The Novel Organic Arsenical Darinaparsin Induces MAPK-Mediated and SHP1-Dependent Cell Death in T-cell Lymphoma and Hodgkin’s Lymphoma Cells and Human Xenograft Models

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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 biological actions and mechanisms of resistance are poorly understood. We examined the effects of darinaparsin in T-cell and Hodgkin lymphoma (HL) by in vitro and in vivo experiments and we examined its biological activity. Darinaparsin induced potent in vitro cell death by apoptosis, and in vivo, it inhibited growth of tumor xenografts in SCID mouse models. Investigations into biological activities of darinaparsin demonstrated ERK phosphorylation and lack of SHP1 as likely mechanisms of resistance, while ERK inhibition or restoration of SHP1 function resulted in sensitizing the resistant L428 HL to darinaparsin treatment. Thus our conclusion is that while darinaparsin is potent as a single agent in T-cell and HL, additional consideration is required on the biological mechanisms resistance to achieve the maximum therapeutic benefit from darinaparsin treatment.
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 regarding 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. Biological effects of darinaparsin on MAPK pathway were investigated using pharmacological inhibitors, RNA interference (RNAi) and transient transfection for overexpression for SHP1 and MEK.

Results: Darinaparsin treatment resulted in dose-dependent cytotoxicity and apoptosis in all TCL and HL cell lines. Additionally, darinaparsin had more rapid, higher, and sustained intracellular arsenic levels compared with arsenic trioxide via mass spectrometry. 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 co-immunoprecipitation showed significant ERK/SHP1 interaction. Furthermore, ERK shRNA knockdown or constitutive overexpression of SHP1 resulted in increased apoptosis, while co-treatment with pharmacologic MEK inhibitors resulted
in synergistic cell death. Conversely, SHP1 blockade (via pharmacologic inhibition or RNAi) as well as 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.
INTRODUCTION

While many patients with Hodgkin’s lymphoma (HL) are cured by conventional chemotherapy, there continues to be a subset of patients with refractory disease or relapse. Further, 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’s lymphoma that are difficult to treat. Long-term survival rates are <30-35% (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). Additionally, early-phase clinical trials in patients with hematological malignancies demonstrated that darinaparsin is safer and effective compared with inorganic arsenic trioxide (ATO).(6-8) Moreover, it is known that darinaparsin and ATO inhibit tumor growth by distinct mechanisms (8).

The biological mechanism of action of darinaparsin in lymphoma is unknown. Our goals were to investigate the potency of darinaparsin in TCL and HL cells lines and related xenograft SCID mouse models. Furthermore, we intended to identify the associated biologic mechanisms of action. We found that darinaparsin induced dose- and time-dependent apoptosis against TCL and HL cell lines, and demonstrated in vivo therapeutic activity of darinaparsin in TCL and HL tumor xenografts grown in SCID research.
mice. Furthermore, we show that darinaparsin treatment resulted in the activation of MAPK pathway by a unique mechanism involving the inhibitory SHP1 protein tyrosine phosphatase.

METHODS

Cell culture, reagents, and transfections

HL cell lines L540, L428, KMH2 and L1236, and TCL cell lines HH, Hut78 and Jurkat were grown in RPMI1640 consisting of 10% heat inactivated fetal bovine serum and 200U of penicillin/streptomycin (Mediatech, Manassas, VA) under 5% CO2 and 37°C. Darinaparsin was kindly provided by Ziopharm Oncology, Inc (Boston, MA). U0126 and AZD6244 was obtained from Selleck Chem. (Houston, TX). Non-targeting or smart pool ERK2 siRNA were obtained from Thermo Fisher Scientific. For L428, transfection of siRNA or plasmid DNA was performed using Amaxa Nucleofector device and Amaxa cell line Nucleofector kit L reagent, (Lonza, Walkersville, MD). For RNAi experiments, lentiviral based pGPIZ expression system (Open Biosystem) was utilized to transduce scrambled non-targeting, ERK2 or SHP1shRNA sequences into lymphoma cells. For induction of ERK activity, Addgene #21193 plasmid construct which express constitutively active form of MEK was used for the transfection experiment in Hut78 using Amaxa Nucleofector device and Amaxa cell line Nucleofector kit R reagent, (Lonza, Walkersville, MD). For SHP1 overexpression experiments, lentiviral supernatants were prepared by transfecting HEK293T cells with pBABE-puro SHP1 WT (Addgene#8575) or pBABE-puro Vector (Addgene#1764) and packaging plasmids pUMVC (Addgene#8449) and PCMV-VSV-G (Addgene#8454), using Fugene 6 reagent (Promega, Inc). High titer lentiviral supernatants were transduced into 0.5x10^6 cells
using retronectin (Clontech Inc) coated 12-well plates and puromycin (2μg/mL) was used as selection antibiotic.

**Mass spectrometry for determination of intracellular arsenic concentration**

Twenty million L428 cells treated with 3μM arsenic trioxide (ATO) or 3μM Darinaparsin for 1, 3 and 6 hours were harvested, washed with PBS and dehydrated in the oven at 60°C overnight followed by digestion in nitric acid at 80°C. 0.1 to 50μg/L of arsenic standards were prepared from a 1mg/L stock, previously prepared from the 10mg/L standard in 2% nitric acid. Internal standard without arsenic at a concentration of 50μg/L. For sample analysis, the internal standard was added to the cell digest and diluted to 4 mL with double distilled and deionized water. Arsenic (As), concentrations were measured using inductively coupled plasma mass spectroscopy (ICP-MS, X Series II, Thermo Electron).

**MTT assay**

For MTT, 10⁴ cells/100μL were plated in a 96-well plate and treated with increasing concentrations of Darinaparsin (1-5μM) for 24-72 hours. MTT assay was performed using Cell Titer Aqueous Non-Radioactive Cell Proliferation assay, Promega Inc. Madison, WI, as per instructions supplied by the manufacturer.

**Apoptosis and cell cycle analysis by flow cytometry**

Annexin-V/propidium iodide (PI)-based estimation of apoptosis by flow cytometry performed using Apoptosis Detection Kit-I (BD Biosciences, San Jose, CA). Briefly, 10⁶ cells per mL of complete RPMI 1640 medium treated with darinaparsin for 24 or 48 hours was harvested, washed with ice cold PBS and stained with Annexin-V-FITC antibody and PI for 15 minutes and the samples were analyzed by flow cytometry within
1 hour. For cell cycle analysis, darinaparsin treated cells were harvested, washed in ice
cold PBS, fixed in ethanol and stained with PI staining, as discussed before (9).

**Western blot analyses**

Protein lysate preparation and Western blot was performed as described
previously.(9) Primary antibodies validated against total or phosho B-Raf (Ser445), c-
Raf (Ser338) MAPK (Erk1/2)Thr202/Tyr204, MEK (Ser217/221),SHP1, AKT, mTOR
(Ser2448), p70S6K (Thr389), PI3K- p85-Tyr458/p55-Tyr199, Foxo3a (Ser253), Bcl-2,
Bim, c-myc, eIF4E (Ser209), 4E-BP1 (Thr37/46), total and cleaved caspase-3, 8, 9 and
PARP were purchased from Cell Signaling Technology (Beverly, MA). For co-
immunoprecipitation experiment, 200μg of protein lysate was used, precleared with
Protein A agarose beads (Cell Signaling Technology, Beverly, MA) followed by
overnight incubation with primary antibody (SHP1 or ERK1/2, 1:50 dilution, validated for
immunoprecipitation, Cell Signaling Technology, Beverly, MA ), followed by precipitation
with 30μl of Protein A agarose beads (50% slurry). Beads were washed five times with
1ml of NaCl lysis buffer by centrifugation, resuspended in 30μl of 3X Lamelli 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 darinaparsin, human lymphoma xenografts
grown in SCID mouse models were utilized. Jurkat (TCL) or L540 (HL) cells were
implanted at a density of 5x10^6 subcutaneously in CB-17 SCID mice, once the tumor
volume reaches 100-250mm^3^. Darinaparsin was administered and compared with
vehicle treatment mice, with approximately 8 mice for each group. Based on clinical
observations and effects on tumor growth with the administration of varying doses of
darinaparsin (500, 200, 100 or 70 mg/kg) in SCID mice bearing L540 (HL) tumor xenografts, in the treatment for Jurkat (TCL) derived xenograft darinaparsin dosage was adjusted to 70 or 40mg/kg. Jurkat (TCL) derived tumor xenograft bearing SCID mice received either saline (control), or darinaparsin (40 or 70mg/kg) by intraperitoneal (i.p.) injections daily for 5 days. L540 (HL)-derived tumor xenograft bearing SCID mice received 100mg/kg Darinaparsin 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 was measured twice weekly for four to six weeks. Tumor volumes were determined using calipers and calculated, as described previously (10). Mice were euthanized when tumors reached 2000 mm³ and were considered as expired at this point for survival analysis. For statistical analysis, a two way repeated measurement ANOVA performed using GraphPad Prism 5.0, (GraphPad Software, Inc. La Jolla, CA) 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

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

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

Cell viability following treatment with darinaparsin 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 darinaparsin (1-5µM) for 72 hours resulted in a dose-dependent decrease in cell viability in all cell lines (Figure 1B). However, the level of sensitivity to darinaparsin at 72 hours varied among TCL and HL cell lines. Jurkat cells were observed to be most sensitive with associated IC$_{50}$ of 2.7µM compared with HH and Hut78 (3.2µM, and 6.7µM, respectively); L540 cell line was the most sensitive HL cell line to darinaparsin (IC$_{50}$ 1.3µM compared with L1236 and L428 at 2.8µM, and 7.2µM, respectively).

**Darinaparsin induce cell cycle arrest and apoptosis HL and TCL cell lines**

Darinaparsin (1-3µM) treatment resulted in accumulation of cells predominantly at G2 cell cycle in both Jurkat and L428 cell lines (Figure 2A). In Jurkat, treatment with 1µM or 3µM resulted in accumulation of cells at G2 with 26% and 13% respectively, compared with presence of 11% G2 cells in the untreated control (Figure 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 1µM or 3µM 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 µM darinaparsin in L428 (HL) resulted only in a moderate effect on cell viability compared with other HL cell lines (Figure 1B). Therefore, these results suggest that perhaps cell cycle arrest with darinaparsin in L428 may impact the cytotoxic response in this cell line. Since 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 µM) resulted in a significant dose-dependent increase in Annexin-V positivity in Jurkat (TCL) and L540, Hut78 and L428 (HL) cell lines (Figure 2B). While treatment with 2µM darinaparsin resulted in >50% Annexin-V positivity in all cell lines, the amount of Annexin positive cells were comparatively lower in L428 cells (Figure 2B), consistent with MTT cell viability findings (Figure 1B). Additionally, 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 (Figure 2B). Treatment of L428 cells with darinaparsin resulted in activation of caspases 3, 8 and 9, while degradation of PARP was not detected (Figure 2B).

**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, 532mm³) and 70 mg/kg (average tumor size, 447mm³), respectively, compared with the average size of tumor (1929mm³) in the vehicle treated control group (p<0.0001, two way repeated ANOVA) (Figure 3A). There was significantly improved survival by Kaplan Meier in Jurkat SCID mice treated with darinaparsin compared with control (p<0.0001) (Figure 3B). While the extent of tumor growth inhibition was similar with 40 or 70mg/kg of darinaparsin, there was no appreciable change in total body weight in the treatment with 40mg/kg dose darinaparsin; however increased dosing to 70mg/kg initially resulted in a 10% loss of total body weight by 5 days and subsequently returned to normal once treatment was completed (Figure 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 (2003 mm³) at four weeks (p=<0.001, two way repeated measurement ANOVA, GraphPad Prism 5) (Figure 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) (Figure 3E). Treatment with 70mg/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 (Figure 3F) in a similar manner as observed with Jurkat-derived xenograft tumors.

Effect of darinaparsin on MAP kinase 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 biological 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 (Figure 4), while 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 down-regulation of BRAF phosphorylation with darinaparsin in Jurkat, HH and Hut78, and L540, while there was no change in L1236 and L428 (Figure 4). While the effect of darinaparsin on MEK phosphorylation followed a similar pattern to BRAF response to darinaparsin (Figure 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 protein 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 (Figure 4). The observed reduction in SHP1 protein levels appeared 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, while 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 biological 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 (Figure 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. Down-regulation 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, while MYC was
down-regulated in Jurkat and L1236. Taken together, these results show that
phosphorylation of c-FOS and down-regulation 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.

**SHP1 and ERK mediates darinaparsin-induced cell death**

While 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 3 of 6 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 MAPK pathway (17, 18). Thus, we focused our
investigation on SHP1 in the context of ERK activation. We performed co-
immunoprecipitation 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 showed presence of SHP1 protein, as detected by Western blot based
analysis (**Figure 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 are demonstrated in the control experiments shown in
**Figure S1**. Furthermore, inhibition of SHP1 using the SHP1/2 inhibitor, NSC87877, or
stable knock down of SHP1 with lentiviral-based shRNA induced phosphorylation of
ERK1/2 in Hut78 TCL cells (**Figure 5B-C**) suggesting a functional role for SHP1 in
modifying ERK1/2 phosphorylation. Similar findings were reported in a previous study
showing that down regulation of SHP1 led to sustained increase in ERK1/2 phosphorylation (19). Treatment with darinaparsin in the presence NSC87877 resulted in a significant reduction in cytotoxicity in Hut78 TCL cells (P<0.001) (Figure 5B). Similarly, treatment with darinaparsin in the presence of SHP1 knock down with shRNA decreased apoptotic cell death compared to control (P<0.0001) (Figure 5C), while there was a slight increase in apoptosis with ERK2 knock down in TCL cells (Figure 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 impact 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 Figure 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 (Figure 5D) suggesting that constitutive ERK activation would reduce darinaparsin sensitivity.

Decreased sensitivity to darinaparsin was observed in L428 HL cells with constitutive ERK activation, while 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) (Figure 6A). These results were further confirmed using shRNA knock out 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 G2 cell cycle arrest with darinaparsin treatment, however, knock-down of ERK2 with siRNA resulted in decreased accumulation of cells at G2 suggesting that ERK2 is the mediator G2 cell cycle arrest with darinaparsin (Figure 6B). Similarly, knock-down of ERK2 with siRNA alone increased cell death by apoptosis, while SHP1 knock-down resulted in a further increase in apoptosis in combination with darinaparsin in L428 cells (Figure 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 1-5 μM of darinaparsin, which are clinically relevant concentrations. In a previous clinical study in acute myelogenous leukemia, it was shown that 1.2 μM of plasma drug concentration was safely and clinically achievable using a 300mg/m² IV 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. Further, 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 down-regulated 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 hematological 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 concentrations compared with ATO (21, 22). We showed that darinaparsin induced time- and dose-dependent cytotoxicity in HL and TCL cells lines and that by mass spectrometry, darinaparsin resulted in significantly higher intracellular arsenic concentrations compared with ATO. Darinaparsin 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 et al have shown that darinaparsin significantly decreased the expression of a number of genes up regulated 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, pharmacological targeting of 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,
while there was minimal effect on AKT. ERK1/2 is the only known physiological substrate for MEK activity, while 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. Darinaparsin 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 darinaparsin 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). While epigenetic silencing and functional loss of SHP1 is 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 (29),(19) 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. The observed reduction in SHP1 protein levels correlated with increased accumulation of phosphorylated ERK. Further, down regulation of ERK by RNAi or constitutive over-expression of SHP1 resulted in increased apoptosis in the presence of darinaparsin. Additionally, co-treatment with pharmacologic MEK inhibitors combined
with darinaparsin resulted in synergistic cell death. Conversely, SHP1 shRNA knock
down resulted in decreased apoptosis with darinaparsin treatment. From these results,
it is evident that the ERK1/2 response to darinaparsin does not follow the pattern of
phosphorylation events that is associated with canonical Ras-RAF-MEK signaling
cascade. In the context of darinaparsin treatment for hematological 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 up regulation 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
appeared to be in part related to a SHP1-dependent mechanism, and in tumors lacking
SHP1 function, co-treatment 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.
AUTHORSHIP CONTRIBUTIONS

D.R. designed and performed research, analyzed data, and wrote the paper; S.B. designed and performed research, analyzed data, and wrote the paper; R.B.G. analyzed data, and wrote the paper; J.C. performed research and analyzed data; I.K. performed research and analyzed data; J. S performed research, A.M. designed research and analyzed data; and A.M.E. designed and performed research, analyzed data, and wrote the paper.

CONFLICT OF INTEREST DISCLOSURES

There is no relevant conflict of interest to disclose.

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REFERENCES


FIGURE LEGENDS

Figure 1. Darinaparsin treatment induces accumulation of intracellular Arsenic and reduces cell viability in lymphoma cell lines. (A) Treatment of Jurkat (TCL) or L428 (HL) with darinaparsin (3µM) results in rapid intracellular accumulation of arsenic compared to ATO (3µM) as detected by mass spectrometry based analysis. (B) MTT based cell viability assay show a dose dependent increase in cytotoxicity with darinaparsin (1-5µM) in a panel of TCL and HL cell lines at 72 hours.

Figure 2. Darinaparsin induce cell cycle arrest and increased apoptosis. (A) Histograms show cell cycle arrest with darinaparsin treatment (24 hours) in Jurkat (TCL) or L428 (HL) stained with propidium iodide and analyzed by flow cytometry (top panel), and changes in the percentage cell cycle specific population is represented as bar graph (bottom panel). The error bars represent standard deviations of mean and statistical significant differences (by student T test) in between control and darinaparsin treatment is indicated by an asterisk [(**)] P<0.001 and [(***)] P<0.0001]. (B) Annexin-V staining with darinaparsin treated TCL or HL cell lines showed a dose dependent increase in the percentage of apoptotic cell population, at 72 hours, as detected by flow cytometry based analysis. (C) Western blot analysis of darinaparsin treated TCL or HL cells show activation of caspases 3, 8 and 9, and cleavage of PARP at 24 hours.

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 show 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 to 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, F).

**Figure 4. AKT and MAPK response to darinaparsin.** Western blot analysis of protein lysates from lymphoma cell lines treated with increasing concentrations of darinaparsin (1-5µM) for 24 hours, show lack of canonical RAF-MEK-ERK response but resulting only in the activation of ERK. A correlation between activation of ERK with darinaparsin treatment with a decrease in the levels of SHP1 phosphatase is observed along with activation of downstream components of the ERK pathway.

**Figure 5. SHP1 and ERK mediate darinaparsin sensitivity.** (A) Immunoprecipitation of protein lysates from Hut78 (TCL) performed using anti-ERK1/2 antibody and analysis by Western blot show presence of SHP1 protein, and in the converse experiment immunoprecipitation with anti-SHP1 antibody and Western blot analysis show presence of ERK1/2 protein. (B-C) Inhibition of SHP1, by NSC87877 or knock-down of SHP1, using shRNA results in increased phosphorylation of ERK1/2, (detected by Western blot), and treatment with darinaparsin results in decreased cell death (D). Induction of ERK phosphorylation by transient MEK expression also decreased darinaparsin induced cell death. The error bars represent standard deviations of mean and statistical significant differences (by student T test) is indicated by an asterisk (*** P<0.0001).
Figure 6. Inhibition of MEK and ERK or constitutive expression of SHP1 restores darinaparsin sensitivity. (A) Inhibition of MEK using either U0126 with 3μM darinaparsin, or using AZD6244 with 5μM darinaparsin in L428 leads to increased apoptosis, as detected by Annexin-V staining and analysis by flow cytometry. The error bars represent standard deviations of mean and statistical significant differences (by student T Test) in treatments comparing darinaparsin 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 show inhibition of ERK phosphorylation with either inhibitor alone or in the presence of darinaparsin. (B) Western blot analysis show stable overexpression of WT-SHP1 in L428 cells and transient knock down of ERK2 with siRNA. The bar graph represents results from flow cytometry based cell cycle analysis with propidium iodide stained cells, show a decrease in accumulation of cells at G2 with ERK2 knock down and darinaparsin treatment (top panel). The bar graph represents results from flow cytometry based analysis of Annexin-V stained cells, show increased apoptosis with darinaparsin treatment in L428 cells with SHP1 overexpression combined with ERK2 knock down and (bottom panel).
Figure 1

**A**

Bar graph showing intracellular arsenic levels in Jurkat and L428 cells with and without Darinaparsin treatment over 6 hours.

**B**

Graph showing percentage control viability against concentration of Darinaparsin in different cell lines (Jurkat, Hut78, HH, L428, L1236, L540).
Figure 2

A

B

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Figure 3
Figure 4
Figure 5

**A**
- ERK: IP
  - Control
  - Darinaparsin
  - WB
  - SHP1
  - ERK1/2

- SHP1: IP
  - Control
  - Darinaparsin
  - WB
  - ERK1/2
  - SHP1

**B**
- Control
- NSC87877
- Darinaparsin
- NSC87877 + Darinaparsin
- P-ERK1/2
- SHP1
- ERK1/2
- β-actin

**C**
- Scrambled
- ERK2 shRNA
- SHP1 shRNA
- P-ERK1/2
- ERK1/2
- β-actin

**D**
- Control
- MEK-CA
- MEK
- P-ERK1/2
- ERK1/2
- β-actin

**Legend**
- **Control**
- **SHP1/2 Inhibitor (NSC87877)**
- **% Control Cell viability**
- **% Annexin-V positivity**
- **Concentration of Darinaparsin (µM)**
- **Percent Annexin Positivity**

**Figure 5**
Figure 6
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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