-1; γH2Ax, phosphorylated histone 2Ax

☆ One Sentence Summary: Pharmacological ascorbate in combination with chemotherapy or radiation increases local control in pre-clinical sarcoma models.

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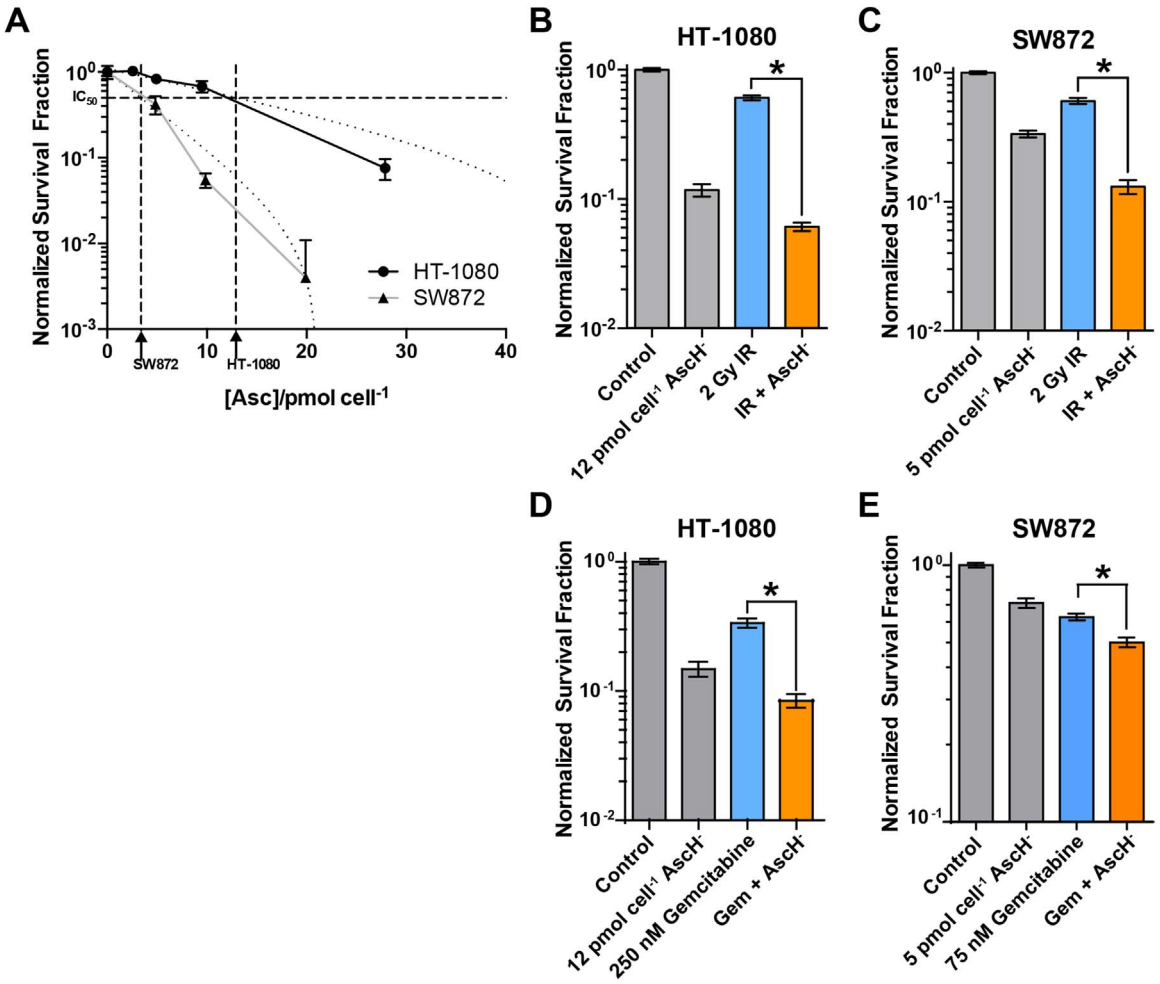

Fig. 1. AscH⁻ sensitizes sarcoma cells to radiation and gemcitabine *in vitro*. (A) Clonogenic survival of HT-1080 and SW872 sarcoma cells after exposure to increasing concentrations of AscH⁻ for 1 h. **(B-C)** Clonogenic survival of (B) HT-1080 and (C) SW872 cells after exposure to AscH⁻ for 1 h followed by 2 Gy IR. **(D-E)** Clonogenic survival of (D) HT-1080 and (E) SW872 cells after exposure to gemcitabine (IC50) for 3 h prior and 1 h during exposure to AscH⁻. For all, $n \geq 3$ biological replicates with $n \geq 6$ technical replicates per sample. Data are as mean represented ± SEM. * represents significant difference, at least $p < 0.05$.

sensitizes cancer cells, as compared to normal cells, to radiation and chemotherapy by a mechanism involving redox active metal ions but the generality of these findings to sarcomas is not known [9–12,16,17].

The current study shows that pharmacological ascorbate (systemic high dose vitamin C achieving ≥ 20 mM plasma levels) is an easily integrable and potentially efficacious addition to current standard of care treatment strategies in preclinical models of fibrosarcoma and liposarcoma both *in vitro* and *in vivo*. Furthermore, enhanced ascorbate-mediated toxicity and DNA damage in these sarcoma models was found to be dependent upon H_2O_2 and intracellular labile iron pools. Together, these data support the hypothesis that pharmacological ascorbate may represent an easily implementable and non-toxic adjuvant to conventional sarcoma therapies based on taking advantage of fundamental differences in cancer cell oxidative metabolism.

2. Materials and methods

2.1. Chemicals and reagents

Unless noted, reagents were obtained from Sigma-Aldrich (St. Louis, MO). L-Ascorbic acid was purchased from Macron Chemicals (Center Valley, PA). Gemcitabine HCl was purchased from Hospira, Inc. (Lake Forest, IL). Ketamine was purchased from Mylan Institutional (Galway, Ireland) and xylazine was purchased from Lloyd Laboratories (Shenandoah, IA).

2.2. Ascorbate and ascorbate exposure

L-Ascorbic acid stock solution (approx. 1 M) made in Nanopure® Type 1 water (18 MΩ) with the pH adjusted to 7.0 with 1 M NaOH was stored in sealed glass tubes with minimal head space. The precise concentration was confirmed spectrophotometrically as previously described (265 nm, $\varepsilon = 14.5\ mM^{-1}\ cm^{-1}$) [18]. For all experiments, ascorbate is dosed per cell in identical media preparations due to previous literature demonstrating that H_2O_2 and ascorbate toxicity is dependent on these metrics (*i.e.* cell density, pH, serum, pyruvate and other α-ketoacids, metal ions, *etc.*) [12,15,18–24].

2.3. Cell culture

Sarcoma cell lines HT-1080 (fibrosarcoma) and SW872 (liposarcoma) were obtained from and authenticated by the American Type Culture Collection (ATCC). Cells were grown in MEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (HyClone). All cells were incubated at 21% O_2 in a humidity controlled environment (37 °C, 5% CO_2; Forma Scientific). All cell lines were utilized before passage 20.

2.4. Ionizing radiation

Ionizing radiation (IR) was delivered in the radiation facility at The University of Iowa using a Pantak Therapx DXT 300 X-ray machine

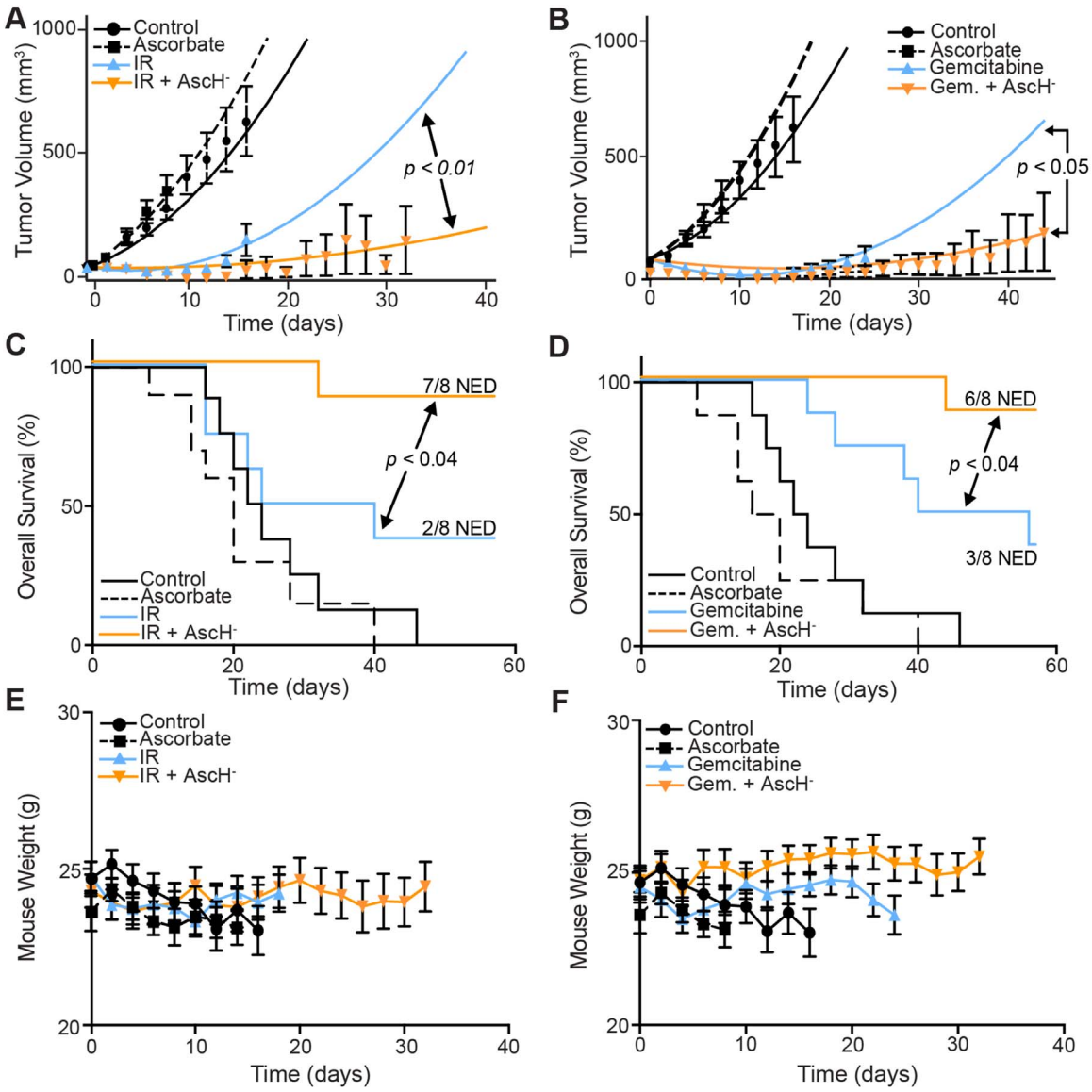

Fig. 2. Pharmacological ascorbate selectively sensitizes fibrosarcoma tumors to radiation or gemcitabine in an orthotopic murine xenograft model. An orthotopic fibrosarcoma model was utilized by injecting 1×10^6 HT-1080 cells into the dermis of the right rear flank of athymic nude female mice. Once tumors were established, therapy was initiated with daily ascorbate (4 g kg^{-1} or sodium chloride control; IP) in combination with either 12 Gy IR in 2 fractions (days 2 and 4) or two cycles of gemcitabine (60 mg kg^{-1} or equivalent dose PBS, IP; days 1 and 4). (A, B) Tumor volume measurements (points ± error) fitted with a logistical regression (line) for mice treated with daily ascorbate in combination with (A) radiation or (B) gemcitabine. (C, D) Overall survival in mice treated with ascorbate in combination with (C) radiation or (D) gemcitabine. Mice were sacrificed when any tumor dimension reached 1.5 cm. NED = no evidence of macroscopic disease at end of experiment. (E, F) Mouse weight in mice treated with ascorbate in combination with (E) radiation or (F) gemcitabine. For each treatment group, $n = 8$ mice.

operated at 200 kVp with added filtration of 0.35 mm Cu + 1.5 mm Al, resulting in a beam quality of 0.95 mm Cu. For *in vitro* studies, cells were irradiated in 60 mm cell culture dishes. For *in vivo* murine xenograft studies, mice were anesthetized using an 87.5 mg kg^{-1} ketamine and 12.5 mg kg^{-1} xylazine mixture and placed in lead boxes with only their right flank exposed to irradiate the sarcoma xenograft.

2.5. Clonogenic survival assays

Cells ($1–2 \times 10^5$) cells were plated in 60 mm cell culture dishes and grown in their respective media for 48 h before exposure to experimental conditions. For ascorbate alone experiments, cells were given fresh media, treated with ascorbate for 1 h at 37 °C, immediately trypsinized, and plated for clonogenic survival at room temperature (RT). For gemcitabine experiments, the cells were given fresh media with gemcitabine for 3 h at 37 °C, prior to the addition of ascorbate for 1 h at 37 °C, followed by trypsinization and clonogenic survival assay. For radiation experiments, fresh media was added for 3 h at 37 °C prior to the addition of ascorbate for 1 h at 37 °C, and then exposure to 2 Gy ionizing radiation at RT, followed by clonogenic survival assay. For catalase experiments, 150 mkU mL^{-1} bovine catalase was added to the media immediately prior to ascorbate exposure. For chelation studies, cells were treated with 250 μM desferrioxamine (DFO) for 3 h prior to and for 1 h during ascorbate exposure.

After exposure, cells were washed with PBS and clonogenic assays were initiated with fresh full respective media. Briefly, floating and attached cells were collected and total cells per plate were counted. An experimentally derived number of cells were plated into each well of a 6-well cell culture plate in 4 mL of media. After sufficient time (7–14 days, cell type-dependent), cells were fixed in 70% ethanol and stained with a Brilliant Blue methanol solution. Cell colonies containing greater than 50 cells were counted and utilized to calculate plating efficiency

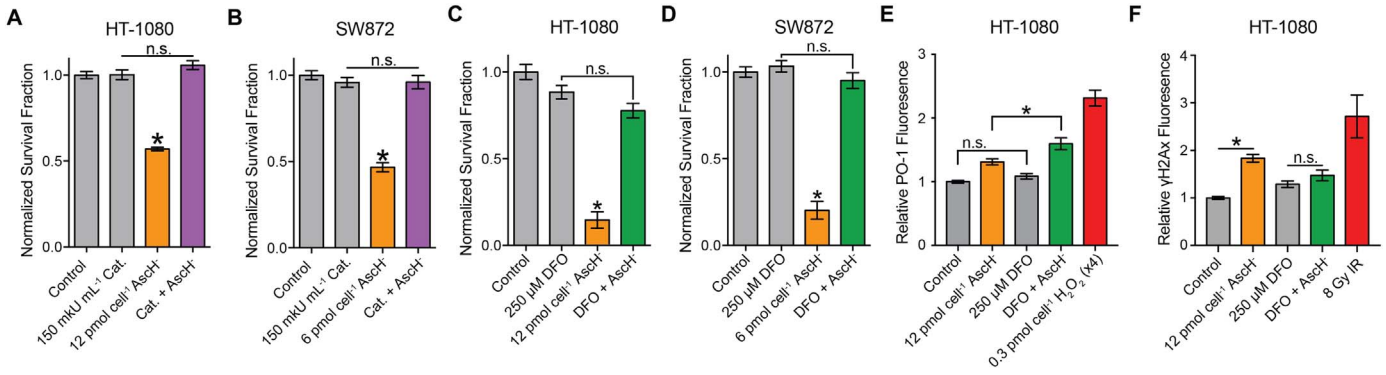

Fig. 3. Redox-active iron mediates ascorbate toxicity through pro-oxidant Fenton-chemistry *in vitro*. **(A, B)** Clonogenic survival of (A) HT-1080 or (B) SW872 cells exposed to ascorbate in the presence of 150 mkU mL^{-1} exogenous bovine catalase. **(C-F)** HT-1080 and SW872 cells were pre-chelated with 250 μM desferrioxamine (DFO) for 3 h prior to and for 1 h during ascorbate exposure. (C) HT-1080 and (D) SW872 cells were then assayed for clonogenic cell survival, (E) relative oxidation of intracellular H_2O_2-sensitive fluorescent probe PO-1 in HT-1080 cells as quantified by flow cytometry [bolus addition of H_2O_2 (0.3 pmol cell^{-1}) delivered every 30 min was used as a positive control], and (F) relative levels of γH2Ax as quantified by flow cytometry [8 Gy ionizing radiation was utilized as a positive control]. For all, $n \geq 3$ biological replicates with $n \geq 6$ technical replicates per sample. N.S. = not significant. * represents significant differences by ANOVA, $p < 0.05$.

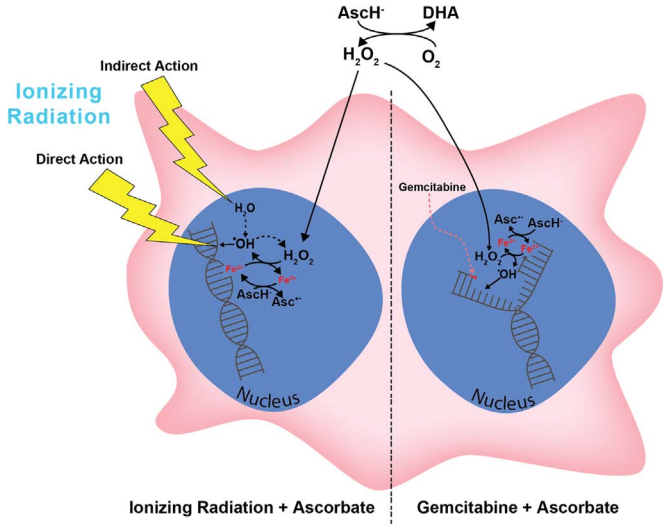

Fig. 4. Model of pharmacological ascorbate enhancing susceptibility of sarcoma cells to radiation or genotoxic chemotherapy *via* increased DNA damage. Ascorbate auto-oxidation, either extracellular or intracellular, generates H_2O_2 that can freely diffuse into sarcoma cells to increase cellular DNA damage in combination with radiation- or gemcitabine-mediated DNA damage. (Left) Ionizing radiation causes DNA damage through both direct oxidation of DNA macromolecules (direct effect) or through initiation of free radical mechanism resulting from the radiolysis of water (indirect effect; multistep process represented by dotted line). The resulting H_2O_2 from the radiolysis of water and the oxidation of ascorbate can react with redox-active labile iron weakly chelated by DNA to produce site-directed DNA damage *via* Fenton-chemistry catalyzed hydroxyl radical (˙OH) production or *via* ˙OH produced directly through the radiolysis of water. (Right) Gemcitabine induces DNA damage through a variety of proposed mechanisms, including, but not limited to the major mechanism as a nucleoside analog, stalling DNA replication after incorporation into a new DNA strand (red), and inhibiting ribonucleotide reductase. Combination of gemcitabine- and ascorbate-mediated DNA damages increase sarcoma cell killing. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for each treatment group. Normalized survival fractions were calculated by comparing plating efficiencies of each treatment group against the control group within a given experiment.

2.6. Murine xenograft models

Female 6–8-week-old female athymic-nu/nu mice were purchased from Envigo (previously Harlan Laboratories) and housed in the Animal Care Facility at The University of Iowa (Iowa City, IA). All procedures were approved by the University of Iowa Institutional Animal Care and Use Committee and conformed to NIH guidelines. HT-1080 cells (1×10^6) were injected subcutaneously into the right rear flank. Once tumors were established, treatment was initiated with daily ascorbate (4 g kg^{-1} or equivalent dose of NaCl, intraperitoneal [IP]), gemcitabine on days 1 and 4 (60 mg kg^{-1} or equivalent dose PBS, IP) and/or IR on days 2 and 4 (12 Gy/ 2 frx). Ascorbate/NaCl was continued for the full extent of the study. Tumors were measured every other day with Vernier calipers (volume = (length × width × (width/2))) and mice were euthanized and sacrificed when tumor length exceeded 1.5 cm in any dimension.

2.7. Quantification of intracellular H_2O_2 with PeroxyOrange-1

To visualize intracellular H_2O_2 levels, the selectively sensitive fluorescent probe PeroxyOrange-1 was utilized as previously described with modifications [25]. HT-1080 cells (1×10^6) were plated and grown in their respective media for 48 h. The complete protocol was conducted in the dark with minimal ambient lighting. Cells were washed with PBS and incubated with 10 μM PO-1 in phenol red-free serum-free MEM for 1 h at 37 °C. Cells were washed and placed back in phenol red-free MEM + 10% FBS. At that time, exposure to ascorbate or 100 μM H_2O_2 (every 30 min as a positive control) was initiated and cells were placed back at 37 °C for 2 h. Cells were then placed on ice for the remainder of the protocol, washed, and trypsinized with phenol red-free 0.5% trypsin. After 15 min, cells still attached were scraped and all cells were collected in 15 mL conical vials containing cold PBS + 10% FBS, centrifuged at 4 °C, and resuspended in cold PBS prior to analysis on a LSR II Flow Cytometer (BD Biosciences) with λ_{ex} = 561 nm, λ_{em} = 585/20 nm. The mean fluorescence intensity (MFI) was analyzed (FlowJo™) and corrected for auto-fluorescence against unlabeled cells. Normalized MFI was calculated by comparing MFI for a given treatment group against control.

2.8. γH2Ax DNA damage

One hour after specified exposures, sarcoma cells were trypsinized, washed twice in PBS, and fixed in 70% at 4 °C overnight. Cells were resuspended in cold PBST (PBS-tween) to rehydrate for 10 min on ice, centrifuged at 4 °C and resuspended at 4 °C overnight in Rabbit α-γH2AX polyclonal antibody at 1:800 in PBST (Cell Signaling Technology; Beverly, MA; Cat # 2577). Cells were washed with 2% FBS in PBS and resuspended in a FITC-conjugated goat α-mouse secondary antibody at 1:200 in PBST for 1 h at ambient room temperature. Cells were then rinsed with PBS, centrifuged at 4 °C, resuspended in fresh PBS and analyzed by flow cytometry (LSR II Flow Cytometer (BD

Biosciences)). The mean fluorescence intensity (MFI) of 10,000 cells was analyzed per sample and corrected for auto fluorescence from unlabeled cells and secondary antibody-only stained cells. Normalized MFI was calculated by comparing MFI for a given treatment group against control.

2.9. Statistical analysis

Data are expressed as mean ± 1 S.E.M., unless otherwise specified. All analyses were 2-sided and assessed for significance at $p < 0.05$. All analyses, unless specified elsewhere, were performed in GraphPad Prism® (GraphPad Software, Inc.). For analyses limited to two groups, Student's *t*-test was utilized. To study differences between three or more groups, one-way ANOVA analysis with Tukey's post hoc test was used. Regression analysis was utilized to model xenograft tumor growth as a non-linear function of follow-up time in order to make comparisons between treatment groups. Actual tumor volume means and standard errors are superimposed on the modeled curves up until the first mouse in the respective group was sacrificed. For survival analyses, the log-rank Mantel-Cox test was used and χ^2-test was utilized to determine any significant difference in mice with no evidence of disease at the end of the xenograft studies.

3. Results and discussion

3.1. Pharmacological ascorbate sensitizes sarcoma cell lines to chemotherapy or radiation in vitro

The *in vitro* sensitivity of sarcoma cells to pharmacological doses of ascorbate was assessed by clonogenic cell survival assays. Exposure to increasing doses of ascorbate demonstrates a dose-dependent increase in clonogenic cell death in both HT-1080 fibrosarcoma (IC_{50} = 12.9 pmol $cell^{-1}$) and SW872 synovial sarcoma (IC_{50} = 3.4 pmol $cell^{-1}$) cell lines (Fig. 1**A**). To investigate pharmacological doses of ascorbate as a potential anti-cancer therapy in combination with standard-of-care therapy modalities, two pre-clinical models were utilized: radiation ± ascorbate as a model of locoregional disease therapy and gemcitabine ± ascorbate as a model of metastatic disease therapy. Ascorbate significantly sensitized HT-1080 and SW872 cells to both radiation (2 Gy/ 1 frx) and gemcitabine (IC_{50} = 250 nM for HT-1080 or 75 nM for SW872) as measured by clonogenic cell killing (Fig. 1**B-E**). These *in vitro* results suggest that adjuvant pharmacological ascorbate may increase sarcoma cell susceptibility to traditional radiation or chemotherapy therapies.

3.2. Pharmacological ascorbate sensitizes murine sarcoma xenografts to chemotherapy or radiation in vivo

To investigate whether the *in vitro* results would translate to an *in vivo* model, an orthotopic murine sarcoma xenograft models system was developed with HT-1080 cells. HT-1080 cells were utilized due to their relative resistance to ascorbate *in vitro* as compared to SW872 cells (Fig. 1**A**). For this model, 1×10^6 exponentially growing HT-1080 cells were introduced by injection into the right rear flank of female athymic nu/nu mice. Once tumors were established, therapy studies were initiated with daily intraperitoneal (IP) administration of ascorbate (4 g kg^{-1} or equivalent dose of NaCl), gemcitabine on days 1 and 4 (60 mg kg^{-1} or equivalent dose PBS, IP) and/or IR on days 2 and 4 (12 Gy in 2 frx). The combination of pharmacological ascorbate with gemcitabine or IR significantly inhibited tumor growth and increased mouse overall survival as compared to chemotherapy or radiation alone (Fig. 2**A-D;** $p < 0.05$). Furthermore, the addition of ascorbate to radiation significantly increased the number of mice with no evidence of macroscopic disease at the end of the study (NED; Fig. 2**C;** $p = 0.01$). Importantly, as seen in pre-clinical and clinical investigations in a variety of disease sites [11,12,16,17], the addition of ascorbate at pharmacological doses did not increase treatment toxicity, as measured broadly by overall mouse weight, demonstrating the cancer cell selective toxicity of pharmacological ascorbate *in vivo* (Fig. 2**E, F**).

3.3. Pharmacological ascorbate enhances therapy-induced DNA damage in a redox-active metal ion dependent mechanism

It has been proposed that the primary mechanism of ascorbate-mediated toxicity is dependent upon the metal-ion catalyzed- or auto-oxidation of ascorbate to produce H_2O_2 [11,13,15,26]. Consistent with this mechanism, exposure of sarcoma cells to ascorbate in the presence of exogenous bovine catalase completely abolished ascorbate-mediated toxicity (Fig. 3**A, B**). However, H_2O_2 mediated pro-oxidant chemistry is thought to be dependent on redox-active metal ions, such as the Fe^{3+}/Fe^{2+} couple, to initiate oxidative DNA damage and induce cell death [12,27,28]. Confirming the role of redox-active iron in the mechanism of ascorbate-mediated toxicity in sarcoma cells, chelation of intracellular iron by desferrioxamine (DFO), an iron chelator that inhibits iron redox activity [18,29], also abolished ascorbate toxicity (Fig. 3**C, D**).

Since both ascorbate oxidation to generate H_2O_2 and the pro-oxidant chemistry of H_2O_2 can be facilitated by redox-active metal ions, the predominate action of labile iron in ascorbate toxicity appears to be multifactorial. Chelation of iron may either inhibit ascorbate oxidation, thereby limiting H_2O_2 generation, and/or inhibit Fenton chemistry mediated formation of $HO^{\bullet}$ formation and subsequent oxidative damage. To assess these possibilities, relative intracellular H_2O_2 levels were quantified utilizing the H_2O_2-sensitive fluorescent probe PeroxyOrange-1 (PO-1) under basal and iron chelation conditions. Interestingly, chelation conditions that inhibited ascorbate toxicity, exacerbated, not inhibited, the oxidation of PO-1 after ascorbate exposure (Fig. 3**E**). We hypothesize that the increase in PO-1 oxidation seen in the presence of desferrioxamine likely represents a reduction in Fenton chemistry inside the cell leading to a greater direct oxidation of PO-1 by H_2O_2. The increased oxidation of PO-1 by H_2O_2 when intracellular redox active labile iron is chelated with desferrioxamine would occur because the rate constant of H_2O_2 reacting with PO-1 (k = 1–2 M^{-1} s^{-1}) is several orders of magnitude slower than the reaction of H_2O_2 with labile iron [30–32]. Consistent with this explanation, under identical conditions, ascorbate-mediated DNA damage, as measured by phosphorylated histone 2Ax (γH2Ax) levels, was inhibited by iron chelation (Fig. 3**F**). Taken together, these data suggest that, at least *in vitro*, pro-oxidant Fenton chemistry with H_2O_2 resulting in oxidative DNA damage may represent the predominant action of labile iron contributing to ascorbate toxicity and radio-chemo-sensitization.

4. Conclusions

Despite major advances in understanding the biological differences between heterogeneous sarcoma subtypes, minimal progress has been made in improving therapeutic outcomes for sarcoma patients in the past two decades [8]. Recently, pharmacological ascorbate has emerged as an adjuvant to radio-chemotherapy that may represent an efficacious and easily implementable addition to current treatment strategies without the addition of significant toxicities. Additionally, ≈50% of patients with loco-regional disease will recur [1]. The data presented here suggest that the addition of ascorbate to neoadjuvant radiation has the potential to enhance local tumor control of some subtypes of sarcoma (Fig. 4).

The exact role of labile iron in ascorbate toxicity has been controversial and conflicting reports have attributed ascorbate toxicity to be dependent upon intracellular labile iron, extracellular labile iron, both extracellular or intracellular, or neither [12,13,26,28]. However, with careful consideration of experimental conditions, particularly the cell-permeability of the specific chelator utilized and the experimental timeframe, these seemingly conflicting studies can be resolved. The

current data clearly show that *in vitro*, labile iron plays a major role in ascorbate-mediated cancer cell toxicity by catalyzing Fenton or Fenton-like chemistry to produce hydroxyl radicals and induce oxidative DNA damage as well as cell death. It remains unclear, however, which action of labile iron predominates under *in vivo* conditions; the lower pH often attributed to the tumor environment may limit the rate of auto-oxidation of ascorbate to form H_2O_2 therefore increasing the apparent role of for metal ion-catalyzed ascorbate oxidation [18]. Interestingly, the oxidative damage caused by redox-active iron with hydroperoxides can actually increase at lower pH [33]. Further studies are needed to more clearly elucidate the precise *in vivo* mechanistic details of ascorbate-mediated toxicity in sarcoma models, but this should not impede the translation of these preclinical studies into clinical trials.

In summary, the current data demonstrate that the addition of pharmacological ascorbate enhances sarcoma susceptibility to radiation or chemotherapy both *in vitro* and *in vivo* and clearly support the continued investigation of the efficacy of pharmacological ascorbate in combination with standard-of-care sarcoma therapy in phase I and II clinical trials. However, the generality of ascorbate anti-cancer activity also needs to be extended beyond the two sarcoma subtypes (fibrosarcoma and liposarcoma) presented here.

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