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# Disulfiram modulates ROS accumulation and overcomes synergistically cisplatin resistance in breast cancer cell lines

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ABSTRACT

The chemotherapeutic agent cisplatin typically induces apoptosis by inhibiting the cell cycle. Cancer Stem Cells (CSCs), which are a proliferative quiescent and slowly-cycling cell population, are less sensitive and therefore frequently spared from toxic effects. Thus, it remains a priority to increase the sensitivity of CSCs to cisplatin-based chemotherapy, or to specifically target CSCs to improve the therapeutic outcome in breast cancer. Disulfiram (DSF) is a drug used clinically for alcoholism treatment that has displayed promising anti-cancer activity in vitro and in cancer xenografts in breast cancer. Our study provides evidence that DSF inhibits Aldehyde dehydrogenase (ALDH) enzyme activity, inhibits the expression of stemness-related transcription factors (Sox, Nanog, Oct) in CSC derived from breast cancer cell lines, and modulates intracellular reactive oxygen species (ROS) generation. Importantly, our research proved that ALDH + stem-like cells play important roles in the resistance to the conventional chemotherapeutic agent cisplatin. DSF enhances the cytotoxic effect of cisplatin through inhibiting the stemness and by overcoming cisplatin resistance of ALDH + stem-like cells. A quantitative measurement showed the synergistic effect of DSF and cisplatin. Further, we show that ALDH + cancer stem-like cells and ALDH- bulk cancer cells have different intrinsic ROS levels, what may explain differences in susceptibility to cisplatin treatment. Importantly, this difference is eliminated by DSF treatment making both cell types similarly susceptible for cytotoxic effects by cisplatin. These findings may influence chemotherapeutic treatment approaches in the future.

## 1. Introduction

Breast cancer has become one of the most common malignancies among the female population and its prevalence has also increased in women ≤40 years of age [1]. It accounts for 22.9% of all female cancers worldwide [2]. Despite the impressive clinical improvements of breast cancer therapies, mortality of breast cancer patients is mostly related to late diagnosis and resistance to adjuvant systemic therapy, leading to metastasis and recurrence.

Cancer stem cells (CSCs) are thought to contribute to drug resistance and cancer recurrence as they are proliferative quiescent and self-renewing tumor cell populations. These cells have stem cell characteristics, and were first isolated in breast tumors in 2003 [3]. Conventional chemotherapies are targeting preferentially the proliferating cancer cells. However, the undifferentiated populations of CSCs are often unaffected [4,5] and retain their self-renewal properties. A number of different intrinsic and extrinsic mechanisms have been reported to correlate with resistance of CSCs, including increased DNA repair capability, enhanced scavenging of reactive oxygen species (ROS), and activation of cell survival pathways [6,7]. Thus, it would be promising to develop alternative adjuvant methods that could target self-renewing and resistant CSCs directly [8].

ALDH activity has been well-accepted and successfully used as a CSC marker in a number of solid tumors, including breast, lung, prostate and others [9–11]. Recent studies have demonstrated that ALDH activity correlates with resistance to both radiation and cisplatin in vitro [6,12,13]. Cells with higher ALDH activity have enhanced resistance to treatment and maintain their tumorigenic properties

*Abbreviations:* ALDH, aldehyde dehydrogenase; CIS, cisplatin; CSCs, cancer stem cells; DSF, disulfiram; MDCs, monolayer-derived cells; ROS, reactive oxygen species; SDCs, spheroid-derived cells

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compared to ALDH negative cells [11]. Additionally, some studies have shown that ALDH is a ROS scavenger which has the potential to decrease oxidative stress and protect stem cells against its toxic effects [14,15]. Thus, targeting ALDH positive tumor cell subpopulations, characterized by other biomarkers as CSCs, might provide a new approach to improve the efficacy of breast cancer treatment.

Currently, cisplatin is one of the most effective chemotherapeutic agents used for breast cancer treatment. Cisplatin unfolds its cytotoxicity by binding to nuclear DNA and thereby interferes with the normal DNA replication mechanisms [16]. Resistance or insensitivity of tumor cells to cisplatin treatment is an essential reason causing relapse. The combination of cisplatin with other chemotherapeutic drugs has obtained satisfactory clinical effects, but also caused greater toxicity for patients. Thus, it is a significant need to explore a combination of cisplatin with new agents, to overcome chemotherapy resistance while minimizing their toxic effects on patients.

Disulfiram (DSF), also known as the medication Antabuse, has been used to treat alcoholism in patients for more than 60 years [17,18]. DSF has been demonstrated to have strong anticancer activities in vitro and in cancer xenografts in breast cancer [19,20]. In the present study, we explored effects of DSF on ALDH + stem-like cells and demonstrate a synergistic effect with cisplatin by different mechanisms. This provides evidence that enhanced therapeutic susceptibility in breast cancer could be expected.

## 2. Materials and methods

### 2.1. Cell culture and drug preparation

Breast cancer cell lines MCF-7, SKB-R3, and MDA-MB-435S were cultured as adherently growing monolayer cells in DMEM medium with L-glutamine (Invitrogen, Heidelberg, Germany) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (both from Biochrom, Berlin, Germany) in a humidified atmosphere at 37 °C containing 5% $CO_2$. All experiments were performed at a confluency of around 70%.

Free DSF (Sigma-Aldrich, St. Louis, US) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, US) at a stock concentration of 10 mM, stored at −20 °C and diluted into working concentrations in medium before use.

### 2.2. Spheroid formation assay

Adherent monolayer cells were detached with Trypsin/EDTA (Biochrom) and recultured in Quantum 263 medium (PAA, Cöllbe, Germany) supplemented with 10 ng/ml Epidermal Growth Factor (EGF) and 10 ng/ml Fibroblast Growth Factor-basic (bFGF) (Biochrom). To generate spheroids, single cells were plated in Ultra-Low Attachment plates (Corning, New York, USA) at a specific density of $2 \times 10^4$ cells/ml in the incubator at 37 °C in a humidified atmosphere with 5% $CO_2$.

### 2.3. MTT assay

Cells were seeded in 96-well plates at a density of 3000 cells/well in 100 µl medium and allowed to attach overnight. Cells were then treated with DSF at indicated concentrations for further 72 h. The MTT (Cell Proliferation Kit I, Roche, Mannheim, Germany) assay was performed after 72 h incubation to determine cellular proliferation and viability. Absorbance was measured at wavelength of 590 nm using an enzyme-linked immunosorbent assay plate reader (BioRad, München, Germany). Relative viability of cells was calculated as the percentage of DSF-treated cells from DSF-free control cell cultures.

### 2.4. Spheroid formation inhibitory assay

To further explore the inhibitory effect of DSF on spheroid formation, spheroid-derived cells (SDCs) were re-seeded into a 96-well ultra-low attachment plate at a density of 100 cells or 200 cells in 100 µl Quantum 263 medium per well (100 cells per well for MCF-7 and SKB-R3 cell lines; 200 cells per well for MDA-MB-435S cell line). Cells were exposed to DSF (1 µM or 5 µM) and cultured for 10 days. Cells without DSF exposure were used as control. Spheroids with 100 µm or more in diameter were counted and photographed at 50 fold* magnification.

### 2.5. Flow cytometric (FACS) analysis of ALDH activity and cell sorting

The identification of aldehyde dehydrogenase (ALDH) activity from spheroid-derived cells (SDCs) and monolayer-derived cells (MDCs) was conducted using the ALDEFLUOR kit (StemCell Technologies, Köln, Germany) following the manufacturer's instructions. For FACS sorting, SDCs were suspended in PBS buffer at a concentration of $1 \times 10^7$ cells/ml and sorted on an BD FACSAria II SORP cell sorter (BD Biosciences). The sorting gates were established with negative controls which were treated with DEAB.

### 2.6. Quantitative real-time PCR

Four different groups of cells for each breast cancer cell line were included in this experiment. MDCs and SDCs without any drug treatment cultured for 4 days and then harvested. SDCs treated with DSF (1 µM) for 48 h and then collected. SDCs exposed to DSF (1 µM) for 48 h, followed by washing and re-cultured in DSF-free medium for further 48 h, and then collected. Stemness-related nuclear transcription factor Sox2, Oct3/4, and Nanog were determined in the present study. MDCs without any drug treatment were used as reference. Primer sequences for Oct3/4, Sox2, and Nanog are described in Table 1.

### 2.7. Apoptosis assessment

Apoptotic status was determined by FLUOS-conjugated Annexin-V and propidium iodide Kit (Roche, Mannheim, Germany) by flow cytometry on a FACS Calibur following the manufacturer's instructions. Briefly, $2*10^4$ cells were seeded in 12-well plates and incubated overnight. Cells were then treated with drugs at indicated concentration for further 72 h. All cells were then harvested by trypsinisation and re-suspended in 100 µl binding buffer containing FITC-conjugated Annexin-V/PI and incubated at RT for 15 min. Apoptosis and necrosis were evaluated using FL3 (PI) and FL1 (Annexin-V) by FACS analysis by FlowJo software (Treestar, Ashland, OR, USA).

### 2.8. Flow cytometric analysis of the cell cycle and ROS content

Cells were seeded in 24-well plates at a density of $3 \times 10^4$ cells in 1 ml medium per well. After overnight incubation, cells were treated by indicated concentrations of DSF, or cisplatin, or DSF/cisplatin combinations for 72 h. Cells without treatment were used as controls. Analysis of cell cycle distribution was performed using flow cytometric analysis of DNA staining with propidium iodide (PI) [21]. FlowJo software (Treestar) was used to quantitate the percentage of cells in each cell cycle phase.

Mitochondrial ROS were measured following the manufacturer's instructions of MitoSOX Red kit (Thermo Fisher Scientific). Samples

**Table 1**
Primer sequences for Oct3/4, Sox2 and Nanog.

| | | |
|---|---|---|
| Oct3/4 | FWD | 5′-GACAGGGGGAGGGGAGGAGCTAGG-3′ |
| | REV | 5′-CTTCCCTCCAACCAGTTGCCCCAAAC-3′ |
| Sox2 | FWD | 5′-GGGAAATGGGAGGGGTGCAAAAGAGG-3′ |
| | REV | 5′-TTGCGTGAGTGTGGATGGGATTGGTG-3′ |
| Nanog | FWD | 5′-AATACCTCAGCCTCCAGCAGATG-3′ |
| | REV | 5′-TGCGTCACACCATTGCTATTCTTC-3′ |

without MitoSOX Red Reagent were used to substract background. Samples treated with MitoSOX Red Reagent, but without any drug treatment, were used as controls. Mean fluorescence intensity was determined and all samples were normalized to untreated control samples.

### 2.9. Statistical analysis

All data presented were representative of three independent experiments. Statistical analyses were performed using GraphPad Prism 6 and FlowJo. The group data were presented as the mean ± standard error of the mean. Differences were considered significant at $P < 0.05$. Quantitative analysis was used for the combination effect to calculate combination indices (CI) of drugs with CompuSyn software.

## 3. Results

### 3.1. Disulfiram exhibits dose-depend cytotoxicity on breast cancer cell lines in vitro

To explore the inhibitory effect of DSF in vitro, we initially examined the cytotoxicity of DSF on three breast cancer cell lines by MTT assay. As shown in Fig. 1A, no severe cytotoxicity (percentage of cytotoxicity < 20%) was observed when cells were exposed to 0.04 μM, 0.2 μM, 1 μM, or 5 μM DSF in MDA-MB-435S and SKB-R3 cell lines. In MCF-7 cell line, DSF showed mild cytotoxicity at the concentration of 0.04 μM, 0.2 μM, or 1 μM, and a moderate cytotoxicity at a concentration of 5 μM. However, there was a significant decrease in cellular relative viability when the concentration of DSF was increased to 25 μM. The results indicated that DSF exhibited its cytotoxicity on the investigated breast cancer cell lines in a dose-dependent manner and at higher concentrations (> 10 u M).

### 3.2. DSF inhibits spheroid formation

Spheroid formation assay is a well-accepted assay to enrich for cells exhibiting positivity for established stem cell markers. As shown in Fig. 1B and C, 1 μM and 5 μM of DSF were found to be adequate to inhibit spheroid formation while avoiding too much direct cytotoxicity on cellular growth. The results showed that the number of spheroids significantly decreased when cells were exposed to 1 μM DSF (the average spheroid number decreased from 5 to 1 in MCF7, from 5 to 0 in MDA-MB-435S, and from 3 to 1 in SKB-R3 per x100 seeded). In addition, the individual size of the spheroids that formed also was reduced in all cell lines not reaching the cut off of 100 μM. No growth of spheroids was observed when the cells were exposed to 5 μM DSF in all three cell lines investigated. This demonstrated that at this concentration, despite very low direct toxicity, a stemness inhibitory effect could be achieved.

### 3.3. DSF inhibits ALDH activity

To further determine the effective targeting of DSF on stem-like cells, ALDH activity in both MDCs and SDCs exposed to DSF (10 μM) was analyzed by ALDEFLUOR assay. As shown in Fig. 2A and B, all SDCs had an increased proportion of ALDH-positive cells as compared to parental MDCs by FACS analysis. The proportion of ALDH-positive cells increased from 10.27 ± 0.636% to 45.7 ± 2.031% in MCF-7, from 12.13 ± 1.162% to 36.03 ± 2.31% in MDA-MB-435S, from 27.03 ± 0.5783% to 30.65 ± 0.65% in SKB-R3 cell line, in MDC and SDC, respectively. The proportion of ALDH positive cells was decreased in both MDCs and SDCs after exposure to DSF: from 10.27 ± 0.636% to 0.05 ± 0.005% in MDCs and from 45.7 ± 2.031% to 0.4573 ± 0.2022% in SDCs in MCF-7 cell line; from 12.13 ± 1.162% to 0.033 ± 0.033% in MDCs and from 36.03 ± 2.31% to 0.0171 ± 0.0089% in SDCs in MDA-MB-435S cell line; and from 27.03 ± 0.5783% to 0.04 ± 0.023% in MDCs and from 30.65 ± 0.65% to 1.15 ± 0.055% in SDCs in SKB-R3 cell line. Importantly, the inhibitory effect of DSF was even stronger than the one of DEAB, used as a control inhibitor, in both MDCs and SDCs. These results proved that DSF could target cancer stem-like cells in the investigated cell lines by inhibiting ALDH enzymatic activity.

### 3.4. DSF inhibits stemness-related gene expression in SDCs

Next, we investigated mRNA expression of nuclear transcription factors (TF) Sox2, Oct3/4, and Nanog by which CSCs are also sharing the stemness characteristics of embryonic stem cells. The mRNA levels detected by RT-PCR of Sox2, Oct3/4, and Nanog were all found to increase in SDCs of all three cell lines from 2-fold to almost 7-fold as compared to MDCs. The results indicated that spheroids could enrich cancer stem cells and non-adherent 3D sphere models as well as SDCs are reasonable to be used for evaluating stem cell activities in these breast cancer cell lines. After DSF (1 μM) exposure for 48 h, mRNA levels of Sox2, Oct3/4, and Nanog in SDCs were all significantly decreased, and even lower than those observed in MDCs. Further, the expression of these markers recovered swiftly when the cells were recultured in DSF-free medium for further 48 h (Fig. 3).

### 3.5. DSF sensitizes breast cancer cells for cisplatin treatment and acts synergistically combined with cisplatin

As DSF inhibited the stemness of CSCs, we wanted to further explore the combination effect of DSF with cisplatin, which rather targets the proliferating non-stemness cancer cells. We chose to treat the cells with a low concentration of DSF (1 μM) in order to judge synergistic effects with cisplatin (used at 5 μM) treatment. As shown in Fig. 4A, the early and late apoptosis and necrosis together increased from 45.9% by treatment with cisplatin alone to 61.6% by the cisplatin/DSF combination treatment in MCF-7 cells, from 19.1%–42.3% in MDA-MB-435S cells, and from 18.3%–25.2% in SKB-R3 cells, respectively. Our results revealed that the combination of treatment with cisplatin and DSF induced more cellular apoptosis than either single drug alone. Fig. 4B shows that cisplatin/DSF combination treatment significantly reduced cell viability compared to cisplatin-treated cells ($P < 0.05$). Cellular viability dropped by 50% in MCF-7 cell line, and 20%–30% in MDA-MB-435S and SKB-R3 cell lines. These results indicate that DSF sensitizes breast cancer cells to cisplatin treatment even at a concentration where DSF is not toxic by itself.

The combination index (CI) value was used in this study as a quantitative measurement for drug interaction in terms of synergism and antagonism for a given endpoint of the effect measurement [22]. CI < 0.1, CI 0.1 − 0.3, CI 0.3 − 0.7, CI 0.7 − 0.85, CI 0.85 − 0.9, and CI 0.90–1.10 indicate very strong synergism, strong synergism, synergism, moderate synergism, slight synergism, and nearly additive effects, respectively [22,23]. As shown in Table 2, the combination of DSF and cisplatin yielded a synergistic effect in all three tested cell lines at a broad concentration range from $IC_{50}$ to $IC_{90}$. Especially in MCF-7 cells, the CI value was 0.16 at the $IC_{90}$ level, and even less than 0.1 at $IC_{50}$ and $IC_{75}$ levels, indicating that a strong or very strong synergism was present. Synergism and moderate synergism were shown on MDA-MB-435S and SKB-R3 cells, respectively. Dose-reduction index (DRI) values is a measurement of how many fold the dose of each drug in a synergistic combination may be reduced at a given effect level when compared with the doses of each drug alone [22]. The results showed that the dosage of each drug was reduced by 2-fold to several hundred-fold when compared with the dosage of each drug alone while maintaining the equal cytotoxic effect in combinations due to their synergistic effect.

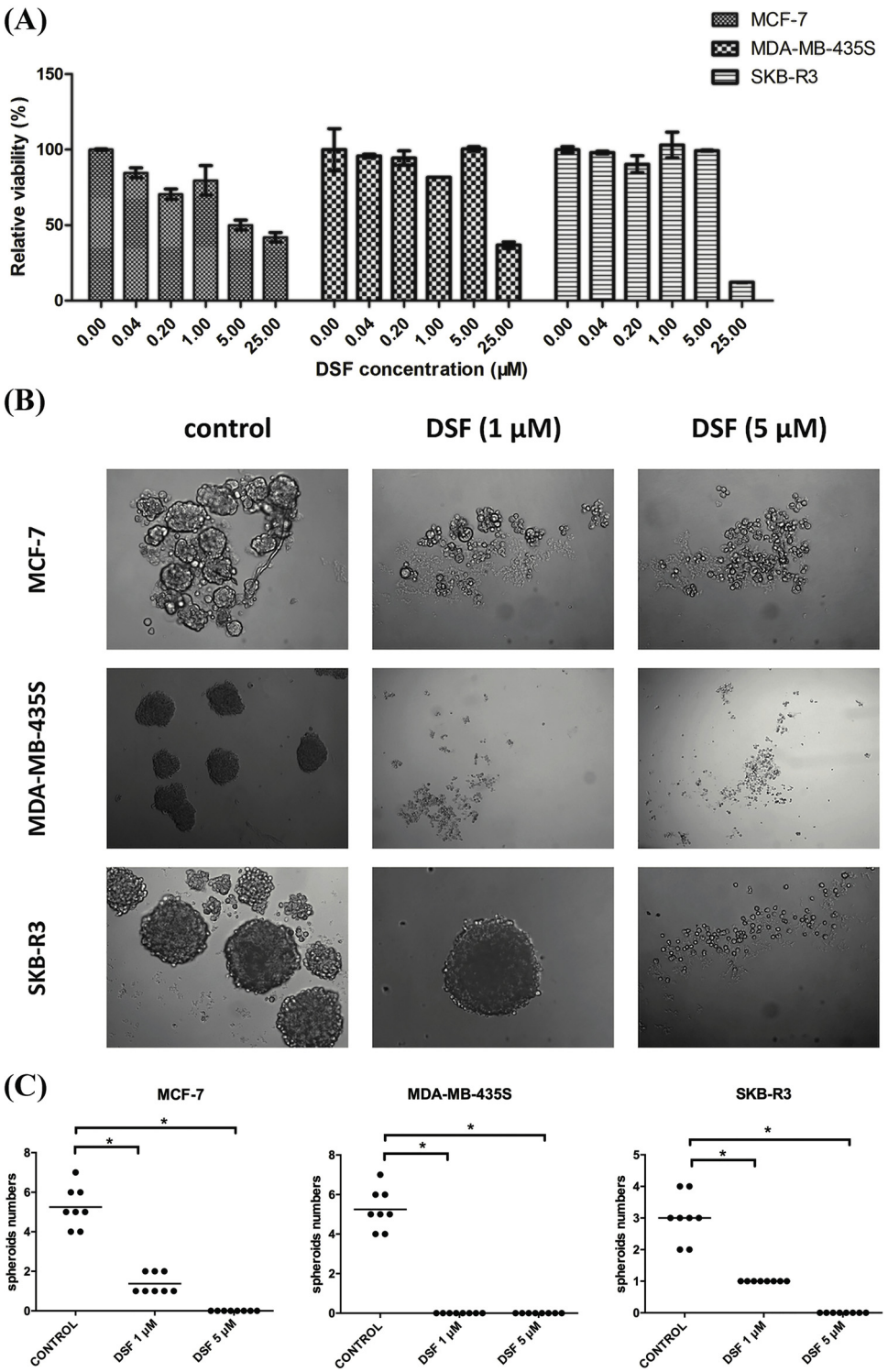


**Fig. 1.** Inhibitory effect of DSF on breast cancer cell lines. A). MTT assay. The breast cancer cell lines were exposed to different concentrations of DSF for 72 h and viability related to untreated control. B, C) DSF inhibited spheroid formation. Cells were cultured in 96-well ultra-low attachment plates without or with 1 μM or 5 μM DSF for 10 days and photographed (magnification 50-fold *). Spheroids with ≥ 100 μm in diameter were counted, and their numbers per well (n = 8) were plotted. * $P < 0.05$ ** $P < 0.01$.

### *3.6. DSF overcomes cisplatin resistance in ALDH + cells*

Since DSF is an irreversible inhibitor of ALDH [24], and ALDH + stem-like cells may play a role in cisplatin resistance [25], we next studied whether DSF combined with cisplatin could overcome cisplatin resistance. ALDH + and ALDH- cells were sorted by flow-cytometry from MCF-7 and were treated with different concentrations of cisplatin with or without DSF for 72 h, and were then subjected to a MTT assay. As shown in Fig. 4C and D, there was a significant difference of cellular viability between ALDH + cells and ALDH- cells when they were treated with cisplatin alone. ALDH + cells were more resistant to cisplatin treatment compared with ALDH- cells. However, when a low concentration of DSF (0.3 μM) was added, cellular viability decreased significantly (around 40%–50%) compared to cells treated with

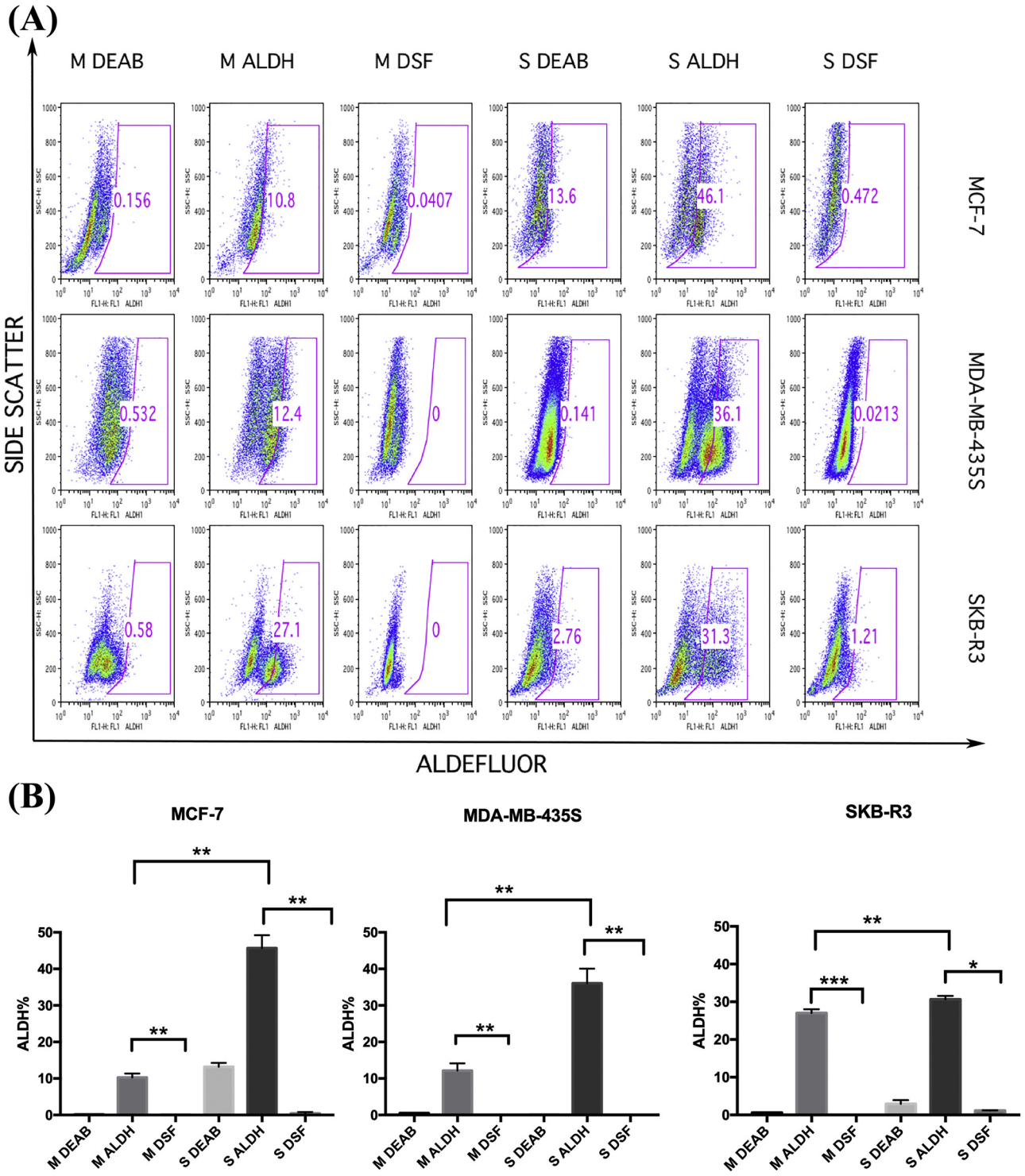


**Fig. 2.** DSF inhibits ALDH activity. A) MDC (M) and SDC (S) were exposed to diethylaminobenzaldehyde (DEAB, a specific ALDH inhibitor, that was used as control), control medium, or DSF (10 μM), and ALDH activity was detected by ALDEFLUOR assay. Numbers represent ALDH + cells (%). B) Graphical representation of the statistical analysis of ALDH activity(n = 3). One representative of three independent experiments is shown. * $P < 0.05$ ** $P < 0.01$ ***$P < 0.001$.

cisplatin only, and there was no difference between ALDH + cells and ALDH- cells anymore, indicating that both ALDH + cells and ALDH- cells were equally sensitive to DSF/cisplatin combination treatment. These results confirmed the potential efficacy of DSF in overcoming the cisplatin resistance of ALDH + cells in this experimental setting.

### *3.7. Cell cycle and ROS generation in ALDH + versus ALDH- cells*

To further explore whether DSF has different effects on ALDH + and ALDH- cells, both FACS-sorted ALDH + cells and ALDH- cells were treated with DSF (0.3 μM) and cisplatin (2 μM). The effect of cisplatin on cell cycle changes and the effect of DSF on ROS generation was measured. As shown in Fig. 5A, 5B and 5C, ALDH- cells were more sensitive to cisplatin treatment, with G0/G1 decreasing from

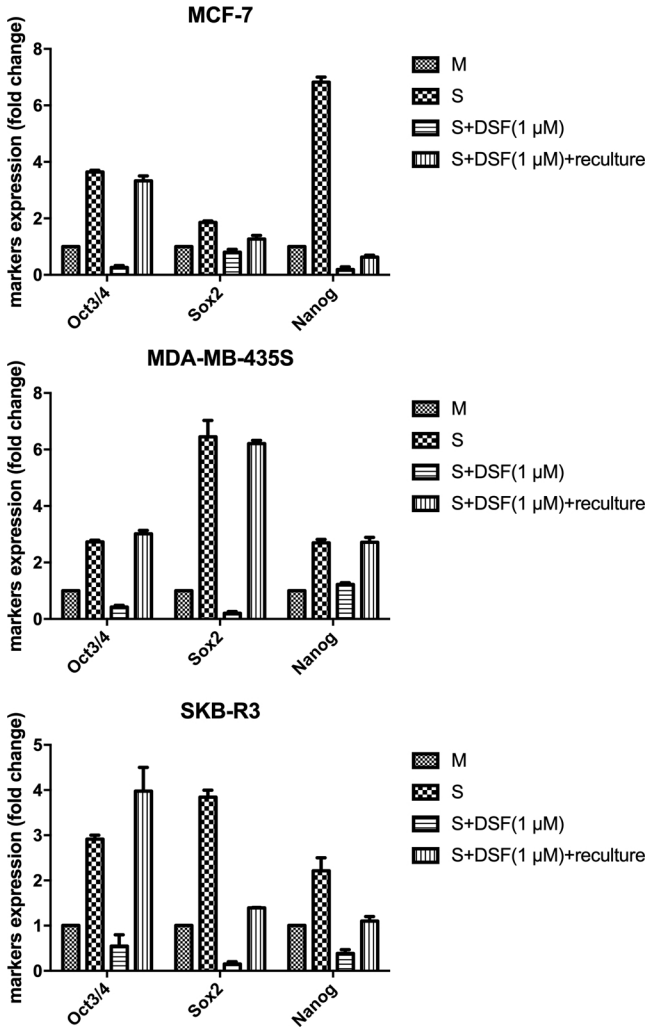


**Fig. 3.** DSF inhibits mRNA expression of stemness-related transcription factors. The expression of stemness-related TF in MDC, SDC, DSF-treated SDC (1 μM, 48 h), and SDC treated by DSF-recultured (1 μM, 48 h, followed by a chase to reculture in DSF-free medium for further 48 h) was quantified by qRT-PCR. M: MDC; S: SDC. One representative of three independent experiments is shown. * $P < 0.05$.

59.4 ± 0.5568% to 39.7 ± 0.7% and G2 increasing from 25.47 ± 0.6173% to 42.17 ± 0.2963%. ALDH + cells were more resistant to cisplatin treatment, with G0/G1 decreasing only from 62.83 ± 1.974% to 59.2 ± 3.126% and G2 increasing from 26.23 ± 1.071% to 27.2 ± 2.053% and there. As shown in Fig. 5D and 5E, ALDH- cells contained higher levels of ROS mean fluorescence intensity (MFI) of 229.3 ± 6.566, compared with lower levels of ROS MFI of 90.73 ± 2.423 in ALDH + cells ($P < 0.0001$). ROS accumulation was increased in both ALDH + and ALDH- cells when they were treated with DSF compared to non-drug treated control cells. ROS MFI by ALDH + cells and ALDH- cells reached to 524 ± 34.7 and 535 ± 75.26 respectively after DSF treatment and there was no significant difference. Concerning the relative increase, ALDH + cells showed a greater increase than ALDH- cells as they contain lower basal levels of ROS.

## 4. Discussion

In this study, we firstly confirmed that DSF itself exhibits dose-dependent cytotoxicity in the three breast cancer cell lines investigated. The ALDH activity was significantly inhibited by DSF, supporting a role for DSF in a targeted treatment of the ALDH positive subpopulation of CSC. Importantly, a suppressive effect of DSF on stemness of ALDH + CSC, as shown by inhibition in a sphere formation assay, was shown. Notably, DSF at 1 μM markedly suppressed the mRNA level of Oct3/4, Sox2, and Nanog in SDC. We speculate that the decreased expression of CSC markers could be due to two reasons: firstly, CSC were specifically killed, resulting in the reduction of stemness transcription factor positive cells in the population, or secondly stemness factor transcription by CSC was inhibited by DSF while the cells were still alive and lost their stem cell character. In order to discriminate these options, we re-cultured the cells for two more days in drug-free fresh medium after DSF exposure. We found that the expression of CSC markers quickly recovered after this phase, mostly back to the original level of SDCs. Since DSF is an irreversible inhibitor of ALDH [24], the results indicate that the stem cells were not killed at these conditions during a short term treatment but the stemness characteristics of cells were rather inhibited.

Next, we explored the combination effect of DSF and cisplatin. This study demonstrated that DSF sensitized breast cancer cell lines to cisplatin treatment and the combination treatment induced more cellular apoptosis than treatment with each drug individually. Our results have also shown that ALDH + cells were more resistant to cisplatin treatment than ALDH- cells. This is in agreement with studies from other labs which demonstrated that cancer cells expressing stem cell markers are highly resistant to radio- and chemotherapy [26]. Based on these results, we examined the resistance-reversing effect of DSF in this study. Our results showed that ALDH + cells exhibited high sensitivity to DSF/cisplatin combination treatment, indicating that DSF overcomes cisplatin resistance. One explanation could be that ALDH activity of ALDH + cells is inhibited by DSF, leaving ALDH + cells and ALDH- cells equally sensitive to cisplatin. Thus cisplatin resistance was reversed by DSF/cisplatin combination treatment. We used a quantitative method to determine the combination effect of DSF and cisplatin. The results showed that DSF and cisplatin yielded a synergistic effect at broad effect levels ranging from $IC_{50}$ to $IC_{90}$ in all three breast cancer cell lines. This synergistic effect in a DSF/cisplatin combination may provide many therapeutic benefits in clinical treatment regimens against breast cancer. The most important consequence is that it could increase or at least maintain the same efficacy but decrease the dosage of each drug to reduce toxicity, thereby potentially reducing the toxicity toward normal tissues.

To explore the possible mechanism of DSF and cisplatin in combination treatment, cell cycle analysis and ROS generation was determined in this study. Studies have shown that cisplatin induces cross-linking of DNA [16], therefore inducing a G2/M arrest. Our results also showed that cisplatin arrested cell cycle distribution in the G2 phase. However, DSF had no significant effect on cell cycle distribution. Cell cycle arrest in DSF/cisplatin combination was mainly due to the cisplatin effect. The results showed that DSF exerted its cytotoxicity by inducing ROS accumulation in a dose-dependent and time-dependent manner. Interestingly, high concentrations of DSF could also induce ROS accumulation in a very short time interval of hours, indicating that DSF can cause instant killing of cells via ROS [27]. In the DSF/cisplatin combination treatment at high concentration in a short time period, DSF was responsible for most of the ROS accumulation. Lower concentration of DSF needed longer time to induce cells to accumulate ROS, and both DSF and cisplatin contributed to the ROS accumulation at lower concentration of the drug combinations.

The effect of cisplatin on cell cycle distribution and the effect of DSF on ROS accumulation was further determined after cell sorting. The results indicated that ALDH + cells showed cancer stem-like properties, with low levels of ROS, while ALDH- cells were more proliferating with higher levels of ROS due to rapid metabolism. Upon treatment, more ROS accumulated in ALDH + cells, ultimately reaching the same levels of ROS as in ALDH- cells. These results indicated that DSF conferred an equal cytotoxicity to ALDH + and ALDH- cells. Concerning the relative increase, however, ALDH + cells showed a greater increase than ALDH-

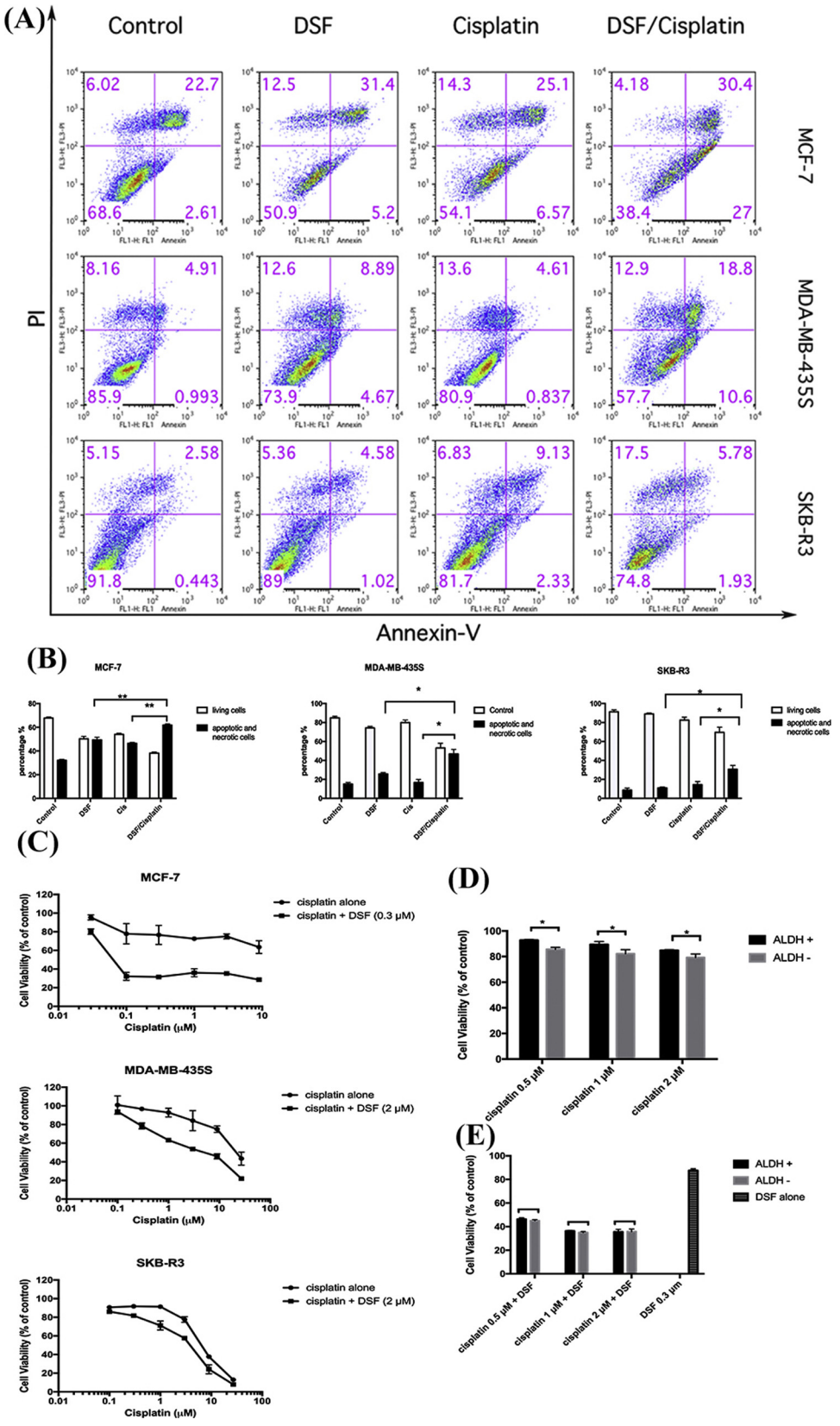


**Fig. 4.** Combination effect of DSF with cisplatin. A) Flow cytometric analysis of an apoptosis assay. Cells were exposed to DSF (1 μM), Cisplatin (5 μM), or DSF (1 μM) plus Cisplatin (5 μM) for 48 h and the percentage of apoptotic cells was determined by Annexin-V/PI dual staining by flow cytometric analysis. Percentage of cells were determined in the 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. B) Graphical representation of the statistical analysis of the Annexin-V/PI dual staining results. C) DSF sensitizes breast cancer cells for cisplatin treatment. MCF-7, MDA-MB-435S and SKB-R3 cells were treated with cisplatin alone or with cisplatin/DSF combination at indicated concentrations for 72 h, followed by MTT assay for cellular viability. D, E) DSF overcomes cisplatin resistance on ALDH + cells. ALDH + /- cells from MCF-7 were treated with (D) cisplatin alone, (E) cisplatin/DSF or DSF at indicated concentrations for 72 h. Cellular viability was determined by MTT assay. Cells without any drug treatment were used as control. One representative of three independent experiments is shown. * $P < 0.05$ ** $P < 0.01$.

cells as they contain lower basal levels of ROS, indicating that ALDH + cells might be more sensitive to DSF treatment or have less compensatory scavenging possibilities.

In consideration of all results we got in this study, we summarize the possible mechanisms for the synergistic effect of DFS and cisplatin combination as follows: 1) DSF suppresses ALDH activity and stemness, reverses cisplatin resistance on ALDH + cells, and sensitizes a cisplatin-resistant CSC population to cisplatin treatment, consequently improving the ability of cisplatin to kill resistant or less sensitive cancer cells. This is in agreement with a study by Song et al. [28] that DSF

**Table 2**
Computer-simulated CI and DRI values for drug combinations at different levels of inhibition.

| Cis + DSF Combination | Combination Index at | | | Dose-Reduction Index at | | |
|---|---|---|---|---|---|---|
| | IC50 | IC75 | IC90 | IC50 | IC75 | IC90 |
| MCF-7 | 0.0023 | 0.014 | 0.16 | 454.89[a] | 84.67 | 15.76 |
| | | | | 13586.7[b] | 382.40 | 10.76 |
| MDA-MB-435S | 0.35 | 0.36 | 0.38 | 2.96 | 2.81 | 2.67 |
| | | | | 66.36 | 115.65 | 201.56 |
| SKB-R3 | 0.64 | 0.75 | 0.88 | 2.34 | 1.85 | 1.47 |
| | | | | 4.78 | 4.82 | 4.87 |

[a] fold reduction compared to single dose cisplatin.
[b] fold reduction compared to single dose DSF.

improved the effectiveness of cisplatin in resistant lung cancer cell lines. 2) DSF and cisplatin exert their cytotoxicity based on different mechanisms. DSF induces cellular apoptosis by accumulation of ROS, including instant killing and delayed cytotoxicity, while cisplatin induces apoptosis based on DNA damage, leading to cell cycle distribution changes, and increased ROS generation during longer treatment periods. 3) Cisplatin and DSF exert their cytotoxicity on different targeted cell types. The conventional chemotherapy agent cisplatin targets proliferating cells with little effect on CSCs, while DSF is equally cytotoxic to both proliferating cells and ALDH + stem-like cells and renders CSCs more sensitive to DSF/cisplatin treatment. Taken together, this study provides evidence that DSF may be used as a novel adjuvant and could be tested for incorporation into conventional chemotherapy regimens, to improve the efficacy of targeted chemotherapy for breast cancer treatment in future.

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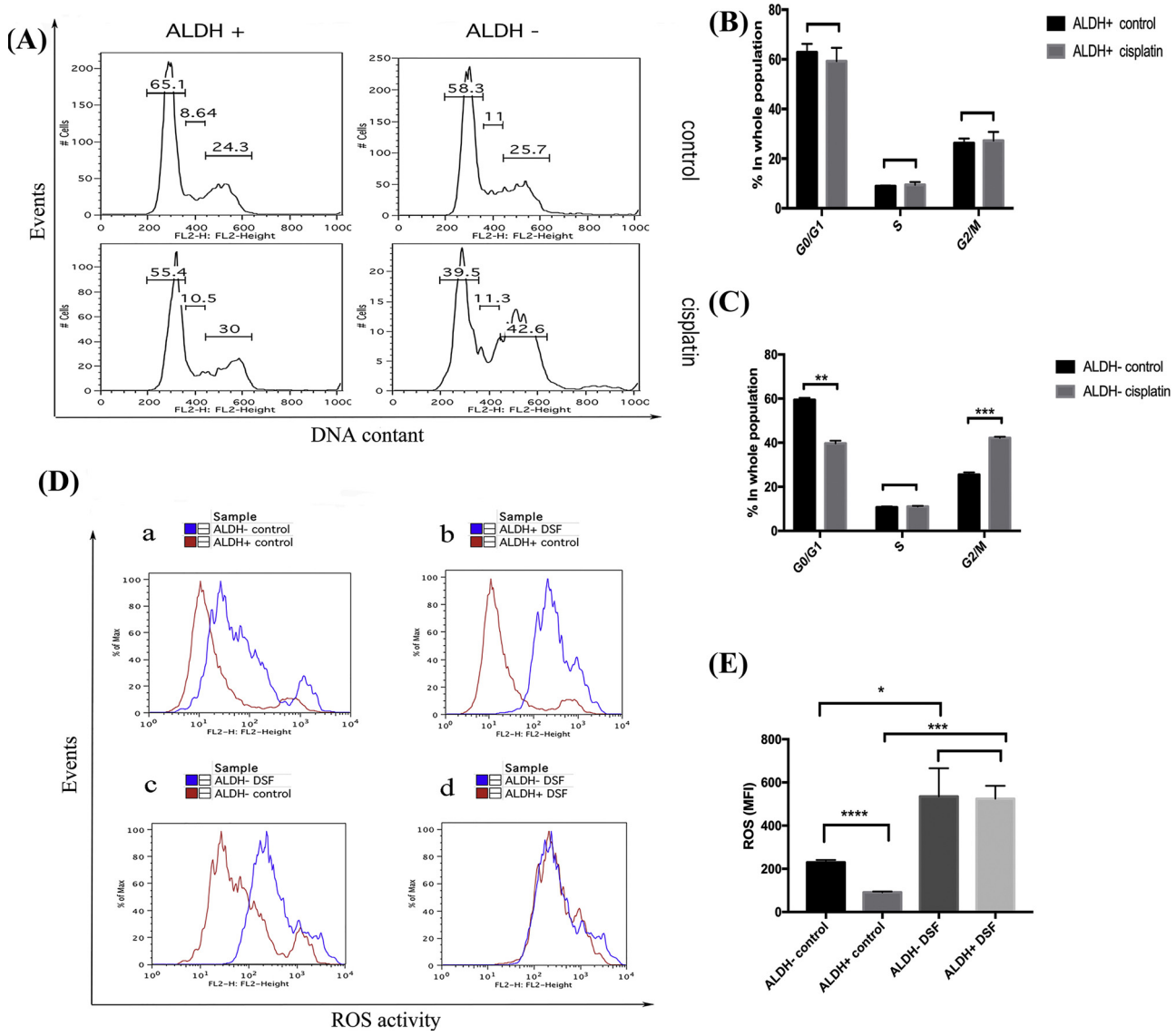


**Fig. 5.** Cell cycle analysis and ROS production of ALDH + and ALDH- cells by FACS. A) The effect of cisplatin (2 μM, 72 h) on cell cycle alterations in ALDH + /- cells. B,C) Graphical representation of the percentage of cells in each cell cycle phase was quantitated and analyzed (n = 3) D) The effect of DSF (0.3 μM, 72 h) on ROS production in ALDH + /- cells. Comparison of a) ALDH + /- control; b) ALDH + DSF-treated/control; c) ALDH- DSF-treated/control; d) ALDH + /- DSF-treated. E) Graphical representation of the ROS mean fluorescence intensity (MFI) was quantitated and analyzed (n = 3) One representative of three independent experiments is shown * P < 0.05 **P < 0.01 *** P < 0.001 ****P < 0.0001.

## Conflict of interest

The authors have no conflict of interest.

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