The Novel Organic Arsenical Darinaparsin Induces MAPK-Mediated and SHP1-Dependent Cell Death in T-cell Lymphoma and Hodgkin Lymphoma Cells and Human Xenograft Models

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Abstract

**Purpose:** Darinaparsin (Zio-101) is a novel organic arsenical compound with encouraging clinical activity in relapsed/refractory T-cell lymphoma (TCL) and Hodgkin lymphoma (HL); however, little is known about its mechanism of action.

**Experimental Design:** TCL cell lines (Jurkat, Hut78, and HH) and HL cell lines (L428, L540, and L1236) were examined for *in vitro* cell death by MTT assay and Annexin V–based flow cytometry. Jurkat and L540-derived xenografts in SCID mice were examined for *in vivo* tumor inhibition and survival. Biologic effects of darinaparsin on the MAPK pathway were investigated using pharmacologic inhibitors, RNAi and transient transfection for overexpression for SHP1 and MEK.

**Results:** Darinaparsin treatment resulted in time- and dose-dependent cytotoxicity and apoptosis in all TCL and HL cell lines. In addition, darinaparsin had more rapid, higher, and sustained intracellular arsenic levels compared with arsenic trioxide via mass spectrometry. In *in vivo* experiments with Jurkat (TCL) and L540 (HL)-derived lymphoma xenografts showed significant inhibition of tumor growth and improved survival in darinaparsin-treated SCID mice. Biologically, darinaparsin caused phosphorylation of ERK (and relevant downstream substrates) primarily by decreasing the inhibitory SHP1 phosphatase and coimmunoprecipitation showed significant ERK/SHP1 interaction. Furthermore, ERK shRNA knockdown or constitutive overexpression of SHP1 resulted in increased apoptosis, whereas cotreatment with pharmacologic MEK inhibitors resulted in synergistic cell death. Conversely, SHP1 blockade (via pharmacologic inhibition or RNAi) and MEK constitutive activation decreased darinaparsin-related cell death.

**Conclusions:** Altogether, these data show that darinaparsin is highly active in HL and TCL and its activity is dependent primarily on MAPK mechanisms. Clin Cancer Res; 20(23); 6023–33. ©2014 AACR.

Introduction

Although many patients with Hodgkin lymphoma (HL) are cured by conventional chemotherapy, there continues to be a subset of patients with refractory disease or relapse. Furthermore, there remains a need to continue to identify targeted, less toxic therapy for the treatment of HL. T-cell lymphomas (TCL) are uncommon and aggressive non-Hodgkin lymphoma that are difficult to treat. Long-term survival rates are <30% to 35% (1). New and targeted therapeutics are desired for the treatment of these lymphomas.

Darinaparsin (S-dimethylarsino-glutathione) is a novel organic arsenical compound reported to have encouraging clinical activity, including several complete remission, in patients with relapsed or refractory TCL and HL (2). Darinaparsin was initially discovered as a cytotoxic product derived from arsenic metabolism (3). Subsequent *in vitro* and *in vivo* experiments with leukemia showed that darinaparsin was a potent antineoplastic agent (4, 5). In addition, early-phase clinical trials in patients with hematologic malignancies demonstrated that darinaparsin is safer and effective compared with inorganic arsenic trioxide (ATO; refs. 6–8). Moreover, it is known that...
Translational Relevance

The organic arsenical, darinaparsin, has shown promising anticancer activity in multiple tumor types and is at various stages of clinical trials, however, its biologic actions and mechanisms of resistance are poorly understood. We examined the effects of darinaparsin in T cell and Hodgkin lymphoma (HL) cell lines and demonstrated ERK phosphorylation and lack of SHP1 as likely mechanisms of resistance, whereas ERK inhibition or restoration of SHP1 function resulted in sensitizing the resistant TCL and HL tumor xenografts in SCID mice. Investigations into biologic activities of darinaparsin demonstrated ERK phosphorylation and lack of SHP1 as likely mechanisms of resistance, whereas ERK inhibition or restoration of SHP1 function resulted in sensitizing the resistant TCL and HL tumor xenografts in SCID mice. Furthermore, we show that darinaparsin treatment resulted in in vivo tumor xenografts in SCID mice. Investigations into biologic activities of darinaparsin demonstrated ERK phosphorylation and lack of SHP1 as likely mechanisms of resistance, whereas ERK inhibition or restoration of SHP1 function resulted in sensitizing the resistant TCL and HL tumor xenografts in SCID mice. Furthermore, we show that darinaparsin treatment resulted in in vivo tumor xenografts in SCID mice. Investigations into biologic activities of darinaparsin demonstrated ERK phosphorylation and lack of SHP1 as likely mechanisms of resistance, whereas ERK inhibition or restoration of SHP1 function resulted in sensitizing the resistant TCL and HL tumor xenografts in SCID mice. Furthermore, we show that darinaparsin treatment resulted in in vivo tumor xenografts in SCID mice. Investigations into biologic activities of darinaparsin demonstrated ERK phosphorylation and lack of SHP1 as likely mechanisms of resistance, whereas ERK inhibition or restoration of SHP1 function resulted in sensitizing the resistant TCL and HL tumor xenografts in SCID mice. Furthermore, we show that darinaparsin treatment resulted in in vivo tumor xenografts in SCID mice. Investigations into biologic activities of darinaparsin demonstrated ERK phosphorylation and lack of SHP1 as likely mechanisms of resistance, whereas ERK inhibition or restoration of SHP1 function resulted in sensitizing the resistant TCL and HL tumor xenografts in SCID mice. Furthermore, we show that darinaparsin treatment resulted in in vivo tumor xenografts in SCID mice. Investigations into biologic activities of darinaparsin demonstrated ERK phosphorylation and lack of SHP1 as likely mechanisms of resistance, whereas ERK inhibition or restoration of SHP1 function resulted in sensitizing the resistant TCL and HL tumor xenografts in SCID mice. Furthermore, we show that darinaparsin treatment resulted in in vivo tumor xenografts in SCID mice. Investigations into biologic activities of darinaparsin demonstrated ERK phosphorylation and lack of SHP1 as likely mechanisms of resistance, whereas ERK inhibition or restoration of SHP1 function resulted in sensitizing the resistant TCL and HL tumor xenografts in SCID mice. Further...
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Bim, c-myc, eIF4E (Ser209), 4E-BP1 (Thr37/46), total and cleaved caspase-3, -8, -9, and PARP were purchased from Cell Signaling Technology. For coimmunoprecipitation experiment, 200 μg of protein lysate was used, precleared with Protein A agarose beads (Cell Signaling Technology) followed by overnight incubation with primary antibody (SHP1 or ERK1/2, 1:50 dilution, validated for immunoprecipitation, Cell Signaling Technology). Followed by precipitation with 30 μL of Protein A agarose beads (50% slurry). Beads were washed five times with 1 mL of NaCl lysis buffer by centrifugation, resuspended in 30 μL of 3 × Laemmli sample buffer, heated at 95°C for 5 minutes, and used for Western blot analysis.

Lymphoma xenograft experiments

For determining the in vivo effect of dariparsin, human lymphoma xenografts grown in SCID mouse models were utilized. Jurkat (TCL) or L540 (HL) cells were implanted at a density of 5 × 10^6 subcutaneously in CB-17 SCID mice, once the tumor volume reaches 100 to 250 mm^3. Dariparsin was administered and compared with vehicle treatment mice, with approximately 8 mice for each group. On the basis of clinical observations and effects on tumor growth with the administration of varying doses of dariparsin (500, 200, 100, or 70 mg/kg) in SCID mice bearing L540 (HL) tumor xenografts, in the treatment for Jurkat (TCL)-derived xenograft, dariparsin dosage was adjusted to 70 or 40 mg/kg. Jurkat (TCL)-derived tumor xenograft-bearing SCID mice received either saline (control) or dariparsin (40 or 70 mg/kg) by intraperitoneal (i.p.) injections daily for 5 days. L540 (HL)-derived tumor xenograft-bearing SCID mice received 100 mg/kg dariparsin subcutaneously daily for initial 5 days and 70 mg/kg i.p. on days 9, 10, and 11. Animals were observed daily and tumor volume and body weight were measured twice weekly for 4 to 6 weeks. Tumor volumes were determined using calipers and calculated, as described previously (10). Mice were euthanized when tumors reached 2,000 mm^3 and were considered as expired at this point for survival analysis. For statistical analysis, a two-way repeated measurement ANOVA was performed using GraphPad Prism 5.0, (GraphPad Software) to analyze tumor growth in both the control and treatment groups. A log-rank statistical analysis was used for comparison of survival benefit between the two groups. Kaplan–Meier graph was used to plot survival curve; P values <0.05 were considered statistically significant.

Results

Dariparsin enhances intracellular accumulation of arsenic and induces cytotoxicity in HL and TCL cell lines

We investigated intracellular levels of arsenic comparing dariparsin with ATO by mass spectrometry; we observed a rapid increase in the intracellular accumulation of arsenic (27.4 ng/million cells) with dariparsin compared with ATO (arsenic, 1.7 ng/million cells) in Jurkat cells. In L428 HL cells, we noted a 4.7-fold increase in intracellular accumulation of arsenic (42 ng/million cells) with dariparsin compared with ATO (arsenic, 8.9 ng/million cells); this accumulation occurred within one hour and remained sustained for more than 6 hours (Fig. 1A). ATO treatment resulted in only a gradual and less prominent increase in intracellular arsenic concentration, suggesting that dariparsin achieves much more rapid and sustained intracellular arsenic levels compared with ATO.

Cell viability following treatment with dariparsin in TCL cell lines (Jurkat, Hut78, and HH) and HL cell lines (L428, L540, L1236) was determined via MTT assay. Treatment with increasing concentrations of dariparsin (1–5 μmol/L) for 72 hours resulted in a dose-dependent decrease in cell viability in all cell lines (Fig. 1B). However, the level of sensitivity to dariparsin at 72 hours varied among TCL and HL cell lines. Jurkat cells were observed to be most sensitive with associated IC_{50} of 2.7 μmol/L compared with HH and Hut78 (3.2 and 6.7 μmol/L, respectively); L540 cell line was the most sensitive HL cell line to dariparsin (IC_{50} 1.3 μmol/L compared with L1236 and L428 at 2.8 and 7.2 μmol/L, respectively).
Darinaparsin induces cell-cycle arrest and apoptosis HL and TCL cell lines

Darinaparsin (1–3 μmol/L) treatment resulted in accumulation of cells predominantly at G2 cell cycle in both Jurkat and L428 cell lines (Fig. 2A). In Jurkat, treatment with 1 or 3 μmol/L resulted in accumulation of cells at G2 with 26% and 13%, respectively, compared with presence of 11% G2 cells in the untreated control (Fig. 2A). The decline in G2 with increased darinaparsin concentrations is likely due to decreased cell viability and associated increased apoptosis at higher dosing. In L428, treatment with 1 or 3 μmol/L darinaparsin resulted in a significant accumulation of 29% (P<0.001) and 74% (P<0.0001) cells, respectively, at G2 compared with 10% in untreated control. Notably, treatment with 1 or 3 μmol/L darinaparsin in L428 (HL) resulted only in a moderate effect on cell viability.
compared with other HL cell lines (Fig. 1B). Therefore, these results suggest that perhaps cell-cycle arrest with darinaparsin in L428 may affect the cytotoxic response in this cell line. Because darinaparsin resulted in a cytotoxic response in TCL and HL, we next investigated whether the observed cytotoxic response was mediated by apoptosis.

Treatment with increasing concentrations of darinaparsin (1–5 μmol/L) resulted in a significant dose-dependent increase in Annexin V positivity in Jurkat (TCL) and L540, Hut78 and L428 (HL) cell lines (Fig. 2B). Although treatment with 2 μmol/L darinaparsin resulted in >50% Annexin V positivity in all cell lines, the amount of Annexin-positive cells were comparatively lower in L428 cells (Fig. 2B), consistent with MTT cell viability findings (Fig. 1B). In addition, darinaparsin resulted in activation of apoptotic caspases and cleavage of PARP. In Jurkat and Hut78, darinaparsin resulted in dose-dependent activation of both extrinsic and intrinsic pathways, which was evident by increasing levels of cleaved caspase-8 and cleaved caspase-9; in HH cells, there was less caspase activation, but PARP degradation was present (Fig. 2C). Treatment of L428 cells with darinaparsin resulted in activation of caspases-3, -8, and -9, while degradation of PARP was not detected (Fig. 2C).

Treatment with darinaparsin inhibits in vivo growth of tumor xenografts and improves survival in SCID mice

The in vivo efficacy of darinaparsin was investigated using tumor xenografts derived using either Jurkat (TCL) or L540 (HL) cell lines. Results from Jurkat-derived xenograft experiments showed with darinaparsin administration a significant 3.6- and 4.3-fold reduction in the average approximate size of the tumor with 40 mg/kg (average tumor size, 532 mm3) and 70 mg/kg (average tumor size, 447 mm3), respectively, compared with the average size of tumor (1,929 mm3) in the vehicle-treated control group ($P$ < 0.0001, two-way repeated ANOVA; Fig. 3A). There was significantly improved survival by Kaplan–Meier in Jurkat, HH, and Hut78, darinaparsin treated with darinaparsin with control ($P$ < 0.0001; Fig. 3B). Although the extent of tumor growth inhibition was similar with 40 or 70 mg/kg of darinaparsin, there was no appreciable change in total body weight in the treatment with 40 mg/kg dose darinaparsin; however, increased dosing to 70 mg/kg initially resulted in a 10% loss of total body weight by day 5 and subsequently returned to normal once treatment was completed (Fig. 3C).

L540-derived SCID xenograft mice initially received 100 mg/kg darinaparsin for 5 days, and based on clinical observations, the dosage subsequently adjusted to 70 mg/kg for days 9, 10, and 11. Treatment with darinaparsin in L540 xenograft SCID mice resulted in a 3.2-fold reduction in the average size of the tumor (tumor size, 619 mm3) compared with control (2,003 mm3) at 4 weeks ($P$ = < 0.001, two-way repeated measurement ANOVA, GraphPad Prism 5; Fig. 3D). Kaplan–Meier survival analysis showed a significant increase in survival with darinaparsin treatment compared with control groups ($P$ < 0.0001, log-rank, Sigma Pad Prism5; Fig. 3E). Treatment with 70 mg/kg initially resulted in 10% loss of total body weight by day 5 and the observed body weight returned to normal once the treatment was completed (Fig. 3F) in a similar manner as observed with Jurkat-derived xenograft tumors.

Effect of darinaparsin on MAPK signaling and other oncogenic pathways

Considering that RAS–RAF–MEK–ERK and PI3K/AKT are major oncogenic pathways involved in lymphoma and that there have been prior data in leukemia linking these biologic pathways to ATO (11, 12), we investigated the impact of darinaparsin on these pathways. Results showed that darinaparsin resulted in increased phosphorylation of AKT in Jurkat cells (Fig. 4), whereas in other TCL lines, there was only minimal increase in phosphorylation of AKT. It must be noted that AKT is constitutively activated in Jurkat due to the lack of PTEN expression, an inhibitory phosphatase that prevents AKT activation (13). In HL cell lines, there were no changes in phosphorylation of AKT.

RAS–RAF–MEK–ERK is another major oncogenic pathway associated with lymphoma (14). Activation of RAS induces RAF-mediated activation of MEK and ERK to promote cell growth and survival through transcriptional regulation of the downstream effectors in this pathway (15). We observed downregulation of BRAF phosphorylation with darinaparsin in Jurkat, HH, and Hut78, and L540, whereas there was no change in L1236 and L428 (Fig. 4). Although the effect of darinaparsin on MEK phosphorylation followed a similar pattern to BRAF response to darinaparsin (Fig. 4), we observed increased ERK1/2 phosphorylation in Jurkat, HH, L1236, and L428 cell lines. In Hut78, ERK1/2 is constitutively present as phosphorylated form and darinaparsin did not result in further activation of ERK1/2 phosphorylation. In L540, although total MEK and ERK1/2 proteins were detectable, there was no apparent phosphorylation under basal condition or with darinaparsin suggesting that the MEK-ERK signaling cascade could be defective in L540.

There are several members of the MAPK phosphatase family including SHP1 (SH2 domain containing protein tyrosine phosphatase), MKP3 (MAPK phosphatase), and PP2A (protein phosphatase) that are known to regulate ERK1/2 phosphorylation (15). Considering such phosphatases could be substrates for darinaparsin activity, we next examined the effect of darinaparsin on SHP1, MKP3, and PP2A. We found that levels of SHP1 were decreased with increasing amounts of darinaparsin in all cell lines, except for L428, where SHP1 was barely detectable (Fig. 4). The observed reduction in SHP1 protein levels seemed to correlate with increased accumulation of phosphorylated ERK. In L428, lack of SHP1 was accompanied by increases in ERK1/2 phosphorylation. MKP3 levels were also decreased in a concentration-dependent manner with darinaparsin in Jurkat, HH, and L428 cell lines, whereas MKP3 protein was barely detectable in Hut78 and L540. The levels of PP2A were decreased with darinaparsin in HH, Hut78, and L540 cells with no effect in Jurkat, L1236, and L428 cells.
We investigated next the functional role of ERK1/2 phosphorylation in the biologic activation of its downstream substrates including c-FOS, Elk, p90 RSK, MYC, and FOXO3a. Darinaparsin resulted in a mild increase in phosphorylation of c-FOS in Jurkat and a strong increase in Hut78. Similarly in HL cell lines, a mild increase phosphorylation of c-FOS was noted in L1236 with a strong increase in L428 (Fig. 4). Darinaparsin did not result in detectable change in the phosphorylation status of Elk protein in any lymphoma cell line (data not shown). Besides Jurkat, there was no effect of darinaparsin on p90 RSK. Downregulation of FOXO3a was observed at higher concentrations of darinaparsin in Jurkat, HH, Hut78, and L428 with FOXO3a being not detectable in L540. Increase in MYC was observed only in Hut78, whereas MYC was downregulated in Jurkat and L1236. Taken together, these results show that phosphorylation of c-FOS and downregulation of FOXO3a in most cell lines correlated with ERK1/2 activation suggesting that ERK1/2 activation might have a functional role in the cellular response to darinaparsin treatment.

Figure 3. Treatment of SCID xenografts mice with darinaparsin inhibits tumor growth and improves survival. Darinaparsin was administered subcutaneously daily for 3 weeks at doses indicated in the chart and further monitored up to 60 days. Compared with control, darinaparsin-treated groups showed significant reduction in the volume of xenograft tumors derived from (A) Jurkat (TCL) or (D) L540 (HL; P < 0.001, two-way repeated ANOVA), represented as line graph Kaplan–Meier survival analysis shows a significant increase in survival benefit with darinaparsin treatment (P < 0.001, log rank, GraphPad Prism5) in the SCID mice bearing xenograft tumors derived from (B) Jurkat (TCL) or (E) L540 (HL) compared with untreated control. Darinaparsin treatment resulted in an initial decrease in total body weight in the tumor-bearing SCID mice and the loss in body weight recovered by the end of the treatment cycle (C and F).
SHP1 and ERK mediate darinaparsin-induced cell death

Although the effect of darinaparsin showed an overall consistent effect on SHP1 levels in all lymphoma cell lines, the effect on MKP3 was observed in only three of six cell lines. Previous studies have shown that SHP1 is a master regulator of several signaling kinases present in the hematopoietic cells (16) and the sensitivity of SHP1 to oxidative stress results in the activation of the MAPK pathway (17, 18). Thus, we focused our investigation on SHP1 in the context of ERK activation. We performed coimmunoprecipitation experiments to determine whether SHP1 and ERK1/2 interact with each other. Results from using anti-ERK1/2 antibody with protein lysates prepared from Hut78 TCL cells showed presence of SHP1 protein, as detected by Western blot-based analysis (Fig. 5A). Conversely, using anti-SHP1 antibody, we detected ERK1/2 protein from the immunocomplex precipitated in Hut78 TCL cell lysates confirming that SHP1 interacts with ERK1/2. The specificity of protein-antibody binding in these immunoprecipitation experiments is demonstrated in the control experiments shown in Supplementary Fig. S1. Furthermore, inhibition of SHP1 using the SHP1/2 inhibitor, NSC87877, or stable knockdown of SHP1 with lentiviral-based shRNA induced phosphorylation of ERK1/2 in Hut78 TCL cells (Fig. 5B and C) suggesting a functional role for SHP1 in modifying ERK1/2 phosphorylation. Similar findings were reported in a previous study showing that downregulation of SHP1 led to sustained increase in ERK1/2 phosphorylation (19). Treatment with darinaparsin in the presence NSC87877 resulted in a significant reduction in phosphorylation (19). Treatment with darinaparsin in the presence SHP1 knockdown with shRNA decreased apoptotic cell death compared with control (P < 0.0001; Fig. 5C), while there was a slight increase in apoptosis with ERK2 knock down in TCL cells (Fig. 5C). These results suggested that SHP1 regulates ERK phosphorylation and SHP1 is required for darinaparsin-induced cell death. Next, we sought to determine whether constitutive ERK activation would affect darinaparsin sensitivity, for this investigation, we performed the following experiment with transient overexpression of constitutively active MEK (MEK-CA) to stimulate ERK activity. As shown in Fig. 5D, forced overexpression with MEK-CA increased basal levels of ERK phosphorylation in Hut78 TCL and together with darinaparsin treatment, there was a significant decrease in apoptosis (P < 0.0001) observed (Fig. 5D).
suggested that constitutive ERK activation would reduce darinaparsin sensitivity.

Decreased sensitivity to darinaparsin was observed in L428 HL cells with constitutive ERK activation, whereas SHP1 was barely detectable in this cell line. We sought to determine whether inhibition of ERK1/2 or overexpression of SHP1 in L428 would restore sensitivity to darinaparsin treatment in L428 HL cells. Using selective MEK small-molecule inhibitors (i.e., U0126 and AZD6244) to block ERK1/2 phosphorylation followed by subsequent darinaparsin, we noted a significant increase in apoptosis using either agent compared with darinaparsin alone ($P < 0.005$; Fig. 6A). These results were further confirmed using shRNA knockout against ERK2; we established a L428 cell line with stable SHP1 expression using lentivirus-based pBABE-SHP1 WT expression vector. Interestingly, expression of SHP1 had no effect on $G_2$ cell-cycle arrest with darinaparsin treatment; however, knockdown of ERK2 with siRNA resulted in decreased accumulation of cells at $G_2$ suggesting that ERK2 is the mediator $G_2$ cell-cycle arrest with darinaparsin (Fig. 6B). Similarly, knockdown of ERK2 with siRNA alone increased cell death by apoptosis, whereas SHP1 knockdown resulted in a further increase in apoptosis in combination with darinaparsin in L428 cells (Fig. 5B). Collectively, these results strongly suggest that both ERK2 and SHP1 are intimately involved in mediating the cellular response to darinaparsin treatment in lymphoma.

**Discussion**

In this study, we examined the biologic activity of the novel organic arsenical agent, darinaparsin, in TCL and HL cell lines and in associated SCID xenograft models. Our studies were performed using 1 to 5 $\mu$mol/L of darinaparsin, which are clinically relevant concentrations. In a previous clinical study in acute myelogenous leukemia, it was shown that 1.2 $\mu$mol/L of plasma drug concentration was safely and clinically achievable using a 300 mg/m$^2$ i.v. infusion (20). We identified that darinaparsin achieved significantly higher intracellular concentration of arsenic compared with ATO and induced potent cell death and apoptosis in a diverse panel of TCL and HL cell lines. Furthermore, we observed that darinaparsin was relatively less toxic in isolated normal (CD19, positive) T and (CD3, positive) B cells compared with lymphoma cells. Darinaparsin also downregulated a number of proteins including ERK and related substrates. We identified that ERK and the inhibitory phosphate, SHP1, interacted closely and that ERK had a functional role in darinaparsin-related cell death. Furthermore, darinaparsin cell death in HL and TCL cells was shown to be dependent on MEK/ERK and SHP1 via pharmacologic inhibitors, genetic deletions, and overexpression studies.

Arsenic derivatives have long been shown to have therapeutic potential in the treatment of hematologic malignancies; however, outside of acute promyelocytic leukemia, its therapeutic index/window is too narrow to allow appropriate dose escalation (6). Darinaparsin is a novel organic arsenical being studied as a novel agent for the treatment of cancer. Darinaparsin was synthesized by conjugating dimethylarsenic to glutathione. In part related to this, it has been shown to induce high intracellular levels of ROS in treated cancer cells leading to comparatively more prominent antioxidant response and higher intracellular drug
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Figure 6. Inhibition of MEK and ERK or constitutive expression of SHP1 restores dariparsin sensitivity. A, inhibition of MEK using either U0126 with 3 μmol/L dariparsin, or using AZD6244 with 5 μmol/L dariparsin in L428 leads to increased apoptosis, as detected by Annexin V staining and analysis by flow cytometry. Error bars, SDs of mean and statistically significant differences (by Student t test) in treatments comparing dariparsin alone and in combination with U0126 or AZD6244, indicated by an asterisk (***, P < 0.005). Western blot analysis of L428 (HL) cells pretreated with U0126 or AZD6244 shows inhibition of ERK phosphorylation with either inhibitor alone or in the presence of dariparsin. B, Western blot analysis shows stable overexpression of WT-SHP1 in L428 cells and transient knockdown of ERK2 with siRNA. The bar graph represents results from flow cytometry-based cell-cycle analysis with PI-stained cells, showing a decrease in the accumulation of cells at G2 with ERK2 knockdown and dariparsin treatment (top). The bar graph represents results from flow cytometry-based analysis of Annexin V–stained cells, showing increased apoptosis with dariparsin treatment in L428 cells with SHP1 overexpression combined with ERK2 knockdown (bottom), indicated by an asterisk (**, P < 0.05).

concentrations compared with ATO (21, 22). We showed that dariparsin induced time- and dose-dependent cytotoxicity in HL and TCL cell lines and that by mass spectrometry, dariparsin resulted in significantly higher intra-cellular arsenic concentrations compared with ATO. Dariparsin also resulted in dose-dependent increases in apoptosis in all cell lines by Annexin V positivity and cleavage of PARP and caspases-3, -8, and -9.

The RAS/RAF/MEK/ERK is a known oncogenic pathway in lymphoma. Tian and colleagues have shown that dariparsin significantly decreased the expression of a number of genes upregulated by RAS in solid tumor cells (22). Previous studies with ATO show that MEK inhibition with ATO treatment potentiates cell death in leukemia (11, 23) and multiple myeloma (24). Furthermore, pharmacologic targeting of the PI3K/AKT pathways with ATO has been shown to potentiate cell death in T-cell leukemia, chronic lymphocytic leukemia, and acute promyelocytic leukemia (12, 25). We interrogated this signaling pathway and observed increased phosphorylation of ERK1/2 in most cell lines, whereas there was minimal effect on AKT. ERK1/2 is the only known physiologic substrate for MEK activity, whereas ERK1/2 has multiple downstream substrates (15) that are involved in the regulation of cell growth and metabolism with the related phosphatases playing a critical role in the negative regulation of ERK1/2 activity. Dariparsin also resulted in phosphorylation of c-FOS and down-regulation of FOXO3a in most cell lines, which correlates with ERK1/2 activation and suggests that ERK1/2 activation might have a functional role in the cellular response to dariparsin treatment. In searching for a mechanism of ERK activation, we examined the MAPK phosphatase, SHP1.

SHP1 is primarily expressed in hematopoietic cells and a key regulator of multiple signaling kinases in the hematopoietic cells (26). In a previous study, lack of SHP1 resulted in sustained increase in ERK1/2 phosphorylation (19). SHP1 is predominantly expressed in hematopoietic cells and negatively regulates MAPK signaling in lymphocytes (27). Although epigenetic silencing and functional loss of SHP1 are reported in the majority of leukemia and lymphoma, it is thought to be involved in malignant transformation and tumor aggressiveness (28). Emerging evidence shows that functional loss of SHP1 leads to defective apoptosis and poor response to treatment (19, 29). We found that levels of SHP1 were decreased with increasing amounts of dariparsin in all cell lines, except for L428, where SHP1 was barely detectable. The observed reduction in SHP1 protein levels correlated with increased accumulation of phosphorylated ERK. Furthermore, downregulation of ERK by RNAi or constitutive overexpression of SHP1 resulted in increased apoptosis in the presence of dariparsin. In addition, cotreatment with pharmacologic MEK inhibitors combined with dariparsin resulted in synergistic cell death. Conversely, SHP1 shRNA knockdown resulted in decreased apoptosis with dariparsin treatment. From these results, it is evident that the ERK1/2 response to dariparsin does not follow the pattern of phosphorylation events that is associated with canonical Ras–RAF–MEK signaling cascade. In the context of dariparsin treatment for hematologic malignancies, presence of SHP1 might be essential to prevent inadvertent activation of ERK1/2.
In summary, the novel organic arsenical compound, darinaparsin, was associated with cell death in TCL and HL cell lines and related human SCID xenograft models. Cell death was associated with dose-dependent apoptosis and upregulation of the MEK/ERK signaling cascade that appeared primarily as a result of decreasing the inhibitory SHP1 phosphatase. Furthermore, induction of cell death by darinaparsin seemed to be in part related to a SHP1-dependant mechanism, and in tumors lacking SHP1 function, cotreatment with MEK inhibition might be required to achieve optimal response to darinaparsin treatment. Continued clinical investigation of darinaparsin is warranted in lymphoma and rational combinations targeting MAPK signaling should be explored.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D. Ravi, A.P. Mazar, A.M. Evens

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