Biomedicine & Pharmacotherapy 118 (2019) 109371

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Inhibitory effect on ovarian cancer ALDH+ stem-like cells by Disulfiram and Copper treatment through ALDH and ROS modulation

Fang Guo[a,1], Zhi Yang[a,1], Hagen Kulbe[a], Andreas E. Albers[b], Jalid Sehouli[a], Andreas M. Kaufmann[a,*]

[a] Clinic for Gynecology, Charité-Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

[b] Department of Otolaryngology, Head and Neck Surgery, Charité-Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

ARTICLE INFO

Keywords:
Adjuvant chemotherapy
ALDH enzyme activity
Cellular apoptosis
Cytotoxicity
Novel chemotherapeutic agent

ABSTRACT

Background: Disulfiram (DSF) is a drug used for treatment of alcoholism that has also displayed promising anti-cancer activity. It unfolds its effects by inhibiting the enzyme activity of aldehyde dehydrogenase (ALDH) isoforms.

Methods: MTT assay, spheroid formation, clonogenicity assay, qRT-PCR, and ALDH enzyme activity analysis were performed using ovarian cancer cell lines IGROV1, SKOV3 and SKOV3IP1. Cell cycle analyses and measurement of intracellular reactive oxygen species (ROS) were carried out by flow cytometry. ALDH+ and ALDH− cells were isolated by FACS sorting.

Results: ALDH activity was inhibited in ovarian cancer stem cells (the proportion of ALDH+ cells was reduced from 21.7% to 0.391%, 8.4% to 0%, 6.88% to 0.05% in cell lines IGROV1, SKOV3, and SKOV3IP1, respectively). DSF with or without the cofactor copper (Cu^{2+}) exhibited cytotoxicity dose- and time-dependent and enhanced cisplatin-induced apoptosis. DSF + Cu^{2+} increased intracellular ROS levels triggering apoptosis of ovarian cancer stem cells (CSC). Significantly more colony and spheroid formation was observed in ALDH+ compared with ALDH- cells ($P < 0.01$). Moreover, ALDH+ cells were more resistant to cisplatin treatment compared with ALDH-cells ($P < 0.05$) and also exhibited a lower basal level of ROS. However, no significant difference in ROS accumulation nor in cellular viability was observed in ALDH + cells in comparison to ALDH- cells after pre-treatment with DSF (0.08 μM).

Conclusion: Our findings provide evidence that DSF might be employed as a novel adjuvant chemotherapeutic agent in combination with cisplatin for treatment of ovarian cancer.

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynaecological cancer [1]. Although many patients initially benefit from surgery and chemotherapy [2,3], recurrent disease develops in more than 80% of patients with advanced stage and 25% of early stage ovarian cancer [4]. Currently, systemic administration of platinum-based chemotherapy following surgery is currently the best standard of care. Unfortunately, cancer cells, either intrinsically have or acquire resistance to cisplatin-based chemotherapy, leading to relapse and therapeutic failure [5]. It has been observed that around 50% of the patients relapse within 5 years [6]. Cancer stem cells (CSCs), which are defined by two key characteristics, enhanced tumorigenicity and the capacity for self-renewal/differentiation [7], are thought to contribute to drug resistance and cancer recurrence. There are several suggested mechanisms for the chemo-resistance of CSCs. First, traditional chemotherapeutic agents typically induce apoptosis by damaging DNA and inhibiting cell cycle distribution. However, CSCs which are a proliferative quiescent and slowly-cycling cell population are therefore less sensitive to treatment. Second, there is increased activation of the DNA damage checkpoint response in CSCs and third, CSCs show enhanced expression of transporters, which help efflux chemotherapeutic drugs. Since CSCs account for the unsatisfactory low response rate of tumors to chemotherapy, it remains a priority to increase the sensitivity of cisplatin-based

* Corresponding author at: Charité-Universitätsmedizin Berlin, Clinic for Gynecology, Hindenburgdamm 30, 12203 Berlin, Germany.
E-mail address: andreas.kaufmann@charite.de (A.M. Kaufmann).

[1] These authors have contributed equally to this work.

https://doi.org/10.1016/j.biopha.2019.109371
Received 15 June 2019; Received in revised form 18 August 2019; Accepted 21 August 2019

chemotherapy or to recognize and target specifically CSCs to improve the outcome of ovarian cancer patients.

Although most ovarian cancer patients initially respond to chemotherapy, there is a small subpopulation of cells (generally less than 2% of the tumor cells) which are resistant to initial adjuvant therapy and hence finally leads to recurrence of the disease [8,9]. Bapat et al. were the first who cultured ovarian cancer stem cells from the ascites of an ovarian cancer patient [10]. They isolated a single tumorigenic clone from the mixed ascites cells, which showed differentiation features and was able to give rise to new tumors when transplanted into mice. Increasing evidence suggested that CSCs might be an explanation for chemo-resistance and the recurrence of ovarian cancer. Several potential CSC markers have been used to identify CSCs in ovarian cancer, such as CD44, c-kit, CD133, and ALDH, and there is certainly an overlap in the expression of these markers [11]. However, ALDH holds the attractive distinction among these markers as ALDH may be more than just a CSC marker but may have a potential functional role in CSC biology [12]. Studies have shown that ALDH enzyme expression and activity may be associated with particular cell types in ovarian tumor tissues and vary according to cellular states [13], indicating that ALDH isozymes may play essential roles in lineage differentiation and pathogenesis for ovarian cancer pathophysiology [14].

The ALDH enzymes are a family comprised of 19 isoforms that are localized in the cytoplasm, mitochondria, or nucleus. The ALDH enzymes in immunohistochemistry (IHC) and ALDH activity in flow cytometry are widely used as a CSC marker for many malignancies such as leukemia, lung, liver, bone, prostate, head and neck, ovarian cancer, and cervical cancer [15–17]. Increased ALDH activity, as measured by ALDEFLUOR assay, is also commonly used for the isolation of CSCs.

ALDH family members play essential roles in a variety of biological activities in CSCs, including differentiation, oxidative stress response, and drug resistance [18]. Tumor cells with higher ALDH activity or ALDH positive cells sorted form primary tumor cells have been demonstrated to have enhanced tumorigenicity, and increased migratory capacity [15–17]. Additionally, studies have shown that ALDH can decrease intracellular oxidative stress as it functions as a ROS scavenger [17,19]. Thus, ALDH activity is required to maintain sufficiently low ROS levels and prevent triggering CSC apoptosis [20]. Potentially, ALDH could protect cancer stem cells against oxidative stress induced by alcohol, UV radiation, and some chemotherapeutic agents [21]. Moreover, there is a potential role for ALDH in CSC resistance to chemotherapy. Studies have indicated that some CSC populations express significantly elevated levels of ALDH in conjunction with resistance to conventional chemotherapy [22].

Disulfiram (DSF), a member of the dithiocarbamate family, is a FDA-approved drug that has been used for the clinical treatment of alcoholism for over 60 years [23]. Recent studies demonstrate that DSF has potent anticancer activity *in vitro* and in cancer xenografts [24–28], highlighting it as a potential novel chemotherapeutic agent. However, the exact mechanism of action as a cytotoxic agent in cancer cells is still unclear. Importantly, Chen et al. initially reported that DSF-copper complexes could potently inhibit the activity of the proteasomes and induce apoptosis in cultured breast cancer cells [24]. Here we explored the inhibitory effect of DSF on ALDH, and the effect of DSF on ALDH + stem-like cells after cell sorting, and the potential underlying mechanisms. The presented experiments demonstrated that ALDH could be a therapeutic target in CSCs. The results showed that ALDH plays an essential role in cisplatin resistance. DSF combined with cisplatin overcomes cisplatin-resistance in ALDH + stem-like cells and achieves the same level of ROS accumulation in ALDH + and ALDH- cells.

2. Materials and methods

2.1. Cell culture

The ovarian cell lines IGROV1, SKOV3, and SKOV3IP1 were cultured in RPMI 1640 medium with L-glutamine (Invitrogen, Heidelberg, Germany) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (both from Millipore, Darmstadt, Germany) in a humidified incubator at 37 °C and 5% CO_2. All experiments were performed on cultures that were 70% confluent. Three cell lines were chosen due to their different degrees of sensitivity to DSF treatment.

2.2. Spheroid formation assay

To generate spheroids, cells were seeded in 24-well Ultra-Low Attachment plates (Corning, New York, USA) at a specific density of 2×10^4 cells/ml in Quantum 263 medium (PAA, Cöllbe, Germany) supplemented with 10 ng/ml Epidermal Growth Factor (EGF) and 10 ng/ml Fibroblast Growth Factor-basic (FGF) (Millipore). Cells were allowed to grow for 10–14 days to form spheroids. For passaging, all spheroids were collected with a 40 µm mesh filter and centrifuged at 1500 rpm for 5 min. Spheroids were dissociated into single cells using trypsin/EDTA (Millipore) at 37 °C at 5% CO^2 for 5 min, followed by washing with PBS twice. Single cells were reseeded in fresh culture medium under the same conditions. After passaging the spheroids into 2nd or 3rd generation, spheroid-derived cells were treated with DSF or DSF + Cu^{2+} in a 96-well ultra-low attachment plate (200 cells in 0.2 ml medium/well) for 7–10 days and photographed at 50 fold magnification. Spheroids were counted when the diameter of spheroids was more than 100 µm.

2.3. MTT assay

Cells were seeded in 96-well plates at a density of 4000 cells per well in 100 µl cell culture medium and incubated overnight. Then culture medium was removed, and cells were exposed to drugs at various concentrations. Free DSF was dissolved in DMSO at a storage concentration of 10 mM and diluted to working concentrations in medium before use. The absorbance was measured at a wavelength of 590 nm with a Bio-Rad microplate reader after 72 h incubation. Relative viability (%) of cells was calculated according to the following equation: Relative viability (%) = (A sample/A control) *100%, where A sample and A control were the absorbance of the sample and control wells, respectively. Dose-response curves and IC_{50} were calculated using GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA).

2.4. Flow cytometric analysis of cell cycle

Analysis of cell cycle progression and detection of apoptosis was performed using flow cytometric analysis of DNA staining. All drug-treated and untreated cells were harvested by trypsinization. Cells ($4*10^4$/well) were suspended in 100 µl PBS and fixed in 900 µl 70% ethanol overnight. The cells were then incubated with RNase (100 µg/ml, Sigma, Darmstadt, Germany) and propidium iodide (50 µg/ml, Sigma, Darmstadt, Germany) for 30 min. The data from 10,000 cells for each sample were acquired by FACS Scan (BD Bioscience, Heidelberg, Germany) and DNA content and cell cycle distribution was analyzed.

2.5. Apoptosis assessment

Apoptotic status was determined by FLUOS-conjugated Annexin-V and propidium iodide Kit (Roche, Mannheim, Germany) using flow cytometry following the manufacturer's instructions. Cells ($4*10^4$/well) were incubated in a 24-well plate overnight and treated with drugs for further 72 h. All cells were then harvested and suspended in 100 µl binding buffer containing FITC-conjugated Annexin-V (2 µl)/PI (2 µl) and incubated at RT for 15 min. Apoptosis and necrosis were evaluated using FL3 (PI) and FL1 (Annexin-V) by FACS analysis. The percentage of cells were determined in four quadrants: live cells, (Annexin-V −/PI −, lower/left quadrant); early apoptotic cells (Annexin-V +/PI −,

lower/right quadrant); late apoptotic cells (Annexin-V + /PI + , upper/right quadrant); and necrotic cells (Annexin-V − /PI + , upper/left quadrant), respectively.

2.6. Clonogenicity assay

Cells were exposed to DSF (1 μM), Cu^{2+} (1 μM), or DSF (1 μM)/Cu^{2+} (1 μM) for 24 h. The drug-treated cells were then collected and cultured in drug-free medium in a 6-well plate at a density of 2000 cells/well for 7–10 days. Fixation and staining of colonies were done by adding 2–3 ml of a mixture of 6.0% glutaraldehyde and 0.5% crystal violet. Colonies of at least 50 cells were counted and compared with non-treated cells as controls.

2.7. Flow cytometric analysis of ALDH activity and sorting

ALDH activity was assessed using the ALDEFLUOR kit (Stem Cell Technologies, Durham, NC, USA). Briefly, cells ($4*10^4$/well) were incubated with ALDH substrate BAAA for 30 min at 37 °C following the manufacturer's instructions. Tested cells were exposed to DSF (10 μM), Cu^{2+} (1 μM), DSF (10 μM) + Cu^{2+} (1 μM). Cells treated with diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, were used as a control to establish the baseline fluorescence and define the cut off for ALDEFLUOR-positive cells.

For FACS sorting, cells were re-suspended in PBS buffer at a concentration of 1×10^7 cells/ml and separated on an Aria cell sorter (BD Biosciences). The sorted cells were exposed to DSF for 30 min, and reactive oxygen species (ROS) activity was analyzed by FACS (methods described below). The sorting gates were established with negative controls that were treated with DEAB.

2.8. Measurement of ROS

Mitochondrial ROS was measured using the Mitosox Red kit (Invitrogen, Paisley, UK). Cells were treated with Cu^{2+} (1 μM), DSF (10 μM), DSF (10 μM) + Cu^{2+} (1 μM), DSF (100 μM), or DSF (100 μM) + Cu^{2+} (1 μM) for 30 min, harvested and analyzed by flow cytometry. Cells with Mitosox Red reagent, but no drug treatment, were used as control. All samples were normalized to untreated control, and relative ROS activity was determined. ROS activity after cells sorting was also determined. Gates were set for the percentage of mitochondrial ROS in control cells, and the same gate was used on treated cells to quantitate the induction of ROS.

2.9. Statistical analysis

The statistical analysis of data in this study was performed with GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA) using one-way ANOVA test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. DSF + Cu^{2+} displays cytotoxic effects on ovarian cancer cell lines

Initially, the cytotoxic effect of DSF on three ovarian cancer cell lines was examined using MTT assay to determine the IC_{50} of cytotoxicity to each cell line. The reduction in viability, as measured by MTT assay, represents the reduced metabolic rate after DSF treatment. As shown in Fig. 1A, the proliferation of cells was significantly inhibited after exposure to concentrations of DSF between 0.001 μM and 100 μM for 72 h. Dose-dependent cytotoxicity was observed in all three ovarian cancer cell lines. DSF showed a linear increase of cytotoxicity with increasing concentration of the drug in SKOV3 cells, and biphasic cytotoxicity in IGROV1 and SKOV3IP1 cell lines, with the relative viability of cells increasing slightly at DSF 10 μM (Fig. 1A). IC_{50} values for these three cell lines were IGROV1 IC_{50}: 2.01 ± 0.11 μM; SKOV3 IC_{50}: 0.19 ± 0.09 μM; SKOV3IP1 IC_{50}: 10 ± 2.48 μM, showing variable sensitivity of the three cell lines investigated towards treatment (Table 1).

We next examined the apoptosis induced over time after treatment with DSF in a time-course experiment. The cells were exposed to a specific concentration according to their IC_{50} (IGROV1 and SKOV3IP1: DSF 1 μM; SKOV3: DSF 0.1 μM) for 4–72 h. The fraction of apoptotic/necrotic cells is represented by Annexin V / PI positive cells, and this fraction contributes to overall lower metabolic activity. As shown in Fig. 1B, both early apoptosis (Annexin-V + /PI − , lower/right quadrant) and late apoptosis (Annexin-V + /PI + , upper/right quadrant) increased with time of treatment. The control cells were cultured for 72 h without treatment showed less apoptosis than drug-treated cells. These results indicate that DSF itself is cytotoxic in a dose- and time-dependent manner around the concentration of their IC_{50} with some variation between the three investigated cell lines.

Moreover, the DSF + Cu^{2+} combination enhances the cytotoxicity on ovarian cancer cell lines. As shown in Fig. 1F, no significant cytotoxicity was observed in cells treated with Cu^{2+} alone until the concentration of Cu^{2+} increased from 10 μM to 100 μM. Therefore, a concentration of 1 μM of Cu^{2+} in combination with DSF was chosen. Although DSF alone had significant effects, the cytotoxicity of DSF was significantly enhanced in Cu^{2+} (1 μM)-supplemented medium in all ovarian cancer cell lines. Treatment with DSF at different doses in combination with 1 μM Cu^{2+} for 72 h significantly inhibited the cell viability as compared to DSF treatment alone. Interestingly, in the presence of 1 μM Cu^{2+}, as low as 0.1 μM DSF showed strong cytotoxicity (Fig. 1F). Fig. 1G shows that both early apoptosis (Annexin-V + /PI − , lower/right quadrant) and late apoptosis (Annexin-V + /PI + , upper/right quadrant), as well as necrosis (Annexin-V − /PI + , upper/left quadrant), increased with DSF + Cu^{2+} treatment compared to DSF exposure alone. Cu^{2+} reduced IC_{50} at 72 h of treatment with DSF about 10–100 fold (Table 1).

3.2. Inhibitory effect of DSF + Cu^{2+} on ovarian cancer cell lines

One of the accepted assays to evaluate the stemness of cells is the ability of epithelial cells to grow anchorage independently. In this study, spheroid culture models were used to enrich CSCs. Results showed that the expression of stemness-related nuclear transcription factor (TF) Oct3/4, Sox2, and Nanog is higher in spheroid-derived cells than monolayer-derived cells (supplemental Fig. 1), indicating that spheroid culture models could be used to assess CSCs. Also, spheroid-derived cells showed higher IC_{50} of cisplatin treatment, demonstrating that spheroid-derived cells were more resistant to cisplatin (Table 2). Fig. 2A shows an abundance of large spheroids grown from untreated control cells. However, the ability of ovarian cancer cells to form spheroids when exposed to DSF was reduced, showing only cellular aggregates, suggesting reduced proliferative potential of the stem cells. When DSF combined with 1 μM Cu^{2+} was applied, only diffuse individual cells were seen in DSF + Cu^{2+}-treated samples and spheroid formation in all three cell lines was completely abolished (Fig. 2A). Fig. 2B shows that the number of spheroids was significantly reduced from average 71 to 0 in IGROV1, from average 16 to 0 in SKOV3, and from 38 to 0 in SKOV3IP1 when the cells were exposed to DSF or DSF + Cu^{2+} ($P < 0.01$) as compared to the untreated control cells. Although the number of spheroids was slightly reduced in Cu^{2+}-treated cells, there was no statistically significant difference compared to control cells.

Next, a clonogenicity assay was performed to test the ability of every cell in the population to undergo "unlimited" divisions to determine cell reproductive capacity after drug treatment. Cells were pretreated with DSF (1 μM) or Cu^{2+} (1 μM), respectively, and in combination with DSF (1 μM) + Cu^{2+} (1 μM) for 24 h, and sub-cultured in drug-free medium for another 7–10 days. As shown in Fig. 2C, the

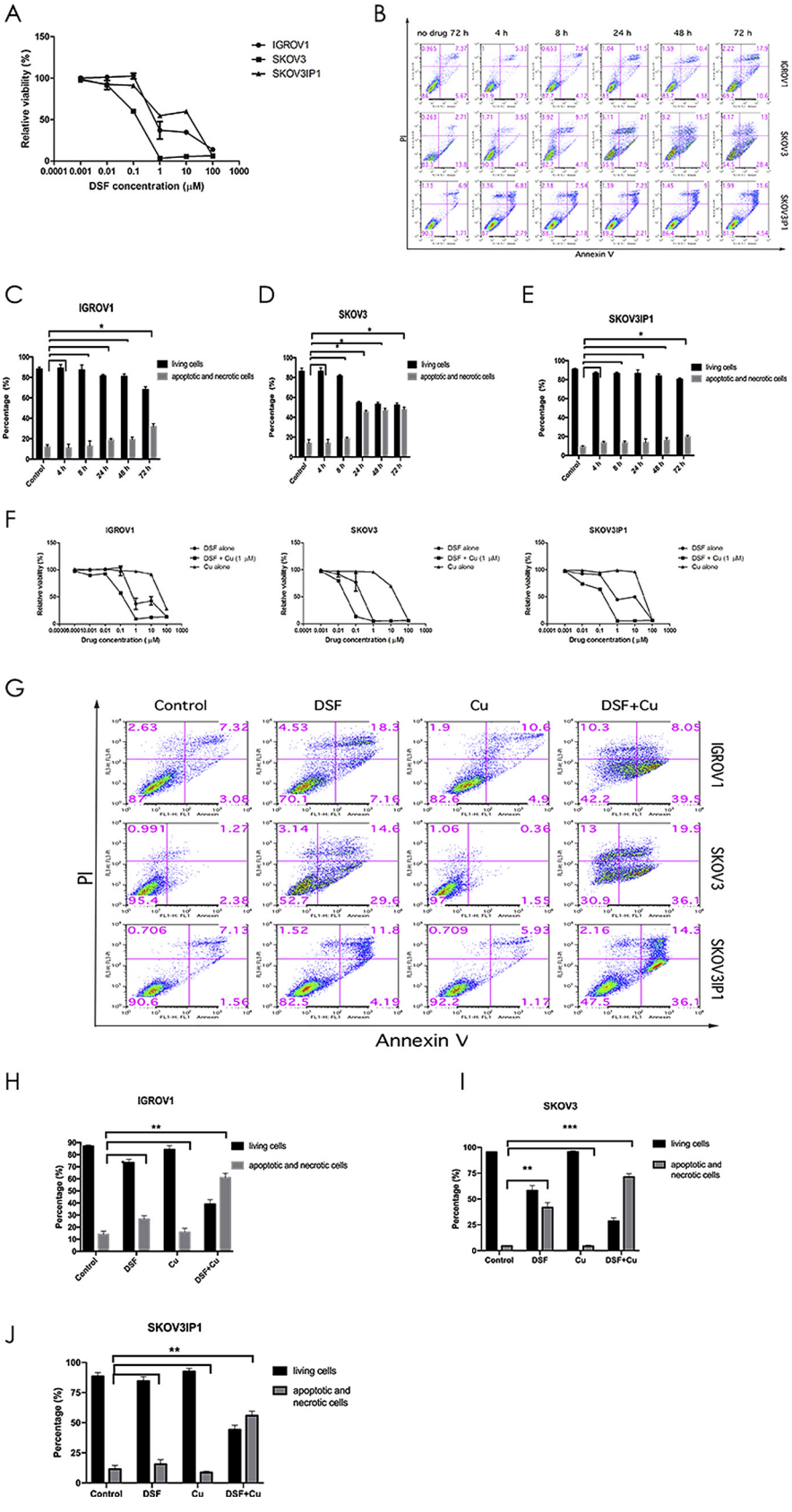

Fig. 1. DSF + Cu^{2+} displays cytotoxic effects on ovarian cancer cell lines. A) MTT assay. The ovarian cancer cell lines were exposed to different concentrations of DSF for 72 h and viability related to untreated control. B) Representative data for flow cytometric analysis of Annexin-V/PI staining after exposure to DSF for different treatment intervals. C–E) Graphical representation of the statistical analysis of the Annexin-V/PI dual staining results. F) MTT assay. The ovarian cancer cell lines were exposed to different concentrations of DSF combined with 1 μM Cu^{2+} for 72 h and viability related to untreated control. G) Annexin-V/PI assay. IGROV1 and SKOV3IP1 cells were exposed to 1 μM DSF alone, 1 μM Cu^{2+} alone, and 1 μM DSF + 1 μM Cu^{2+} for 72 h. SKOV3 cells were exposed to 0.1 μM DSF alone, 1 μM Cu^{2+} alone, and 0.1 μM DSF + 1 μM Cu^{2+} for 72 h. H–J) Graphical representation of the statistical analysis of the Annexin-V/PI dual staining results. Cells without any drug treatment were used as control. One representative of three independent experiments is shown. Statistical analyses are based on three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.0001.

colony number in the Cu^{2+} (1 μM) treated group was decreased as compared to control cells that had not been treated. This was caused by a slowed growth of surviving cells and resulted in smaller colonies that did not reach the counting threshold (diameter of spheroids had to be more than 100 μm per colony to be counted). The colony number was significantly reduced by DSF (1 μM) treatment of cells with colony-forming units from around 700 in control to 200 in SKOV3 cell line and from around 1000 in control to 500 in IGROV1 and SKOV3IP1 cell lines

Table 1
IC_{50} of three ovarian cancer cell lines treated by DSF or DSF/Cu^{2+} for 72 h.

	DSF	DSF/Cu
IGROV1	2.01 ± 0.11 μM	0.2 ± 0.07 μM
SKOV3	0.19 ± 0.09 μM	0.02 ± 0.01 μM
SKOV3IP1	10 ± 2.48 μM	0.1 ± 0.09 μM

Table 2
IC_{50} of MDCs and SDCs treated by cisplatin for 72 h.

	MDCs	SDCs
IGROV1	10 ± 1.8 μM	40 ± 2 μM
SKOV3	1.0 ± 0.5 μM	3.0 ± 0.1 μM
SKOV3IP1	12 ± 0.1 μM	15 ± 1.0 μM

($P < 0.05$). The colony-forming ability of ovarian cancer cells was almost totally eradicated by DSF (1 μM) + Cu^{2+} (1 μM) treatment in SKOV3 and SKOV3IP1 cell lines. There is also a significant difference between cell cultures treated by DSF alone and DSF + Cu^{2+} treated cultures (Fig. 2C). These results indicated that DSF or DSF in conjunction with Cu^{2+} was able to suppress the clonogenicity of ovarian cancer cells.

As ALDHs are essential for maintenance and differentiation of stem cells as well as normal development, and increased ALDH activity has been found to relate to stemness of CSCs as well as chemotherapy resistance, we wanted to determine whether DSF or DSF + Cu^{2+} could inhibit the activity of the aldehyde dehydrogenase enzymes measured by ALDEFLUOR assay. Fig. 2D shows that DSF significantly reduced the proportion of ALDH + cells (from 21.7% to 0.391% in IGROV1 cell line, 8.4% to 0 in SKOV3 cell line, 6.88% to 0.05% in SKOV3IP1 cell line), while Cu^{2+} alone did not affect the ALDH + population. In comparison with control cells that had not been drug treated, the ALDH + population was abolished in ovarian cancer cell lines incubated with DSF (10 μM) + Cu^{2+} (1 μM) (Fig. 2D). The result also demonstrated that ALDH activity in ovarian cancer cells was not only inhibited by diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, but also inhibited by DSF with or without Cu^{2+} supplementation. Moreover, the inhibitory effect by DSF was better than that of DEAB, while Cu^{2+} supplementation even enhanced this inhibitory effect compared to DSF treatment alone.

3.3. No significant cell cycle changes are induced by DSF + Cu^{2+}

Cell cycle analysis was performed by flow cytometry and assessed by propidium iodide staining of DNA. Firstly, the cells were treated with a higher concentration of DSF at the same concentration like in the Annexin-V/PI assay (1 μM DSF alone, 1 μM Cu^{2+} individually, and 1 μM DSF + 1 μM Cu^{2+} for IGROV1 and SKOV3IP1 cells; 0.1 μM DSF alone, 1 μM Cu^{2+} individually, and 0.1 μM DSF + 1 μM Cu^{2+} for SKOV3 cells). As shown in Fig. 3A (upper rows a and b), a more massive sub-G1 peak was induced by the DFS + Cu^{2+} combination than by DSF alone in the treated cells. These results were in agreement with the Annexin-V/PI assay showing that the DFS + Cu^{2+} combination induced more apoptosis than DSF alone. However, since the cell cycle phases were compromised and disappeared at this high drug concentration analysis of the cell cycle was not possible. In order to display the cell cycle distribution while avoiding excessive apoptosis during prolonged exposure, cells were treated with DSF at a lower concentration (0.01 μM DSF alone, 1 μM Cu^{2+} alone, and 0.01 μM DSF + 1 μM Cu^{2+} for 72 h for three cell lines). The DFS concentration was selected according to the sensitivity of the cell lines in the specific assays to be able to measure and determine the different effects on apoptosis and proliferative activity. As shown in Fig. 3A (lower rows c–f) the proportion of cells in the G1 phase, S phase, and G2-M phase did not significantly change although there is some variation with different treatments compared to untreated control cells (Fig. 3B–D). Cells treated with other concentrations of DSF were also assessed to further illustrate the cell cycle distribution. Cells treated with cisplatin, which arrests cell cycle at G2/M phase were used as a positive control (Supplemental Fig. 2). These results indicated that DSF with or without Cu^{2+} had no significant effect on cell cycle at relatively lower concentrations.

3.4. DSF + Cu^{2+} enhances the generation of ROS

ROS are involved in cancer development and metastasis. We have shown that DSF inhibits ALDH activity which generally acts as a ROS scavenger to protect cells against oxidative stress. Consequently, it is meaningful to further investigate if the underlying mechanisms of DSF toxicity are based on ROS accumulation. Cells were treated as described in the method section, and mitochondrial ROS was measured by flow cytometry. The results showed that DSF, with or without Cu^{2+} supplementation, significantly induced more ROS activity in all three ovarian cancer cell lines. The relative ROS activity after normalization to untreated control cells was increased by 19.4 fold in IGROV1 cells, 2.98 fold in SKOV3 cells, and 2 fold in SKOV3IP1 cells by treatment with 10 μM DSF (Fig. 4A). More ROS was generated when the DSF concentration was increased from 10 μM to 100 μM. The relative ROS activity after normalization to untreated control cells was significantly increased by 64.9 fold in IGROV1 cells, 99.4 fold in SKOV3 cells, and 51.2 fold in SKOV3IP1 cells when incubated with 100 μM DSF. With the same concentration of DSF, more ROS was induced by supplementation with Cu^{2+} (1 μM) as compared to DSF treatment alone (Fig. 4A–D). As shown in Fig. 4E, ALDH + cells exhibited a lower basal level of ROS due to higher levels of ALDH expression. However, no significant difference in ROS generation in ALDH+/− cells was observed after DSF (10 μM) treatment. When DSF concentration was increased to 100 μM, ROS levels decreased in ALDH + cells (Fig. 4E). So the explanation could be that ALDH + cells were probably more vulnerable to DSF and were swiftly killed by this concentration.

3.5. DSF sensitizes cancer cells to cisplatin treatment and enhances cisplatin-induced apoptosis

To investigate whether DSF sensitizes cancer cells to cisplatin treatment, we tested the cell viability after treatment with DSF alone (IGROV1: 0.1 μM; SKOV3: 0.08 μM; SKOV3IP1: 1 μM) or cisplatin alone (IGROV1: 1 μM or 5 μM; SKOV3: 0.2 μM or 0.4 μM; SKOV3IP1: 1 μM or 5 μM) or in combination. Fig. 5A shows that exposure to either DSF or cisplatin alone for 72 h only slightly reduced the cellular viability. However, a significant decrease of cellular viability was induced by the drug combinations compared to individual treatment with DSF or cisplatin ($P < 0.01$) at these concentrations. The results indicate that DSF sensitizes cancer cells to cisplatin treatment even at lower doses, with decreased cell viability of 50–80% in the three cell lines. To further determine whether DSF sensitizes for cisplatin treatment and suppresses cell viability by inducing apoptosis, we tested the apoptotic status by flow cytometry using Annexin-V (FITC) and propidium iodide (PI) staining. Cells were treated with DSF alone (IGROV1 and SKOV3IP1: 1 μM; SKOV3: 0.05 μM) or cisplatin alone (IGROV1 and SKOV3IP1: 5 μM; SKOV3: 0.3 μM) or in a combination for 72 h. The results showed that in the IGROV1 cell line early apoptosis increased from 0.893% to 6.42%, late apoptosis increased from 4.15% to 19.8%, and necrosis increased from 2.11% to 2.74% in control cells and in cells treated with cisplatin alone, respectively. However, a significant increase in cellular apoptosis and necrosis was induced when 1 μM DSF was combined with cisplatin, with early apoptosis and late apoptosis reached to 16.7% and 31.4%, respectively. Importantly, treatment with 1 μM DSF did not induce more cellular apoptosis and necrosis compared to control cells. Similar results were observed in the SKOV3 and SKOV3IP1 cell lines. In SKOV3 cells, early apoptosis, late apoptosis, and

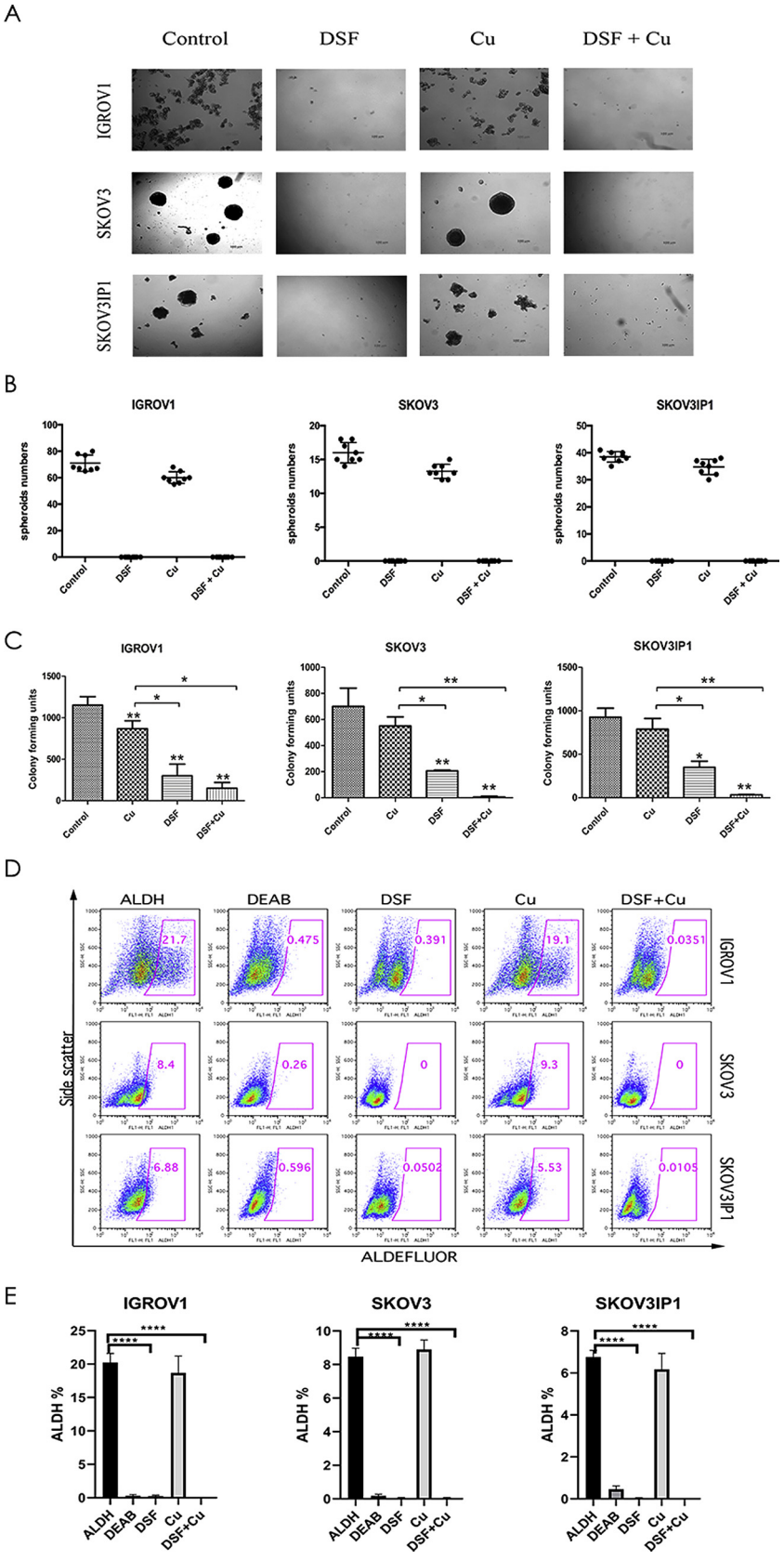

Fig. 2. Inhibitory effect of DSF + Cu^{2+} on ovarian cancer cell lines. A) DSF inhibited spheroid formation. Cells were treated with 0.1 μM DSF or 1 μM Cu^{2+} or 0.1 μM DSF + 1 μM Cu^{2+} in ultra-low attachment 96-well plates for 7–10 days and photographed (magnification 50 fold). B) Cells were exposed to drugs for 10 days, and spheroids with ≥ 100 μm in diameter were counted, and their numbers per well (n = 8) were plotted. C) Clonogenicity assay. Cells exposed to 1 μM Cu^{2+} alone, 1 μM DSF alone, or 1 μM DSF + 1 μM Cu^{2+} for 24 h were cultured in drug-free medium in six-well plates at a cell density of 2000 cells per well for 7–10 days. The colonies were counted. D) DSF + Cu^{2+} inhibits ALDH activity. Cells were exposed to DSF (10 μM), Cu^{2+} (1 μM), or DSF (10 μM) + Cu^{2+} (1 μM). Cells treated with diethylaminobenzaldehyde (DEAB), which is a specific ALDH inhibitor, were used as control. Numbers represent ALDH+ cells (%). One representative of three independent experiments is shown. E) Graphical representation of the statistical analysis of ALDH activity (n = 3). Statistical analyses are based on three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001.

necrosis increased from 6.52%, 5.02%, and 2.45% in cisplatin-treated cells to 30.4%, 13.9% and 2.61%, respectively, in the drug combination of DSF plus cisplatin. In SKOV3IP1 cells, early apoptosis, late apoptosis, and necrosis increased from 4.77%, 9.34%, and 0.617% in cisplatin-treated cells to 8.53%, 23%, and 1.76%, respectively, in the drug combination (Fig. 5B). These results indicate that DSF enhanced cisplatin-induced cellular apoptosis in ovarian cancer cell lines.

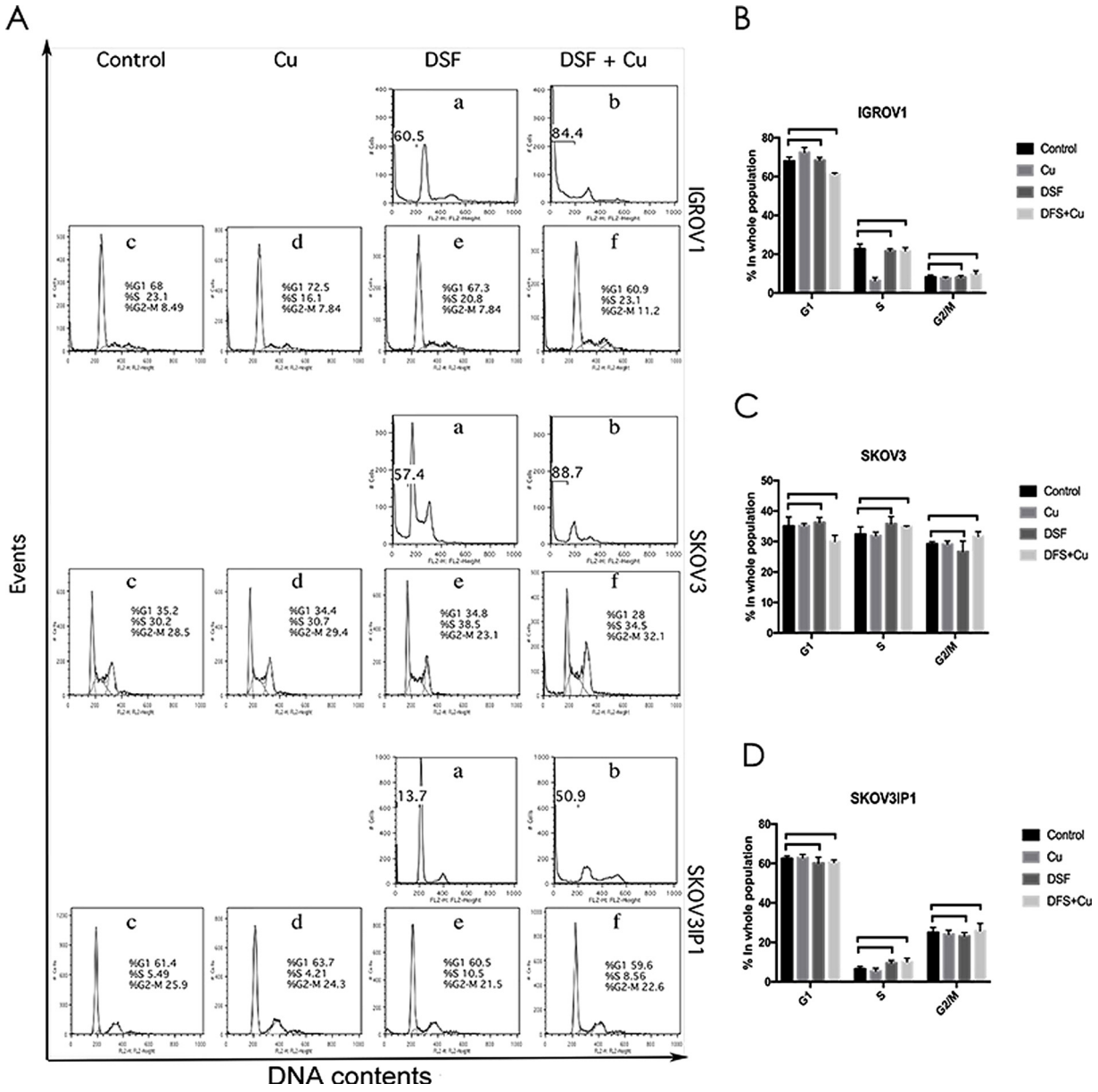

Fig. 3. Flow cytometric analysis of cell cycle. A) Cells were treated with Cu^{2+} alone or DSF alone or DSF + Cu^{2+} for 72 h. The upper row (a and b) concentration is 1 μM DSF alone and 1 μM DSF + 1 μM Cu^{2+} for IGROV1 and SKOV3IP1; 0.1 μM DSF alone and 0.1 μM DSF + 1 μM Cu^{2+} for SKOV3. The lower row (c–f) concentration is control cells, 1 μM Cu^{2+} alone, 0.01 μM DSF alone, and 0.01 μM DSF + 1 μM Cu^{2+} for three cell lines. Cells without any drug treatment were used as control. Histograms were generated from propidium iodide-stained cells. The sub-G1 region was gated in the upper row of each cell line. The percentage of cells in each cell cycle phase was quantitated and expressed as percent of all cells acquired. One representative of three independent experiments is shown. B–D) Graphical representation of the percentage of cells in each cell cycle phase was quantitated and analyzed (n = 3). Statistical analyses are based on three independent experiments.

3.6. DSF enhances cisplatin cytotoxicity in ALDH + cells

To assess the impact of ALDH expression in CSCs further, FACS-sorted ALDH + and ALDH − cells from cell line SKOV3 were cultured for 10 days and the colony and spheroid formation assays were performed. As shown in Fig. 6A–D, significantly more colony and spheroid formation was observed in ALDH + cells compared to ALDH- cells ($P < 0.01$). Remarkably, the number of colonies and spheroids was increased in ALDH + cells compared to ALDH- cells. These results confirm the importance of ALDH activity in CSCs.

ALDH + cells and ALDH- cells were then treated with cisplatin at indicated concentrations for 72 h and subjected to MTT assay. As shown in Fig. 6E, ALDH + cells were more resistant to cisplatin treatment compared to ALDH − cells at any concentration investigated. The relative cellular viability was significantly higher in ALDH + cells than ALDH − cells after cisplatin treatment at the same concentration ($P < 0.05$). These results indicate that ALDH + cells display stem-like characteristics, such as sustained proliferation capability and resistance to chemotherapy. However, when the cells were pre-treated with DSF (0.08 μM) for 24 h, following incubation with cisplatin plus DSF, no significant difference in cellular viability was observed in ALDH + cells in comparison to ALDH − cells, indicating that DSF inhibited the ALDH + population, reversed cisplatin-resistance of ALDH + cells, and enhanced cisplatin cytotoxicity on ALDH + cells (Fig. 6F).

4. Discussion

Since a remarkable series of transplant experiments initiated in the

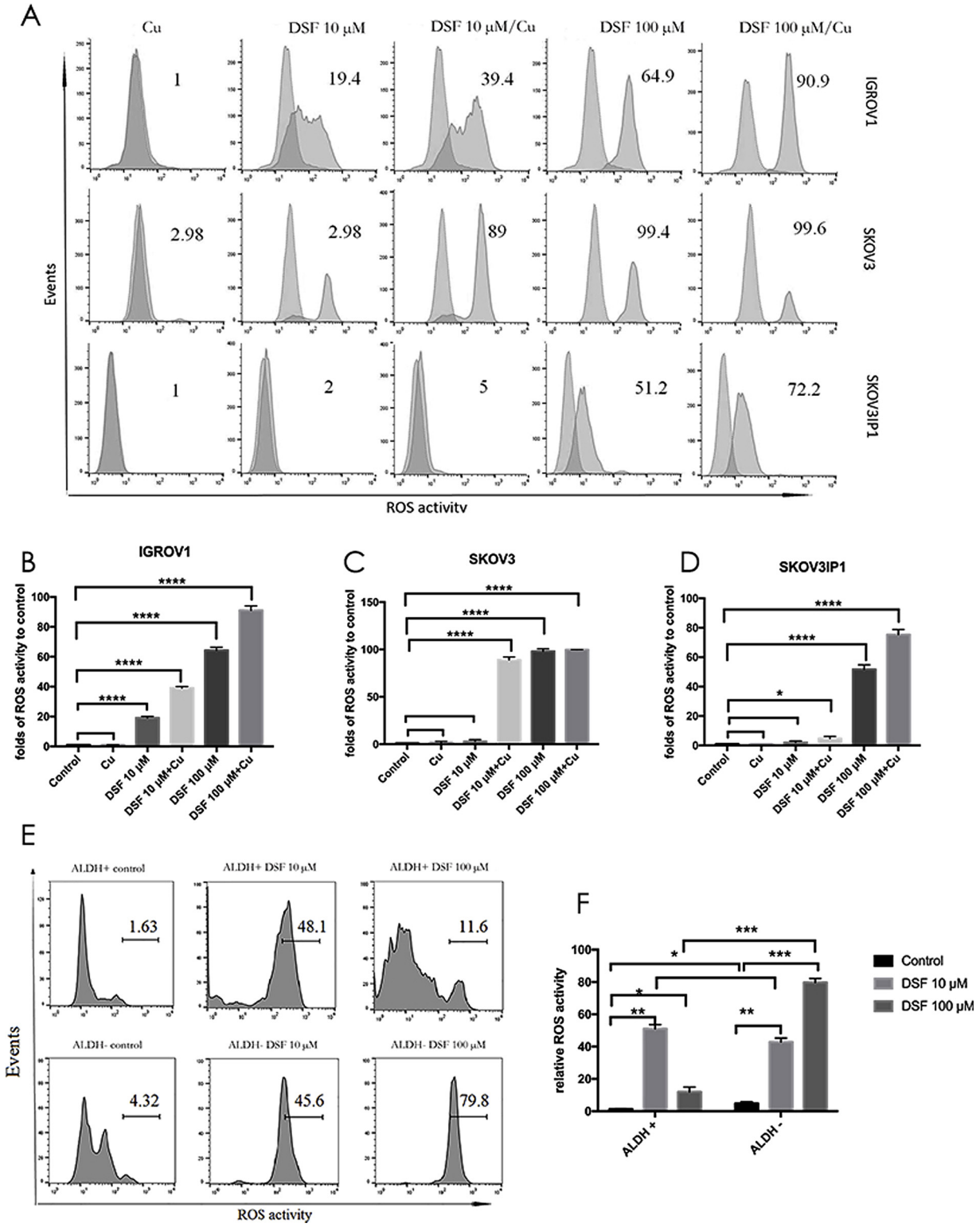

Fig. 4. DSF + Cu^{2+} triggers ROS generation in ovarian cancer cell lines. A) Cancer cells were exposed to the indicated reagents and concentrations for 30 min. The dotted lines represent the untreated cells and the solid lines represent drug-treated cells, respectively. The relative ROS activity was calculated and normalized to untreated control cells. One representative of three independent experiments is shown. B–D) Graphical representation of the relative ROS activity was quantitated and analyzed (n = 3). E). ALDH + and ALDH- FACS-sorted cells from SKOV3 cell line were exposed to 10 μM DSF and 100 μM DSF. The relative ROS activity was gated. One representative of three independent experiments is shown. F) Graphical representation of the relative ROS activity was quantitated and analyzed (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

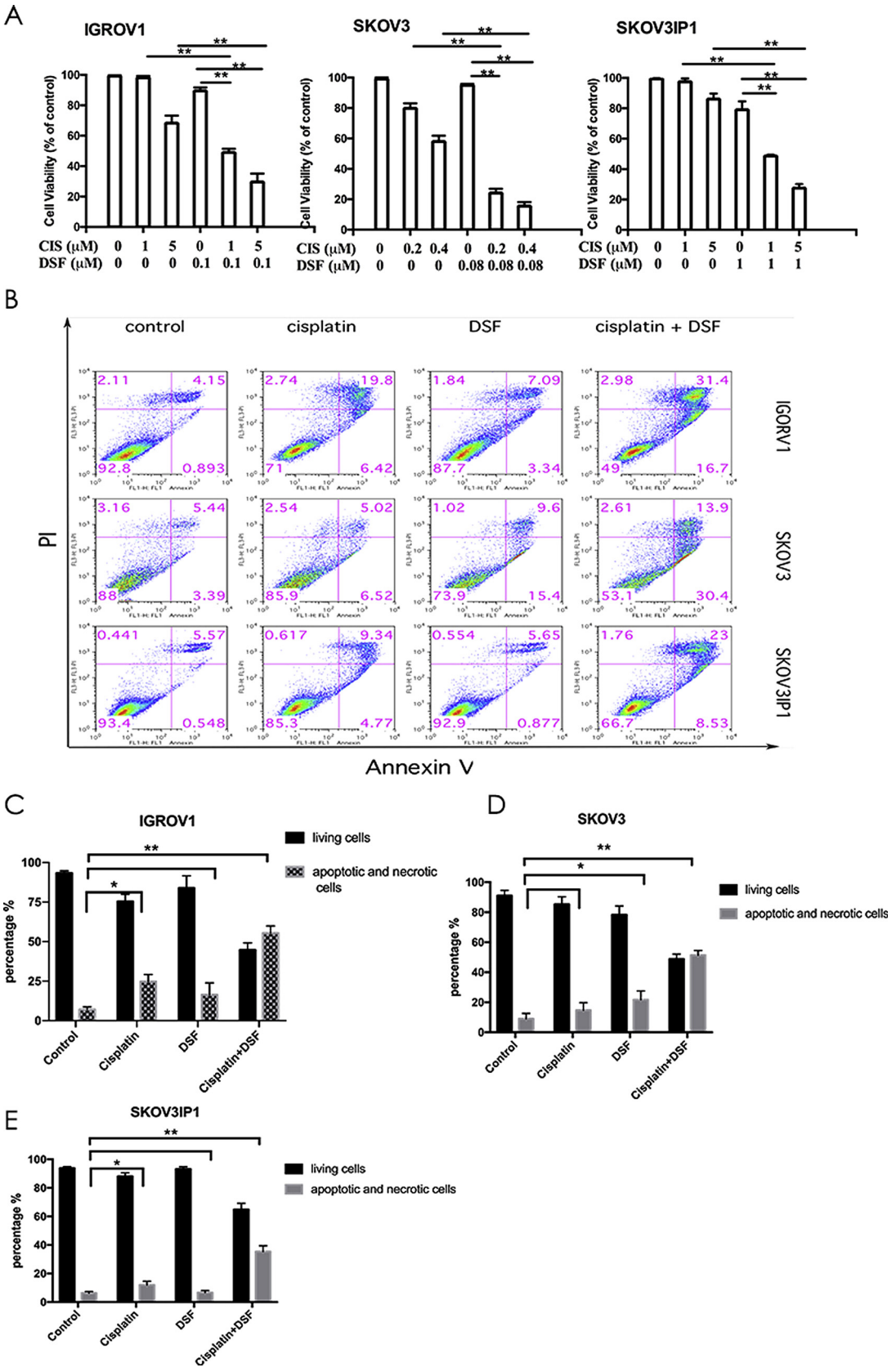

Fig. 5. DSF sensitizes ovarian cancer cells to cisplatin treatment and induces more apoptosis. A) Cells were treated with either cisplatin alone or DSF alone or the combinations at indicated concentrations for 72 h. Cellular viability was detected by MTT assay. B) Flow cytometric analysis exhibits the cellular apoptotic status. Cells were treated with cisplatin alone or DSF alone or their combination for 72 h. LL, LR, UR and UL are representative for live cells, early apoptotic cells, late apoptotic cells, and necrotic cells, respectively. One representative of three independent experiments is shown. C–E) Graphical representation of the statistical analysis of the Annexin-V/PI dual staining results from 3 independent experiments. Cells without any drug treatment were used as control. DSF, Disulfiram; CIS, Cisplatin. *P < 0.05, **P < 0.01.

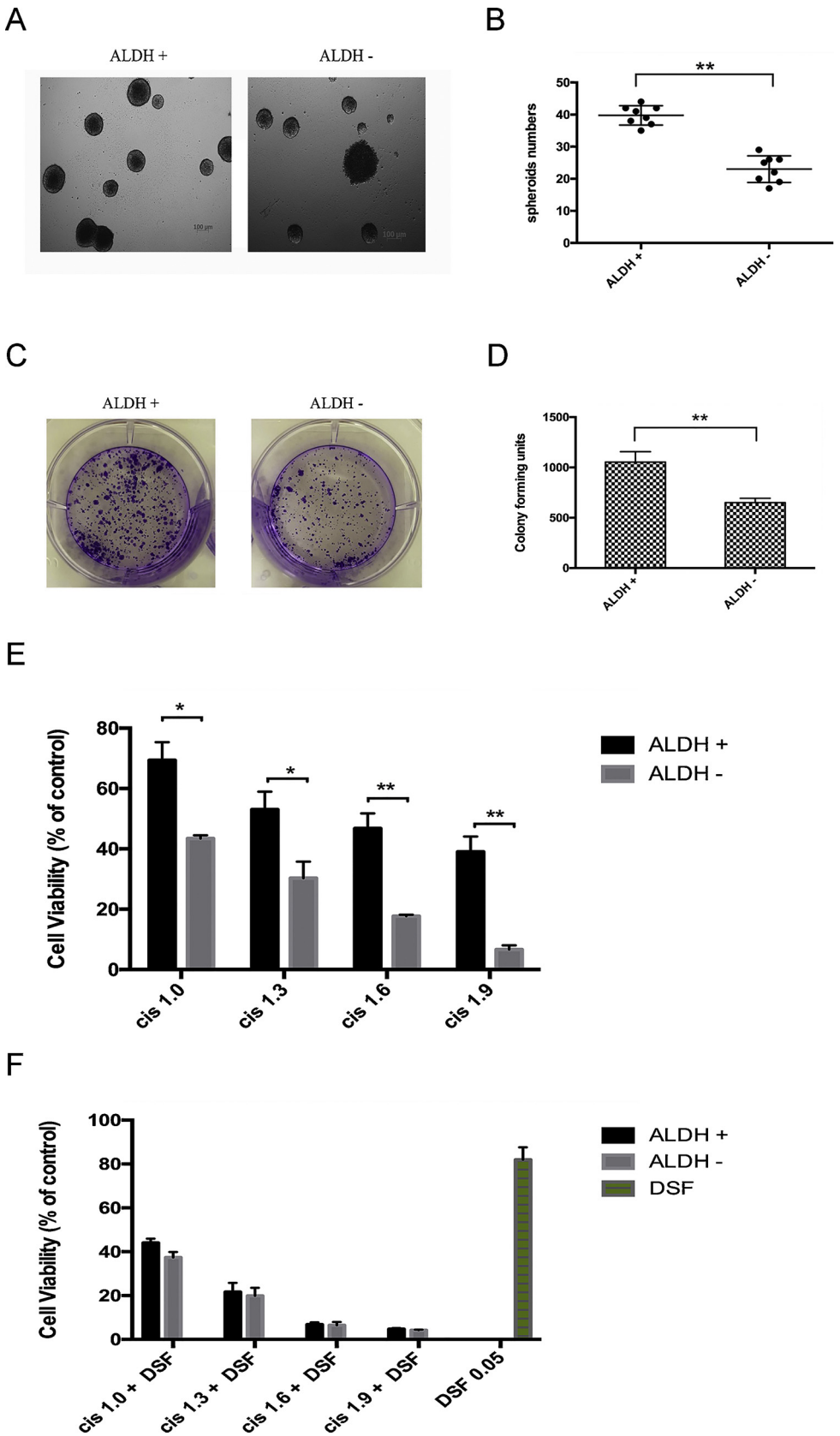

Fig. 6. Characterization of ALDH+/− cells sorted from SKOV3 and treatment by cisplatin/DSF. A) Spheroid formation assay of FACS-sorted ALDH+ and ALDH− cells. B) Quantitative analysis of spheroid formation of ALDH+/− cells. C) Colony formation assay of FACS-sorted ALDH+ and ALDH− cells. D) Quantitative analysis of the number of colonies formed by ALDH+/− cells. E) ALDH+/− cells were treated with cisplatin and cell viability was analyzed by MTT assay. F) ALDH +/− cells were treated with cisplatin combined with DSF and cell viability was analyzed by MTT assay.

1950s [29], which reflected that only a limited number of cells, defined as cancer stem cells, are capable of initiating the tumor, increasing evidence suggested that the characteristics of cancer stem cells might be a promising explanation for the recurrence and metastasis of cancer [30]. Therefore, identification of drugs targeting CSCs is urgently required to improve the therapeutic outcomes in cancer and in particular of ovarian cancer patients. Our study provides strong evidence that DSF can inhibit cancer stem cells partly due to its modulation of ALDH and ROS generation that can be enhanced by the addition of Cu^{2+}.

This study shows that DSF itself exhibits dose-dependent and time-dependent cytotoxicity in ovarian cancer cells and the DSF-mediated cytotoxicity was enhanced by exogenous Cu^{2+} supplementation in ovarian cancer cell lines. It has been proven that DSF can react with redox-sensitive sulfhydryl groups (thiols) and binds Cu^{2+} [31]. Cu^{2+}, that can activate some critical proteins such as superoxide dismutase, tyrosinase and cytochrome oxidase, plays an important role in biological pathways in the human body [32]. Therefore, the Cu^{2+} concentration in the human body is tightly regulated. However, the average concentration of Cu^{2+} in cancerous tissues is $1.7 \pm 0.6\,\mu g/g$, which is significantly ($P < 0.025$) higher than the average concentration of $1.3 \pm 0.3\,\mu g/g$ in the normal tissues [33]. This may help to enable selective treatment by DSF in cancer cells with enhancement of cytotoxicity by the higher level of Cu^{2+} concentrations in cancers while sparing normal tissues. One scenario could be that lipophilic DSF can penetrate cancer cells and bind with intracellular Cu^{2+} and form the DSF + Cu^{2+} complex (diethyldithio-carbomate (DDC)-Cu^{2+}) which is more cytotoxic and induces apoptosis. Studies are showing that Cu^{2+}-chelating DSF, but not DSF alone, evokes the unfolded protein response and heat shock protein activation [34]. Another scenario is that when exogenous Cu^{2+} was added into the medium, an instant and short-term action between DSF and Cu^{2+} happened, producing some certain chemical species, such as ROS, which in turn is toxic and causes instant killing of cancer cells [35].

ALDH activity has been shown to be associated with the stem cell population. Landen et al. have proven that ALDH+ ovarian cancer cells are resistant to a wide range of classical cytotoxic anticancer drugs. However, these cells can become re-sensitized to chemotherapy by ALDH1A1 silencing using nanoliposomal siRNA in ovarian cancer cell line SKOV3TRip2 and A2780cp20 [19]. Researches reported recently demonstrated that DSF + Cu^{2+} treatment eliminated the stem cell-like ALDH + cells pool in non-small cell lung cancer and multiple myeloma [36,37]. Our study also showed that ALDH+ cells display stem-like characteristics such as enhanced expression of stem cell transcription factors (supplemental Fig. 1), clonogenicity, sustained proliferation, and resistance to chemotherapy. Other than ALDH, the expression of stemness-related transcription factors Oct3/4, Sox2, and Nanog were also 2 to 10 fold higher in spheroid-derived compared to monolayer-derived cells. Our results are in agreement with results from other labs supporting that there is an enrichment of CSCs according to ALDH and stemness-related transcription factor expression by anchorage-independent culture techniques and that therefore spheroid cell culture models might be useful for evaluating CSCs [38,39]. Our study further demonstrated that DSF with or without Cu^{2+} supplementation inhibited ALDH activity which is associated with many properties of ovarian cancer stem cells, such as spheroid and colony formation, as well as chemo-resistance, indicating that DSF may target ALDH+ stem-like cells in ovarian cancer.

ROS are broadly defined as oxygen-containing chemical species with reactive properties. These include the superoxide ($O_2\bullet-$) and hydroxyl (HO•) free radicals as well as non-radical molecules such as hydrogen peroxide (H_2O_2) [40]. Cancer cells contain higher levels of ROS due to rapid metabolism and signalling [41]. To compensate for increased oxidative stress, cancer cells enhance their ability to detoxify ROS by activating adaptive redox mechanisms, including increased expression and activity of ROS-scavenging systems and antioxidants [42]. Additionally, ALDH enzyme has been proven to act as a ROS scavenger, and that could also explain why ALDH+ stem-like cells, which have higher levels of ALDH, exhibit lower basal levels of ROS [20]. Our data demonstrate that ROS, which contributes to a wide variety of cell and tissue injury, may have a crucial role in DSF + Cu^{2+} induced cytotoxicity and apoptosis in ovarian cancer cell lines. DSF + Cu^{2+} treatment increased intracellular ROS levels, which typically triggered ALDH+ stem-like cells apoptosis. Simultaneously, DSF + Cu^{2+} treatment inhibited ALDH enzyme activity and subsequently leads to a loss of ALDH-mediated protection against oxidative stress. These results are in agreement with the reports that ROS inhibitor (N-acetylcysteine) could suppress antitumor potentiation of DSF by inhibiting H_2O_2 generation, and ROS promotor superoxide dismutases (SODs) could increase the toxicity of DSF [43]. There was a similar amount of ROS generated in ALDH+/− cells after DSF (10 μM) treatment. A lower level of ROS in ALDH+ cells than ALDH- cells was shown after 100 μM DSF treatment. One explanation could be that ALDH+ cells were probably more vulnerable to DSF and were swiftly killed by this concentration.

Further, we explored the combined effect of DSF and cisplatin. We choose cisplatin in this study because cisplatin can arrest the cell cycle in the G2/M phase [20] while DSF induces ROS accumulation. Therefore, these two drugs may potentiate the combination effect based on different mechanisms. We found that DSF effectively sensitized cancer cells to cisplatin treatment even at its lower dose, and significantly enhanced cisplatin-induced apoptosis. Based on these results, we further examined the resistance-reversing effect of DSF on ALDH+ stem-like cells. ALDH+ and ALDH− cells were explored after cell sorting to avoid heterogeneous cell populations. Our results showed that ALDH+ cells displayed stem-like characteristics and were more resistant to cisplatin treatment than ALDH− cells. However, ALDH+ cells exhibited high sensitivity to DSF/cisplatin combination treatment, indicating that DSF overcomes cisplatin resistance. This is in agreement with a study by Song et al. [44] that cisplatin, in combination with DSF, decreased the cell survival rate of cisplatin-resistant lung cancer cell line A549DDP. Our results showed that DSF reversed the cisplatin resistance of ALDH+ cells, leaving no difference between ALDH+ and ALDH− cells when treated with cisplatin combined with DSF. The explanation could be that DSF suppresses ALDH activity and stemness, reverses cisplatin resistance on ALDH+ cells, and sensitizes a cisplatin-resistant ALDH+ stem-like population to cisplatin treatment, consequently improving the ability of cisplatin to kill resistant or less sensitive cancer cells. Due to its chemo-sensitizing effects, DSF is very promising to be combined with cisplatin-based chemotherapy to improve the therapeutic impact.

Together, these results indicate that DSF with or without Cu^{2+} supplementation is toxic in ovarian cancer cell lines *in vitro* by mechanisms including targeting oxidative stress response and ALDH+ stem-like cells. There are additional effects of Cu^{2+} described that might support the induction of apoptosis and contribute to the effect of DSF. The clarification of their contribution, however, was not the scope of this manuscript and should be investigated separately. DSF effectively suppressed ALDH activity and modulated ROS generation. It is attractive to consider DSF as an adjuvant treatment combined with cisplatin to establish a new chemotherapy protocol for targeting CSCs. In this novel combination, DSF could suppress the ALDH activity and sensitize the cisplatin-resistant ALDH+ stem-like population to cisplatin treatment. At the same time, the cytotoxicity of cisplatin could be increased once combined with DSF, inducing apoptosis in ovarian cancer cells. However, more research is still needed to further explore the effect of DSF on tissues and primary tumors from patients.

Disclosure statement

The authors declare that they have no competing interests.

Acknowledgments

Cell sorting was technically supported by Berlin-Brandenburg Center for Regenerative Therapies (BCRT).

We acknowledge support from the German Research Foundation (DFG) and the Open Access Publication Fund of Charité – Universitätsmedizin Berlin.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109371.

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