Darinaparsin: solid tumor hypoxic cytotoxin and radiosensitizer
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Abstract

Purpose: Hypoxia is an important characteristic of the solid tumor microenvironment, and constitutes a barrier for effective radiotherapy. Here we studied the effects of Darinaparsin (DPS, an arsenic cytotoxin) on survival and radiosensitivity of tumor cells in vitro under normoxia and hypoxia and in vivo using xenograft models, compared to effects on normal tissues.

Experimental Design: The cytotoxicity and radiosensitization of DPS were first tested in vitro in a variety of solid tumor cell lines under both normoxia and hypoxia, and compared with arsenic trioxide (ATO, an arsenical with reported cytotoxic and radiosensitizing activities on tumor cells). The effects were then tested in mouse models of xenograft tumors derived from tumor cell lines and clinical tumor specimens. The potential mechanisms of DPS effects, including ROS generation, cellular damage, and changes in global gene expression, were also investigated.

Results: In comparison to ATO, DPS had significantly higher in vitro cytotoxic and radiosensitizing activities against solid tumor cells under both normoxia and hypoxia. In vivo experiments confirmed these activities at doses that had no systemic toxicities. Importantly, DPS did not radiosensitize normal bone marrow, and actually radioprotected normal intestinal crypts. The DPS-mediated anti-tumor effects under hypoxia were not dependent on ROS generation and oxidative damage, but were associated with inhibition of oncogene (RAS and MYC)-dependent gene expression.

Conclusion: DPS has significant and preferential cytotoxic and radiosensitizing effects on solid tumors as compared with normal cells. DPS may therefore increase the therapeutic index of radiation therapy and has near term translational potential.
Translational relevance

Darinaparsin (DPS), an organic arsenic compound, is currently in clinical trials as a single agent for the treatment of lymphohematopoietic malignancies and solid tumors. Here we report that DPS is a more potent cytotoxin and radiosensitizer under hypoxia than arsenic trioxide (ATO). At well-tolerated doses, DPS sensitized tumors, but not normal radiosensitive bone marrow, and protected gastrointestinal epithelium from radiation. These findings therefore suggest that DPS may have the potential to significantly increase the therapeutic index of radiotherapy for the treatment of solid tumors.
Introduction

Solid tumors are often relatively chemo- and radiation-resistant, due in part to the presence of hypoxia (1). Inorganic arsenic trioxide (ATO) is an established chemotherapy drug for treatment of acute promyelocytic leukemia (APL) that inhibits the APL-specific oncoprotein, AML-RXRα. We and others have previously reported that ATO has cytotoxic and radiosensitizing effects in solid tumor models, at least partially attributable to the induction of oxidative stress (2-5). Unfortunately, ATO was less efficacious in clinical trials of solid tumors than leukemia, and has dose-related risks of cardiac and hepatic toxicity (6). Recently, a number of organic arsenics with anti-cancer activities have been developed (7-11). Their mechanisms of action are different from those of ATO, and include effects on tumor angiogenesis (11), metabolism (12), and cell signaling (NF-kappaB) (9). As a result, unlike ATO, these organic arsenicals do not depend on AML-RARα inhibition for their anti-leukemic activities (8, 13, 14), and may be useful for treatment of other cancers including solid tumors.

Dariparsin (DPS) is an organic arsenical with potent activity against leukemia and multiple myeloma, with approximately 3-10 times greater cytotoxicity than ATO in vitro (15). Remarkably, much higher doses of DPS can be safely used in animal models with significantly less systemic toxicity than ATO (16). These favorable toxicity profiles have been confirmed in phase I and II clinical trials (17, 18). Like other organic arsenics, DPS does not induce differentiation of APL cells, and does not cause PML-RARα degradation and rearrangement of PML nuclear bodies (19). Neither myeloma nor APL ATO-resistant cell lines are resistant to DPS (20). In addition, DPS has been reported to have in vitro cytotoxic activity against a variety of solid tumor cell lines (19). These findings suggest that DPS may have different mechanisms of action than ATO, and may be an effective anti-solid tumor agent. Since hypoxia is an important determinant of chemo- and radio-resistance, we assessed the in vitro activities of DPS under both hypoxia and normoxia,
alone and in combination with radiation. Further experiments were conducted to test these DPS activities \textit{in vivo} on xenograft tumors and normal tissues, and to elucidate the underlying mechanisms of action.
Materials and Methods

Cell culture and reagents. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) unless otherwise specified. All cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. In the hypoxic experiments, the cells were placed in hypoxia chambers (0.5% O₂) overnight before drug or irradiation treatment. DPS was provided by ZIOPHARM Oncology (Boston, MA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. A ¹³⁷Cs γ-ray irradiator (dose rate 250 cGy/min) and an X-ray irradiator (dose rate 126 cGy/min) were used to irradiate cells and animal tumors, respectively.

Animal tumor models. Subconfluent HI-LAPC-4 (3x10⁶/mouse) and PANC-1 (2.5x10⁶/mouse) cells were suspended in MatriGel (BD Bioscience, Franklin Lakes, NJ) and injected subcutaneously on the lower back of immunodeficient (nude) mice. DPS treatment was started when xenograft tumors reached ~100 - 200 mm³ in volume, as calculated by π/6 x length x width². The tumor volume (TV) was normalized with the starting volume (T0), and fitted by Exponential Growth modeling (Prism, La Jolla, CA), which determined the P value and tumor volume doubling time with 95% confidence interval (CI).

The in vivo model of primary prostate cancer using tissue slice grafts (TSGs) has been previously described (21). The TSGs maintain the normal and cancer tissue histology as evidenced by immunohistochemical staining, and respond to androgen ablation. Briefly, a prostate cancer specimen (Gleason score 4+3) obtained from radical prostatectomy was precision-cut into TSGs of 5-mm diameter and 300-μm thickness, which were subsequently implanted under the renal capsules of male RAG2⁻/⁻γC⁻/⁻ mice (Fig 1D). After one month of establishment, mice bearing consecutive TSGs were paired and treated with DPS (100 mg/kg, IP, TIW) or saline as a control. The TSGs were recovered,
fixed, serially sectioned and stained for p63 (marker for normal basal cells) and AMACR (marker for prostate cancer cells) (PIN cocktail, Biocare Medical, Concord, CA), and the area of tumor and TSG in each section was quantified using ImagePro (Media Cybernetics, Bethesda, MD).

For the study of the DPS effect in combination with radiation on normal intestine, we used an established microcolony assay that measured the intestinal stem cell survival (as the number of crypts/cross-section) after radiation (22).

**Cytotoxicity, cell death, apoptosis, and clonogenic survival assays.** *In vitro* cytotoxicity was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and expressed as the drug concentration causing 50% inhibition of cell growth (IC50). Cell death was measured by Trypan Blue (0.04%, Invitrogen, Carlsbad, CA) exclusion after trypsinization and expressed as the % of positively stained cells divided by total number of cells. Apoptosis was measured by staining with Alexa Fluor 488-conjugated annexin V (Invitrogen) and propidium iodide (PI, 2 μg/mL) followed by flow cytometry. For clonogenic assays, the cells were maintained in fresh medium after treatment for 2 - 3 weeks before staining with 0.25% crystal violet in 95% ethanol. Colonies with ≥50 cells were scored. The surviving fraction was calculated as the colony-forming efficiency (CFE) of treated cells divided by the CFE of untreated cells.

**ROS measurement.** Cellular ROS were measured by fluorescent probe CM-H2DCFDA (5 μM for 30 minutes at 37°C, Invitrogen). Mitochondria superoxide (O2−) was determined using the mitochondria-specific fluorescent probe MitoSox Red (10 μM for 10 min at 37°C, Invitrogen). The fluorescence intensity was measured by flow cytometry, and expressed as the mean fluorescent unit (MFU).

**Western blot.** After cell lysis, sample proteins were separated by 10% SDS-PAGE, and transferred onto PVDF membranes. The membranes were incubated overnight at 4°C with anti-cleaved caspase-3, anti-γH2AX, or anti-β-actin (1:1,000, Cell Signaling
Technology, Beverly, MA) antibodies, and then with goat anti-rabbit (1:2,000, Cell Signaling) for 1 hour at room temperature.

**RNA isolation and microarray analysis.** Total RNA was isolated using TRIzol (Invitrogen) and purified using a ‘RNeasy MinElute Kit’ (Qiagen, Valencia, CA). The microarray hybridization was conducted by Stanford Functional Genomics Facility using array chips (SurePrint G3 Human GE 8x60k, Agilent Technologies, Santa Clara, CA). A Universal Human Reference RNA (Agilent) was used as the control for each sample. The acquired fluorescence intensities for each of the 62,976 features (containing 27,958 Entrez gene RNAs) were obtained with an Agilent G2505C Microarray Scanner System after labeling, and available at GenBank (accession no: GSE33001). We selected gene features whose expression levels varied ≥2.5-fold from the untreated controls (CON) in both 4 and 8 hr treatment groups under normoxia; and ≥1.5-fold from CON under hypoxia. The resulting datasets (listed in Suppl Table 1) were analyzed with Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA), Transcription Factor Target Enrichment Analysis (http://bioinfo.vanderbilt.edu/webgestalt), oncogene-dependent gene expression signature analysis, and Connectivity Map analysis (http://www.broadinstitute.org/cmap).

**Other assays.** Intracellular total GSH concentrations were determined using the GSH reductase recycling assay (23) and normalized with protein concentration. DNA damage was assessed using the neutral comet assay (24). One hundred cells were scored for each sample and the data were expressed as the mean moment (tail length * intensity of DNA in the tail ± SE). Mitochondrial membrane potential (MMP) was measured by JC-1 (Invitrogen, 10 μg/mL, 1 hr) staining and flow cytometry (25). Senescence was measured using SA (senescence associated)-β-Gal-staining (26), and expressed as the ratio of positive stained cells to total number of cells.
Results

DPS inhibited solid tumor cell growth \textit{in vitro} and \textit{in vivo}. Using a panel of cell lines derived from prostate [hormone-independent (HI)-LAPC-4 and PC-3], pancreas (PANC-1), brain (SNB-75), and cervical (HeLa) tumors, the \textit{in vitro} cytotoxicity of DPS and ATO was determined using the MTT assay at 3 days after incubation with DPS or ATO for 4 hr under normoxia or hypoxia. DPS had IC$_{50}$s of ~ 3-8 $\mu$M in different cell lines (Fig 1A). The IC$_{50}$s were similar under either normoxia or hypoxia, indicating that the effect of DPS on cell proliferation was O$_2$-independent. In comparison, IC$_{50}$s of ATO were ~2- to 6-fold higher than DPS, with hypoxic IC$_{50}$s generally higher than corresponding normoxic IC$_{50}$s, indicating that ATO activity against these cell lines was less potent than DPS and O$_2$-dependent to a certain extent. In clonogenic assays which assess the ability of individual cells to proliferate and give rise to colonies, the IC$_{50}$/4hr of DPS was ~1 - 3 $\mu$M for the cell lines studied under both normoxia and hypoxia (Suppl Table 2).

Next the effect of DPS on tumor growth \textit{in vivo} was determined using mouse models with established HI-LAPC-4 and PANC-1 subcutaneous xenograft tumors. The mice were injected with DPS intraperitoneally 3 times/wk with a dose of 100 mg/kg for 4 wks. Toxicology studies, including blood chemistry panels, did not indicate any systemic toxicity with similar doses except for changes in physical activity and body weight (16) (Suppl Fig 1A and Suppl Table 3). In pharmacokinetic studies with human subjects (18), this regimen achieved blood arsenic concentrations (C$_{\text{max}}$, ~10 $\mu$M) above the IC$_{50}$s observed in our \textit{in vitro} studies (Fig 1A and Suppl Table 2). In both HI-LAPC-4 and PANC-1 tumor models, DPS significantly inhibited tumor growth (P < 0.0001) (Fig 1 B1 and B2), with the average tumor volume doubling time increased from 4 to 13 days in HI-LAPC-4 tumors (P < 0.001), and 4 to 7 days in PANC-1 tumors (P < 0.001).

Finally, the activity of DPS in a novel and clinically relevant tumor slice graft (TSG) model of primary prostate cancer was tested. Mice that received consecutive TSGs
(Gleason score 4+3) were paired and treated with DPS (100 mg/kg, IP, 3 times/week for 4 weeks) or saline as a control (Fig 1C). After recovery, the TSGs were fixed and serially sectioned. The presence of cancer in each section was evaluated by AMACR staining, and AMACR-positive areas were imaged and quantitated. The total areas of the TSG sections from DPS- and saline-treated mice that were evaluated were similar (data not shown). In three of the four TSG pairs, DPS decreased the total tumor area to less than 50% of the control. Although the difference (P = 0.139 by paired t-test) was not statistically significant, possibly due to the small sample size of this pilot study, overall the findings suggest DPS-induced cytotoxicity in primary prostate cancer as well as advanced cancer as modeled by the cell lines.

**DPS sensitized solid tumor cells to radiation.** Since ATO has been reported to sensitize solid tumors to radiation (2-5), we compared the *in vitro* radiosensitizing effect of DPS to ATO using a panel of relatively radioresistant cell lines (HI-LAPC-4, PC-3, SNB-75, HeLa, and PANC-1). Prior to irradiation, the cells were incubated with 3 μM DPS or ATO for 4 hr. Fig 2A shows the clonogenic survival of γ-irradiated cells after normalization for the effect of drug alone. As expected, all cell lines were more sensitive to radiation under normoxia as compared to hypoxia. Under hypoxia, ATO had little or no effect on the clonogenic survival of the irradiated cells; in contrast, DPS increased radiation-induced cell killing by approximately one log, which was even greater than the effect of normoxia (oxygen) under normoxic conditions.

Next, the *in vivo* effect of DPS in combination with local tumor irradiation was evaluated. Nude mice bearing subcutaneous HI-LAPC-4 and PANC-1 xenograft tumors were treated with DPS (100 mg/kg, IP) 4 hr before a single dose (5 Gy) of irradiation. In both models, DPS significantly enhanced the radiation-induced tumor growth inhibition, with an increase in the tumor doubling time from 11 to 20 days in HI-LAPC-4 tumors and 16 to 20 days in PANC-1 tumors (Fig 2B 1-2, P < 0.0001). The effect of DPS in combination
with a clinically relevant fractionated radiation regimen was also tested. In this regimen, DPS (100 mg/kg, IP) was administered 4 hr before radiation (2 Gy/day) for 3 consecutive days using the HI-LAPC-4 tumor model. A significant delay of tumor volume doubling time was observed from 7 days with radiation alone and 6 days with DPS alone to 19 days for the combined treatment of radiation and DPS (Fig 2B3, P < 0.0001).

**DPS did not radiosensitize normal radiosensitive tissues.** The body weight loss observed in the radiosensitization regimen, either by DPS alone or in combination with radiation, was minimal (< 5%, Suppl Fig 1A). Serial complete blood counts (CBCs, indicators of bone marrow function) were obtained following total body radiation (3.5 Gy) with or without DPS treatment (100 mg/kg, 4 hr, IP). As expected, the white blood cells (WBC), platelet, red blood cells (RBC), and hemoglobin (HGB) significantly decreased after the radiation and recovered over time. DPS did not affect these CBC changes (Fig 3A). Using the established intestinal microcolony assay, we observed a significant decrease in viable duodenal crypt cells following radiation. However, to our surprise, DPS treatment significantly enhanced the survival of crypt cells following irradiation (Fig 3B). In comparison to saline-treated controls, the DPS-treated mice had nearly 10 times more viable crypts at 3.5 days after radiation (10-12 Gy). Similar results were observed in the jejunum and ileum (data not shown). The data therefore indicate that DPS does not sensitize, and may even protect, normal radiosensitive tissues to radiation.

**DPS induced apoptosis and senescence but not autophagy in HI-LAPC-4 cells.** DPS caused HI-LAPC-4 cell death in a time (24 and 48 hr)-dependent manner, as demonstrated by Trypan Blue exclusion assay (Fig 4A). We therefore assessed the modes of cell death after DPS treatment. After 24 hrs of DPS treatment (10 μM, 8 hr), there was a significant increase in caspase-3 cleavage (Fig 4B) and annexin V externalization (Fig 4C) under both normoxia and hypoxia, indicating cell death by apoptosis. Importantly, after 24 hr incubation in hypoxia, DPS was more cytotoxic to HI-
LAPC-4 cells, which is in contrast to equivalent effects of DPS in normoxia and hypoxia after only 4 hr incubation (Fig 1A). In addition, a small proportion of cells that survived DPS treatment had morphologic features of senescence with increased expression of SA-β-galactosidase (Fig 4D). Finally, morphological (vacuole formation) and LC-3 activation (an autophagy marker) (Suppl Fig 2) suggested autophagy was not involved in DPS-induced cell death. A cell cycle analysis was also conducted and revealed no apparent cell cycle arrest after 24 hr of DPS treatment (Suppl Fig 3).

**DPS cytotoxicity was independent of ROS generation and DNA or mitochondria damage.** Since DPS-induced cytotoxicity in hematologic cancer cell lines is mediated by ROS generation, with subsequent mitochondrial damage (19), we determine the effect of DPS on cellular ROS in solid tumor cell lines. Using the fluorescent probe CM-H2DCFDA, we observed a dose-dependent increase of cellular ROS in DPS-treated HI-LAPC-4 cells under normoxia but not hypoxia (Fig 5A). In addition, DPS depleted cellular antioxidant GSH in normoxia, but not hypoxia (Fig 5B). Since ROS generation disrupts normal protein folding in the endoplasmic reticulum (ER), and subsequently induces ER stress and unfolded protein response (UPR) (27), the level of ER stress following DPS treatment was assessed using an established luciferase reporter model for UPR-activated transcription factors XBP-1 or ATF-4 (28). As expected, DPS caused a dose-dependent activation of XBP-1 and ATF-4 under normoxia, but not under hypoxia (Fig 5C). Finally, in HI-LAPC-4 cells under normoxia (but not hypoxia), there was a moderate increase of DNA damage (Comet assay, Fig 5D) and DNA damage response (γH2AX, Fig 5E) following DPS treatment. However, when cells were pretreated with DPS for 4 hours and then irradiated, there was no synergistic increase in γH2AX at either 0.5 or 12.5 hours after irradiation (Suppl Fig 4). In summary, the data above demonstrate that DPS did not induce oxidative stress under hypoxia, indicating the existence of ROS-independent mechanisms of DPS cytotoxicity.
While DPS significantly decreased (28%, \( P < 0.01 \)) mitochondrial membrane potential (MMP) in an APL cell line, HL-60 (19, Suppl Fig 5A), it did not significantly affect the MMP in the five solid tumor cell lines tested, with the exception of HI-LAPC-4 under normoxia (31%, \( P < 0.05 \)). In addition, there was no increase of mitochondrial ROS under either normoxia or hypoxia (Suppl Fig 5B), indicating that ROS generation did not cause detectable mitochondrial damage in the majority of the solid tumor cell lines studied, and is not a primary mechanism of DPS cytotoxicity in these cells.

**The anti-tumor effect of DPS was associated with inhibition of oncogenic signaling pathways.** The lack of immediate DNA and mitochondrial damage suggested that DPS-induced cell death was mediated by signaling events. In addition, cycloheximide (CHX, a translation inhibitor) significantly decreased DPS cytotoxicity under both normoxia and hypoxia (\( P < 0.0001 \) for the interaction of CHX and DPS by 2-way ANOVA, Suppl Fig 6), indicating that DPS activity required de novo protein expression. Next, DPS effects on Hypoxia-induced factor Iα (HIF1α) and c-Jun NH2-terminal kinase (JNK) were studied because of their roles as a regulator of cell survival and radiosensitivity under hypoxia (1) and a mediator of DPS cytotoxicity in leukemia cells (15), respectively. The data indicate that neither factor likely plays a significant role in DPS-mediated cytotoxicity in solid tumor cells under hypoxia (Suppl Fig 7-8).

Gene expression profiling was performed with HI-LAPC-4 cells after 4 and 8 hrs incubation with DPS or ATO at the concentrations of IC\(_{50}/4\)hr (Fig 1A) under normoxia. Among the 16,433 genes analyzed, 343 (up) and 113 (down) genes were regulated by DPS, and 139 (up) and 99 (down) genes were regulated by ATO, with 104 (up) and 57 (down) overlapping genes. Several of the upregulated genes were validated by RT-qPCR assay of independently-treated samples (Suppl Table 4A and B). An Ingenuity Pathway analysis demonstrated that DPS affected multiple biological processes and pathways that involve cancer, inflammation, and apoptosis (Suppl Table 4C).
Transcription Factor Target Enrichment Analysis identified transcription factors whose
target gene expression was significantly affected by DPS (DPS/CON) and ATO
(ATO/CON), many of which were associated with oncogene regulated pathways (Suppl
Table 4D).

To further assess the effect of DPS on oncogenic pathways, we interrogated the
microarray data against established oncogene-dependent gene expression signatures
(29-33), including oncogenes such as RAS, RAF, MEK, MAPK, AKT, MYC, β-catenin,
SRC, E2F, androgen receptor (AR), Cyclin D, and ERBB2. The analysis showed
significant overlap of RAS and MYC target genes with DPS-affected genes (Fig 6).
Specifically, DPS significantly decreased the expression of genes that were upregulated
by RAS, and increased the expression of genes that were down-regulated by MYC. In
contrast, ATO only affected AR signaling. Next, we compared RAS and MYC status in
HI-LAPC-4 and PC-3 (both prostate cancer) cells with normal fibroblasts, and found
increased expression of both proteins in the cancer cell lines (Suppl Fig 9). The data are
consistent with previous reports of increased MYC and RAS expression in solid tumor
cells (34), and suggest a selective effect of DPS on cancer cells via oncogenic
pathways.

Connectivity Map analysis (Suppl Table 4E) was used to compare gene expression
changes induced by DPS and ATO to those induced by hundreds of other molecules of
known function. Of note, there was no overlap of the top 20 molecules connected with
DPS or ATO, again suggesting differential mechanisms of action for these two
arsenicals. Many of the DPS-connected molecules are known cancer drugs and affect
oncogenic pathways, such as HSP90 inhibitors [tanespimycin (35), geldanamycin (36),
and alvespimycin (37)], withaferin A (38), and wortmannin, further supporting the
involvement of oncogenic pathways in DPS activity.
Discussion

Resistance of many solid tumors to currently available cancer therapies is an important clinical problem, and is determined in part by the presence of hypoxia in the tumor microenvironment. In this study, we report that DPS effectively inhibited the growth of a variety of tumor cell lines *in vitro* under hypoxia and *in vivo* in xenograft tumor models. Importantly, DPS significantly radiosensitized tumor cells *in vitro* under hypoxia and *in vivo* with a clinically relevant fractionated radiation regimen, but did not enhance the sensitivity of normal radiosensitive intestine and bone marrow to radiation. In fact, DPS actually radioprotected the GI epithelium, suggesting that DPS has the potential to significantly enhance the therapeutic index of radiation therapy. With higher cytotoxic and radiosensitizing potency under hypoxia than ATO, and a more favorable toxicity profile, DPS is more promising for the treatment of solid tumors. Although DPS, a dimethylarsenic conjugated to glutathione, may be generated as an intermediate in the inorganic arsenic metabolic transformation pathway (19, 39), our data suggest that the cellular generation of DPS from ATO is insufficient to fully explain the anti-tumor effects observed with DPS alone.

Currently, most of the information about the mechanism of action of DPS comes from malignant lympho-hematopoietic cell lines, in which oxidative stress plays an important role. However, under hypoxia (0.5% O₂), DPS did not induce oxidative stress and DNA damage in solid tumor cells. We therefore performed mechanistic studies to elucidate other possible mechanisms of action for DPS. In contrast to ATO, DPS caused significant changes of gene expression that were associated with the oncogenes RAS and MYC. This targeted effect may help to explain the differential effect of DPS on cancer as compared to normal cells, as well as the decreased systemic toxicity of DPS compared with ATO. In addition, since oncogenes are important for cancer cell survival after irradiation, and oncogene inhibition can radiosensitize cells (40), it is therefore
possible that the selective radiosensitizing effect of DPS is due to inhibition of
dysregulated oncogenic pathways in cancer, but not normal, cells.

Of particular interest and potential clinical utility is the observation that DPS enhanced
the survival of radiosensitive intestinal cells, while sensitizing tumor cells to radiation.
This differential effect may be, in part, due to differences in signaling factors and signal
transduction pathways between intestinal and tumor cells. p53, a tumor suppressor that
is mutated or silenced in many tumor cells (including the 5 cell lines used in this study)
(41-44), has been reported to protect intestinal cells from radiation damage via cell cycle
arrest (45). It is therefore possible that DPS activates p53 and downstream p21, which
enhances the radiation-induced cell cycle arrest in crypt cells. Experiments are ongoing
to further elucidate the differential effect of DPS on normal cells as compared to tumor
cells.

In summary, the results presented here demonstrate that DPS has significant cytotoxic
and radiosensitizing activities against normoxic and hypoxic solid tumor cells in vitro and
in vivo. Moreover, DPS does not sensitize bone marrow, and protected normal intestinal
epithelium from the effects of radiation in vivo, suggesting that DPS has the potential to
significantly increase the therapeutic index of radiation therapy. The favorable toxicity
profile of DPS and the data presented here are very promising and may have broad
clinical applicability for the treatment of solid tumors, in which hypoxia enhances tumor
resistance to radiation therapy. Since DPS is already in clinical trials, these results have
near term translational potential.
Acknowledgement

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Reference

Fig 1. DPS inhibited tumor cell growth *in vitro* and *in vivo*. A, Cells were treated with DPS for 4 hr under normoxia (NO) or hypoxia (HO), and kept in fresh media for 3 days under NO before MTT assay. Data were analyzed by Sigmoidal dose-response modeling, and expressed as IC$_{50}$s with standard error. *, P < 0.01 for NO/DPS vs. NO/ATO and HO/DPS vs. HO/ATO; §, P < 0.01 for NO/ATO vs. HO/ATO. B, Nude mice with subcutaneous HI-LAPC-4 (B1) and PANC-1 (B2) xenograft tumors were given DPS (100 mg/kg, 3 times/wk, IP) or saline (CON). N = 9 (DPS) and 7 (CON) in B1, and N = 10 (DPS) and 11 (CON) in B2. *, P < 0.0001 for CON vs. DPS by exponential growth modeling. C, Rag2$^{-/-}$γC$^{-/-}$ mice bearing paired xenograft human prostate cancer TSGs were treated with DPS as in 1C for 4 wks, and stained for AMACR (cancer, arrowheads) and p63 (normal basal cell, arrows). P = 0.139 by paired t-test.

Fig 2. DPS was a potent radiosensitizer *in vitro* and *in vivo*. A, Cells were treated with DPS or ATO (3 μM, 4 hr) under NO or HO before irradiation and colony survival assay. §, P < 0.01 of NO vs. NO/DPS and HO vs. HO/DPS in cells with 5 Gy radiation. B, Mice with subcutaneous xenograft tumors were treated with DPS (IP, 100 mg/kg) and 4 hr later with irradiation (XRT) of 5 Gy (B1 and B2) or 2 Gy (B3) at days indicated by the arrows. *, P < 0.0001 vs. all other groups by exponential growth modeling. N = 5-7/group.

Fig 3. DPS did not radiosensitize normal radiosensitive tissues. A, Balb/C mice were treated with DPS (100 mg/kg, IP, 4 hr) alone, total body irradiation (IR) alone, or IR following DPS (IR/DPS). Each group (N = 8) comprised 2 subgroups (N =4). Each subgroup was retroorbitally bled alternatively (every other week) and counted for WBC, RBC, HGB, and platelets. B, Balb/C mice were divided into 4 groups, including untreated control (CON), DPS (100 mg/kg, IP), IR, and IR following 4 hr DPS treatment (IR/DPS). N = 3/group. The intestinal samples were collected 3.5 days after IR, and viable crypts/cross-section were quantified. Data presented are from duodenum.
samples. *, P < 0.05 vs. CON. Data are representative of three independent experiments.

**Fig 4. DPS induced apoptosis and senescence in HI-LAPC-4 cells.** A, Cells were treated with DPS (10 μM) for the time indicated and analyzed with Trypan blue exclusion assay. B-C, Cells were treated with DPS (24 hr) under NO and HO before measurements of cleaved caspase-3 (B, Western blot) and annexin V externalization (C, flow cytometry). D, Cells were treated with DPS (24 hr) under NO or HO, and maintained in fresh medium for 24 hrs before SA-β-Gal staining. Few cells survived the DPS treatment (6 μM) under HO, and therefore were not counted. P < 0.001 by one way ANOVA, and *, P < 0.001 vs. 3 and 6 μM by Tukey’s multiple comparison test. The arrow of the inset indicates a cell with senescence morphology and positive stain of SA-β-Gal.

**Fig 5. DPS induced oxidative stress under normoxia but not hypoxia.** A, HI-LAPC-4 cells were treated with DPS (8 hr) and assayed with fluorescent probe CM-H2DCFDA. Inset, ROS level of cells with or without H₂O₂ (positive control) treatment (1 mM, 10 min) under HO. B, HI-LAPC-4 cells were treated with DPS (4 hr) before analysis for the total cellular GSH content. C, Pancreatic cancer (MIA PaCa-2) cells that stably express a luciferase reporter of ATF-4 or XBP-1 were treated with DPS (8 hr) and thapsigargin (TG, 30 nM, 24 hr, positive control). The luciferin production was expressed as the relative fluorescence intensity (RFU) over untreated control, and normalized by protein concentration. Inset: both HO and TG induced XBP-1 and ATF-4 activity. D and E, HI-LAPC-4 cells were treated with DPS for 4 hr and used for comet assay (D) and γH2AX Western blot (E). IR, irradiation (positive control). Inset, representative comet micrograph. P < 0.0001 for one way ANOVA in A-E comparing conditions under NO. *, P < 0.01 for comparison with NO untreated control by Tukey’s multiple comparison test.
Fig 6. Gene microarray analysis of DPS effect on oncogenes. HI-LAPC-4 cells were treated with DPS or ATO at IC\textsubscript{50} under NO or HO for 4 and 8 hr. The gene expression was assayed by cDNA microarray, and the data analyzed by oncogene-dependent gene expression signature analysis. The heatmap presents the overlaps of DPS-regulated genes and oncogene (RAS and MYC)-dependent signatures under each condition. “up” and “down”, oncogene up- or down-regulated signature gene set. The superscripts denote the references of the signature set. P < 0.001 by Chi Square test or Yate’s Corrected Chi Square test for enrichment of RAS\_up genes in DPS/CON\_NO and DPS/CON\_HO, and MYC\_down genes in DPS/CON\_NO.
Fig. 1. DPS inhibited tumor growth in vitro and in vivo.
Fig 2. DPS is a potent radiosensitizer in vitro & in vivo
Fig 3. DPS did not enhance the radiosensitivity of normal radiosensitive tissues

A

- WBC
- RBC
- HGB
- Platelet

Week after treatment

B

- CON
- IR
- DPS
- IR/DPS

Crypts/section

IR (Gy)
Fig 4. DPS-induced apoptosis and senescence in HI-LAPC-4 cells

A

B

C

D

β-actin

Total caspase 3

Cleaved caspase 3

Annexin V

SA-β-Gal stained/total cells

0 μM  3 μM  6 μM
Fig 5. DPS induces oxidative stress under NO but not HO
Fig 6. DPS inhibited oncoprotein-dependent signaling