Darinaparsin: Solid Tumor Hypoxic Cytotoxic 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 (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 darinaparsin 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 darinaparsin effects, including reactive oxygen species (ROS) generation, cellular damage, and changes in global gene expression, were also investigated.

Results: In comparison with ATO, darinaparsin 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, darinaparsin did not radiosensitize normal bone marrow and actually radioprotected normal intestinal crypts. The darinaparsin-mediated antitumor 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: Darinaparsin has significant and preferential cytotoxic and radiosensitizing effects on solid tumors as compared with normal cells. Darinaparsin may therefore increase the therapeutic index of radiation therapy and has near term translational potential. Clin Cancer Res; 1–11. ©2012 AACR.

Introduction

Solid tumors are often relatively chemotherapeutic 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 oncprotein, AML-RARα. 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 it has dose-related risks of cardiac and hepatic toxicity (6). Recently, a number of organic arsenics with anticancer 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-kB; ref. 9). As a result, unlike ATO, these organic arsenicals do not depend on AML-RARα inhibition for their antileukemic activities (8, 13, 14), and may be useful for treatment of other cancers including solid tumors.

Darinaparsin is an organic arsenical with potent activity against leukemia and multiple myeloma, with approximately 3 to 10 times greater cytotoxicity than ATO in vitro (15). Remarkably, much higher doses of darinaparsin can be safely used in animal models with significantly less systemic toxicity than ATO (16). These favorable toxicity profiles have been confirmed in phases I and II clinical trials (17, 18). Like other organic arsenics, darinaparsin 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 darinaparsin (20). In addition, darinaparsin has been reported to have in vitro cytotoxic activity against a variety of solid tumor cell lines (19). These findings suggest that darinaparsin may have different mechanisms of action than ATO and may be an effective antitumor agent. Because hypoxia is an important determinant of radioresistance, we assessed the in vitro activities of darinaparsin under both hypoxia and normoxia, alone and in...
Darinaparsin, 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 darinaparsin is a more potent cytotoxin and radiosensitizer under hypoxia than arsenic trioxide (ATO). At well-tolerated doses, darinaparsin sensitized tumors, but not normal radiosensitive bone marrow, and protected gastrointestinal epithelium from radiation. These findings therefore suggest that darinaparsin may have the potential to significantly increase the therapeutic index of radiotherapy for the treatment of solid tumors.

**Combination with Radiation.** Further experiments were conducted to test these darinaparsin activities 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) 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% O$_2$) overnight before drug or irradiation treatment. Darinaparsin was provided by ZIOPHARM Oncology. All other reagents were obtained from Sigma-Aldrich unless otherwise specified. A $^{137}$Cs 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 hormone-independent (HI)-LAPC-4 (3 × 10^6 per mouse) and PANC-1 (2.5 × 10^6 per mouse) cells were suspended in Matrigel (BD Bioscience) and injected subcutaneously on the lower back of immunodeficient mice. Darinaparsin treatment was started when xenograft tumors reached approximately 100 to 200 mm$^3$ in volume, as calculated by $\pi/6 \times \text{length} \times \text{width}^2$. The tumor volume was normalized with the starting volume and expressed as the drug concentration causing 50% survival (IC$_{50}$). Cell death was measured by trypsin blue (0.04%; Invitrogen) exclusion 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 (2 μg/mL) followed by flow cytometry. For clonogenic assays, the cells were maintained in fresh medium after treatment for 2 to 3 weeks before staining with 0.25% crystal violet in 95% ethanol. Colonies with 50 cells or more were scored. The surviving fraction was calculated as the colony-forming efficiency (CFE) of treated cells divided by the CFE of untreated cells.

**Reactive oxygen species measurement**

Cellular reactive oxygen species (ROS) were measured by fluorescent probe CM-H2DCFDA (5 μmol/L for 30 minutes at 37°C; Invitrogen). Mitochondria superoxide (O$_2^-$) was determined using the mitochondria-specific fluorescent probe MitoSox Red (10 μmol/L for 30 minutes at 37°C; Invitrogen). The fluorescence intensity was measured by flow cytometry and expressed as the mean fluorescent unit.

**Western blot**

After cell lysis, sample proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with antibody against cleaved caspase-3, anti-γH2AX, or anti–β-actin (1:1,000; Cell Signaling Technology) 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 an RNaseasy MinElute kit (Qiagen). The microarray hybridization was conducted by Stanford Functional Genomics Facility using array chips (SurePrint G3 Human GE 8 × 60 k; Agilent Technologies). 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 in which expression levels varied 2.5-fold or more from the untreated controls (CON) in both 4- and 8-hour treatment groups under normoxia; and 1.5-fold or more from CON under hypoxia. The resulting data sets (listed in Supplementary Table S1) were analyzed with Ingenuity Pathway Analysis (Ingenuity Systems), 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 glutathione (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 \( \times \) intensity of DNA in the tail \( \div \) SE). Mitochondrial membrane potential (MMP) was measured by JC-1 (10 \( \mu \)g/mL, 1 hour; Invitrogen) staining and flow cytometry (25). Senescence was measured using senescence-associated (SA)-\( \beta \)-Gal staining (26) and expressed as the ratio of positive stained cells to total number of cells.
Results  

Darinaparsin inhibited solid tumor cell growth in vitro and in vivo  

Using a panel of cell lines derived from prostate (HI-LAPC-4 and PC-3), pancreas (PANC-1), brain (SNB-75), and cervical (HeLa) tumors, the in vitro cytotoxicity of darinaparsin and ATO was determined using the MTT assay at 3 days after incubation with darinaparsin or ATO for 4 hours under normoxia or hypoxia. Darinaparsin had IC₅₀ values of approximately 3 to 8 μmol/L in different cell lines (Fig. 1A). The IC₅₀ values were similar under either normoxia or hypoxia, indicating that the effect of darinaparsin on cell proliferation was O₂ independent. In comparison, IC₅₀ values of ATO were approximately 2- to 6-fold higher than darinaparsin for O₂ independent to a certain extent. In clonogenic assays against these cell lines was less potent than darinaparsin regimen achieved blood arsenic concentrations (0.139 ± 0.015 mg/L for the cell lines studied under both normoxia and hypoxia (Supplementary Table S2).

Next, the effect of darinaparsin on tumor growth in vivo was determined using mouse models with established HI-LAPC-4 and PANC-1 subcutaneous xenograft tumors. The mice were injected with darinaparsin intraperitoneally 3 times per week with a dose of 100 mg/kg for 4 weeks. 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; Supplementary Table S2). The body weight loss observed in the radiosensitization regimen, either by darinaparsin alone or in combination with radiation, was minimal (<5%, Supplementary Fig. S1A). Serial complete blood counts (CBC, indicators of bone marrow function) were obtained following total body radiation (3.5 Gy) with or without darinaparsin treatment (100 mg/kg, 4 hours, i.p.). As expected, the white blood cells (WBC), platelet, red blood cells (RBC), and hemoglobin (HGB) significantly decreased after the radiation and recovered over time. Darinaparsin 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, darinaparsin treatment significantly enhanced the survival of crypt cells following irradiation (Fig. 3B). In comparison with saline-treated controls, the darinaparsin -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 darinaparsin did not radiosensitize normal radiosensitive tissues.

Darinaparsin sensitized solid tumor cells to radiation  

Because ATO has been reported to sensitize solid tumors to radiation (2–5), we compared the in vitro radiosensitizing effect of darinaparsin to ATO using a panel of relatively radioresistant cell lines (HI-LAPC-4, PC-3, SNB-75, HeLa, and PANC-1). Before irradiation, the cells were incubated with 3 μmol/L darinaparsin or ATO for 4 hours. Figure 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 with hypoxia. Under hypoxia, ATO had little or no effect on the clonogenic survival of the irradiated cells; in contrast, darinaparsin 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 darinaparsin in combination with local tumor irradiation was evaluated. Nude mice bearing subcutaneous HI-LAPC-4 and PANC-1 xenograft tumors were treated with darinaparsin (100 mg/kg, i.p.) 4 hours before a single dose (5 Gy) of irradiation. In both models, darinaparsin 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. 2B1 and B2, P < 0.0001). The effect of darinaparsin in combination with a clinically relevant fractionated radiation regimen was also tested. In this regimen, darinaparsin (100 mg/kg, i.p.) was administered 4 hours 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 darinaparsin alone to 19 days for the combined treatment of radiation and darinaparsin (Fig. 2B3, P < 0.0001).

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Darinaparsin does not sensitize, and may even protect, normal radiosensitive tissues to radiation.

Darinaparsin induced apoptosis and senescence but not autophagy in HI-LAPC-4 cells

Darinaparsin caused HI-LAPC-4 cell death in a time (24 and 48 hours)-dependent manner, as shown by trypan blue exclusion assay (Fig. 4A). We therefore assessed the modes of cell death after darinaparsin treatment. After 24 hours of darinaparsin treatment (10 μmol/L, 8 hours), 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 hours incubation in hypoxia, darinaparsin...
was more cytotoxic to HI-LAPC-4 cells than in normoxia, which is in contrast to equivalent effects of darinaparsin in normoxia and hypoxia after only 4 hours incubation (Fig. 1A). In addition, a small proportion of cells that survived darinaparsin treatment had morphologic features of senescence with increased expression of SA-β-galactosidase (Fig. 4D). Finally, morphologic (vacuole formation) and LC-3 activation (an autophagy marker; Supplementary Fig. S2) suggested autophagy was not involved in darinaparsin-induced cell death. A cell-cycle analysis was also conducted and revealed no apparent cell-cycle arrest after 24 hours of darinaparsin treatment (Supplementary Fig. S3).

**Darinaparsin cytotoxicity was independent of ROS generation and DNA or mitochondria damage**

Because darinaparsin-induced cytotoxicity in hematologic cancer cell lines is mediated by ROS generation, with subsequent mitochondrial damage (19), we determine the effect of darinaparsin on cellular ROS in solid tumor cell lines. Using the fluorescent probe CM-H2DCFDA, we observed a dose-dependent increase of cellular ROS in darinaparsin-treated HI-LAPC-4 cells under normoxia but not hypoxia (Fig. 5A). In addition, darinaparsin depleted cellular antioxidant GSH in normoxia, but not hypoxia (Fig. 5B). Because ROS generation disrupts normal protein folding in the endoplasmic reticulum (ER), and subsequently induces ER stress and unfolded protein response (UPR; ref. 27), the level of ER stress following darinaparsin treatment was assessed using an established luciferase reporter model for UPR-activated transcription factors XBP-1 or ATF-4 (28). As expected, darinaparsin 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 darinaparsin treatment. However, when cells were pretreated with darinaparsin for 4 hours and then irradiated, there was no synergistic increase in γH2AX at either 0.5 or 12.5 hours after...
Figure 5. Darinaparsin induced oxidative stress under normoxia but not hypoxia. A, HI-LAPC-4 cells were treated with darinaparsin (8 hours) and assayed with fluorescent probe CM-H2DCFDA. Inset, ROS level of cells with or without H2O2 (positive control) treatment (1 mmol/L, 10 minutes) under HO. B, HI-LAPC-4 cells were treated with darinaparsin (4 hours) 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 darinaparsin (8 hours) and thapsigargin (TG, 30 nmol/L, 24 hours, 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 darinaparsin for 4 hours and used for comet assay (D) and γH2AX Western blot (E). IR, irradiation (positive control). Inset, representative comet micrograph. *P < 0.0001 for 1-way ANOVA in A–E comparing conditions under NO. **P < 0.01 for comparison with NO untreated control by the Tukey multiple comparison test.
irradiation (Supplementary Fig. S4). In summary, the data above show that darinaparsin did not induce oxidative stress under hypoxia, indicating the existence of ROS-independent mechanisms of darinaparsin cytotoxicity.

Although darinaparsin significantly decreased (28%, \( P < 0.01 \)) MMP in an APL cell line, HL-60 (19, Supplementary Fig. S5A), it did not significantly affect the MMP in the 5 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 (Supplementary Fig. S5B), 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 darinaparsin cytotoxicity in these cells.

The antitumor effect of darinaparsin was associated with inhibition of oncogenic signaling pathways

The lack of immediate DNA and mitochondrial damage suggested that darinaparsin-induced cell death was mediated by signaling events. In addition, cycloheximide (CHX, a translation inhibitor) significantly decreased darinaparsin cytotoxicity under both normoxia and hypoxia (\( P < 0.0001 \) for the interaction of CHX and darinaparsin by 2-way ANOVA, Supplementary Fig. S6), indicating that darinaparsin activity required de novo protein expression. Next, darinaparsin effects on hypoxia-induced factor I\( \alpha \) and c-jun—NH\(_2\)—kinase were studied because of their roles as a regulator of cell survival and radiosensitivity under hypoxia (1) and a mediator of darinaparsin cytotoxicity in leukemia cells (15), respectively. The data indicate that neither factor likely plays a significant role in darinaparsin-mediated cytotoxicity in solid tumor cells under hypoxia (Supplementary Figs. S7 and S8).

Gene expression profiling was carried out with HI-LAPC-4 cells after 4 and 8 hours incubation with darinaparsin or ATO at the concentrations of IC\(_{50}/4 \) hours (Fig. 1A) under normoxia. Among the 16,433 genes analyzed, 343 (up) and 113 (down) genes were regulated by darinaparsin, 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 reverse-transcription and quantitative PCR assay of independently treated samples (Supplementary Table S4A and S4B). An Ingenuity Pathway analysis showed that darinaparsin affected multiple biological processes and pathways that involve cancer, inflammation, and apoptosis (Supplementary Table S4C). Transcription Factor Target Enrichment Analysis identified transcription factors in which target gene expression was significantly affected by darinaparsin (DPS/CON) and ATO (ATO/CON), many of which were associated with oncogene regulated pathways (Supplementary Table S4D).

To further assess the effect of darinaparsin 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 darinaparsin-affected genes (Fig. 6). Specifically, darinaparsin significantly decreased the expression of genes that were upregulated by RAS, and increased the expression of genes that were downregulated 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 (Supplementary Fig. S9). The data are consistent with previous reports of increased MYC and RAS expression in solid tumor cells (34) and suggest a selective effect of darinaparsin on cancer cells via oncogenic pathways.
Connectivity Map analysis (Supplementary Table S4E) was used to compare gene expression changes induced by darinaparsin 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 darinaparsin or ATO, again suggesting differential mechanisms of action for these 2 arsenicals. Many of the darinaparsin-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 darinaparsin 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 darinaparsin effectively inhibited the growth of a variety of tumor cell lines in vitro under hypoxia and in vivo in xenograft tumor models. Importantly, darinaparsin 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, darinaparsin actually radioprotected the GI epithelium, suggesting that darinaparsin 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, darinaparsin is more promising for the treatment of solid tumors. Although darinaparsin, 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 darinaparsin from ATO is insufficient to fully explain the antitumor effects observed with darinaparsin alone.

Currently, most of the information about the mechanism of action of darinaparsin comes from malignant lymphohematopoietic cell lines, in which oxidative stress plays an important role. However, under hypoxia (0.5% O₂), darinaparsin did not induce oxidative stress and DNA damage in solid tumor cells. We therefore conducted mechanistic studies to elucidate other possible mechanisms of action for darinaparsin. In contrast to ATO, darinaparsin 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 darinaparsin on cancer as compared with normal cells, as well as the decreased systemic toxicity of darinaparsin compared with ATO. In addition, because 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 darinaparsin 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 darinaparsin 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; ref. 41–44), has been reported to protect intestinal cells from radiation damage via cell-cycle arrest (45). It is therefore possible that darinaparsin 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 darinaparsin on normal cells as compared to tumor cells.

In summary, the results presented here show that darinaparsin has significant cytotoxic and radiosensitizing activities against normoxic and hypoxic solid tumor cells in vitro and in vivo. Moreover, darinaparsin does not sensitize bone marrow, and protected normal intestinal epithelium from the effects of radiation in vivo, suggesting that darinaparsin has the potential to significantly increase the therapeutic index of radiation therapy. The favorable toxicity profile of darinaparsin 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. Because darinaparsin is already in clinical trials, these results have near term translational potential.

Disclosure of Potential Conflicts of Interest

J. Tian received commercial research grant from ZIOPHARM Oncology. S.J. Knox received commercial research grant from ZIOPHARM Oncology, Inc. and is a consultant and an advisory board member of InterWest Partners. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Acquisition of data: J. Tian, H. Zhao, S.W. Reese, S.R. Young, X. Li, D.M. Peehl, S.J. Knox

Analysis and interpretation of data: J. Tian, H. Zhao, D.M. Peehl, S.J. Knox

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