Darinaparsin Inhibits Prostate Tumor-Initiating Cells and Du145 Xenografts and Is an Inhibitor of Hedgehog Signaling

Nitu Bansal1, Nadine Johnson Farley1, Lisa Wu1, Jonathan Lewis2, Hagop Youssoufian2, and Joseph R. Bertino1

Abstract

Prostate cancer is the leading cause of cancer-related death in men in the United States. A major cause of drug resistance in prostate and other epithelial tumors may be due to the presence of a fraction of tumor cells that retain the ability to initiate tumors and hence are termed tumor-initiating cells (TIC) or cancer stem cells. Here, we report that darinaparsin, an organic derivative of arsenic trioxide, is cytotoxic to prostate cancer cell lines as well as fresh prostate cancer cells from patients at low micromolar concentrations, and importantly inhibits the TIC subpopulations. It also inhibits growth of the castrate-resistant Du145 prostate tumor propagated as xenograft in mice and inhibits the tumor-initiating potential of prostate cancer cells. Although the mechanism by which darinaparsin acts is not completely known, we show that it kills prostate cancer cells by blocking cells in the G2–M phase of the cell cycle and inhibits Hedgehog signaling by downregulating Gli-2 transcriptional activity. These data provide a rationale for evaluating darinaparsin in patients with castrate-resistant prostate cancer. Mol Cancer Ther; 14(1); 23–30. ©2014 AACR.

Introduction

Arsenic derivatives have been therapeutically used for more than 2,000 years. In the early 20th century, use of arsenic trioxide (ATO) in treating leukemia was first reported, and by the mid-20th century, its effectiveness in patients with relapsed acute promyelocytic leukemia (APL) was demonstrated (1). A randomized clinical trial in United States led to FDA approval of ATO for relapsed or refractory APL (1). Recently, the combination of ATO and all trans-retinoic acid has been recommended as first-line treatment of APL (2). ATO has been investigated in the treatment of other non-APL cancers; however, it was less effective at clinically relevant doses and was highly toxic at higher concentrations (3). Therefore, other arsenicals with antitumor activity and with less toxicity and oral availability have been sought. Darinaparsin is an organic arsenic (S-dimethylarsinosiglutathione; Z-101) made by conjugating dimethylarsenic to glutathione (4, 5). Screening of the NCI-60 panel of cells indicated that Ic50 concentrations with a 50-fold higher than that of ATO. Darinaparsin in patients with advanced refractory solid tumors showed that 300 mg/m2 i.v. for 5 consecutive days every 4 weeks was well tolerated (6). Phase II studies in both hematologic malignancies and solid cancers are currently under way (7, 8). In vitro studies showed that darinaparsin more potently induces growth arrest, apoptosis, and oxidative stress than ATO in several hematologic malignancies (8). Also, unlike ATO, it induces growth arrest, apoptosis, and oxidative stress than ATO in several hematologic malignancies (8). Also, unlike ATO, it does not increase bcl-2 protein levels. Importantly, darinaparsin is effective in ATO-resistant leukemic cell lines that over-express multidrug-resistant protein 1/ATP-binding cassette, subfamily C, member 1 (MRP1/ABCC1; refs. 3, 9). Darinaparsin showed increased antiangiogenic activity in both in vitro human umbilical vascular endothelial cell microtubule formation and in vivo Matrigel plug models (10). Recent investigations have also shown that darinaparsin is a multivalent molecule that can induce an incomplete stress response by disrupting microtubules and sonic hedgehog (Shh) signaling (11).

In this study, we examined the cytotoxic effects of darinaparsin in both established prostate cancer cell lines and in fresh prostate cancer cells from patients. Here, we show that darinaparsin is a potent cytotoxic against various human prostate cancer cell lines as well as primary prostate cancer cells and is effective in inhibiting prostate tumor-initiating cells (TIC). Studies with the Du145 cell line also showed synergistic cell kill with taxotere. Du145 tumors propagated in nude mice were also sensitive to darinaparsin. Prompted by studies showing that ATO inhibited Hedgehog signaling (12), we show that darinaparsin also inhibits Hedgehog signaling in prostate cancer by downregulating Gli-2 transcriptional activity.

Materials and Methods

Reagents

Collagen I (rat tail collagen) was purchased from BD Biosciences, darinaparsin/Z101 was obtained from Ziopharm Oncology Inc., NOD/SCIDγ mice were purchased from The Jackson Laboratory, Gli-2, GAPDH, and β-tubulin antibodies...
were purchased from Cell Signaling Technology, α2-FITC, CD44-APC (BD Biosciences), SAG (Smoothened agonist), and ATO were purchased from Sigma-Aldrich. Gli-2 luciferase reporter plasmid was obtained as a kind gift from P.A. Beachy (Stanford University School of Medicine, Stanford, CA).

Cell culture
Du145, LnCap, PC3, and CWR22 cells were purchased from the ATCC in 2010. The ATCC uses isoenzymology method for species determination and short-tandem repeats methods for identity verification of the cell line. The cells were also tested for Mycoplasma (http://www.atcc.org/~/media/PDFs/Technical%20Bulletins/tb08.ashx). Upon receipt, the cells were maintained at low passage numbers in RPMI (Gibco BRL) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. The cells were routinely tested for Mycoplasma contamination in the laboratory using the Lonza Mycoplasma Testing Kit. No other method was used to authenticate the cells.

Isolation of primary prostate cells from prostate tumor tissues
Human primary prostate tumors were obtained from the Robert Wood Johnson Hospital (New Brunswick, NJ) after prostatectomy on an Institutional Review Board (IRB)–approved protocol. To obtain single cells from fresh prostate tumors, the specimen was minced in small pieces and incubated in 200 µg/ml of collagenase I in RPMI for 2 to 4 hours, the longer time for larger size specimens. After 4 hours, the media containing the minced tissue was strained and the supernatant was collected and washed twice in 1× PBS at 250 × g for 30 seconds (this step eliminates the fibroblasts from the epithelial cells). The pellet was suspended in PROSTAlife medium (Life Science Technologies) and plated in a flask. The cells were incubated at 37°C for 3 to 4 days to allow the epithelial organoids to attach. The media was replaced and cells passaged upon 70% confluence. The cells were stained with anti-EPCAM and anti-androgen receptor antibodies and analyzed with flow cytometry to confirm that the isolated cells were prostate epithelial cells.

Collagen attachment assay
For enrichment of prostate TICs from early-passage Du145 cells, a collagen I attachment assay was used to harvest 5-minute attached cells (13). These cells, enriched for TICs, were maintained in keratinocyte serum-free medium (KSFm) supplemented with epidermal growth factor (EGF) and bovine pituitary extract (13). Briefly, tissue culture plates were coated with 70 µg collagen I

Figure 1.
Prostate cancer cells are sensitive to Z101 (darinaparsin) and ATO in micromolar concentrations. A, prostate cancer cell lines LnCap, Du145, and PC3 were plated in 96-well plates. Twenty-four hours later, cells were treated with darinaparsin and ATO at different concentrations. Seventy-two hours after treatment, MTS reagent was added and color change was monitored at 490 nm. Data were analyzed using prism software. B, primary prostate tumor cells isolated from the prostate of 5 different patients with Gleason score 8–9 were also plated in 96-well plates and treated with darinaparsin (see Materials and Methods). After 72 hours, MTS reagent was added and color change was monitored at 490 nm. Data were analyzed using prism software.
IC50 concentrations were added to the spheroids. After 48 or 72 hours were collected and counted. Equal numbers of untreated and treated cells were plated on collagen I-coated dishes for 5 minutes. Attached cells were counted and calculated as a percentage of total cells. The graph represents the average values of three independent experiments. Error bars, ±SEM; P ≤ 0.05 is considered statistically significant. A, darinaparsin decreases the number of cells that attach on collagen I-coated dishes for 5 minutes. The 5-minute attached cells were collected after trypsinization (0.5% trypsin). The unattached cells after 20 minutes were also collected and used as a control (non-TICs).

For three-dimensional spheroid formation, 5-minute attached tumor cells enriched for TIC’s and 20-minute nonattached cells were suspended and plated in 1% agarose-coated 6-well plates. Every 3 days, half of the media was supplemented with fresh KSFM. For drug treatment assays, drugs at specified IC50 concentrations were added to the spheroids. After 48 or 72 hours, spheroids were collected and dissociated and used for colony-forming assays. For colony formation assay from the spheroids, 48 or 72 hours after drug treatment, floating cell spheroids were collected, washed, and dissociated with 0.1% trypsin to form single cells. Equal numbers of single cells (25–300 cells) were then plated in 6-well tissue culture plates. After 14 days, colonies were stained with 0.25% crystal violet in 95% ethanol. Colonies consisting of more than 50 cells were counted.

Cytotoxicity assay

Darinaparsin, PC3, and LnCap prostate cancer cells and primary prostate cells were plated at a density of 3,500 cells/200 µL in 96-well plates. Twenty-four hours after plating, darinaparsin, taxotere and other drugs were added at various concentrations. Seventy-two hours after treatment, the MTS reagent was added (Promega) to the cells. Color change was monitored at 490 nm and data were acquired by SOFTmax pro. Cell viability and IC50 concentrations were determined using PRISM software. For synergy experiment, CalcuSyn software was used to determine the combination index (CI) values between taxotere and darinaparsin (14).

Flow cytometry

Flow cytometry using FACS was used to determine cell-surface markers: α2-FITC and CD44-APC. Data were analyzed using the CellQuest software. For cell-cycle analysis, the cells were collected at 72 hours, washed, and fixed in ethanol overnight. The fixed cells were then washed and stained with PI-RNase (BD Pharmigen) and cells were acquired using flow cytometry. The data were analyzed using MODFIT software.
Sixteen hours after plating, cells were cotransfected with 0.5 μg pcDNA-Gli2 and 0.5 μg 8xGli-luciferase reporter. After 8 hours of transfection, cells were treated with a Shh agonist (10 nmol/L SAG from Sigma-Aldrich) with or without darinaparsin for 24 hours. Luciferase assays were performed with the Promega Luciferase dosage and schedule as described above for Du145 cells. Results involving PC3 cells, 2 × 10^6 cells in 100 μL of PBS were injected subcutaneously into the right flank of NOD/SCIDBy mice. Once tumors were palpable, the mice were randomized into two groups (n = 4), control and darinaparsin. Mice were treated with the same dosage and schedule as above for Du145 cells. Results are presented as mean ± SEM.

Statistical analysis
All experiments were performed three times, and each experiment was done in triplicate. Statistical analysis was performed using Prism software (GraphPad). In all cases, ANOVA followed by two-tailed, paired Student t tests were performed to analyze statistical differences between groups. P values of <0.05 were considered statistically significant.

Results
Darinaparsin is toxic to prostate cancer cells
Prostate cancer cell lines LnCap, Du145, and PC3 cells were treated with darinaparsin or ATO at various concentrations for 72 hours. After 72 hours, cell viability was measured using the MTS, and as shown in Fig. 1A, all three cell lines were sensitive to both darinaparsin and ATO; IC_{50} concentrations of Du145, PC3, and LnCap cells ranged from approximately 5 to 10 μmol/L. Du145 and LnCap cells were more sensitive to darinaparsin than ATO, while PC3 cells were equally sensitive to both darinaparsin and ATO. Similar to the prostate cancer cell lines, primary prostate cancer cells isolated from five different patients (Gleason score 8) were equally sensitive to darinaparsin with IC_{50} concentrations ranging from 2.5 to 20 μmol/L (Fig. 1B). Primary prostate cancer cells isolated from primary prostate tumors were confirmed for the prostate epithelial cells by testing for EpCam and AR markers (Supplementary Fig. S1A and S1B).
Darinaparsin reduces the number of the tumor-initiating subpopulation of cells

Relapse in most solid tumors after conventional chemotherapy has been attributed to the existence of drug-resistant TICs. We previously developed a novel assay for TIC prostate tumor enrichment based on rapid adherence of TICs to collagen I (13). In brief, cells that attach on collagen I have high expression of CD44 and α2β1 (α2β1hi/CD44hi) as compared with 20-minute unattached cells (α2β1low/CD44low). We also showed that α2β1low/CD44hi cells have increased colony forming, migration, and adhesion ability and can form tumors in nude mice and are the TICs. As shown in Fig. 2A, darinaparsin treatment at an IC50 concentration or 2-fold higher reduced the number of Du145 cells and PC3 cells that attached on collagen I after 5 minutes. Darinaparsin-treated cells as compared with taxotere or ATO-treated cells had fewer α2β1low/CD44hi cells when grown as monolayer (Fig. 2B) or as spheroids (Fig. 2C; IC50 of darinaparsin in spheroids from Du145 was determined by the MTS assay; Supplementary Fig. S2). Dot plots are in Supplementary Figs. S3 and S4. Darinaparsin treatment also reduced secondary colonies formed from dissociating α2β1low/CD44hi spheroids (Fig. 3A), indicating an effect on the TICs. Darinaparsin also decreased levels of CD44hi and α2β1hi cells (Fig. 3B and C) from primary prostate cells, further validating the inhibitory effect of darinaparsin on TICs.

Effect of darinaparsin on Shh signaling

Darinaparsin induces an incomplete stress response with disruption of microtubules and Shh signaling (11). As darinaparsin is a potent inhibitor of growth of Du145 cells, we tested the effect of darinaparsin on Shh signaling. Prostate cancer-initiating cells from Du145 cultures (early-passage cells) isolated by the collagen attachment assay showed an increase in Gli-2 levels as compared with the parental or 20 minutes unattached β1low/CD44low cells, at both the protein and mRNA level (Fig. 4A, i and ii). Furthermore, treatment of cells with darinaparsin decreased the protein levels of Gli-2 (Fig. 4B). Interestingly, Gli-2 levels were decreased at 1.5 μmol/L concentration but not at 3 μmol/L. This experiment was repeated three times and the same pattern was observed. The mechanism by which Gli2 levels come back may be due to some feedback mechanism. We also investigated whether darinaparsin inhibits Gli-2 activity by measuring its effect on the Gli responsive luciferase promoter pGL38xGli, which contains 8 GLI DNA-binding sites attached to the chicken lens crystalline promoter followed by the luciferase gene (12). Du145 cells were transfected with the luciferase plasmid and cotransfected with pcDNA-Gli-2 plasmids. After 8 hours of transfection, cells were treated with a SAG and cotreated with darinaparsin or ATO or left untreated. Twenty-four hours later, cells were harvested and luciferase activity was assayed. The luciferase levels were normalized with total protein. As shown in Fig. 5A, such as ATO (15), darinaparsin also inhibited Gli transcriptional activity compared with vehicle control. The effect of darinaparsin on the cell cycle was also examined. Darinaparsin blocks the cells in G2–M phase of cell cycle and but did not induce apoptosis (Fig. 5B). Therefore, the cytotoxic effect of darinaparsin may be attributed to the block in the G2–M phase as well as inhibition of Gli2.

Darinaparsin inhibits growth of the Du145 and PC3 prostate tumors

To show the antitumor effect of darinaparsin and to determine whether the combination of darinaparsin with taxotere produced additive or synergistic antitumor effects in vivo, nude mice bearing the Du145 tumor were injected with darinaparsin...
Effect of darinaparsin on tumor growth initiated by TICs

Every other day for 5 times, taxotere every once a week for three times, or with both taxotere and darinaparsin in the same doses and schedule to determine toxicity. Interestingly, darinaparsin inhibited tumor growth as effectively as taxotere. However, unlike the in vitro study (Supplementary Fig. S5), the combination of taxotere and darinaparsin did not have more of an effect than either of the drugs alone (Fig. 6A). Neither drug, nor the combination caused toxicity, was measured by weight loss (Fig. 6B). We also tested the efficacy of darinaparsin in human PC3 xenografts, also castrate resistant, to determine whether other prostate cancers also were inhibited by darinaparsin. As shown in Fig. 6C, darinaparsin treatment inhibited tumor growth without evidence of toxicity, as measured by a decrease in body weight (Fig. 6D).

Effect of darinaparsin on tumor growth initiated by TICs

Mice were injected with 20,000 α2β1hi/CD44hi cells (enriched for TICs) or with 20,000 α2β1low/CD44low cells (depleted of TICs) and treated with darinaparsin or with taxotere before tumors were palpable (day 1 after implantation). As shown in Table 1, only 3 of 8 darinaparsin-treated mice developed tumors, whereas 5 of 8 taxotere-treated mice developed tumors. Six of 8 untreated mice formed tumors (Table 1).

### Table 1. Darinaparsin inhibits tumor growth initiated by TICs

<table>
<thead>
<tr>
<th>Cells (10,000)</th>
<th>Tumors remaining</th>
<th>Drugs injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2β1hi/CD44hi</td>
<td>6/8</td>
<td>Untreated</td>
</tr>
<tr>
<td>α2β1low/CD44low</td>
<td>5/8</td>
<td>Taxotere-6 mg/kg</td>
</tr>
<tr>
<td>α2β1low/CD44low</td>
<td>3/8</td>
<td>DAR-100 mg/kg</td>
</tr>
</tbody>
</table>

NOTE: 20,000 α2β1hi cells (TICs fraction) mixed with Matrigel were injected s.c. into the abdominal flank of nude mice. Staring on day 2, the drugs were injected as described; taxotere was given once a week for 3 weeks and darinaparsin was administered every other day for six times. The tumor growth was monitored every day for 45 days. (n = 8, see in Materials and Methods).

Discussion

In this study, we report that darinaparsin is effective in killing both prostate cancer cells from cell lines as well as cells from fresh prostate tumors at low micromolar concentrations. The IC_{50} concentration of darinaparsin was lower than ATO in Du145 and LnCaP cells, whereas in PC3 cells, IC_{50} of both darinaparsin and ATO were similar. Importantly, darinaparsin was efficient in reducing the number of α2β1hi/CD44hi and reducing the number of primary and secondary colonies formed from α2β1hi/CD44hi spheroids, indicating that darinaparsin and ATO are cytotoxic to TICs, and darinaparsin is more potent. We previously showed that clinically used chemotherapeutic drugs did not affect TICs from Du145 TICs (12). ATO antagonizes Shh signaling by reducing Gli2, a transcriptional effector (12). ATO treatment inhibits the cytosolic accumulation of Gli2 and hence blocks trafficking required for activation. In addition, ATO caused a reduction of overall levels of Gli2. Moreover, in SMO inhibitor–resistant cells, ATO efficiently inhibited tumor growth either alone or in combination with iraconazole, a compound that also inhibited Shh signaling (16). In this study, we show that darinaparsin also inhibited Shh signaling. We also show that darinaparsin blocks cell in G2–M. Reports showing that darinaparsin inhibits microtubule polymerization and induces stress granule formation (11) may be the mechanism by which it blocks cells in G2–M (Fig. 3D).

The mechanism by which darinaparsin kills cells appears to be complex. In hematopoietic cell lines, an increase in oxidative stress was shown to play an important role in darinaparsin action. In solid cancers, gene expression studies comparing darinaparsin to ATO (17) showed that darinaparsin decreased the expression of genes that were upregulated by Myc. In this study, we show that darinaparsin inhibited growth of two human prostate cancer xenografts in nude mice and reduced the number of mice with tumors that were initiated with TICs. However, in contrast to the in vitro results that...
showed synergistic cell kill between taxotere and darinaparsin (Supplementary Fig. S3), the in vivo study with a combination of taxotere and darinaparsin was not even additive. A possible explanation for lack of additivity or synergy is that darinaparsin and taxotere both block cells in G2–M. It is also possible that different doses and schedules would cause additive or synergistic effects.

Itraconazole, also inhibits Shh signaling by inhibiting downstream gli-2 transcription, such as ATO and darinaparsin, (16). In a phase II study, itraconazole showed only modest antitumor activity, and dose escalation was limited by toxicity (15).

The development of an oral form of darinaparsin and evidence of antitumor activity in phase I and II trials (6, 18), together with the results of this study, provide a rationale for evaluating darinaparsin in patients with castrate-resistant prostate cancer.

Disclosure of Potential Conflict of Interest

J. Lewis has ownership interest (including patents) in Ziopharm Oncology Inc. J.R. Bertino reports receiving a commercial research grant from and is a consultant/advisory board member for Scientific Review Committee of Ziopharm Oncology, Inc. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

The authors thank Dr. P.A. Beachy for providing them with 8xGli-luciferase reporter plasmid construct.

Figure 6.

Xenograft studies with darinaparsin. NOD/SCIDγ were injected with Du145 cells, once the tumor was palpable; mice were randomized into four groups (n = 8 per group). Control (group 1), darinaparsin (group 2), taxotere (group 3), and darinaparsin and taxotere (group 4). Darinaparsin group was injected every other day 7 days with darinaparsin at 100 mg/kg; taxotere group was injected with taxotere at dose of 6 mg/kg once a week for 2 weeks; and the combination group got darinaparsin and taxotere as mentioned above. A, darinaparsin suppresses growth of a Du145 tumor xenograft; tumor volume was measured three times a week, and the tumor volume calculated using the formula width2 × length/2. Results are presented as mean ± SEM. B, the mice were weighed every day for 28 days; the graph represents average weight of 8 mice in each group. C, NOD/SCIDγ were injected with PC3 cells, once the tumor was palpable; mice were randomized into two groups. Control and Darinaparsin. Darinaparsin group was injected with similar dosage and schedule as mentioned for Du145 cells. Darinaparsin also suppresses growth of a PC3 tumor xenograft; tumor volume was measured and calculated as mentioned for Du145 cells. D, the mice were weighed every other day for 15 days; the graph represents average of weight of 8 mice in each group. Results are presented as mean ± the SEM. Error bars, ±SEM. *, P ≤ 0.05.
Grant Support

This work was supported by a grant from Ziopharm Oncology Inc. to J.R. Bertino.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 17, 2013; revised October 21, 2014; accepted October 25, 2014; published OnlineFirst November 7, 2014.

References

Molecular Cancer Therapeutics

Darinaparsin Inhibits Prostate Tumor–Initiating Cells and Du145 Xenografts and Is an Inhibitor of Hedgehog Signaling


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-1040

Cited articles
This article cites 17 articles, 8 of which you can access for free at:
http://mct.aacrjournals.org/content/14/1/23.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.