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The novel arsenical darinaparsin is transported by cystine importing systems

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Running Title:

Link between darinaparsin and cystine import

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List of Abbreviations:

ABCC1, ATP-binding cassette, sub-family C member 1; AQP, Aquaporins; APL, Acute Promyelocytic Leukemia; ARE, Antioxidant response elements; ATO, Arsenic trioxide (As_2O_3); BSO, L-Buthionine Sulfoximine; DAR, Darinaparsin (ZIO-101, S-dimethylarsino-glutathione); DMA III, Trivalent dimethylarsinic acid; DMA V, Pentavalent dimethylarsinic acid; DMAC, Dimethylarsino-cysteine; γ-GT, γ-glutamyl-transpeptidase; γ-GPN, L-Glutamic acid γ-(p-nitroanilide) hydrochloride; GCS, γ-glutamyl-cysteine synthetase ; GLUT, Glucose transporter; GSAO, 4-(N-(S-glutathionylacetyl)amino) phenylarsonous acid; GCAO, 4-(N-(S-cysteinylglycylacetyl)amino) phenylarsonous acid; GSH, Glutathione (γ-glutamylcysteinylglycine); ICP-MS, Inductively coupled plasma-mass spectrometry; MRP1, Multidrug resistance protein 1; NAC, N-acetyl-cysteine; NB4, Human APL cell line; NRF2, NF-E2-related factor-2; PI, Propidium iodide; ROS, Reactive oxygen species; *SLC1A1,* Gene coding for xAG; *SLC7A11,* Gene coding for xCT; *SMARCA4,* Gene coding for BRG1

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Abstract:

Darinaparsin (ZIO-101; S-dimethylarsino-glutathione; Dar) is a promising novel organic arsenical currently undergoing clinical studies in various malignancies. Dar consists of dimethylarsenic conjugated to glutathione (GSH). Dar induces more intracellular arsenic accumulation and more cell death than the FDA-approved arsenic trioxide (ATO) *in vitro*, but exhibits less systemic toxicity. Here, we propose a mechanism for Dar import that might explain these characteristics. Structural analysis of Dar suggests a putative breakdown product: dimethylarsino-cysteine (DMAC). We show that DMAC is very similar to Dar in terms of intracellular accumulation of arsenic, cell cycle arrest and cell death. We found that inhibition of γ-glutamyl-transpeptidase (γ-GT) protects human acute promyelocytic leukemia cells (NB4) from Dar, but not from DMAC, suggesting a role for γ-GT in the processing of Dar. Overall, our data supports a model where Dar, a GSH-S-conjugate, is processed at the cell surface by γ-GT leading to formation of DMAC, which is imported via xCT, xAG or potentially other cystine/cysteine importing systems. Further, we propose that Dar induces its own import via increased xCT expression. These mechanisms may explain the enhanced toxicity of Dar towards cancer cells compared with ATO.

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Introduction:

Arsenic trioxide (ATO) is a proven chemotherapy for acute promyelocytic leukemia (APL) (Lo-Coco et al., 2008; Lo-Coco et al., 2013; Shen et al., 1997; Soignet et al., 2001). In recent years, 90 trials have been initiated to test the safety and efficacy of ATO in a wide array of other hematologic and solid malignancies. However, most of these studies showed substantially reduced anti-tumor effects of clinically achievable levels of ATO in non-APL malignancies. This led to the development of candidate arsenic-based, anti-cancer drugs with more potent anti-tumor effects.

Darinaparsin (Dar, ZIO-101, S-dimethylarsino-glutathione) is a newly developed organic arsenical, currently in clinical development. Dar was designed by conjugating dimethylarsenic to glutathione (GSH) and shows significant activity against multiple cancers *in vitro* (Diaz et al., 2008). Dar has a maximum tolerated dose that is 50-fold higher than ATO in mice (Camacho et al., 2006) and is active against xenograft tumors (Hussein et al., 2007). Although currently undergoing clinical studies in both hematologic malignancies and solid tumors, the mechanism of Dar's anti-tumor effects has not been totally elucidated (Mann et al., 2009). We have shown previously that Dar is significantly more potent than ATO at mediating apoptosis in various malignant cell lines and is highly active against APL cells. Moreover, consistent with others, we have shown that Dar induces significantly more intracellular arsenic accumulation than ATO when both are given at equimolar arsenic concentrations (Diaz et al., 2008; Manshouri et al., 2005a; Manshouri et al., 2005b; Matulis et al., 2009).

ATO is imported into cells via glucose transporters and aquaglyceroporins (Rosen and Liu, 2009). We have shown that the ABCC1 exporter is involved in arsenic

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efflux after ATO treatment, but not after Dar treatment (Diaz et al., 2008). However, there have been no reports about Dar cellular uptake. Since Dar is a GSH-s conjugate, where the dimethylarsenic is conjugated to GSH via a sulfide bond (Figure 1), we hypothesized that the GSH moiety of Dar may contribute to its import.

GSH is present in cells at millimolar concentrations and acts as the major intracellular antioxidant, protecting cells from oxidative stress (Meister, 1995; Sies, 1999; Zhang and Forman, 2012). Cystine/cysteine is the rate-limiting substrate for GSH synthesis (Bannai and Tateishi, 1986; Ishii et al., 1987; Zhang and Forman, 2012). Cystine (Cys-S-S-Cys), the disulfide form of cysteine, is the prevailing form in the extracellular space. The cystine/cysteine redox cycle generally consists of cystine uptake, intracellular reduction to cysteine and either cysteine efflux or *de novo* synthesis of GSH, if needed (Banjac et al., 2008). In order to increase cysteine uptake, extracellular GSH can be cleaved by γ-glutamyl-transpeptidase (γ-GT), then by a cysteinylglycine dipeptidase, both at the cell surface. Only then is cysteine/cystine imported (Dilda et al., 2008). This is often referred to as the scavenger pathway (Zhang and Forman, 2012). Moreover, compounds that form GSH conjugates are processed by γ-GT and cysteinylglycine dipeptidase to cysteine S-conjugates (Monks et al., 1990). GSH-conjugates can also be cleaved by γ-GT and a dipeptidase to facilitate uptake. For example, GSH-conjugated methylmercury uptake in the renal proximal tubules is significantly abrogated by the addition of a γ-GT inhibitor (Wang et al., 2012), Therefore, we hypothesized that Dar might enter the cell by this mechanism. Co-treatment with Dar and extracellular thiols can reduce intracellular arsenic accumulation and subsequent cell death in a myeloma cell line (Matulis et al., 2009; Sakurai et al., 2006), supporting

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our hypothesis that Dar uptake utilizes GSH uptake pathways. Structural analysis of Dar suggested that a putative arsenic-containing breakdown product could be dimethylarsino-cysteine (DMAC, Figure 1). Here, we elucidate how and in which form Dar is imported into the cancer cell. We show that Dar is processed before cell entry and that the resulting intermediate product enters the cell via the cystine/cysteine cellular import system. This is of particular relevance regarding the toxicity of Dar against cancer cells, because drug import/export is a key factor in sensitivity or resistance towards cancer therapies. In fact, we provide preliminary evidence in colorectal cancer that expression of the cystine transporter xCT may correlate with sensitivity to Dar.

Materials and methods:

Cell culture: The human APL cell line, NB4, (provided by Dr. M Lanotte) was cultured in RPMI-1640 media (Wisent, St-Bruno, Quebec, Canada) supplemented with 10% fetal bovine serum (FBS; Wisent) and penicillin/streptomycin (Wisent), in a humidified chamber at 37°C with 5% CO_2, as described previously (Diaz et al., 2008). The human colorectal cancer cell lines HCT 116 and RKO 2577 (provided by Dr. Raquel Aloyz) were cultured in RPMI-1640 media (HCT 116) or EMEM (RKO 2577), supplemented with 10% fetal bovine serum. HT1080 human fibrosarcoma cells transduced with empty vector or xCT were described previously (Lastro et al., 2008) and were cultured in DMEM containing 10% fetal bovine serum.

Synthesis of DMAC and DMA III: DMAC was prepared using an adapted method previously described (Cullen et al., 1984).10 ml of milli-Q water was degassed by bubbling nitrogen for 15 min. L-cysteine (0.2414g, 19.92 mmol) was added and

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dissolved to yield a clear solution. Cacodylic acid (DMA V) (0.0917g, 6.64 mmol) was added to the solution, forming a cloudy white precipitate. The precipitate was filtered, dried and purified by re-crystallization in ethanol and stored under nitrogen at -35 ˚C. As DMA (III) is sensitive to oxidation, solutions of DMA (III) were prepared fresh prior to each experiment using an adapted method previously described (Reay and Asher, 1977). DMA (V) (0.113g, 1eq), sodium metabisulfite (0.25, 1.6eq) and sodium thiosulfate pentahydrate (0.02g, 0.05eq) were dissolved in 5 ml of H_2O. Sulfuric acid (0.25ml of 7.5M) was added drop wise. The solution was left stirring for 30 minutes. The literature method for its preparation (Reay and Asher, 1977) is known to give a wide range of thiolated and over-reduced species when a bolus of reductant is used (Suzuki et al., 2004). We have found that exact control of the reductant stoichiometry gives pure DMA III. Purity was ascertained by ^{1}H NMR for separate parallel solutions prepared in D_2O: ^{1}H NMR (400 MHz, D_2O) δ= 1.40 (s, 6H), showing 99% purity of our DMA III preparation. DMA III was stored in the solid state at -20°C and solubilized immediately before each experiment directly in the buffer employed and added to cultures within 10 minutes. Surprisingly, rapid oxidation of DMA III to DMA V has been reported for its dissolution (Yehiayan et al., 2009). However, under the conditions used in our experiments, it has been reported that these solutions only begin to appreciably allow for the oxidation of DMAIII after three days (Gong et al., 2001), times which are well beyond those used in our experiments. **Propidium iodide staining:** Quantification of apoptotic cells was performed as previously described (Hardin et al., 1992). Cells were treated, washed in buffer (phosphate-buffered saline [PBS]/5% FBS/0.01 M NaN_3) at 4°C, pelleted, and resuspended in 0.5ml hypotonic fluorochrome solution containing 50

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µg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Fluorescence was measured on a Becton Dickinson fluorescence-activated cell sorter (FACS) Calibur (San Jose, CA). Cells undergoing DNA fragmentation and apoptosis (those in which PI fluorescence was weaker than the typical G0-G1 cell cycle peak) were quantified using CellQUEST software (BD Bioscience, Mississauga, ON, Canada) and FCS Express (De Novo Software, Los Angeles, CA, USA). A total of 10,000 events per condition were acquired.

Western blot analysis: Whole cell extracts were prepared using lysis buffer (50mM Tris-HCl, pH 8, 150mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10µg/mL each aprotinin, leupeptin and $NaVO_4$) at 4°C. Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Mississauga, ON). To detect xCT or xAG, 50µg of protein was added to an equal volume of 2X sample buffer and run on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Proteins were transferred to nitrocellulose membranes (Bio-Rad), and blocked with 5% milk in PBS containing 0.5% Triton X-100 overnight at 4°C. The membrane was hybridized overnight at 4°C with antibodies against xCT (1:1000; rabbit polyclonal recognizing both human and mouse antigen, Abcam) or xAG (1:750; mouse monoclonal, Invitrogen). Blots were then incubated with goat anti-rabbit or anti-mouse antibody (1:5000; BD PharMingen) for 1 hour at room temperature. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Baie d'Urfe, QC). Immunostaining for β-actin was used to confirm equal protein loading. xCT protein levels were assessed by densitometry using Image J. The intensity of each xCT band is indicated as a ratio

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over the corresponding loading control band (actin), compared to the control condition. Quantification of western blotting was performed using ImageJ software.

Intracellular arsenic content determination: Cells (5 x10^5 per condition) were treated for 6 hours, then harvested and washed with cold PBS. Cells were then resuspended in 500µL PBS and 9.5ml lysis buffer (0.5mM NH_4OH, 5µM EDTA, 0.005% Triton-X). Levels of arsenic ions were measured by inductively coupled plasma-mass spectrometry (ICP-MS, Perkin Elmer Elan 6100 DRC) at the Geochemical Laboratories, Earth & Planetary Sciences, McGill University. Alternatively, to enhance sensitivity of detection, 2 x10^6 or 1 x10^7 cells per condition were treated for 3 hours. Cells were collected and washed with cold PBS. Cell pellets were frozen for shipping, and then resuspended in 4ml lysis buffer. Levels of arsenic ions were measured by ICP-MS (Chemical Solutions Ltd, Mechanicsburg, PA, USA).

shRNA knock-down: Exponentially-growing NB4 cells were cultured in the presence of lentiviral transduction particles designed against human *SLC7A11* (gene encoding xCT), non-targeting any mammalian gene (Santa Cruz), or with lentiviral transduction particles designed against human *SLC1A1* (gene encoding xAG) (MISSION, Sigma) and 5 µg/ml polybrene. Transduced NB4 cells were then selected with 1µg/ml puromycin. Knock-down was then assessed by western blot.

γ-GT activity assay: This assay was adapted from a previously described method (Dilda et al., 2008). Briefly, NB4 cells were incubated with or without acivicin, a γ-GT inhibitor (Sigma) for 1 hour, then incubated for 3 hours or 24 hours in PBS containing 5 mM γ-GPN, a γ-GT specific substrate that leads to proportional color development

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(yellow) upon formation of p-nitroanilide. The activity of γ-GT was measured colorimetrically (λ = 405 nm).

Statistical analysis: Statistical significance was determined by one way analysis of variance followed by Newman–Keuls post-hoc test using Prism version 3.0 (GraphPad software, San Diego, CA, USA). *: $p< 0.05$; **: $p<0.01$, ***: $p<0.001$.

Results:

DMAC recapitulates the effects of Dar

The chemical structure of Dar led us to believe that dimethylarsino-cysteine (DMAC) is a likely breakdown product of Dar's potential processing. We also considered that the dimethylarsenic moiety of Dar might be labile, thus leading to the formation of dimethylarsenite (DMA III) or dimethylarsenate (DMA V), or both. We investigated the effects of these anticipated breakdown products as compared to Dar in an APL cell line, NB4. We have previously shown that the human NB4 cell line is sensitive to Dar-induced cell death and cell cycle arrest (Diaz et al., 2008). NB4 cells were treated with equimolar concentrations of Dar, DMAC, DMA III or DMA V at clinically achievable arsenic concentrations (0.5-2 μM) and the intracellular levels of arsenic, cell cycle and cell death were measured. DMAC treatment resulted in levels of intracellular arsenic similar to Dar, whereas DMA III and DMA V yielded very low intracellular arsenic (Figure 2A). This is consistent with previous reports showing that extracellular DMA V is weakly toxic and that DMA III might be converted to DMA-OH and finally be oxidized to DMA V

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prior to cell entry (Sakurai et al., 2006). However, this is in contrast with another report showing that DMA III can be toxic to NB4 cells when given as DMA (III)-iodide (Chen et al., 2003). DMA (III)-iodide does not appear to yield significant levels of intracellular arsenic (Chen et al., 2003), suggesting an alternative mechanism for its toxicity. Figure 2B also shows that both Dar and DMAC arrest NB4 cells in the G2/M phase of cell cycle. In contrast, DMA III and DMA V treatment did not alter the cell cycle from control treated cells (Figure 2B). Consistently, Figure 2C shows that DMAC and Dar induce similar amounts of cell death, while neither DMA III nor DMA V are cytotoxic. Together with the ICP-MS results, these data indicate that DMA III and DMA V lack the ability to efficiently enter the cell, but that DMAC may be a breakdown product that is efficiently transported in cells.

γ-GT cleaves Dar to produce DMAC

GSH can be cleaved by γ-GT and an aminopeptidase to generate L-cysteine. Through a process of auto-oxidation and disulfide bond formation, L-cystine is generated from L-cysteine, and is subsequently imported. We hypothesized that γ-GT could play a role in the extracellular processing of Dar to DMAC, and thus, on Dar import into the cell (Figure 3A). Therefore, we tested whether inhibition of γ-GT could inhibit Dar or DMAC-induced intracellular arsenic accumulation and cell death. First, we tested the effect of various doses of the γ-GT inhibitor, acivicin (Lantum et al., 2004), and we have found that 50μM acivicin was the highest concentration with limited toxicity in NB4 cells (Supplementary Figure 1A). When NB4 cells were pretreated for an hour with 50μM acivicin, we observed decreased Dar-induced cell death and intracellular accumulation of arsenic. Inhibition of Dar-induced death was dependent upon the

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acivicin concentration (Supplementary Figure 1B). However, acivicin did not inhibit DMAC-induced death or intracellular arsenic accumulation (Figure 3B and 3D). Using a γ-GT activity assay, we confirmed that 50μM acivicin significantly inhibits the activity of γ-GT, whereas higher concentration (100 μM) does not yield higher inhibition (Figures 3C, 3E, and Supplementary Figure 1C). Together, these data support our hypothesis that Dar is cleaved by γ-GT to ultimately produce DMAC, which can enter the cell.

Thiols can prevent Dar-induced cytotoxicity

Intracellular thiols, including GSH and cysteine, are known to reduce ATO-induced cell death, notably by scavenging ROS and increasing GSH-dependent arsenic efflux (Lee et al., 1989; Ochi et al., 1994). Decreasing intracellular GSH potentiates ATO-induced cell death in both ATO-sensitive and resistant cell lines (Dai et al., 1999; Davison et al., 2003). In contrast, decreasing intracellular GSH levels does not alter Dar-induced cell death (Diaz et al., 2008; Matulis et al., 2009). However, extracellular GSH and thiols could compete for cystine transporters, which we hypothesize is critical in the transport of Dar. To test the effects of thiols on Dar's import and activity, we pretreated NB4 cells with pharmacological concentrations of L-cysteine, D-cysteine or L-GSH, prior to Dar treatment. As predicted by our model, thiols prevented Dar-induced intracellular arsenic accumulation and apoptosis (Figure 4A and 4B), as reported in other cellular models (Matulis et al., 2009). Interestingly, previous work has shown that N-acetyl-cysteine (NAC) has the ability to reduce Dar-induced apoptosis, whereas depletion of intracellular GSH level does not potentiate Dar effects (Diaz et al., 2008; Matulis et al., 2009). Furthermore, ATO-resistant NB4 cells, which have increased intracellular GSH levels, remain sensitive to Dar (Diaz et al., 2008). Therefore, we

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postulate that the effect of thiols does not result from an increase in intracellular GSH, but rather, from modulation of Dar import.

In addition, thiol-containing compounds are able to inhibit DMAC-induced intracellular accumulation of arsenic and DMAC-induced cell death to the same extent as with Dar (Figure 4C and 4D). This is consistent with our hypothesis in which DMAC is Dar's active breakdown product, and that DMAC enters the cell via a cystine importing system. Moreover, in cells pre-treated with acivicin, GSH can no longer inhibit DMAC-induced cytotoxicity, suggesting that GSH needs to be cleaved into cysteine, which can then inhibit DMAC at the import level (Supplementary Figure 2A and 2B). Thiols have minimum effect on intracellular arsenic accumulation and related cell death following treatment with ATO in this setting (Figure 4D and 4E), suggesting that our results are specific to Dar and DMAC.

Cystine transporters are implicated in the import of DMAC

xCT is considered the major cystine/cysteine cellular importer (Banjac et al., 2008). The gene coding for xCT, *SLC7A11*, has an antioxidant response element (ARE) in its promoter and is regulated by the nuclear factor (erythroid-derived 2)-like 2 (NRF2) in response to intracellular ROS (Sasaki et al., 2002; Shih et al., 2003; Wang et al., 2007). ATO and Dar induce intracellular ROS production that activates the NRF2 signaling pathway, thereby leading to expression of NRF2 target genes (Diaz et al., 2005; Garnier et al., 2013; Mann et al., 2009). Therefore, we tested the ability of Dar and ATO to induce xCT. Figure 5A and 5B show that both ATO and Dar induce xCT protein and mRNA levels in NB4 cells.

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We then tested whether xCT may serve as an importer for DMAC, as a breakdown product of Dar, in NB4 cells. To test this hypothesis, we performed an shRNA-mediated knock-down of *SLC7A11*, the gene coding for the light chain catalytic subunit of system xCT and based on densitometry, compared to control NB4 cells, shxCT1 and shxCT2 achieved 94% and 93% knock-down, respectively, (Huang et al., 2005) (Figure 5C). NB4 cells harboring shRNA knock-down for *SLC7A11* were less sensitive to Dar- and DMAC-induced intracellular arsenic accumulation and subsequent cell death, but remained equally sensitive to ATO (Figure 5E and 5F). However, the effect was mild, so we postulated that other cystine/cysteine importers might play a role in Dar/DMAC import. Thus, we also performed an shRNA-mediated knock-down of *SLC1A1*, the gene coding for xAG (Figure 5D), another cystine importer (Budy et al., 2006), and compared to control NB4 cells, achieved an 86% and 90% knock-down with shxAG1 and shxAG5, respectively. This led to similar results (Figure 5E and 5F), showing that DMAC can enter NB4 cells via multiple cystine/cysteine importer systems. In contrast with xCT, xAG does not seem to be induced by ATO or Dar (Supplementary Figure 3).

xCT expression predicts response to Dar and DMAC in non-APL malignancies

In order to strengthen and expand our hypothesis beyond NB4 cells, we utilized several cell line models of non-APL malignancies. First, we utilized a previously characterized *in vitro* model of increased xCT activity, HT1080 human fibrosarcoma cells transduced with an empty retroviral vector or one directing the expression of xCT, (Lastro et al., 2008). We compared, and assessed apoptosis in the modified HT1080

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cell lines following 48 hours of treatment with 2-5 μM Dar or DMAC. Cells overexpressing xCT were significantly more sensitive to Dar- and DMAC-induced cell death at both concentrations (Figure 6A). These data support the hypothesis that xCT acts as an importer for both Dar and DMAC, and that xCT may be a marker of sensitivity to Dar.

Thus, we tested if endogenous xCT levels could predict sensitivity to Dar or DMAC in HCT 116 and RKO 2577 cells, two human colon carcinoma cell lines. First, we assessed xCT levels in these cells. RKO 2577 cells have increased endogenous xCT mRNA and protein levels as compared with HCT 116 (Figure 6B and 6C). Interestingly, RKO 2577 cells are also more sensitive to Dar- and DMAC-induced cell death than HCT 116 cells (Figure 6D and 6E).

Discussion:

We describe a cellular import mechanism for a novel organic arsenic-based anti-cancer drug, darinaparsin. Compared with ATO, Dar treatment leads to higher intracellular levels of arsenic and cell death in NB4 cells. As shown in Figure 6, ATO is imported into cells via glucose transporters and aquaglyceroporins (Rosen and Liu, 2009). The higher intracellular arsenic accumulation observed after Dar treatment compared with ATO suggests that Dar has more and/or better modes of entry. Whether Dar and ATO share some of them remains to be investigated.

Interestingly, our data shows that DMA III and DMA V do not enter NB4 cells, and do not result in enhanced toxicity. This is in contrast to previously published reports

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showing that DMA (III)-iodide can decrease NB4 cell number and cell viability (Chen et al., 2003). NB4 cells do not methylate arsenic and when co-cultured with cells that do methylate arsenic (HepG2 hepatoma cells), DMAs represent only a minor fraction (Chen et al., 2003). Many other cell types, including pancreatic islet cells (Douillet et al., 2013) and uroepithelial cells (Drobna et al., 2005), have increased uptake of methylated arsenicals, which leads to increased cytotoxicity, as compared to inorganic arsenicals. Although we cannot fully explain the discrepancies between our data and that published by Chen et al., we do not believe it is due to oxidation of our DMA (III) as it known to be stable for up to three days when pure preparations are used (Gong et al., 2001). It is possible that glutathione concentrations in the serum used could affect import of DMA III, should it be conjugated and imported as DMAC. Alternatively, the use of DMA (III)-iodide instead of the direct reduction method used here may result in differential toxicity.

Numerous studies have shown that mercury, another metal, is transported into some cells as a cysteine conjugate (Bridges et al., 2004; Bridges and Zalups, 2006; Zalups, 1995; Zalups and Lash, 2006). In particular, L-cysteine- and GSH-conjugated, but not N-acetylcysteine-conjugated, methylmercury are efficiently transported into the renal proximal tubules (Wang et al., 2012). In addition, acivicin significantly blocked entry of GSH-conjugated methylmercury into the proximal tubules (Wang et al., 2012). These studies suggest that the GSH processing machinery, as well as the cystine importing systems could be permissive enough to allow GSH S-conjugates to use them in order to achieve cell import (Bridges and Zalups, 2005).

Here, we propose this route of cell entry may also exist for arsenicals and is likely to be specific to cysteine-S conjugated arsenicals. Our data strongly suggest Dar-

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induced intracellular arsenic and cytotoxicity are dependent upon γGT, suggesting Dar may be cleaved at the cell surface into DMAC, and that DMAC is imported via cysteine/cystine transporters, using its cysteine moiety. However, we do not exclude the possibility that a certain amount of DMAC could also arise subsequently from the dimethyl-arsenic being exchanged from Dar to a free cysteine (Bohle and Gu, 2013). Interestingly, another organic arsenical, 4-(N-(S-glutathionylacetyl)amino) phenylarsonous acid (GSAO), also a GSH-s conjugate, is cleaved by γGT to produce 4-(N-(S-cysteinylglycylacetyl)amino) phenylarsonous acid (GCAO) (Dilda et al., 2008). GSH is cleaved by both γGT and a dipeptidase extracellulary and is involved in processing of GSAO (Dilda et al., 2008). Thus, an additional step involving a dipeptidase is likely required to ultimately produce DMAC. Further investigation is required in order to elucidate this aspect of Dar extracellular processing.

We show that down regulation of individual cystine/cysteine importing systems partially impairs Dar entrance (Figure 5), and that saturation of the extracellular media with cystine/cysteine blunts Dar import almost entirely (Figure 4), indicating that Dar/DMAC utilizes multiple cystine/cysteine importers. It has been proposed that most of these transporters, including xCT and xAG, can be involved in import of cysteine or cystine (Budy et al., 2006). This could provide a partial explanation as to why these transporters can recognize and import DMAC based on its cysteine moiety (Figure 1).

Although Dar is a promising novel anti-cancer agent, shedding light on its mechanism of import is critical, as its therapeutic spectrum is incompletely defined. We propose that cystine/cysteine importing systems may serve as biomarkers for predicting response to Dar in the clinic. Indeed, we provide preliminary data suggesting that, in

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colon cancer, xCT could predict response to Dar *in vitro*. Moreover, cancer cells are usually under high oxidative stress, exacerbating their need for GSH and hence, for cystine/cysteine. We believe that this might partially explain the selective toxicity to cancer cells of Dar relative to ATO, as Dar will be preferentially absorbed by cells expressing high levels of γGT and cystine/cysteine importers, i.e. cancer cells. In addition, upregulation of xCT expression, cystine/cysteine uptake and intracellular GSH levels have been associated with resistance to cancer therapy (Diaz et al., 2008; Huang et al., 2005). Indeed, the xCT-overexpressing HT1080 model cells employed in these experiments are resistant to dihydropyridines and cobalt chloride (Lastro et al., 2008). Importantly, xCT expression is induced by both ATO and Dar, leading to cytoprotection against ATO, but leading to increased import for Dar. Therefore, we conclude that Dar could represent an interesting therapeutic alternative for multi-drug resistant tumors, since chemo-resistance mediated by upregulation of the GSH cycle components may translate into hypersensitivity to Dar.

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Authorship contributions:

Participated in research design: Garnier, Gu, Bohle, del Rincon, Conklin, Mann and Miller

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Contributed new reagents or analytic tools: Gu, Sun, Conklin, and Bohle

Performed data analysis: Garnier, Redstone, Dahabieh, Nichol

Wrote or contributed to the writing of the manuscript: Garnier, Gu, Bohle, del Rincon, Mann and Miller

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Footnotes:

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Disclosure of Potential Conflicts of Interest:

Ziopharm Oncology Inc. has provided a research grant to Wilson H. Miller Jr. The other authors disclosed no potential conflicts of interest.

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Figure Legends:

Figure 1: Structural analysis of Dar and potential breakdown products. Darinaparsin consists of a trivalent dimethylarsenic S-conjugated to GSH via cysteine and can give rise to DMAC, a trivalent dimethylarsenic S-conjugated to cysteine, in a multistep process that may involve cell surface enzymes. GSH, a tripeptide, is also cleaved at the cell surface in a multistep process that involves cell surface enzymes to produce cysteine, which is oxidized in cystine. DMAC and cystine have a common residue, cysteine, and are therefore structurally similar, to a certain extent.

Figure 2: DMAC recapitulates the effects of Dar. $5x10^5$ cells were treated for 6 hours with increasing amounts of Dar, DMAC, DMA III or DMA V. Intracellular levels of arsenic were measured by ICP-MS. Statistical significance is indicated for all three doses compared to control (A). Cells were treated for 24 hours with 2μM Dar, DMAC, DMA III or DMA V. Cell cycle (B) and cell death (C) were assessed by propidium iodide staining and quantified by flow cytometry. Statistical significance of increased G2/M compared to control (B) and for increased sub-G0 compared to control (C) are indicated.

Figure 3: γ-GT cleaves Dar to produce DMAC. Dar is cleaved to ultimately produce DMAC by γ-GT, which can be inhibited by acivicin (A). 2 $x10^6$ cells were treated for 3 hours with 2μM Dar or DMAC with or without a 1 hour pre-treatment with 50μM acivicin. Intracellular levels of arsenic were measured by ICP-MS (B). Cells were pre-incubated with acivicin for 1 hour, then γ-GPN was added for 3 hours and γ-GT activity was assessed (C). Cells were treated for 24 hours with 2μM Dar or DMAC with or without a 1 hour pre-treatment with 50μM acivicin. Cell death was assessed by propidium iodide

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staining and quantified by flow cytometry (D). Cells were pre-incubated with acivicin for 1 hour, then γ-GPN was added for 24 hours and γ-GT activity was assessed (E).

Figure 4: Thiols can inhibit Dar-induced cytotoxicity. 1 x10^7 cells were pre-treated for 1 hour with 0.1 mM or 1 mM L-cysteine, D-cysteine or with 0.5mM or 5mM L-GSH. Then, cells were treated with 2μM Dar (A and B), DMAC (C and D) or ATO (E and F). Intracellular levels of arsenic were measured by ICP-MS after 6 hours (A, C and E) and cell death was assessed by propidium iodide staining and quantified by flow cytometry after 24 hours (B, D and F).

Figure 5: Cystine transporters are implicated in the import of DMAC. NB4 cells were treated with 2μM ATO or Dar. xCT protein levels were assessed by western blotting after 6 hours (A) and mRNA levels by qPCR after 3 hours (B). NB4 cells were transfected with shRNA lentiviral particles against *SLC7A11*, *SLC1A1*, or no mammalian target (Ctrl). xCT (C) and xAG (D) protein levels were quantified by western blotting. 2 x10^6 untransfected and transfected NB4 cells were treated with 1μM ATO, Dar or DMAC, intracellular concentration of arsenic were measured by ICP-MS after 3 hours (E) and cell death was assessed by PI staining and flow cytometry after 48 hours (F).

Figure 6: Sensitivity to Dar correlates with xCT expression. (A) HT1080 cells transduced with empty vector or xCT (left immunoblots) were treated with 2 or 5 μM Dar or DMAC for 48 hours. Cell death was assessed by PI staining and flow cytometry. Proteins (B) or mRNA (C) were harvested from HCT 116 and RKO 2577 cells and xCT levels were assessed. HCT (D) and RKO (E) cells were treated with 2μM Dar or DMAC; cell death was assessed by PI staining and flow cytometry after 48 hours.

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Figure 7: Dar *vs* ATO import route and intracellular effects. Dar is cleaved at the cell surface by γ-GT and a dipeptidase to ultimately produce DMAC, which enters the cells via cysteine/cystine importing systems, leading to high intracellular levels of arsenic and cell death. ATO is imported into cells via glucose transporters and aquaporins.

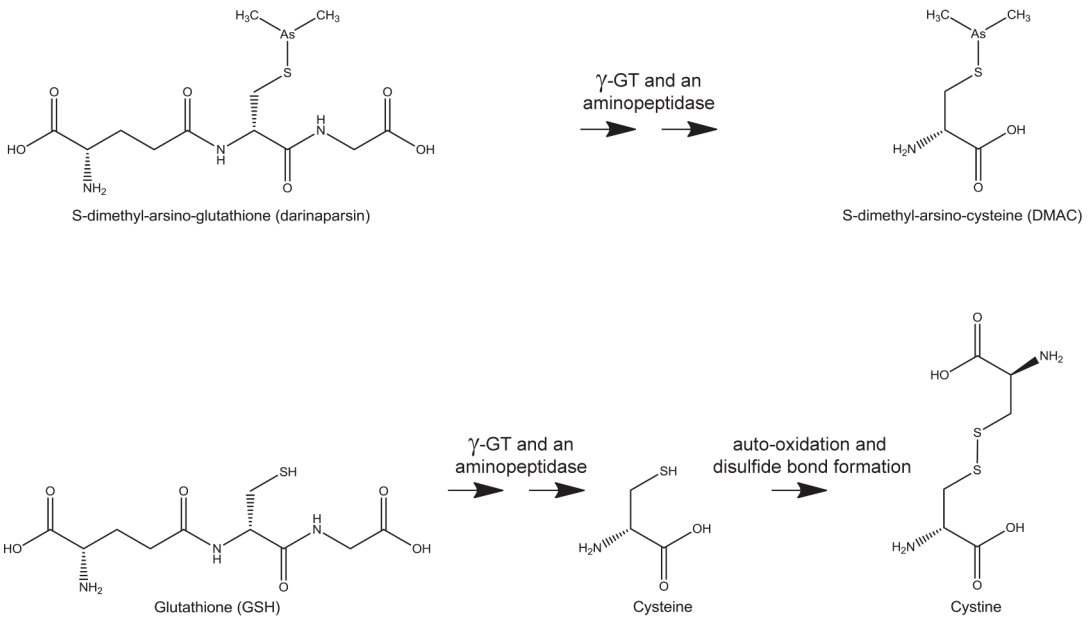
γ-GT and an aminopeptidase
S-dimethyl-arsino-glutathione (darinaparsin)
S-dimethyl-arsino-cysteine (DMAC)
γ-GT and an aminopeptidase
auto-oxidation and disulfide bond formation
Glutathione (GSH)
Cysteine
Cystine

Figure 1

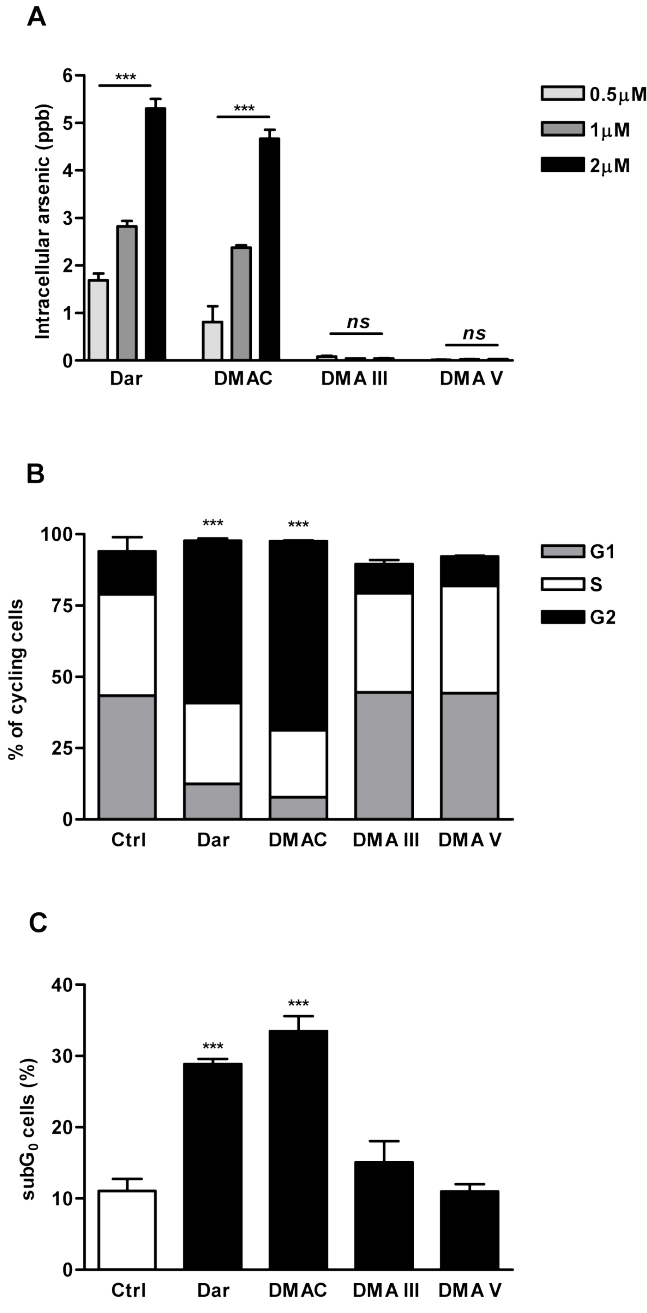
A
Intracellular arsenic (ppb)
0
1
2
3
4
5
6
***
***
ns
ns
Dar
DMAC
DMA III
DMA V
0.5μM
1μM
2μM
B
% of cycling cells
0
25
50
75
100
***
***
Ctrl
Dar
DMAC
DMA III
DMA V
G1
S
G2
C
subG0 cells (%)
0
10
20
30
40
***
***
Ctrl
Dar
DMAC
DMA III
DMA V

Figure 2

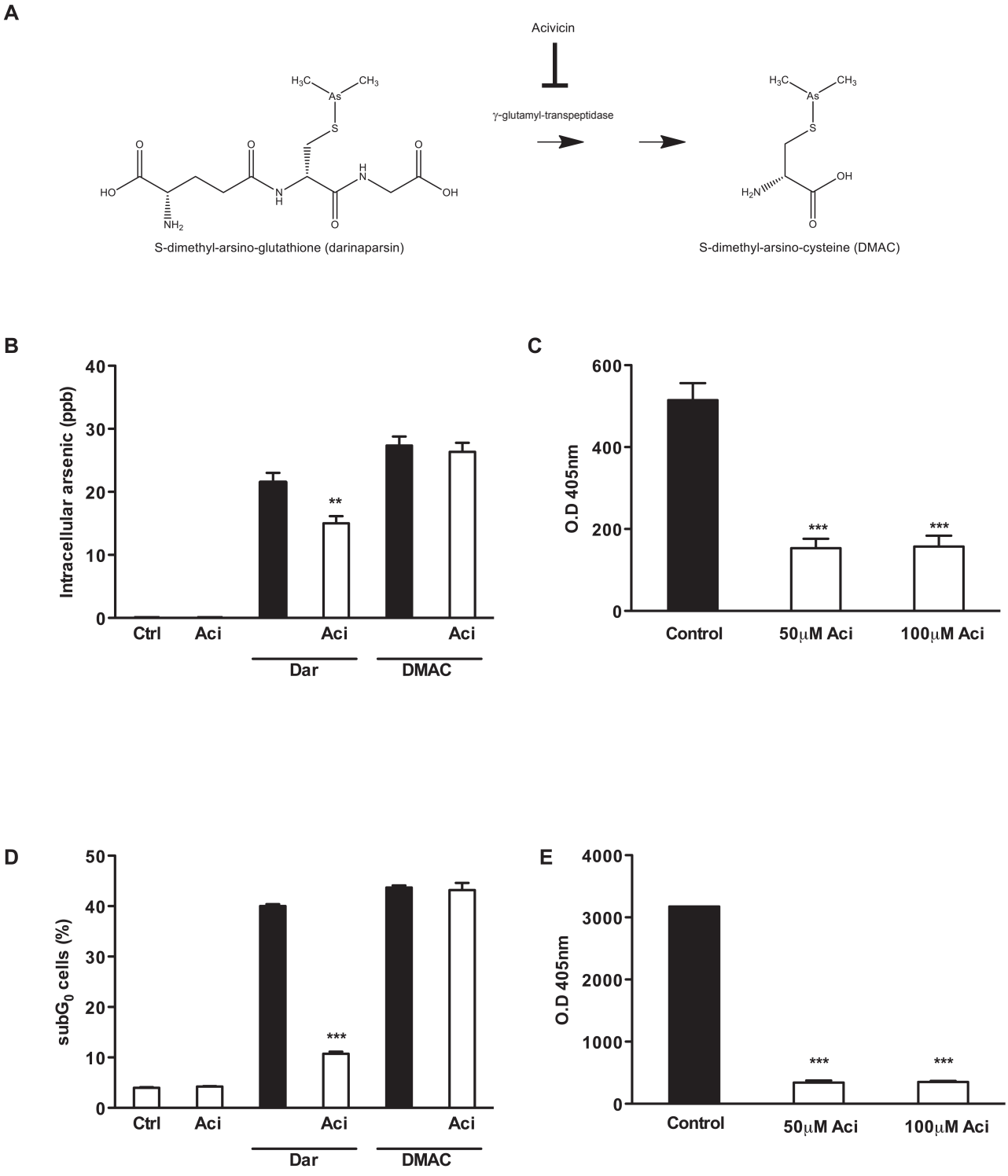
A
Acivicin
γ-glutamyl-transpeptidase
S-dimethyl-arsino-glutathione (darinaparsin)
S-dimethyl-arsino-cysteine (DMAC)
B
Intracellular arsenic (ppb)
Ctrl
Aci
Aci
Aci
Dar
DMAC
C
O.D 405nm
Control
50μM Aci
100μM Aci
D
subG0 cells (%)
Ctrl
Aci
Aci
Aci
Dar
DMAC
E
O.D 405nm
Control
50μM Aci
100μM Aci

Figure 3

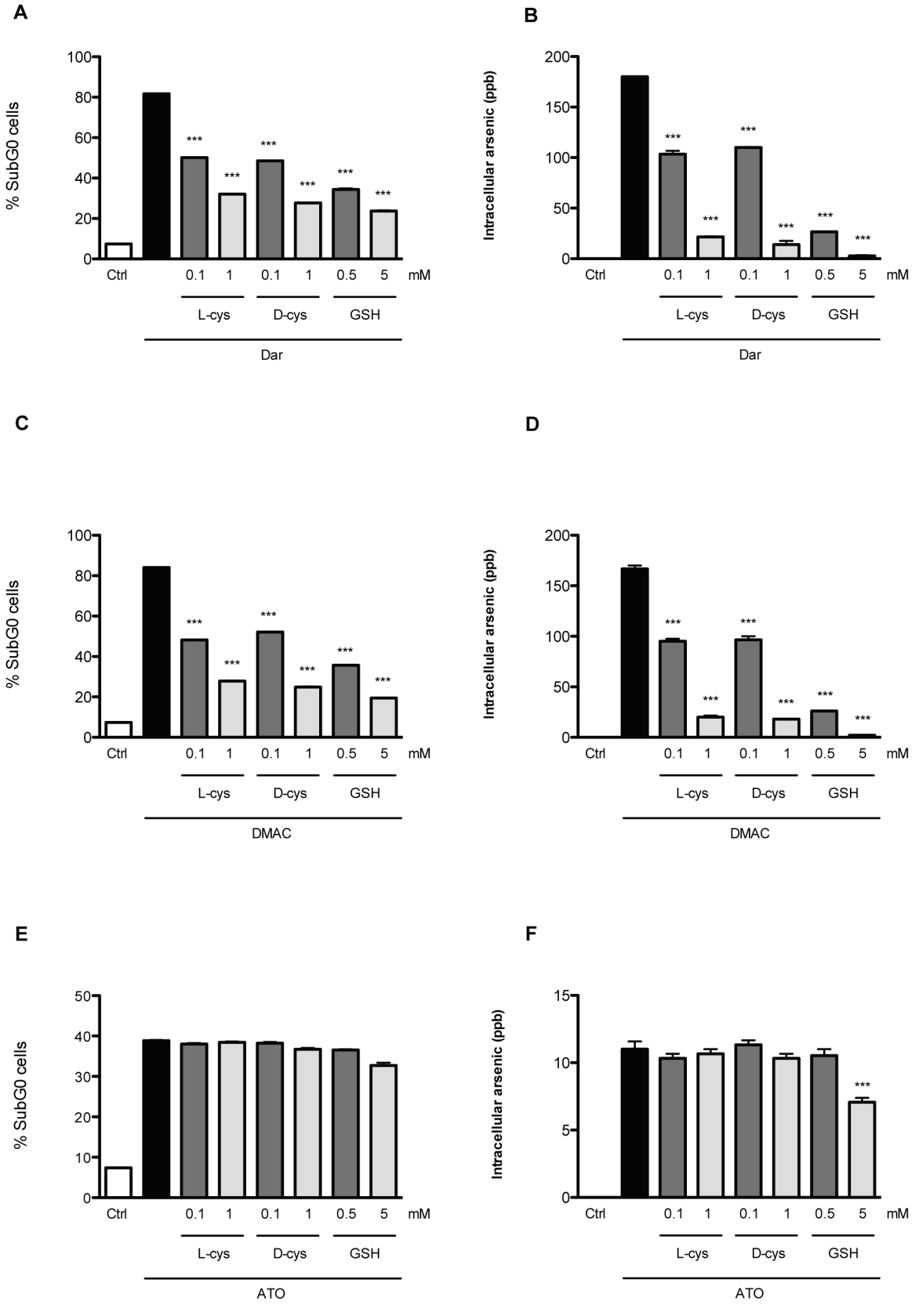
A
% SubG0 cells
100
80
60
40
20
0
***
Ctrl
0.1
1
0.1
1
0.5
5
mM
L-cys
D-cys
GSH
Dar
B
Intracellular arsenic (ppb)
200
150
100
50
0
C
DMAC
D
E
ATO
F
15
10
5

Figure 4

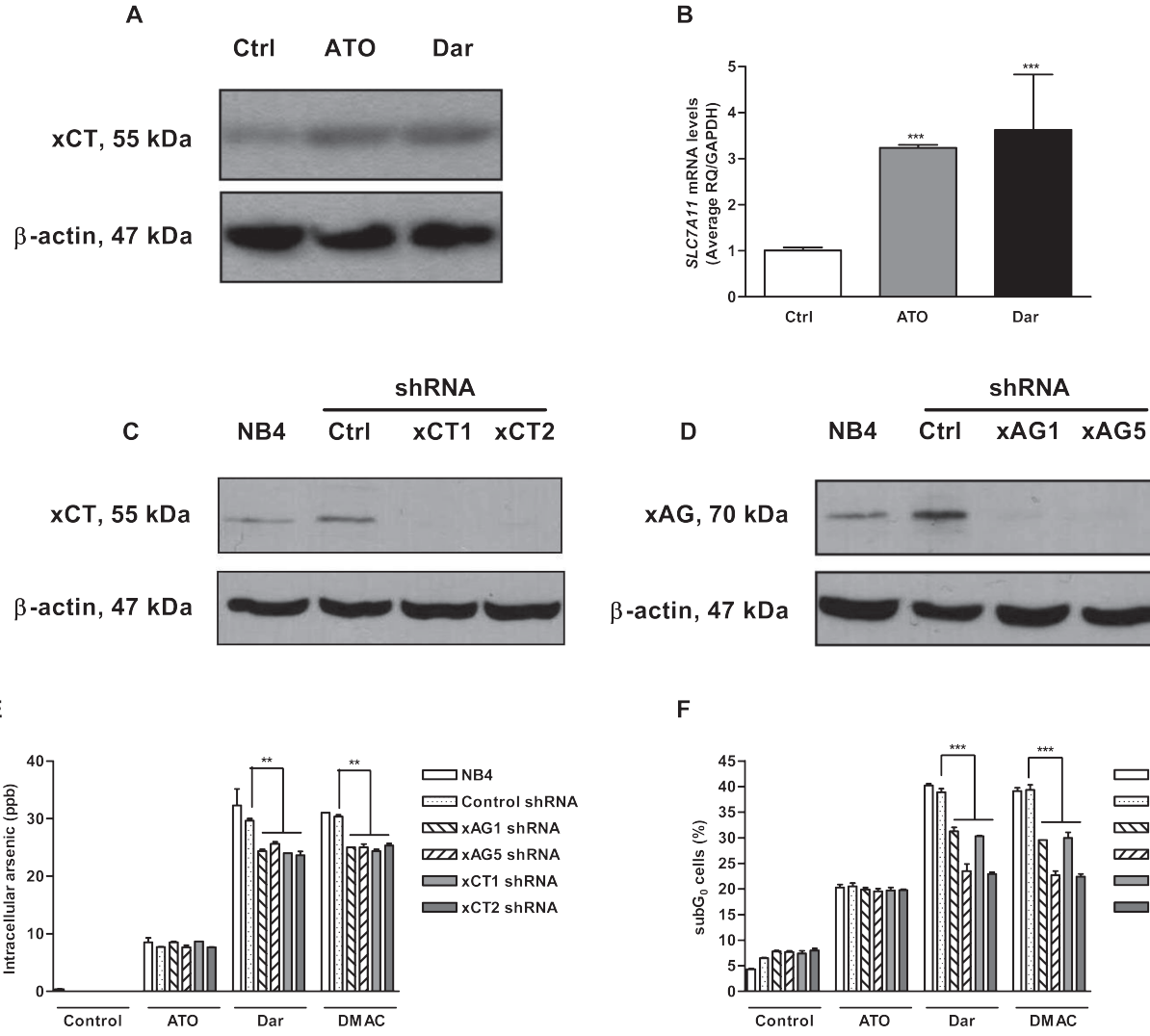
A
Ctrl
ATO
Dar
xCT, 55 kDa
β-actin, 47 kDa
B
SLC7A11 mRNA levels
(Average RQ/GAPDH)
***
***
Ctrl
ATO
Dar
C
shRNA
NB4
Ctrl
xCT1
xCT2
xCT, 55 kDa
β-actin, 47 kDa
D
shRNA
NB4
Ctrl
xAG1
xAG5
xAG, 70 kDa
β-actin, 47 kDa
E
Intracellular arsenic (ppb)
**
**
Control
ATO
Dar
DMAC
NB4
Control shRNA
xAG1 shRNA
xAG5 shRNA
xCT1 shRNA
xCT2 shRNA
F
subG0 cells (%)
***
***
Control
ATO
Dar
DMAC
NB4
Control shRNA
xAG1 shRNA
xAG5 shRNA
xCT1 shRNA
xCT2 shRNA

Figure 5

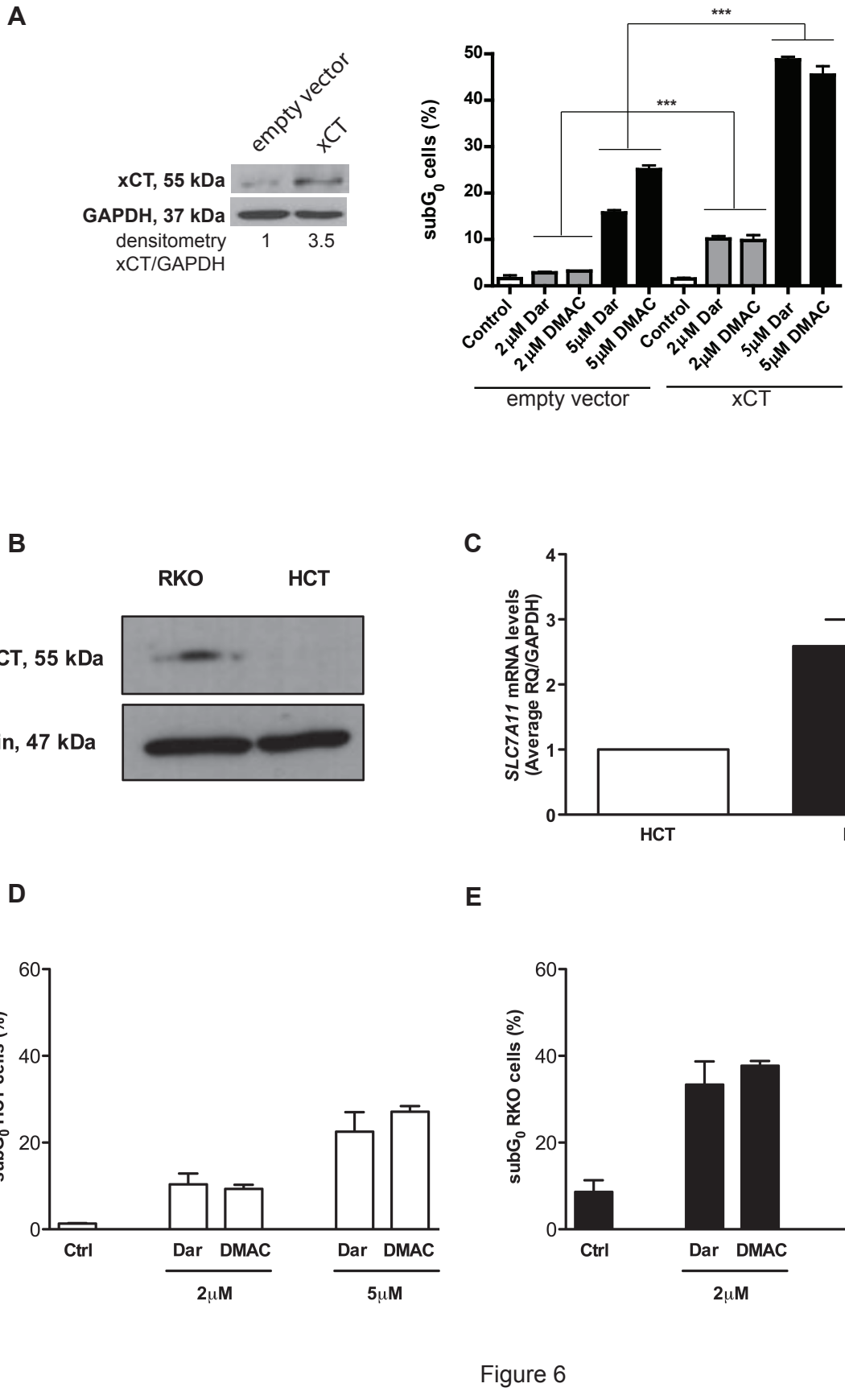
A
empty vector
xCT
xCT, 55 kDa
GAPDH, 37 kDa
densitometry xCT/GAPDH
1
3.5
subG0 cells (%)
***
***
Control
2 μM Dar
2 μM DMAC
5μM Dar
5μM DMAC
empty vector
xCT
B
RKO
HCT
xCT, 55 kDa
β-actin, 47 kDa
C
SLC7A11 mRNA levels (Average RQ/GAPDH)
HCT
RKO
D
subG0 HCT cells (%)
Ctrl
Dar
DMAC
2μM
5μM
E
subG0 RKO cells (%)

Figure 6

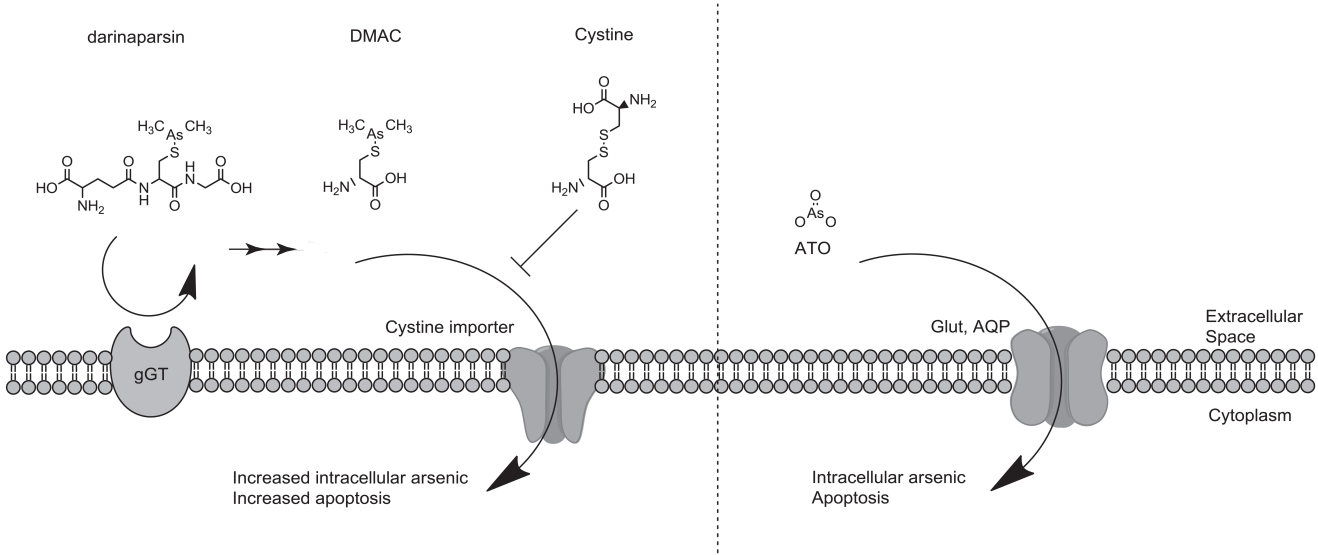
darinaparsin
DMAC
Cystine
ATO
gGT
Cystine importer
Glut, AQP
Extracellular Space
Cytoplasm
Increased intracellular arsenic
Increased apoptosis
Intracellular arsenic
Apoptosis

Figure 7