

Disulfiram's Antineoplastic Effects on Ovarian Cancer

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Abstract

Objective: Aldehyde dehydrogenase (ALDH) enzymatic activity identifies ovarian cancer stem-like cells. We investigated the antineoplastic activity of the ALDH inhibitor Disulfiram on bulk ovarian cancer cells and CD133⁺/ALDH⁺ cancer stem-like cells. **Study Design:** Ovarian cancer cell lines, human ovarian surface epithelial cells, and mesenchymal stem cells were treated with increasing concentrations of Disulfiram and/or Cisplatin *in vitro*. Treated cells were assessed for viability or FACS-analyzed for either percentage of ovarian cancer stem-like cells or induction of apoptosis. Disulfiram's impact on cancer stem-like cells was tested *in vitro* using tumor sphere formation assays and *in vivo* using tumor initiation assays with *in vitro*-treated A2780 cells in NSG mice. Finally, Disulfiram's *in vivo* activity was assessed versus CD133⁺/ALDH⁺ cell-initiated tumor xenografts. **Results:** Disulfiram demonstrated antineoplastic activity against multiple ovarian cancer cell lines. While Disulfiram had limited *in vitro* toxicity against human ovarian surface epithelial cells or mesenchymal stem cells (IC₅₀ of ~15 μ M and >30 μ M, respectively), its antineoplastic activity against cell lines was comparable to Cisplatin (IC₅₀ ~1.5 μ M). Disulfiram-mediated cell death was due, at least in part, to induction of apoptosis. Disulfiram activity was additive with chemotherapy. Disulfiram demonstrated selective depletion of CD44⁺ cells but not the CD133⁺ cancer stem-like cells. Disulfiram had no therapeutic impact on tumor initiation studies or *in vivo* therapy of whole cell line or stem cell-initiated tumor xenografts. **Conclusions:** In biologically relevant concentrations, Disulfiram has clear antineoplastic activity against ovarian cancer cells *in vitro*. Disulfiram selectively depleted CD44⁺ but not CD133⁺ ovarian cancer stem-like cells *in vitro*. However, Disulfiram had no significant activity *in vivo*. Thus, improved and more selective ALDH inhibitors may be required to target ovarian cancer stem cells.

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Keywords

Antineoplastic, ALDH, Disulfiram, NSG Mice, Ovarian Cancer Stem Cells

1. Introduction

Many solid tumors contain a population of cells, termed cancer stem-like cells (CSC). CSC, despite constituting a small percentage of the overall cancer cell population, have increased tumor-initiation capacity and have therefore been proposed as a source of disease recurrence [1]. As such, CSC have been proposed as a therapeutic target. Aldehyde dehydrogenase enzymatic activity (ALDH) has been identified as a marker of CSC in numerous malignancies, including ovarian cancer [2] [3]. ALDH-high (ALDH⁺) epithelial ovarian cancer cells are chemotherapy-resistant; can both initiate and propagate tumors in mice, and can create heterogeneous lineages of ovarian cancer cells [3]. Thus, they meet the American Association for Cancer Research consensus criteria for CSC [4]. Indeed, we have shown that ALDH and CD133 identify an ovarian cancer differentiation hierarchy [5].

There is additional evidence that ALDH⁺ cells are critical in ovarian cancer: 1) the presence of cells positive for ALDH1A1 and prominin-1 (CD133, a CSC marker) in patients' primary tumor specimens correlates with poor outcome [3]; 2) ALDH1A1 is upregulated 100-fold in ovarian cancer cells selected for resistance to either Cisplatin or Paclitaxel; 3) siRNA knockdown of ALDH1A1 restores chemosensitivity [6]; and 4) ALDH1A1 and CD133 are enriched in tumor specimens immediately following chemotherapy [7]. Based on the preferential activity of ALDH in chemotherapy-resistant tumor-propagating cells, ALDH has been implicated as a therapeutic target.

ALDH enzymes catalyze the oxidation of aldehydes to their carboxylic acid forms. These enzymes play an important role in various cellular metabolic pathways including the detoxification of acetaldehyde, the detoxification of free oxygen radicals generated by chemotherapeutic agents (such free oxygen radicals can lead to cancer cell membrane peroxidation and subsequent apoptosis), and the conversion of retinal to retinoic acid to regulate cellular transcription and differentiation pathways [8].

Disulfiram is an orally available ALDH inhibitor which has been used for decades in the treatment of chronic alcoholism. While Disulfiram was primarily developed to inhibit ALDH2, Disulfiram also inhibits ALDH1 isozymes. Based upon its ability to inhibit ALDH1 isozymes, Disulfiram has recently been tested as a CSC-targeting drug. Disulfiram has been reported to have anti-cancer activity in numerous malignancies, including breast, colon and prostate cancers among others [9]-[14]. Studies in breast cancer have suggested that Disulfiram may specifically target the breast CSC population [11]. More recently, it was shown that ALDH activity was necessary to maintain a drug-resistant phenotype in cancer cells and that ALDH inhibition with Disulfiram, via modulation of reactive oxygen species, could overcome this drug-resistant phenotype and delay disease recurrences [15].

Given that we and others have defined ALDH as a marker of ovarian CSC, and given the potential of Disulfiram to target ALDH⁺ CSC, we tested the ability of Disulfiram to target ovarian CSC both *in vitro* and *in vivo* in Nod-Scid-Gamma (NSG) mice.

2. Materials and Methods

2.1. Cell Lines and Cytotoxicity Assays

A2780, OVCAR3, OVCAR8, HEY1, and OVSAHO cells were grown in RPMI media (Thermo Scientific, Rockford, IL) while SKOV3 and human ovarian surface epithelial (HOSE) cells were maintained in DMEM media (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Invitrogen) at 37°C with 5% CO₂. Mesenchymal stem cells (MSC, purchased from Invitrogen) were grown in serum-free MEBM-2 media (Lonza, Walkersville, MD) supplemented with 2% B27 (Gibco), EGF (1:5000 dilution, Invitrogen), 1% penicillin/streptomycin (Invitrogen), hydrocortisone (1:50,000 dilution, Sigma, St. Louis, MO), insulin (1:800 dilution, Invitrogen), BME (1:125 dilution, Sigma), and 0.5% bFGF (BD Biosciences, San Jose, CA). Cell lines were seeded onto 96-well plates at a density of 7500 cells/well. The following day, cells were treated with increasing concentrations (range 0.3 - 30 µM) of Disulfiram or Cis-

platin for 3 days; then cell viability was assessed using the methylthiazoltetrazolium (MTT) assay as previously described [16].

2.2. Tumor Sphere Assays

SKOV3 cells, primary patient ascites, or FACS sorted primary cells were plated in triplicate (2000 cells/well for SKOV3 and 10,000 cells/well for patient samples) on ultra-low attachment plates (Corning, Tewksbury, MA) in serum-free MEBM-2 media (Lonza) supplemented with 2% B27 (Gibco), EGF (1:5000 dilution, Invitrogen), 1% penicillin/streptomycin (Invitrogen), hydrocortisone (1:50,000 dilution, Sigma), insulin (1:800 dilution, Invitrogen), BME (1:125 dilution, Sigma), and 0.5% bFGF (BD Biosciences). Cells were treated with the indicated concentrations of Disulfiram for 3 days. Sphere formation was assessed on day 15 (SKOV3) or 20 (patient samples); triplicate wells were photographed using the Olympus Microsuite Biological Suite software and total sphere number per well was counted. Results were compared using Student t-test.

2.3. In Vivo Studies

Nude mice were obtained from Charles River Laboratories. Animals were maintained in accordance with institutional policies, and all studies were performed with approval of the University Committee on Use and Care of Animals (UCUCA) of the University of Michigan. To determine the tumor-initiating capacity of *in vitro*-treated bulk ovarian cancer cells, A2780 cells were treated with Disulfiram (2 μ M and 10 μ M) or Cisplatin (0.25 μ g/ml) for 3 days. Viable cells (identified as being Annexin-V⁻/propidium iodide⁻) were FACS-isolated and injected subcutaneously into the axillae of female Nu/Nu mice [5000 cells/axilla suspended in 100 μ L of DPBS and 200 μ L of Matrigel (BD Biosciences)]. Tumor volume was assessed periodically using calipers, with volume calculated based on the modified ellipsoid formula ($L \times W \times W/2$), where L represents length and W represents width. The mice were euthanized when tumors reached 1500 to 2000 mm³.

To examine the effect of Disulfiram *in vivo*, we first assessed the impact of Disulfiram on flank tumor growth. 100,000 bulk A2780 cells or 10,000 FACS-isolated CD133⁺/ALDH⁺ A2780 cells were injected into the flank of nude mice. Tumors were allowed to engraft for 1 week; then we initiated therapy with either vehicle, Disulfiram (0.15 mg i.p. daily for 21 days—dose equivalent of 500 mg/day in humans [17]), Cisplatin (0.5 mg/kg daily for 3 days), or the combination of Cisplatin and Disulfiram either concurrently daily for 3 days, or concurrently daily for 3 days followed by an additional 18 days of Disulfiram therapy. Tumor volumes were measured using the $L \times W \times W/2$ formula for ellipsoid tumors. For IP experiments, 10,000 SKOV3 cell or 10,000 FACS-isolated CD133⁺/ALDH⁺ A2780 cells were injected intraperitoneally into nude mice. Cells were allowed to engraft for 1 week then mice were treated daily for 21 days with Disulfiram (0.15 mg i.p.). Controls received vehicle only. Mice were evaluated periodically for physical health and were sacrificed when fulfilling the UCUCA criteria of severe morbidity. Survival was estimated using the Kaplan-Meier plot.

2.4. Flow Cytometric Analysis and Fluorescence-Activated Cell Sorting (FACS)

A2780, OVSAHO, or HEY1 cells were harvested, counted in single-cell suspensions and plated (250,000 cells/well and 500,000 cells/well for the apoptosis induction and the ovarian CSC experiments, respectively) in 60 mm plates, then treated the following day with Disulfiram (7.5 μ M for 3 days and 15 μ M for 2 days for the apoptosis induction and the ovarian CSC experiments, respectively). Controls received vehicle only. Cells were harvested and counted; then FACS-sorted for the Annexin-V-positive cells (which indicates apoptosis induction) or CD133 positivity (which marks ovarian CSC).

In the apoptosis induction assays, cells were stained with Annexin-V and propidium iodide (PI), using the FITC Annexin V Apoptosis Detection Kit II (BD Biosciences) according to the manufacturer's instructions. This was followed by flow cytometric analysis.

In the assays examining the *in vitro* effect of Disulfiram on the ovarian CSC population using the CD133 antigen as a marker for stemness, A2780 cells were stained with anti-CD133/2-APC (MiltenyiBiotec, Auburn, CA), followed by ALDEFLUOR using the ALDEFLUOR assay (Stem Cell Technologies, Vancouver, BC, Canada). As a negative control, for each sample of stained cells an aliquot was treated with 50 mM of diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. This was followed by flow cytometric analysis. PI exclusion gates defined the viable cell population while the negative gates were established using the ALDEFLUOR/

DEAB treated cells as previously described [3].

In the *in vivo* experiments, where applicable, following FACS analysis, sterile sorting for the Annexin-V⁻/PI⁻ cells or the CD133⁺/ALDH⁺ cells was performed under low pressure in the absence of UV light.

For all experiments, flow cytometric analysis was performed by the University of Michigan Flow Cytometry Core using either the BD FACSARIA or FACSDiva instruments (both from Becton Dickinson, San Diego, CA). FACS profiles were subsequently analyzed using FlowJo (Tree Star Inc., Ashland, OR).

2.5. Statistical Analysis

Mean values and standard deviations for all experiments were determined using Microsoft Excel software (Microsoft, Redmond, WA). Groups were compared using Student t-test (Microsoft Excel software). A p value of less than 0.05 was considered statistically significant. The IC₅₀s were estimated from the concentration/response curves. GraphPad Prism version 5.00 for Windows (GraphPad Inc., San Diego, CA) was used to generate Kaplan-Meier survival curves.

3. Results

We first examined the *in vitro* cytotoxicity of Disulfiram against bulk ovarian cancer cells. Using MTT assays, Disulfiram demonstrated an antineoplastic activity against a variety of ovarian cancer cell lines (**Figure 1(a)** & **Figure 1(b)**), but was most effective in the platinum-sensitive (A2780, IC₅₀: ~1.5 μ M) as opposed to the platinum-resistant (SKOV3, IC₅₀: ~40 μ M) cell lines. Consistent with previous results, we observed that Disulfiram induced apoptotic cell death (Supplemental **Figure 1**). We then compared the cytotoxicity of Disulfiram to that of Cisplatin, and found that the antineoplastic activity of the two drugs in the A2780 cell line is comparable (Cisplatin IC₅₀: ~3 μ M, **Figure 1(b)**). We then examined, *in vitro*, the relative sensitivities of ovarian cancer cells or non-neoplastic human cells, including human ovarian surface epithelial cells (HOSE) and mesenchymal

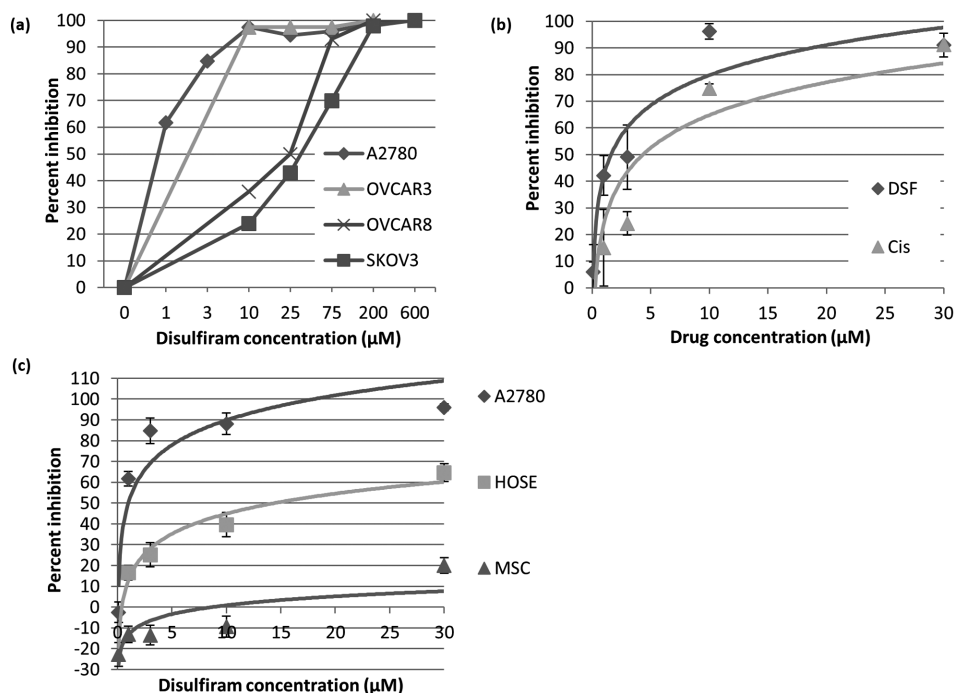


Figure 1. *In vitro* cytotoxicity of Disulfiram against ovarian cancer: (a) MTT assays demonstrating percent inhibition of cell growth with increasing concentrations of Disulfiram (DSF) treatment versus untreated controls; (b) Comparison of percent inhibition of cell growth with Cisplatin (Cis) versus Disulfiram at various concentrations; (c) Comparison of growth inhibitory effects of Disulfiram on A2780 ovarian cancer cells (OCC) versus human ovarian surface epithelial cells (HOSE) versus mesenchymal stem cells (MSC) suggests that Disulfiram preferentially inhibits cancer cells. Results are representative of at least 3 assays with 3 replicates for each group.

stem cells (MSC), to Disulfiram. We observed that ovarian cancer cells are most sensitive to Disulfiram. The HOSE cells were modestly sensitive to Disulfiram, while the MSC were relatively insensitive to Disulfiram (**Figure 1(c)**). This data suggests that ALDH inhibition with Disulfiram preferentially impact cancer cells.

As ALDH inhibition is reported to enhance chemotherapy response in numerous malignancies [10]–[14], we next tested the response of A2780 cells to Cisplatin, Disulfiram, or their combination. While Cisplatin alone had little activity, the combination of Disulfiram to Cisplatin increased activity (**Figure 2(a)**). As Cisplatin can increase the percentage of ALDH⁺ cells [3], it is possible treating with cisplatin could increase resistance to Disulfiram. We therefore also examined the importance of drug sequence. Cells were treated with either drug alone, the two drugs given sequentially, or given concurrently. Treatment with concurrent Cisplatin and Disulfiram was superior to either drug as a single agent. Neither drug sequence (Cisplatin followed by Disulfiram or Disulfiram followed by Cisplatin) was better than single-agent therapy (**Figure 2(b)**).

Disulfiram has been reported to target CSC in breast cancer. We therefore next used flow cytometry to assess the ability of Disulfiram to specifically deplete marker-expressing ovarian CSC *in vitro*. As Disulfiram is an irreversible inhibitor of ALDH, we could not use ALDH expression as a measure of stemness. We therefore used the expression of CD133 and CD44 as an ovarian CSC marker to evaluate the CSC population in different cell lines. At lower doses of Disulfiram, we observed clear induction of cell death but no selective death of CD133⁺ cells (**Figure 3** and data not shown). At higher concentrations of Disulfiram we saw modest (~1.2 fold) preferential depletion of the CD133⁺ cells but up to 1.9–4-fold depletion of CD44⁺ cells (**Figure 3**).

We then tested the impact of Disulfiram on the growth of ovarian tumor spheres, which is a functional assay for tumor CSC growth. Compared to mock-treated cells, cells treated with Disulfiram showed a concentration-dependent reduction in tumor sphere formation. This was demonstrated in both cell lines and primary patient samples (**Figure 4(a)** and **Figure 4(b)**).

We next assessed the ability of Disulfiram to impact the tumor initiation capacity of ovarian cancer cells. We treated the A2780 cell line *in vitro* with Disulfiram, Cisplatin, or vehicle and FACS-isolated viable (Annexin-V⁻/PI⁻) cells. 5000 viable cells from each group were then injected into immunodeficient mice. We observed

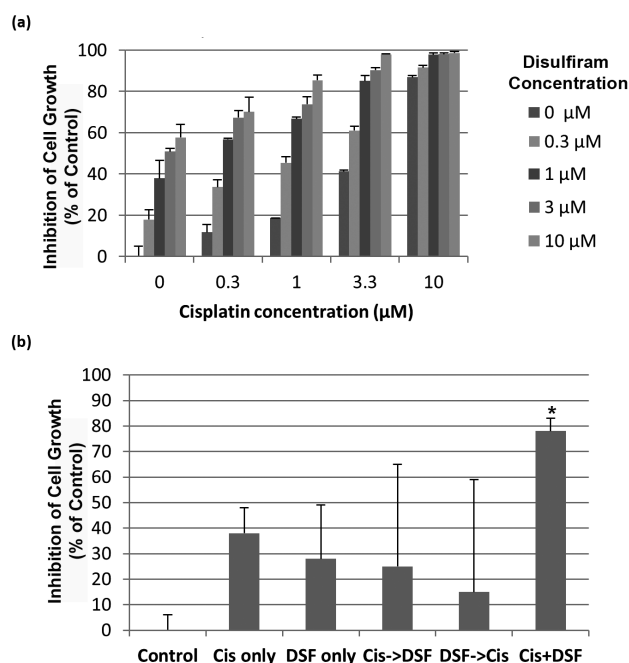


Figure 2. Disulfiram increases the cytotoxicity of Cisplatin. (a) Percent inhibition of A2780 cell growth with increasing doses of Cisplatin combined with increasing doses of Disulfiram (DSF); (b) Drug sequencing study demonstrating that concurrent Cisplatin (Cis) and Disulfiram (Cis + DSF) is superior to Cisplatin therapy followed by Disulfiram therapy (Cis- > DSF), Disulfiram therapy followed by Cisplatin (DSF- > Cis), or either drug as a single agent. Results are representative of at least 2 assays with 3 replicates for each group. *indicates $p < 0.05$.

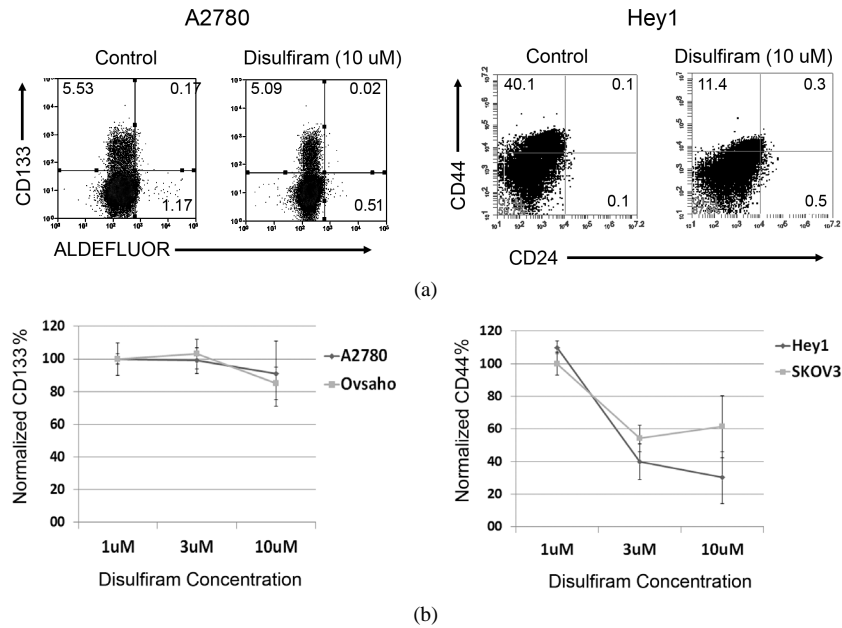
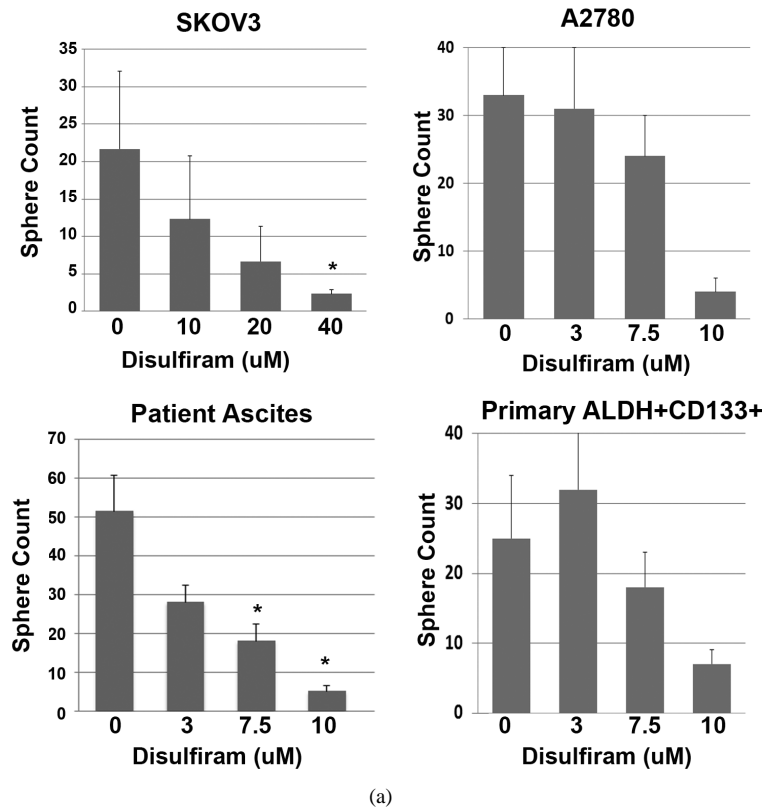


Figure 3. Disulfiram’s impact on ovarian CSC marker expression *in vitro*. (a) CD133 FACS analysis of A2780 and HEY1 cells treated with 10 μM Disulfiram demonstrating a minimal depletion of the CD133⁺ ovarian cancer cell population and 3.9 fold reduction of the CD44⁺ cell population with Disulfiram treatment; (b) Dose dependent effect of Disulfiram on CD133⁺ cell and CD44⁺ ovarian cancer cell populations for the indicated cell lines. The percentage of cells expressing CD133 or CD44 at each treatment dose are presented as normalized to the percent of marker expressing cells in the untreated control.



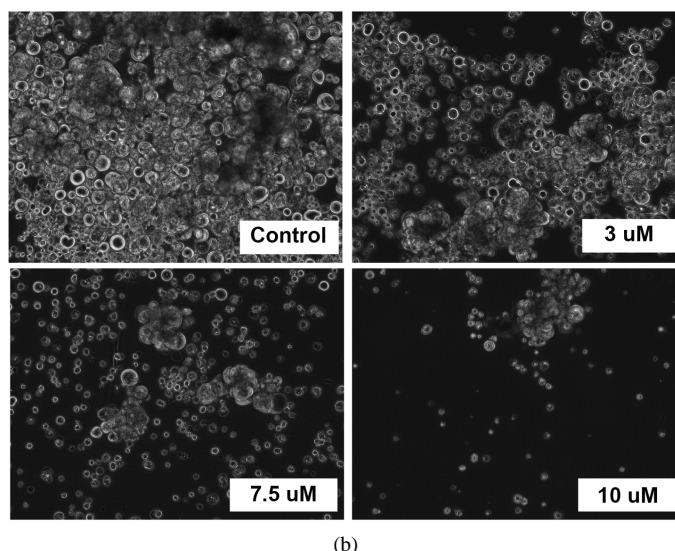


Figure 4. Disulfiram's impact on tumor sphere formation. (a) Tumor sphere count from A2780 and SKOV3 cell lines or FACS isolated primary ALDH⁺ CD133⁺ primary cells or primary patient ascites samples ($n = 3$) treated with the indicated concentrations of Disulfiram (DSF) for 3 days, then maintained for 14 and 20 days respectively; (b) Representative pictures of primary patient ascites samples at the end of the culture period. Results are representative of at least 2 assays with 3 replicates for each group. *indicates $p < 0.05$.

no impact on tumor initiation or growth with either Cisplatin or Disulfiram (**Figure 5(a)**).

We then examined the effect of Disulfiram on flank tumors derived from either whole A2780 cell line or from FACS-isolated ALDH⁺/CD133⁺ A2780 CSC. Disulfiram alone had no statistically significant impact on the growth of either tumor population. Similarly the addition of Disulfiram to a sub-therapeutic dose of Cisplatin (concurrently or concurrently with consolidative Disulfiram) had no impact on tumor growth (**Figure 5(b)**). In order to increase drug exposure to tumors we repeated a therapeutic study using intraperitoneal treatment of FACS-isolated ALDH⁺/CD133⁺ A2780 cells injected intraperitoneally into NSG mice. Xenografts were allowed to establish for 1 week prior to initiation of treatment. Mice were then treated with Disulfiram or vehicle i.p. for 21 days. Survival curves showed no impact on survival with Disulfiram treatment (**Figure 5(c)**). Finally, given the increased CD44 depletion *in vitro* with Disulfiram vs. SKOV and HEY1 cells, we established IP SKOV tumors and repeated dosing. Once again no overall survival effect was observed (**Figure 5(c)**).

4. Discussion

Disulfiram, as an inhibitor of ALDH enzymatic activity, has been reported to selectively target CSC. Given its relatively low cost, ease of administration, and acceptable toxicity profile, Disulfiram would be an attractive CSC-targeting therapeutic agent. Agents that target CSC are reported to overcome chemotherapy resistance and increase survival in murine tumor models. Given studies from our lab and others linking ALDH activity in ovarian cancer cells with increased tumor initiation capacity and chemotherapy resistance, we tested the ability of Disulfiram to act as a CSC-targeting agent in ovarian cancer. We found that Disulfiram demonstrated significant anti-tumor activity *in vitro*, which was additive to chemotherapy. However, while Disulfiram effectively targeted CD44⁺ ovarian cancer cells, Disulfiram demonstrated little selective elimination of ALDH⁺ or CD133⁺ ovarian cancer-initiating cells *in vitro* and lacked efficacy against CSC *in vivo*.

Our results differ from prior studies in breast cancer, in which Disulfiram was reported to target breast cancer stem-like cells [11]. Some studies have used copper to enhance the effects of Disulfiram [9]-[11] [14]. We did not use copper, and this may have reduced efficacy. Similarly, the dosing we used in this study is less than that used in other studies and may impact the lack of efficacy. We chose to use a dose reflective of a 500 mg daily maintenance dose used in humans for the treatment of alcohol addiction. We hypothesize a maintenance dose could prevent or delay disease recurrence. We found higher doses were limited by neurologic side-effects in the

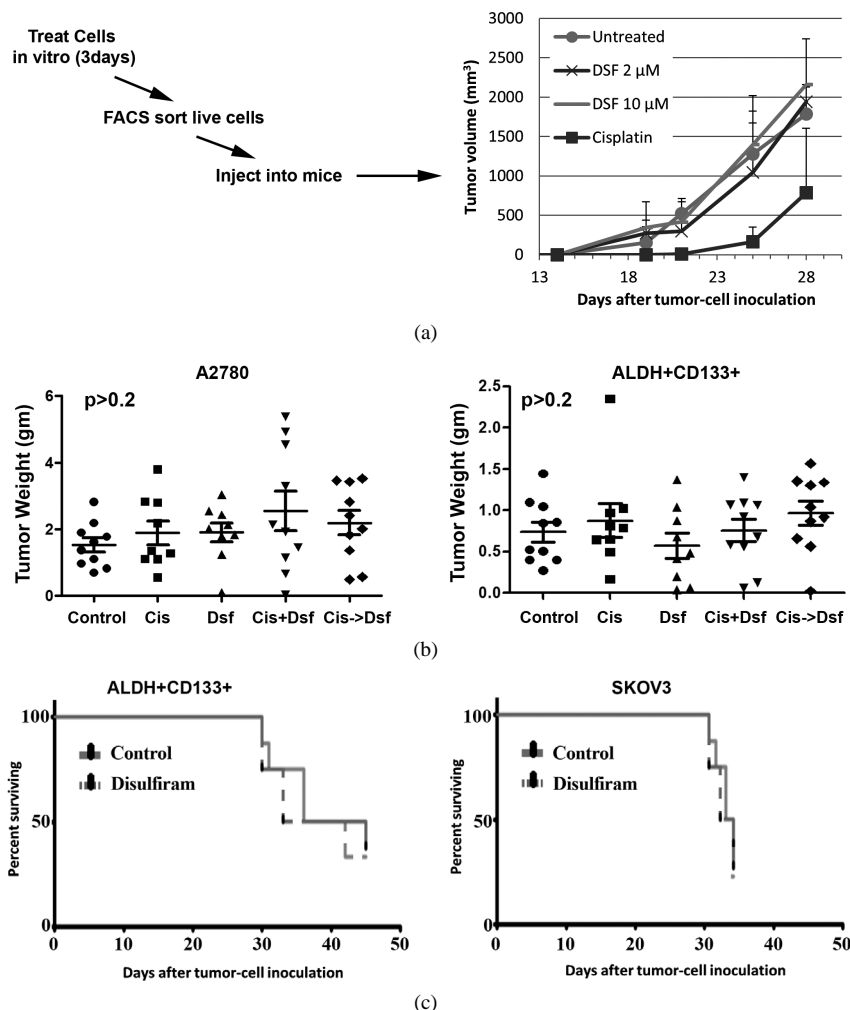


Figure 5. Disulfiram does not impact tumor initiation or cancer stem-like cells (CSC) growth *in vivo*. (a) A2780 cells were mock-treated or treated with Cisplatin (Cis) or Disulfiram (DSF) for 3 days. 5000 FACS-isolated viable (Annexin-V⁻/PI⁻) cells from each group were injected into the axillae of nude mice and tumor initiation and growth were tracked. Disulfiram had no impact on tumor initiation or growth; (b) Nude mice were injected in the flank with bulk A2780 cells or FACS-isolated ALDH⁺/CD133⁺ A2780 cells, then treated with vehicle, Cisplatin (Cis, 0.25 mg/kg i.p. daily for 3 days), Disulfiram (DSF, 0.15 mg i.p. daily for 21 days), concurrent Cisplatin and Disulfiram for 3 days (Cis + DSF), or Cisplatin for 3 days followed by Disulfiram for 18 days (Cis->DSF). Disulfiram had no impact on tumor growth; (c) Nude mice were injected i.p. with 10,000 FACS-isolated CD133⁺/ALDH⁺ A2780 cells, or total SKOV3 cells and then treated daily for 21 days with i.p. Disulfiram or vehicle. Mice were evaluated periodically and were sacrificed when fulfilling University Committee on Use and Care of Animals (UCUCA) criteria of severe morbidity. Kaplan-Meier survival curves showed no impact on survival with Disulfiram treatment in either case.

mice. While higher doses have been used in humans, up to 2 grams/day, this is associated with much higher side-effects and would not be acceptable as a maintenance approach. A likely explanation for the different activity of Disulfiram *in vitro* versus *in vivo* is reduced bioavailability. Disulfiram has a very short half-life *in vivo* and numerous active metabolites [18]-[21], and these multiple metabolites of Disulfiram are associated with numerous off-target effects [9]-[11]. The tumor microenvironment may further restrict bioavailability. In an effort to maximize *in vivo* exposure, we used an intraperitoneal tumor model and intraperitoneal drug administration. Despite this, we still saw limited *in vivo* Disulfiram effect.

The reason for the differential effect of Disulfiram *in vitro* vs. *in vivo* remains unclear. One reason that Disulfiram might have been unsuccessful is that it is inadequately/not targeting the ALDH isozymes selectively expressed in CSC. There are at least 19 ALDH isozymes in the human aldehyde dehydrogenase superfamily [22]. The ALDEFLUOR assay that is used to identify CSC detects the enzymatic activity of multiple ALDH isozymes [23]. The exact isozymes that are linked with ovarian CSC ALDH activity remain unknown. Most studies have implicated ALDH1A1 as a marker of ovarian CSC; however, other ALDH1A family members and ALDH3 family members have also been implicated [24] [25]. Disulfiram was developed clinically based on its high-affinity inhibition of ALDH2 [26]. ALDH2 is a broadly expressed mitochondrial enzyme and, as such, is likely not a CSC-specific target. Although Disulfiram can inhibit ALDH1 *in vitro* [27], the *in vivo* activity of the drug is mainly attributed to its metabolites targeting mitochondrial ALDH2 [18]-[21]. Thus, Disulfiram may result in limited inhibition of ALDH1A1 *in vivo*.

5. Conclusion

In conclusion, ALDH inhibition with Disulfiram shows anti-neoplastic activity *in vitro*. However, *in vivo* activity was limited. Our results suggest that Disulfiram is not the ideal ALDH inhibitor for ovarian cancer and that more selective may be necessary to efficiently target CSC. A more comprehensive examination of the Disulfiram, its pharmacokinetics and combination with chemotherapy *in vivo* are indicated prior to use in clinical trials in ovarian cancer patients.

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