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Inhibitory effect of Disulfiram/copper complex on non-small cell lung cancer cells

Lincan Duan [a,1], Hongmei Shen [b,1], Guangqiang Zhao [a], Runxiang Yang [c], Xinyi Cai [d], Lijuan Zhang [e], Congguo Jin [f], Yunchao Huang [a,*]

[a] Department of Thoracic Surgery, The Third Affiliated Hospital of Kunming Medical University, Kunming, China
[b] Cancer Center of Integrative Medicine, The Third Affiliated Hospital of Kunming Medical University, Kunming, China
[c] Cancer Chemotherapy Center, The Third Affiliated Hospital of Kunming Medical University, Kunming, China
[d] Colorectal Cancer Center, The Third Affiliated Hospital of Kunming Medical University, Kunming, China
[e] Department of Pathology, The Third Affiliated Hospital of Kunming Medical University, Kunming, China
[f] Cancer Institute, The Third Affiliated Hospital of Kunming Medical University, Kunming, China

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ABSTRACT

Non-small cell lung cancer (NSCLC) is the most common cause of cancer-related death in both men and women worldwide. Recently, Disulfiram has been reported to be able to inhibit glioblastoma, prostate, or breast cancer cell proliferation. In this study, the synergistic effect of Disulfiram and copper on NSCLC cell growth was investigated. Inhibition of cancer cell proliferation was detected by 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay and cell cycle analysis. Liquid colony formation and tumor spheroid formation assays were used to evaluate their effect on cancer cell clonogenicity. Real-time PCR was performed to test the mRNA level of cancer stem cell related genes. We found that Disulfiram or copper alone did not potently inhibit NSCLC cell proliferation *in vitro*. However, the presence of copper significantly enhanced inhibitory effect of Disulfiram on NSCLC cell growth, indicating a synergistic effect between Disulfiram and copper. Cell cycle analysis showed that Disulfiram/copper complex caused NSCLC cell cycle arrest in G2/M phase. Furthermore, Disulfiram/copper significantly increased the sensitivity of cisplatin in NSCLC cells tested by MTT assay. Liquid colony formation assay revealed that copper dramatically increased the inhibitory effect of Disulfiram on NSCLC cell colony forming ability. Disulfiram combined with copper significantly attenuated NSCLC cell spheroid formation and recuded the mRNA expression of lung cancer stem cell related genes. Our data suggest that Disulfiram/copper complex alone or combined with other chemotherapy is a potential therapeutic strategy for NSCLC patients.

1. Introduction

Non-small cell lung cancer (NSCLC) is one of the most deadly malignant diseases. The five-year survival rate remains around 15% for several decades without dramatic improvement mainly due to diagnosis at a late disease stage. To date, platinum combination chemotherapy is the standard first-line therapy for advanced NSCLC patients with response rate less than 30% [1]. It is critical to develop novel effective chemotherapy or to improve the efficacy of platinum therapy in order to increase the overall survival. Disulfiram has been widely used as a first-line anti-alcoholism drug in the clinics for over 60 years. Recently, accumulating evidence demonstrates that Disulfiram has strong anticancer activity for certain types of cancer both *in vitro* and in mouse models [2–5]. Disulfiram also enhances the cytotoxicity of several anticancer drugs as well as radiotherapy, suggesting it as a potential chemotherapeutic agent [6]. Reported pharmacological mechanisms include the induction of oxidative stress and inhibition of proteasome activity through JNK, NF-κB, or PI3K pathways [3,7–9]. More importantly, different groups found that the cytotoxicity of Disulfiram is copper dependent [5,9,10]. Copper plays an essential role in redox reactions and triggers generation of reactive oxygen species (ROS) in both normal and tumor cells [8,10]. Since it is a bivalent metal ion chelator, Disulfiram forms a complex with copper and improves the transport of copper into cancer cells. Therefore, Disulfiram/copper complex is a much stronger ROS inducer [11]. In addition, relatively high copper concentration in cancer cells enables Disulfiram to specifically target cancer instead of normal tissues [12].

* Corresponding author. Address: Department of Thoracic Surgery, The Third Affiliated Hospital of Kunming Medical University, 519 Kunzhou Road, Kunming 650118, China. Fax: +86 871 68181942.
E-mail address: daliduanlincan@163.com (Y. Huang).
[1] These two authors contributed equally to this work.

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It has been proposed that certain types of solid tumor contain a subset of stem-like cancer cells which are capable of self-renewal, differentiation, and are involved in radio- and chemoresistance and tumor recurrence [13]. Among different cancer stem cell markers, aldehyde dehydrogenase (ALDH) is considered as not only a surrogate marker but also a functuionally important target [14]. Inhibition of ALDH activity has been suggested as a potential strategy to eliminate cancer stem cells and to overcome drug resistance. Disulfiram has been well known as an ALDH inhibitor, suggesting that Disulfiram may specifically target on cancer stem cell subpopulation. In fact, in glioblastoma cancer cells, Disulfiram reduces ALDH activity detected by Aldefluor assay and decreases ALDH positive cell fraction [5]. In breast cancer, Disulfiram and copper treatment inhibits NF-κB activity, increases ROS and the number of breast cancer stem cells [8]. These findings led us to investigate the effect of Disulfiram and copper in NSCLCs.

In the present work, we examined the inhibitory effect of Disulfiram alone and in combination with copper on NSCLC cell proliferation and cisplatin sensitivity. We also determined that Disulfiram/copper complex reduced lung cancer cell colony formation and spheroid formation. The combination of Disulfiram and copper were capable of reducing lung cancer stem cells using ALDH as a marker.

2. Materials and methods

2.1. Cell lines and materials

Non-small cell lung cancer lines A549 and NCI-H2009 were purchased from *American Type Culture Collection (*Manassas, VA). Cell lines were cultured in RPMI-1640 (Gibco, Carlsbad, CA) supplemented with 5% fatal bovine serum (Gibco), 2 mM L-gluatmine, Penicillin (100 units/ml), and *Streptomycin* (100 μg/ml) (Sigma–Aldrich, St. Louis). All cell lines were maintained at 37 °C in a humidified incubator with 95% air and 5% CO_2. The cultured cells were harvested with 0.25% trypsin (Gibco) and split at 1:5 ratio when cells were 80–90% confluent.

2.2. MTT assay

Lung cancer cells were seeded in triplicates in 96-well plates. Drugs were given as 4-fold dilutions with a maximum dose of 100 μmol/L for Disulfiram, $CuCl_2$ (Sigma), or 50 μmol/L cisplatin (Teva Parenteral, CA). Different doses of Disulfiram or $CuCl_2$ were used in combination assays as indicated. Five days after treatment, the absorbance was measured on an enzyme-linked immunosorbent assay plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. The entire assay was done in triplicates. Dose response curves and IC_{50}s were calculated using GraphPad Prism 5.04.

2.3. Liquid colony formation assay

Cells were seeded at a density of 500 cells/well in 6-well plates. 24 h later, attached cells were continuously treated with different concentration of Disulfiram or $CuCl_2$ for 14 days. Formed colonies were stained with 0.05% crystal violet and counted.

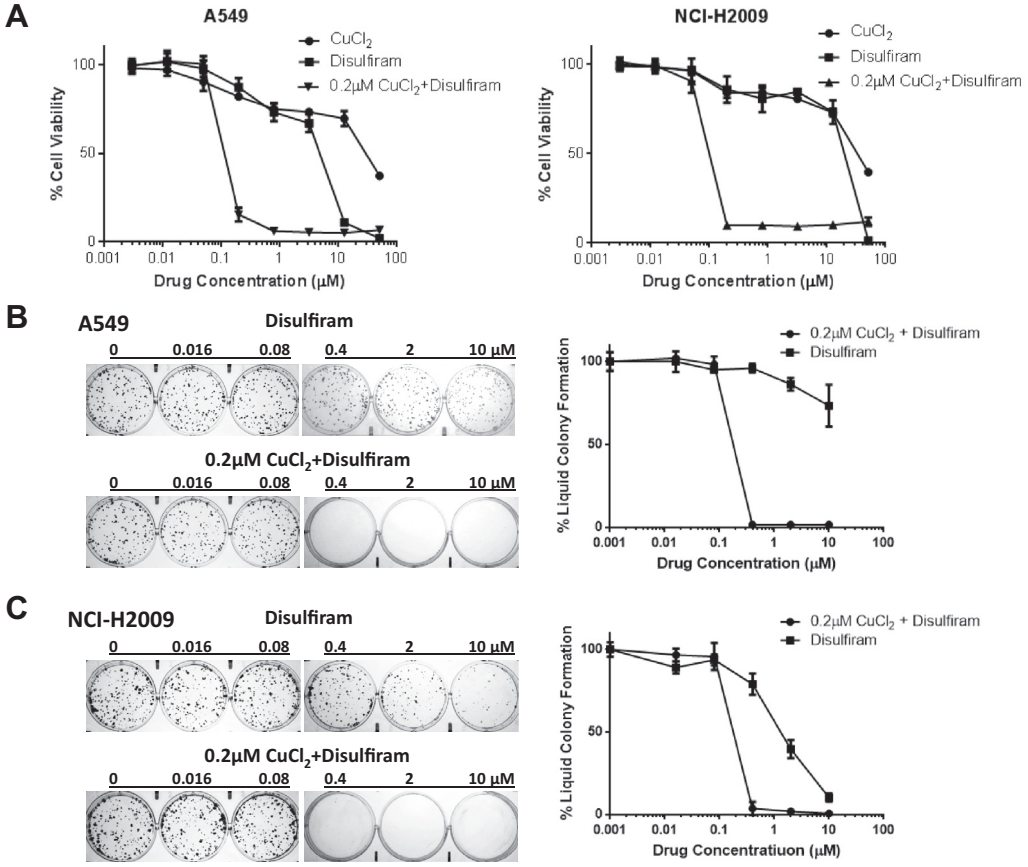

Fig. 1. Inhibition of NSCLC cell proliferation and liquid colony formation by Disulfiram/copper complex. (A) MTS assay revealed that copper synergistically increased the inhibitory effect of Disulfiram on A549 and NCI-H2009 cell proliferation. A549 (B) and NCI-H2009 (C) cells were treated with different concentration of Disulfiram for 2 weeks in the presence or absence of 0.2 μM $CuCl_2$. The complex synergistically reduced anchorage-dependent colony formation of lung cancer cells.

2.4. Cell cycle analysis

Analysis of cell cycle progression and detection of apoptosis was performed using DNA staining. Briefly, lung cancer cells were plated into T-75 flasks at an initial seeding density of 10^6 cells/flask. After a 48 h treatment, trypsinized cells were fixed in 75% ethanol for 15 min at −20 °C, washed with phosphate-buffered saline (PBS) and then incubated with 500 U/ml RNase A (Worthington Biochemical, Freehold, NJ) and 50 mg/ml propidium iodide (PI) solution (Calbiochem, La Jolla, CA) at 37 °C for 45 min. Stained cells were analyzed on an EPICS 752 flow cytometer (Coulter, Hialeah, FL) equipped with 488 nm excitation for the measurement of PI fluorescence. DNA analysis was performed using MPLUS software (Phoenix Flow Systems, San Diego, CA). Data were expressed as percentage distribution of cells in G_0/G_1, S and G_2/M phases of the cell cycle.

2.5. Real-time PCR

Real-time PCR was employed to measure relative mRNA expression levels of ALDH1, NANOG, and OCT-4. Total mRNA was extracted using Trizol (Invitrogen, CA) according to the manufacturer's instructions. mRNA (0.5 μg) was reverse-transcribed using High-Capacity cDNA synthesis kit (Applied Biosystems, California). Real-time PCR was performed using the TaqMen PCR kit (Qiagen, USA) on Light Cycler 480 real-time PCR machine (Roche Diagnostics). GAPDH expression was employed as an internal control. Relative mRNA levels were expressed as fold-change compared to untreated cells.

2.6. Spheroid formation assay

The single lung cancer cells were suspended in serum-free DMEM/F12 (1:1, Gibco) supplemented with 20 ng/ml human recombinant EGF (Sigma), 20 ng/ml bFGF (Sigma) and B27 (Life technologies), cultured at a density of 5×10^4 cells/ultra-low attachment 10 cm dish (Corning). Fresh medium was added every other day. Spheroids were collected and disassociated after 2 weeks. Images of lung cancer spheroids were recorded using a Nikon 2000-S inverted photomicroscope with Nikon digital sight DSFi1 camera.

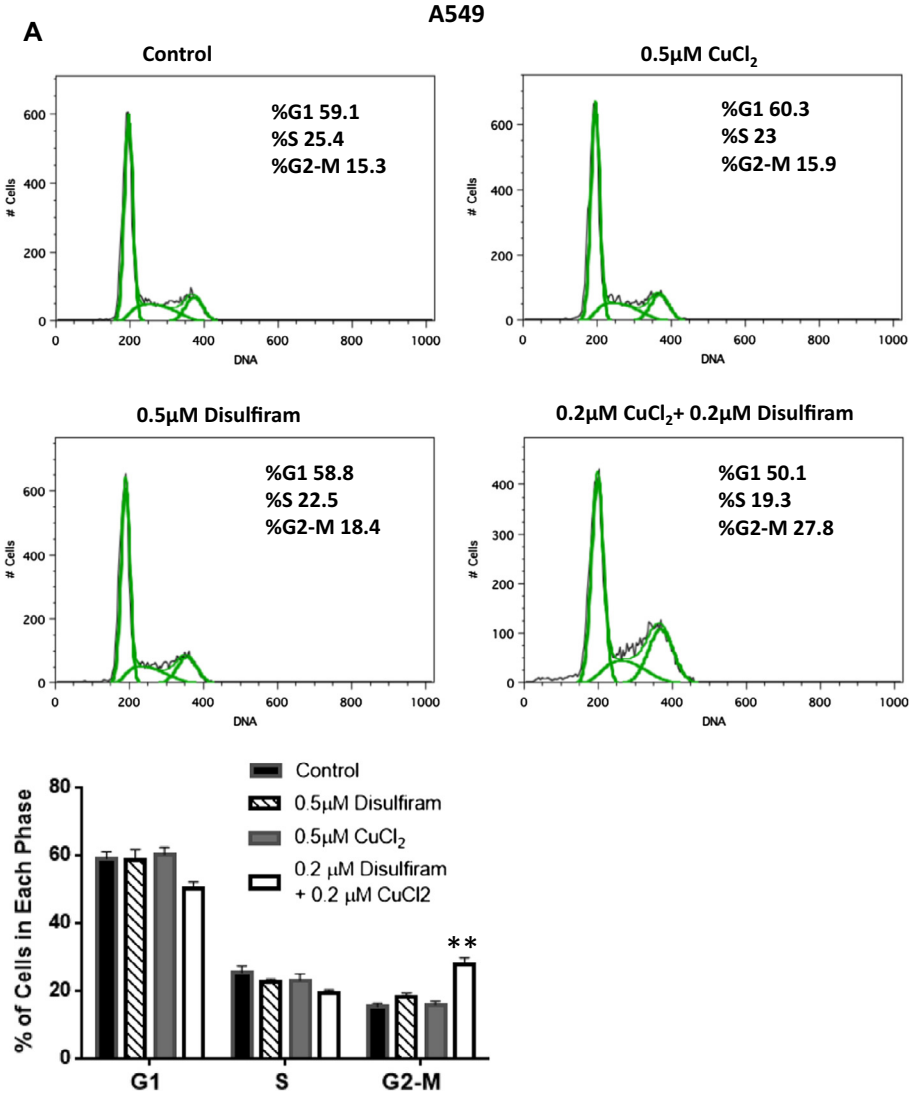

Fig. 2. NSCLC cell cycle arrest caused by Disulfiram/copper treatment. (A) A549 cells were treated with 0.5 μM Disulfiram or 0.5 μM $CuCl_2$ alone, or 0.2 μM Disulfiram + 0.2 μM $CuCl_2$ for 2 days followed by cell cycle analysis. The complex caused lung cancer cell cycle arrest in G2/M phase. Similarly effects were observed in NCI-H2009 cells (B) ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$ compared to control).

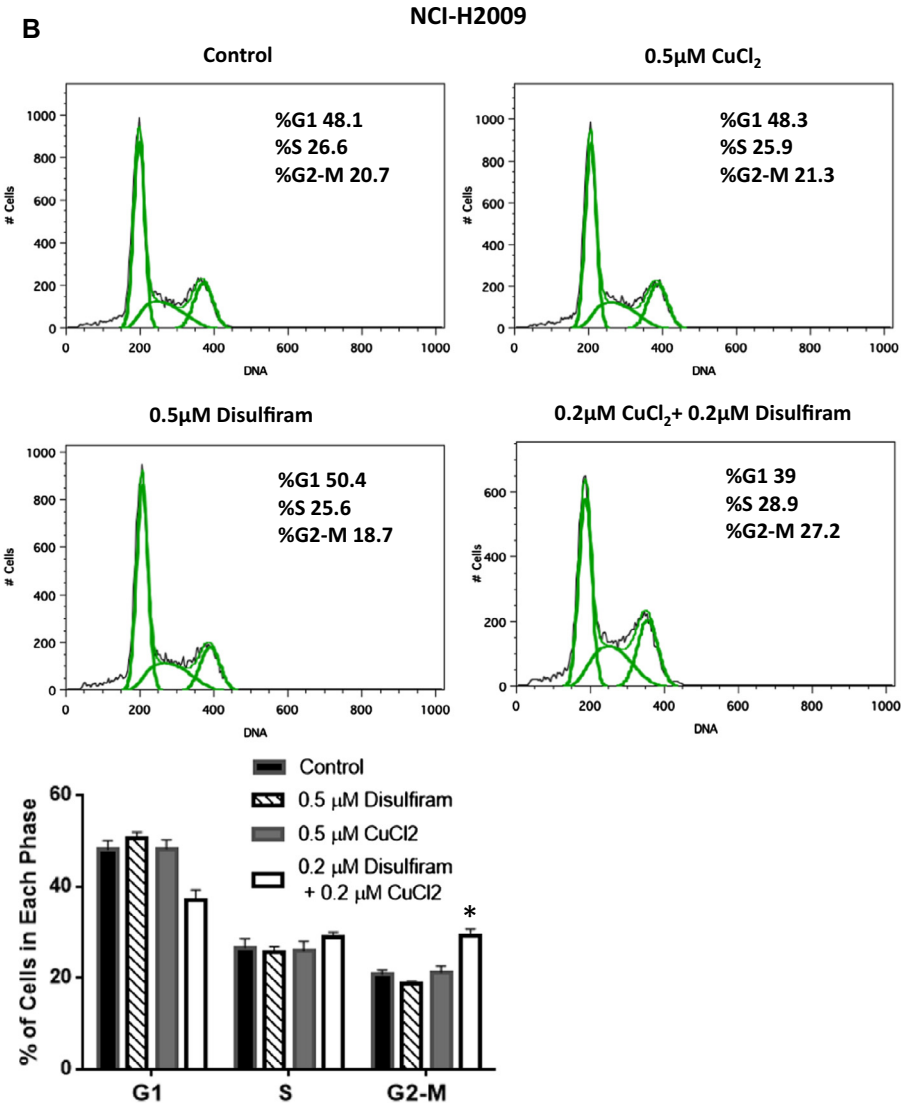

Fig. 2 (*continued*)

2.7. Statistical analysis

All statistical analyses were performed using SPSS 13.0. Data were shown as mean ± standard deviation (SD) calculated from all experiments. All comparisons between experimental groups were performed by Student *t*-test. *P values* less than 5% were considered to be statistically significant. To determine the effects of the combination of Disulfiram and copper on NSCLC cells combination index (CI) values were calculated using the equation

$$CI = (D1/Dx1) + (D2/Dx2) + (D1D2/Dx1Dx2)$$

D1 = concentration of drug A used in the combination to achieve the effect.
D2 = concentration of drug B used in the combination to achieve the effect.
Dx1 = concentration of drug A required to achieve the effect as a single agent.
Dx2 = concentration of drug B required to achieve the effect as a single agent.
From this analysis, CI = 1 indicates additivity; CI < 1 indicates synergism; and CI > 1 indicates antagonism.

3. Results

3.1. Disulfiram and copper synergistically inhibited NSCLC cell proliferation and colony formation

To examine the synergistic cytotoxicity of Disulfiram/copper complex, NSCLC lines were treated with Disulfiram and copper alone or in combination followed by MTS assay. No cytotoxicity was observed in NCI-H2009 cells until the cells were treated with 50 μM Disulfiram or 50 μM $CuCl_2$ alone. However, in the presence of 0.2 μM $CuCl_2$, as low as 0.2 μM Disulfiram showed strong cytotoxicity. Similarly, 12 μM Disulfiram or 50 μM $CuCl_2$ alone caused around 12% and 40% A549 cell death, respectively. Adding 0.2 μM $CuCl_2$ significantly improved cytotoxicity of Disulfiram in A549 cells, i.e. $CuCl_2$ reduced IC_{50} of Disulfiram about 100-fold (Fig. 1A). The combination indices of Disulfiram and $CuCl_2$ in A549 and NCI-H2009 cells are 0.26 and 0.23, respectively, indicating strong synergistic effect. In liquid colony formation assay, 10 μM Disulfiram inhibited 90% colony in H2009, whereas 0.4 μM Disulfiram combined with 0.2 μM $CuCl_2$ caused 100% inhi-

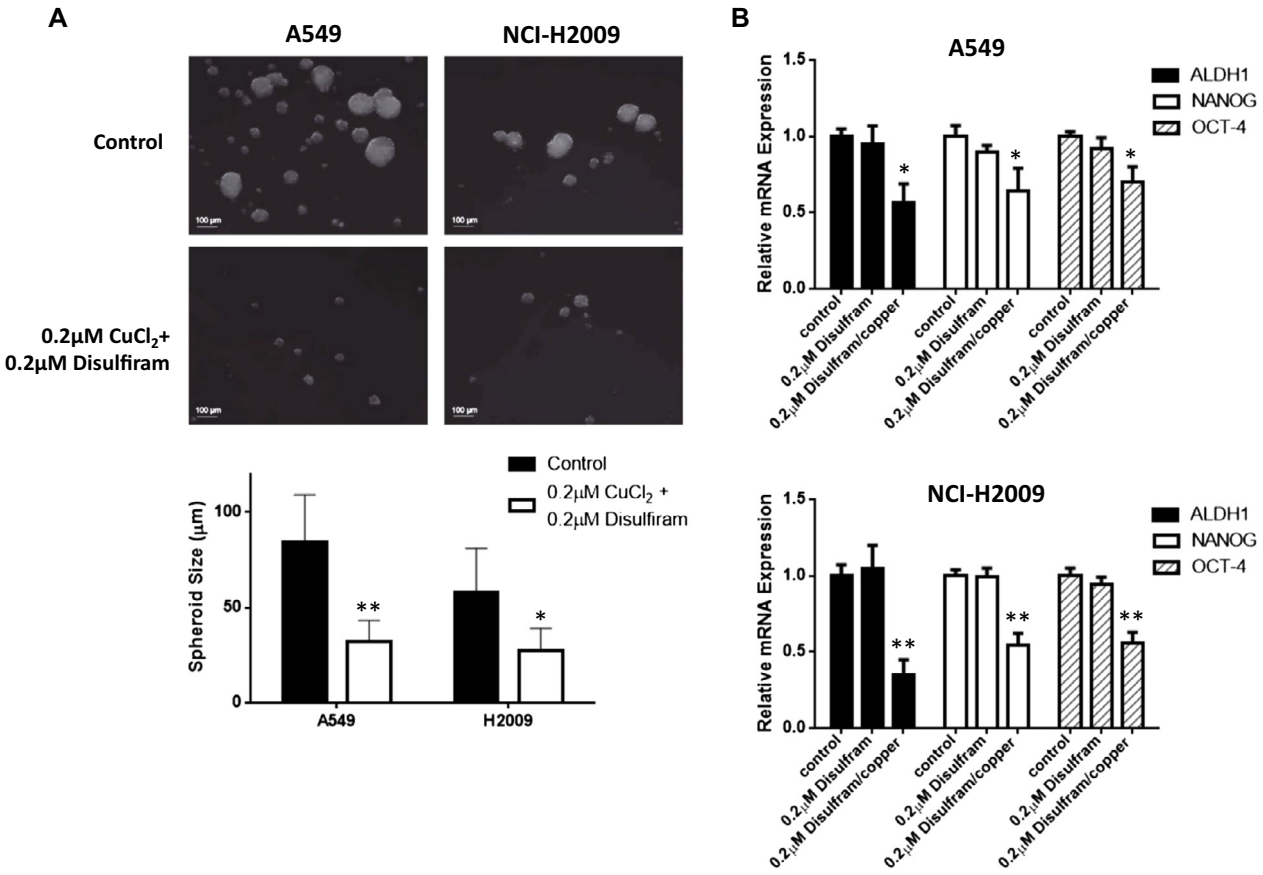

Fig. 3. Disulfiram/copper reduces lung cancer cell spheroid formation. (A) Single A549 or NCI-H2009 cells were cultured in serum-free medium for 2 weeks with or without 0.2 μM Disulfiram + 0.2 μM $CuCl_2$ treatment. The complex significantly reduced the size and number of spheroids. (B) Real-time PCR revealed that the complex inhibited the mRNA expression of ALDH1, OCT-4, and NANOG in A549 or NCI-H2009 spheroids ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control).

bition of colony formation. We observed similar effect of Disulfiram/copper on A549 colony formation ability (Fig. 1B).

3.2. Disulfiram/copper complex caused cell cycle arrest in G2/M phase in lung cancer cells

To investigate the mechanism by which Disulfiram/copper complex inhibits lung cancer cell proliferation, we analyzed cell cycle status before and after treatment. Exposure of A549 cells to 0.5 μM Disulfiram or 0.5 μM $CuCl_2$ alone did not change the distribution of cells in different stage compared to untreated cells. However, co-exposure of A549 cells to 0.2 μM Disulfiram and 0.2 μM $CuCl_2$ caused a significant increase of cells in G2/M phase relative to control (from 15% to 28%, Fig. 2A). Consistently, Disulfiram/copper treatment led to increased NCI-H2009 cells in G2/M phase without induction of apoptotic cells (Fig. 2B). The data suggested that Disulfiram/copper synergistically inhibited lung cancer cell proliferation through G2/M phase arrest.

3.3. Disulfiram/copper complex reduced lung cancer cell spheroid formation in vitro

Because Disulfiram is a traditional ALDH inhibitor, we hypothesized that Disulfiram reduced lung cancer stem cell subpopulation. Sphorid formation assay was performed to investigate the effect of Disulfiram/copper complex on cancer stem cell self-renewal since only cancer stem cells are capable of forming spheroid in serum-free medium. We observed healthy lung cancer spheroids derived from single A549 stem-like cells after 2-week culture. Exposure of A549 cells to 0.2 μM Disulfiram and 0.2 μM $CuCl_2$ significantly decreased the amount and size of lung cancer spheroids (Fig. 3A). Real-time PCR revealed that Disulfiram/copper complex specifically reduced ALDH1 mRNA expression compared to control A549 spheroids. Consistently, the treatment caused a reduction of both OCT-4 and NANOG mRNA expression in the spheroids, which are classical transcription factors in cancer stem cell subpopulation (Fig. 3B). Our data supported the hypothesis that Disulfiram/copper complex specifically targeted lung cancer stem-like cells.

3.4. Disulfiram/copper complex increased the sensitivity of lung cancer cells to cisplatin

Since cancer stem cells are involved in drug resistance, we further examined whether Disulfiram/copper could improve cisplatin sensitivity in lung cancer cells. MTS assay revealed that IC_{50} of cisplatin was 10 μM and 5 μM in NCI-H2009 and A549 cells, respectively. Exposure of two cell lines to Disulfiram or copper alone did not significantly change cisplatin sensitivity. However, co-exposure of NSCLC cells to cisplatin and Disulfiram/copper complex strongly increased the cytotoxicity of cisplatin (Fig. 4A). Cell cycle analysis showed that the enhanced cisplatin cytotoxity was due to both S and G2/M phase arrest without inducing apoptosis (Fig. 4B). Taken together, our findings showed that Disulfiram/copper complex synergistically reduced lung cancer cell proliferation and also specifically targeted lung cancer stem cell subpopulation.

4. Discussion

Previous studies demonstrate that Disulfiram is highly cytotoxic to a wide range of cancer cells both *in vitro* and *in vivo* [15–

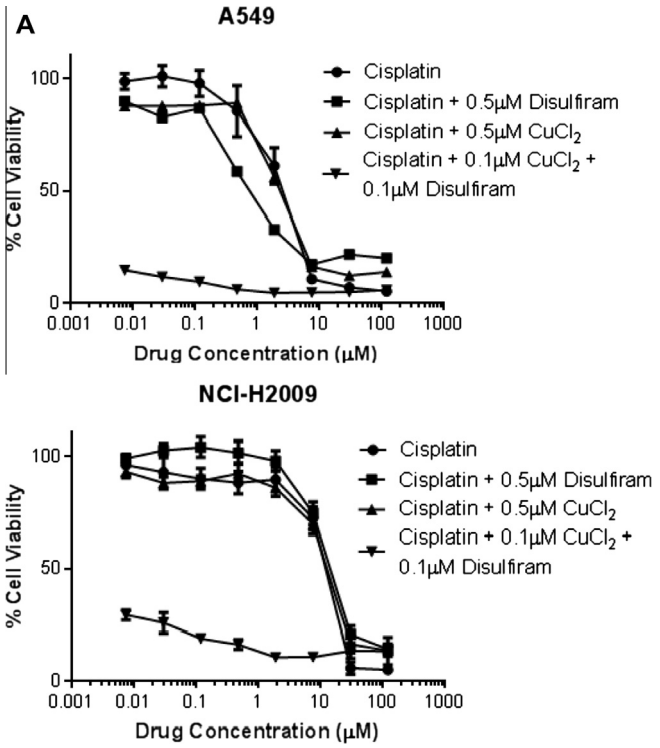

Fig. 4. Disulfiram/copper improves the sensitivity of cisplatin to NSCLC cells *in vitro*. (A) A549 or NCI-H2009 cells were treated with cisplatin alone or different combinations as indicated for 5 days. MTS assay showed that Disulfiram/copper increased the cytotoxicity of cisplatin to A549 and NCI-H2009 cells. (B) Cell cycle analysis showed that Disulfiram/copper treatment led to cell cycle arrest in both S and G2/M phases compared to cisplatin alone in A549 and NCI-H2009 cells ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to 0.1 μM cisplatin).

19]. Here, we examined the cytotoxicity of Disulfiram and copper alone or in combination and found their synergistic inhibitory effect on NSCLC proliferation and colony formation. We observed that Disulfiram/ copper complex significantly reduced lung cancer stem cell subpopulation and increased cisplatin sensitivity to cultured lung cancer cells. Our data also indicated that copper was critical for Disulfiram-induced cytotoxicity in NSCLC cell lines. Proposed underlying mechanism of copper-induced cytotoxicity is that copper ions promote ROS formation, which has been shown in breast cancer, prostate cancer, and melanoma cell lines [10]. ROS are reactive oxygen containing species generated from the mitochondrial respiratory chain reaction. Two forms of intracellular copper, cupric and cuprous, induce the formation of hydroxyl radicals from hydrogen peroxide, which could react with and damage variety of intracellular molecules [20]. However, copper alone is not highly cytotoxic to NSCLC cells since the transport of copper into cells is tightly controlled by the copper transporter. As an efficient bivalent ion chelator, Disulfiram can form a complex with copper, which significantly promotes the transport of copper into cancer cells in a copper transporter independent manner [21]. On the other hand, Disulfiram/copper complex induced ROS activity and cytotoxicity is reversed by ROS inhibitors [6].

Chemo- or radiotherapy resistance remains a serious problem for current cancer treatment. We found that Disulfiram/copper complex dramatically improve the sensitivity of cisplatin to NSCLC cells. Several signaling pathways have been shown to be involved in re-sensitizing cancer cells to the drugs by Disulfiram/copper [22,23]. For example, Xu et al. reported that Disulfiram/copper re-sensitizes doxorubicin resistant leukemia HL60 cells to doxorubicin through activating JNK/c-jun pathway [9]. Disulfiram/copper inhibited breast cancer stem cells and enhanced cytotoxicity of paclitaxol through simultaneous induction of ROS and inhibition of NF-κB pathway [8]. Although the mechanisms are not fully

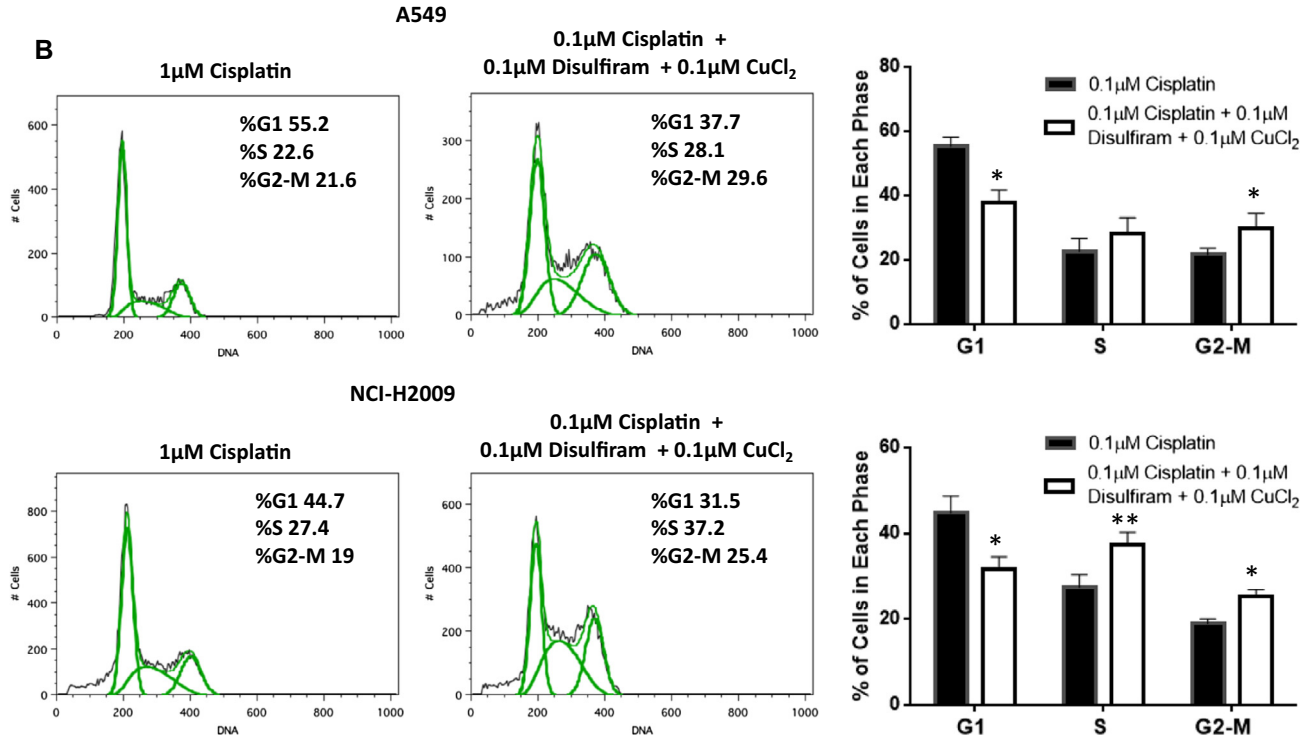

Fig. 4 (*continued*)

understood, further investigation of Disulfiram/copper anti-NSCLC effect will lead to its rapid translation into NSCLC chemotherapy.

Besides the cytotoxicity, Disulfiram is able to target cancer stem cells identified by elevated ALDH activity since Disulfiram is an ALDH inhibitor which has been long-term used to treat alcoholism in the clinics [24,25]. Cancer stem cell theory partially explains tumor recurrence, drug resistence, and tumor metastasis. We found that Disulfiram/copper complex significantly reduced lung cancer spheroid formation and the expression of cancer stem cell transcription factors, suggesting that Disulfiram/copper specifically targeted lung cancer stem cell subpopulation. Therefore, the combination of Disulfiram/copper with classical anti-cancer drugs could elimiate both cancer stem cells and the rest bulk tumor cells. Recently, a Phase I/II clinical trial to evaluate Disulfiram in patients with metastatic melanoma has been completed. However, the results are not available to the public. Other Phase I clinical trials of Disulfiram in hormone refractory cancers with lung and liver metastases (NCT00256230 and NCT00742911) or in prostate cancer (NCT01118741) are undergoing.

In summary, the current study strongly supported that Disulfiram in combination with copper significantly inhibited NSCLC cell proliferation and liquid colony formation. The complex also increased cisplatin sensitivity to NSCLC cells partially through targeting lung cancer stem like cells. More importantly, clinical experience with disulfiram in the treatment of alcoholism shows that side effects of Disulfiram are usually minor. Therefore, developing Disulfiram/copper into therapeutic agent will be of significant clinical importance in NSCLC treatment.

Acknowledgments

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